Biochemical parameters and 18S rRNA gene sequence analysis amongst green microalgal strains from selected aquatic sites of Eastern India

Rashi Vishwakarma, Dolly Wattal Dhar, Mrutyunjay Jena and Madhulika Shukla

ABSTRACT

In the present study, 24 green microalgae strains were isolated from selected aquatic sites of India. These were microscopically identified as *Chlamydomonas* sp., *Scenedesmus* sp., *Chlorella* sp., *Dictyosphaerium* sp. and *Dunaliella* sp. *Nannochloropsis* sp. (MCC 25), was used as a reference strain. Results showed that *Dictyosphaerium* sp. (MCC 10 and MCC 12) showed relatively higher nutritive content. The total soluble proteins in the reference strain was 21.4%, whereas it showed carbohydrate content of 17.2% and the lipids were 3.4% on a dry weight basis. Best performing strains were identified by biochemical characterization. Five genera were selected for molecular identification since they were the most representative based upon their area of isolation and their optimum content of total soluble proteins, carbohydrates and lipids. 18S rRNA sequencing authenticated their identification as *Scenedesmus* sp., *Dictyosphaerium* sp. and *Chlorella* sp. The sequences of these have been submitted in NCBI database with accession numbers as KT808247–KT808251. The correlation matrix showed positive correlation between carbohydrates and lipids, while negative correlation was seen between proteins and carbohydrates and between proteins and lipids. This study emphasizes the need for complete compositional analysis of the biomass for the possible applicability in the area of value addition.

Key words | carbohydrates, identification, lipids, microalgae, proteins

HIGHLIGHTS

- Biochemical characterization in terms of total soluble protein, carbohydrates and lipid content of 24 microalgal strains from different aquatic sites across India was carried out and the best performing strains were identified.
- Aquatic sites of Odisha represent biodiversity hotspot as a large number of green algae strains were isolated from such habitats.
- This study emphasizes the need for complete compositional analysis of the biomass for the possible applicability in the area of value addition and biofuel.

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doi: 10.2166/wst.2020.195

GRAPHICAL ABSTRACT

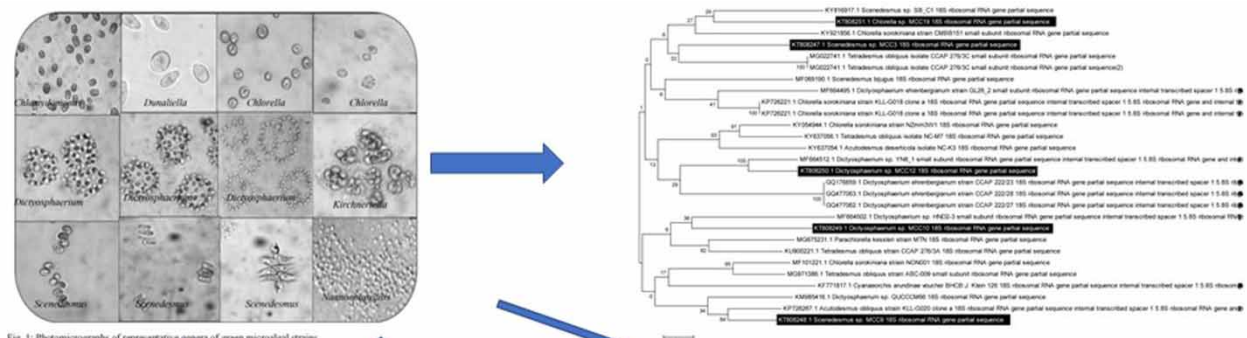

Fig. 1: Photomicrographs of representative genera of green microalgal strains.

Strains	14 days	21 days	28 days	Mean
1	334.6±12.83	376.3±18.02	255.2±4.34	322.0±6.25
2	379.4±24.36	435.5±7.05	443.2±51.16	419.4±6.05
3	28.6±3.77	117.3±3.11	360.1±23.30	168.7±5.96
4	124.5±3.11	193.9±4.56	165.5±7.15	161.3±9.37
5	498.3±13.40	190.3±8.11	520.7±13.20	403.1±15.14
6	223.3±13.27	251.6±8.70	496.4±7.44	323.8±38.39
7	300.1±23.53	205.1±13.17	397.5±4.97	300.9±11.42
8	171.3±1.27	258.7±14.87	139.9±85.79	189.9±10.07
9	281.8±6.58	213.2±12.12	521.9±3.48	338.9±29.36
10	119.5±2.40	394.9±6.71	312.9±15.86	275.7±36.28
11	235.7±15.22	464.0±4.08	419.8±18.20	373.2±18.10
12	228.5±15.19	484.2±3.89	395.5±6.67	369.4±14.33
13	248.2±12.76	472.0±11.33	310.5±14.68	343.6±14.48
14	412.1±13.22	135.8±7.08	122.9±9.51	223.6±6.63
15	185.6±9.28	276.7±6.98	213.0±3.49	225.1±6.44
16	283.6±9.41	418.5±32.64	352.7±3.86	351.6±26.18
17	98.7±4.93	324.4±4.76	385.2±3.20	269.5±20.32
18	191.3±5.80	314.1±15.92	228.6±15.45	244.7±30.82
19	101.5±8.29	309.6±7.98	234.7±4.50	215.3±11.29
20	361.7±16.67	401.8±1.86	313.7±10.13	359.1±7.01
21	232.6±12.8	465.6±10.2	239.9±1.63	312.7±6.32
22	147.7±5.05	422.1±20.58	263.9±3.12	277.9±7.97
23	281.0±2.19	394.6±29.91	340.6±13.14	338.7±40.95
24	278.6±4.51	476.5±6.42	256.2±9.55	337.1±36.86
25	198.5±9.86	297.1±6.41	145.2±8.04	213.6±16.87
Mean	100.4±9.8	116.9±26.5	238.7±15.7	
SE (±)	2.78	1.35	2.45	2.31
CD (p<0.5)	0.2	0.8	1.1	0.9

Strains	14 days	21 days	28 days	Mean
1	73.3±8.19	25.6±6.44	223.8±4.11	107.6±6.25
2	60.9±7.35	36.4±5.73	192.6±5.07	96.7±6.05
3	91.8±7.07	23.4±7.76	185.1±3.06	100.1±5.96
4	119.3±14.43	126.5±6.34	35.0±7.35	93.60±9.37
5	262.6±9.33	22.7±10.89	481.0±25.22	255.44±15.15
6	106.0 ±0.66	114.7±86.26	268.5± 28.25	163.05±38.39
7	119.3±13.57	19.4±17.90	366.8±2.80	168.51±11.42
8	153.9±7.73	179.4±5.57	396.9±16.90	243.38±10.07
9	86.3±12.20	52.7±22.43	664.5±53.46	267.85±29.36
10	35.9±16.80	122.0±73.75	278.0±18.29	145.30±36.28
11	161.7±10.46	114.7±24.53	372.8±19.31	216.40±18.10
12	20.6±1.86	164.5±22.49	337.2±18.65	174.10±14.33
13	25.3±8.55	34.23±8.28	155.8±26.62	71.81±14.48
14	134.9±10.24	153.4±5.32	350.9±4.34	213.07±6.63
15	121.7±6.17	245.9±5.34	135.0±7.82	167.54±6.44
16	136.0±4.87	215.1±39.16	263.0±34.52	204.72±26.18
17	156.7±26.35	25.1±18.83	103.3±15.78	95.03±20.32
18	26.7±4.57	45.0±77.95	200.1±9.94	90.62±30.82
19	23.5±14.36	54.2±14.89	108.0±4.62	61.92±11.29
20	27.9±4.59	319.5±1.40	116.7±15.04	154.68±7.01
21	154.7±7.33	124.9±7.39	213.3±4.23	164.28±6.32
22	45.6±9.037	168.4±6.44	40.9±8.44	85.01±7.97
23	89.6±11.13	52.4±108.84	307.9±2.89	149.95±40.95
24	127.8±20.50	247.2±54.79	37.1±35.29	137.35±36.86
25	147.5±6.98	234.9±23.98	132.6±19.65	171.69±16.87
Mean	237.9±9.99	331.8±10.66	313.4±13.67	
SE (±)	5.35	3.71	4.62	4.76
CD (p<0.5)	2.0	0.4	1.2	3.0

Strains	14 Days	21 Days	30 Days	Mean
1	6.2±0.06	9.4±0.95	3.3±0.11	6.3
2	6.8±0.07	12.0±0.89	3.0±0.05	7.3
3	13.7±0.31	5.7±0.35	5.5±0.02	8.3
4	5.2±0.44	6.9±2.17	2.1±0.18	4.7
5	12.6±0.07	13.5±0.92	6.2±0.88	10.8
6	6.4±0.09	10.7±1.22	3.1±0.03	6.7
7	8.0±0.19	7.8±0.05	14.8±0.10	10.2
8	7.4±0.29	9.1±0.19	3.7±0.06	6.7
9	4.8±0.04	9.5±0.10	4.8±0.07	6.4
10	6.6±0.17	11.0±1.50	2.1±0.02	8.0
11	11.5±1.37	10.8±1.18	3.9±0.05	8.7
12	10.5±0.48	11.0±1.20	6.3±0.10	9.3
13	9.7±0.07	8.0±0.71	1.8±0.13	6.5
14	11.2±1.15	10.3±4.61	2.9±0.07	8.1
15	1.2±2.46	1.9±0.92	3.2±0.12	2.1
16	4.8±0.46	9.8±1.23	4.2±0.004	6.3
17	11.3±1.42	9.8±0.39	1.2±0.01	7.4
18	12.4±0.70	2.4±2.24	1.8±0.06	5.5
19	2.9±0.61	6.9±0.66	0.7±0.03	3.5
20	5.1±1.53	5.7±1.29	1.3±0.02	4.0
21	2.9±0.11	8.1±0.27	0.3±0.003	3.8
22	0.9±0.45	1.2±0.35	0.5±0.01	0.9
23	8.7±0.64	10.3±0.12	3.1±0.03	7.4
24	1.1±0.14	1.4±0.08	0.4±0.01	1.0
25	2.9±0.84	4.2±0.79	3.1±0.08	3.4
Mean	7.0	7.9	3.3	
SE(±)	6.65	1.31	0.90	
CD (P<0.05)	13.30	2.62	0.18	

INTRODUCTION

Microalgae have been studied extensively due to their numerous applications in varied fields. Ecologically, microalgae occupy an important position at the base of aquatic food webs. Nutrient cycling and evolution of oxygen are the critical attributes which govern the equilibrium of an ecosystem, where microalgae play an essential role (Demirbas 2009). They possess a vast repertoire of high value products and can be used as biodiesel feedstock because of high rate of biomass production as compared to oil yielding plants (Chisti 2007; Groom *et al.* 2008; Stephens *et al.* 2010; Arbib *et al.* 2014). Therefore, it is desirable to identify, isolate and characterize new strains from different natural environments with high value content of bioproducts for use in commercial and industrial applications.

Morphological convergence has been reported in several commonly known groups of algae, where taxonomic units with similar morphologies are distantly placed in a phylogenetic tree (Krienitz *et al.* 2004). More than 35,000 species of microalgae are described in literature (Cheng & Ogden 2011) and their diversity is known to be far greater. The divergence of green lineage into distinct clades is established through molecular methods which confirms the structure-based hypothesis. According to this, the clade Chlorophyta comprises the green algae, of which several have been described, and the clade Streptophyta, which comprises a paraphyletic assembly of freshwater algae, has led to the evolution of land plants. A limited number of taxa have been studied for their multigene relationship data, due to which phylogenetic relationships between and within the main clades of the chlorophytes, namely Ulvophyceae, Trebouxiophyceae and Chlorophyceae, have not been completely recognized (Leliaert *et al.* 2012).

Screening and collection of microalgae is therefore vital to select optimal performing strains with desired traits. Moreover, 'indigenous' strains, which are naturally adapted to specific environments and climates, will be more competitive, efficient and sustainable. In this paper, we report an intensive study of 24 microalgal strains isolated from selected aquatic sites and one reference strain (MCC 25), for their specific biochemical parameters.

MATERIALS AND METHODS

Isolation, identification and maintenance of microalgae

Water samples were collected from selected aquatic sites of India with the details mentioned in Table 1. The green microalgal strains were isolated following single cell isolation method using glass capillary tube having straight or curved tip (Andersen & Kawachi 2005). In total 24 green microalgal strains were isolated and purified from various aquatic sites. The reference strain (*Nannochloropsis* sp., MCC 25) was kindly given by Dr. Lothar Krinitz, Germany. These were grown and maintained in BG-11 (+N) medium, pH 7.0 at 28 ± 2 °C under a photoperiod of 16:8 h light–dark cycle with light intensity of 52–55 μmole photon/m^2/s provided with cool white fluorescent tubes. Further, purification of the isolated cultures was attempted through serial dilution and plating. The cultures were hand shaken 2–3 times to avoid adherence to the sides of the flasks and the purity was routinely checked through microscopic observations. The isolated green algae were identified using keys given in standard monographs for morphological parameters (Iyengar & Desikachary 1981; Komárek & Fott 1983; Hindak 1988). The isolated

Table 1 | List of green microalgal strains and area of their isolation

S. no.	Strains	Designation	Origin	Coordinates
1	*Chlamydomonas* sp.	MCC1	Road side Ditch, Nandankanan, Odisha	20.3958° N, 85.8260° E
2	*Chlamydomonas* sp.	MCC2	Rohtang Pass	32.3716° N, 77.2466° E
3	*Scenedesmus* sp.	MCC3	Dal Lake	34.1106° N, 74.8683° E
4	*Scenedesmus* sp.	MCC4	Dal Lake	34.1106° N, 74.8683° E
5	*Chlorella* sp.	MCC5	Nandankanan Lake, Odisha	20.4009° N, 85.8198° E
6	*Chlorella* sp.	MCC6	Ansupa Lake, Odisha	20.4587° N, 85.6036° E
7	*Chlorella* sp.	MCC7	Katitirtha temple pond, Bhuwaneswar,Odisha	20.2961° N, 85.8245° E
8	*Scenedesmus* sp.	MCC8	Pond, Balasore,Odisha	21.4934° N, 86.9135° E
9	*Dictyosphaerium* sp.	MCC9	Pond, (way to Chilika Lake), Khurda, Odisha	19.7751° N, 85.4180° E
10	*Dictyosphaerium* sp.	MCC10	Pond, (way to Chilika Lake), Khurda, Odisha	19.7751° N, 85.4180° E
11	*Dictyosphaerium* sp.	MCC11	Pond, (way to Chilika Lake), Khurda, Odisha	19.7751° N, 85.4180° E
12	*Dictyosphaerium* sp.	MCC12	Nandankanan Lake, Odisha	20.4009° N, 85.8198° E
13	*Dictyosphaerium* sp.	MCC13	Nandankanan Lake, Odisha	20.4009° N, 85.8198° E
14	*Dictyosphaerium* sp.	MCC14	Nandankanan Lake, Odisha	20.4009° N, 85.8198° E
15	*Dictyosphaerium* sp.	MCC15	Nandankanan Lake, Odisha	20.4009° N, 85.8198° E
16	*Chlorella* sp.	MCC16	Pond, Kalijai, Chilika, Odisha	19.6651° N, 85.2167° E
17	*Scenedesmus* sp.	MCC17	Nandankanan Lake, Odisha	20.4009° N, 85.8198° E
18	*Scenedesmus* sp.	MCC18	Pond, Nariyawal, Bareilly U.P.	28.3176° N, 79.4771° E
19	*Kirchneriella* sp.	MCC19	Ansupa Lake, Odisha	20.4587° N, 85.6036° E
20	*Chlorella* sp.	MCC20	Pond, Balasore, Odisha	21.4934° N, 86.9135° E
21	*Dictyosphaerium* sp.	MCC21	Ansupa Lake, Odisha	20.4587° N, 85.6036° E
22	*Dunaliella* sp.	MCC22	Sambhar Lake, Rajasthan	26.9261° N, 75.0962° E
23	*Scenedesmus* sp.	MCC23	Barapani lake, Shilong	25.6654° N, 91.8928° E
24	*Chlorella* sp.	MCC24	Sambhar Lake, Rajasthan	26.9261° N, 75.0962° E
25	*Nannochloropsis* sp.	MCC25	Courtesy: Dr. Lothar Krinitz, IGB-Stechlin, Germany	

and identified strains were maintained in N-enriched BG-11 medium under standard cultural conditions (Andersen & Kawachi 2005).

Estimation of lipids, proteins and carbohydrates

Microalgal biomass was harvested on the 14th, 21st and 28th days of incubation for estimating selected biochemical parameters. Growth of the representative strains was observed based on chlorophyll content with respect to the days of incubation. The analysis of the biochemical parameters was chosen during the late exponential phase until the onset of the stationary phase of growth. A known volume of homogenized suspension was used for dry weight measurements, by filtering through preweighed filter paper (Whatman, Poole, UK) and drying at 80 °C until a constant weight was achieved. Proteins were measured by the method of Lowry *et al.* (1951) using Bovine Serum Albumin as standard and a known volume of suspension was used for estimation of total carbohydrates by the anthrone method using glucose as standard (Yemm & Willis 1954). For extraction of lipids from a known amount of (0.5–1.0 g) dried biomass, aqueous solution of biomass was subjected to pretreatment with microwave (2,450 MHz for 6 min and a high temperature of 100 °C). This was followed by lipid extraction as per standard protocol (Bligh & Dyer 1959). The cell lysate was mixed with 200 mL chloroform/methanol (2:1 v/v) by vigorous shaking for 1 h. The organic phase (extract) was separated and the lower phase containing lipids was collected. Additional chloroform was added for complete extraction of lipids which was expressed on % dry weight basis as per the equation:

$$\frac{\text{Weight of lipid extracted [mg]}}{\text{sample weight [mg]}} \times 100$$

Polymerase chain reaction (PCR) amplification of 18S rRNA gene and gel electrophoresis

Out of 24 microalgal strains used for biochemical parameters, five selected strains, namely MCC 3, MCC 9, MCC 10, MCC 12, and MCC 19 were used for 18S rRNA gene sequencing. These five genera were selected for molecular identification based upon their area of isolation. These also showed desired content of total soluble proteins, carbohydrates and lipids. Homogenised suspension from the exponential phase of incubation (14th day) was disrupted by grinding in a sterile mortar and pestle, and DNA was extracted using the DNeasy Plant Mini Kit (Promega) according to the manufacturer's instructions. Isolated DNA was verified by running in 0.8% agarose gel electrophoresis and quantified by Nanodrop (Thermofisher) and stored at −20 °C. The extracted DNA was used for PCR amplification of 18S rRNA gene with universal primers (Duff *et al.* 2008; Forward Primer 5′- CGAATTCAACCTGGTTGATCCTGCCAGT-3′ and Reverse Primer 5′- CCGGATCCTGATCCTTCTG CAGGTTCACCTAC-3′). The reaction was performed in a total volume of 25 μL having 1X TAE buffer containing 10X Taq buffer, 2 mM of dNTPs (dATP, dCTP, dGTP, dTTP), 18S primers (FP and RP 5 pmole each) and 1 U of Taq polymerase (Fermentas, USA). Amplification was achieved in a Mastercycler gradient program for initial denaturation (94 °C for 5 min) followed by 35 cycles composed of denaturation (94 °C for 30 s), annealing (61 °C for 45 s) and extension (72 °C for 2 min) and final extension of 72 °C for 10 min. The amplified PCR products were kept on hold at 4 °C for 24 h and gel electrophoresis of the PCR product was carried out (Ratha *et al.* 2012). 10 μL of the amplified PCR product was loaded on horizontal 1.2% (w/v) agarose (Pronadisa, Europe) gel in 1X TAE buffer (0.5 M EDTA, 1 M Tris acetate, pH 8.0 and electrophoresed at 75 volts for about 1 h. The standard marker of 1 kb molecular size (Fermentas) was also used as a reference along with the amplified products. Gels were stained with ethidium bromide solution (0.5 μg/mL). The amplified products were visualized and documented on AlphaImager gel documentation system (Alpha Innotech, USA).

18S rRNA gene sequence alignment and phylogenetic analysis

PCR amplified products of 18S rRNA gene was subjected to sequence analysis using Applied Biosystems ABI prism automated DNA sequencer. The partial 18S rRNA gene sequences were analysed and homologous sequences were searched using BLAST available in NCBI database (Altschul *et al.* 1990). Identification of isolates was done on the basis of gene sequence similarity of ≥97% with the sequences in Genbank. Sequence alignment and comparison was performed using multiple sequence alignment tool CLUSTAL W (Thompson *et al.* 1994) with default parameters. The sequence results of 18S rRNA gene of the five strains have been submitted to NCBI GenBank with Accession Numbers as KT808247-KT808251.

The phylogenetic tree was constructed using neighbor-joining method (NJ) as executed in the program MEGA

package version 6.1 (Saitou & Nei 1987; Tamura *et al.* 2011). The NJ stability of the relationships was assessed by bootstrapping (1,000 replicates) (Felsenstein 1985). The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1,000 replicates) are shown above the branches. The tree was drawn to scale, with branch lengths in correlation with the evolutionary distances used to infer the phylogenetic tree. All positions containing gaps and missing data were eliminated from the dataset.

RESULTS AND DISCUSSION

Isolation and identification

Ecological habitats ranging from streams, ponds, Eastern ghats over Odisha along with coastal areas of West Bengal and North Eastern regions have been reported to be the most vibrant biodiversity sites, hence, the selected aquatic sites were used for the isolation of microalgae for their possible economic application (Jena *et al.* 2005, 2008; Prasanna & Kaushik 2005; Rath & Adhikary 2005; Ratha *et al.* 2012). The possibility of obtaining promising isolates from aquatic, terrestrial and extreme environments has been reported in earlier studies as well (Spolaore *et al.* 2006; Ratha & Prasanna 2012).

Green microalgal strains were microscopically identified based on microscopic observations and morphological features and these have been submitted in the germplasm collection of CCUBGA, ICAR-IARI (Figure 1, Table 1). 24 strains along with one reference strain were studied for their specific biochemical parameters namely total soluble proteins, carbohydrates and lipids as these constitute a major proportion of the cellular contents (Um & Kim 2009). Total soluble proteins enhanced with incubation time

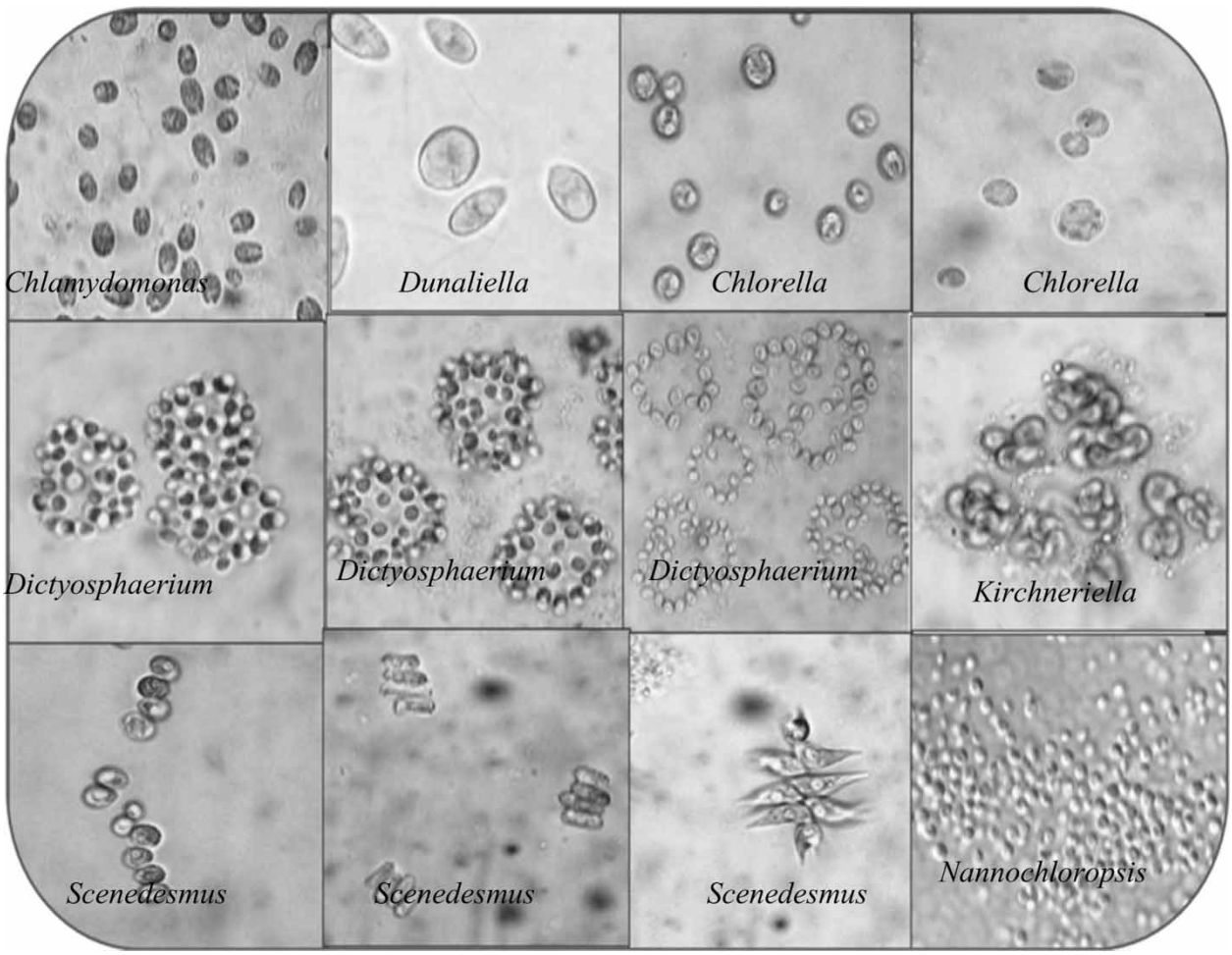

Figure 1 | Photomicrographs of representative genera of green microalgal strains.

with highest recorded at 28th day of incubation. Total soluble proteins were maximum (419.4 μg/mg dry wt.) in *Chlamydomonas* sp. (MCC 2) isolated from Rohtang pass (Table 2). Studies conducted in the isolated green microalgal strains indicated that carbohydrates enhanced from the 14th day until the 21st day of incubation followed by a decline at the 28th day. Mean carbohydrates calibrated during the incubation period indicated that *Scenedesmus* sp. (MCC 9) isolated from Pond, Khurda, exhibited highest carbohydrates (267.85 μg/mg dry wt.) and *Chlorella* sp. (MCC 19) from Anusupa Lake showed the lowest carbohydrates (61.92 μg/mg dry wt.) (Table 3). Lipids were highest on the 21st day of incubation as compared to the 14th and the 28th day of incubation and *Chlorella* sp. (MCC 5) from Nandankanan Lake, Odisha showed the highest value of lipids (10.8%) on a dry wt. basis (Table 4). Such analyses on biochemical parameters have been carried out in different strains of microalgae under selected growing conditions (Becker 2004; Spolaore *et al.* 2006; Rodriguez-Garcia & Guil-Guerrero 2008). Studies have also been conducted showing the applicability of biomass and extracts of microalgae for human consumption (Colla *et al.* 2007; Gouveia *et al.* 2007; Valencia *et al.* 2007). Varied potentials of microalgae have been documented extensively in a review by Shukla & Wattal (2013). The maximum protein content of 41.9% in MCC 2 followed by 40.3% in MCC 5 and 36.9% in MCC 12 showed the

Table 2 | Comparative total soluble proteins (μg/mg dry wt) amongst green microalgal strains

Strains	14 days	21 days	28 days	Mean
1	334.6 ± 12.83	376.3 ± 18.02	255.2 ± 4.34	322.0 ± 6.25
2	379.4 ± 24.36	435.5 ± 7.05	443.2 ± 51.16	419.4 ± 6.05
3	28.6 ± 3.77	117.3 ± 3.11	360.1 ± 23.30	168.7 ± 5.96
4	124.5 ± 3.11	193.9 ± 4.56	165.5 ± 7.15	161.3 ± 9.37
5	498.3 ± 13.40	190.3 ± 8.11	520.7 ± 13.20	403.1 ± 15.14
6	223.3 ± 13.27	251.6 ± 8.70	496.4 ± 7.44	323.8 ± 38.39
7	300.1 ± 23.53	205.1 ± 13.17	397.5 ± 4.97	300.9 ± 11.42
8	171.3 ± 1.27	258.7 ± 14.87	139.9 ± 85.79	189.9 ± 10.07
9	281.8 ± 6.58	213.2 ± 12.12	521.9 ± 3.48	338.9 ± 29.36
10	119.5 ± 2.40	394.9 ± 6.71	312.9 ± 15.86	275.7 ± 36.28
11	235.7 ± 15.22	464.0 ± 4.08	419.8 ± 18.20	373.2 ± 18.10
12	228.5 ± 15.19	484.2 ± 3.89	395.5 ± 6.67	369.4 ± 14.33
13	248.2 ± 12.76	472.0 ± 11.33	310.5 ± 14.68	343.6 ± 14.48
14	412.1 ± 13.22	135.8 ± 7.08	122.9 ± 9.51	223.6 ± 6.63
15	185.6 ± 9.28	276.7 ± 6.98	213.0 ± 3.49	225.1 ± 6.44
16	283.6 ± 9.41	418.5 ± 32.64	352.7 ± 3.86	351.6 ± 26.18
17	98.7 ± 4.93	324.4 ± 4.76	385.2 ± 3.20	269.5 ± 20.32
18	191.3 ± 5.80	314.1 ± 15.92	228.6 ± 15.45	244.7 ± 30.82
19	101.5 ± 8.29	309.6 ± 7.98	234.7 ± 4.50	215.3 ± 11.29
20	361.7 ± 16.67	401.8 ± 1.86	313.7 ± 10.13	359.1 ± 7.01
21	232.6 ± 12.8	465.6 ± 10.2	239.9 ± 1.63	312.7 ± 6.32
22	147.7 ± 5.05	422.1 ± 20.58	263.9 ± 3.12	277.9 ± 7.97
23	281.0 ± 2.19	394.6 ± 29.91	340.6 ± 13.14	338.7 ± 40.95
24	278.6 ± 4.51	476.5 ± 6.42	256.2 ± 9.55	337.1 ± 36.86
25	198.5 ± 9.86	297.1 ± 6.41	145.2 ± 8.04	213.6 ± 16.87
Mean	100.4 ± 9.8	116.9 ± 26.5	238.7 ± 15.7	
SE (±)	2.78	1.35	2.45	2.31
CD ($p < 0.5$)	0.2	0.8	1.1	0.9

SE, Standard error of the mean; CD, Critical difference of the mean and $p < 0.05$.

Table 3 | Comparative carbohydrates (μg/g dry wt) amongst green microalgal strains

Strains	14 days	21 days	28 days	Mean
1	73.3 ± 8.19	25.6 ± 6.44	223.8 ± 4.11	107.6 ± 6.25
2	60.9 ± 7.35	36.4 ± 5.73	192.6 ± 5.07	96.7 ± 6.05
3	91.8 ± 7.07	23.4 ± 7.76	185.1 ± 3.06	100.1 ± 5.96
4	119.3 ± 14.43	126.5 ± 6.34	35.0 ± 7.35	93.60 ± 9.37
5	262.6 ± 9.33	22.7 ± 10.89	481.0 ± 25.22	255.44 ± 15.15
6	106.0 ± 0.66	114.7 ± 86.26	268.5 ± 28.25	163.05 ± 38.39
7	119.3 ± 13.57	19.4 ± 17.90	366.8 ± 2.80	168.51 ± 11.42
8	153.9 ± 7.73	179.4 ± 5.57	396.9 ± 16.90	243.38 ± 10.07
9	86.3 ± 12.20	52.7 ± 22.43	664.5 ± 53.46	267.85 ± 29.36
10	35.9 ± 16.80	122.0 ± 73.75	278.0 ± 18.29	145.30 ± 36.28
11	161.7 ± 10.46	114.7 ± 24.53	372.8 ± 19.31	216.40 ± 18.10
12	20.6 ± 1.86	164.5 ± 22.49	337.2 ± 18.65	174.10 ± 14.33
13	25.3 ± 8.55	34.23 ± 8.28	155.8 ± 26.62	71.81 ± 14.48
14	134.9 ± 10.24	153.4 ± 5.32	350.9 ± 4.34	213.07 ± 6.63
15	121.7 ± 6.17	245.9 ± 5.34	135.0 ± 7.82	167.54 ± 6.44
16	136.0 ± 4.87	215.1 ± 39.16	263.0 ± 34.52	204.72 ± 26.18
17	156.7 ± 26.35	25.1 ± 18.83	103.3 ± 15.78	95.03 ± 20.32
18	26.7 ± 4.57	45.0 ± 77.95	200.1 ± 9.94	90.62 ± 30.82
19	23.5 ± 14.36	54.2 ± 14.89	108.0 ± 4.62	61.92 ± 11.29
20	27.9 ± 4.59	319.5 ± 1.40	116.7 ± 15.04	154.68 ± 7.01
21	154.7 ± 7.33	124.9 ± 7.39	213.3 ± 4.23	164.28 ± 6.32
22	45.6 ± 9.037	168.4 ± 6.44	40.9 ± 8.44	85.01 ± 7.97
23	89.6 ± 11.13	52.4 ± 108.84	307.9 ± 2.89	149.95 ± 40.95
24	127.8 ± 20.50	247.2 ± 54.79	37.1 ± 35.29	137.35 ± 36.86
25	147.5 ± 6.98	234.9 ± 23.98	132.6 ± 19.65	171.69 ± 16.87
Mean	237.9 ± 9.99	331.8 ± 10.66	313.4 ± 13.67	
SE (±)	5.35	3.71	4.62	4.76
CD ($p < 0.5$)	2.0	0.4	1.2	3.0

SE, Standard error of the mean; CD, Critical difference of the mean and $p < 0.05$.

relevance of these as a protein source. The average protein quality of most of the algae examined is equal, sometimes even superior, to that of conventional plant proteins (Becker 2007).

The comparative study on carbohydrates (Table 3) showed the highest content of 26.7% in *Scenedesmus* sp. (MCC 9) followed by 25.5% in *Chlorella* sp. (MCC 5) and 24.3% in *Scenedesmus* sp. (MCC 8). The lowest carbohydrate content of 6.1% was recorded in *Chlorella* sp. (MCC 19). Studies conducted on different microalgal biomass reported proteins in the range of 6–71% and carbohydrates as 4–64%, whereas, lipids ranged from 1 to 70% (Chisti 2007; Becker 2013). *Chlorella pyrenoidosa* also depicted carbohydrate content of 26% whereas *Chlorella vulgaris* showed carbohydrate content of 12–17% on a dry wt. basis (Um & Kim 2009). However, the *Chlamydomonas reinhardtii* reported protein content of 48% on a dry matter basis (Um & Kim 2009; Sydney *et al.* 2010). The protein content of 8–18%, 50–56% and 47% has been reported in different species of *Scenedesmus*, namely *S. dimorphus; S. obliquus* and *S. quadricauda* (Um & Kim 2009).

Strain MCC 5 (*Chlorella* sp.) showed maximum lipid content of 10.8% on a dry wt. basis which was *at par* with the lipid content of 10–22% in *Chlorella vulgaris*. The lowest lipid content of 3.5% recorded in MCC 19 (*Chlorella* sp.) was higher than the reported content of 2% from *Chlorella vulgaris* (Um & Kim 2009; Sydney *et al.* 2010). The compositional analysis of *Chlorella* sp., *Scenedesmus*

Table 4 | Comparative lipids (% dry weight) amongst green microalgal strains

Strains	14 Days	21 Days	30 Days	Mean
1	6.2 ± 0.06	9.4 ± 0.95	3.3 ± 0.11	6.3
2	6.8 ± 0.07	12.0 ± 0.89	3.0 ± 0.05	7.3
3	13.7 ± 0.31	5.7 ± 0.35	5.5 ± 0.02	8.3
4	5.2 ± 0.44	6.9 ± 2.17	2.1 ± 0.18	4.7
5	12.6 ± 0.07	13.5 ± 0.92	6.2 ± 0.88	10.8
6	6.4 ± 0.09	10.7 ± 1.22	3.1 ± 0.03	6.7
7	8.0 ± 0.19	7.8 ± 0.05	14.8 ± 0.10	10.2
8	7.4 ± 0.29	9.1 ± 0.19	3.7 ± 0.06	6.7
9	4.8 ± 0.04	9.5 ± 0.10	4.8 ± 0.07	6.4
10	6.6 ± 0.17	11.0 ± 1.50	2.1 ± 0.02	8.0
11	11.5 ± 1.37	10.8 ± 1.18	3.9 ± 0.05	8.7
12	10.5 ± 0.48	11.0 ± 1.20	6.3 ± 0.10	9.3
13	9.7 ± 0.07	8.0 ± 0.71	1.8 ± 0.13	6.5
14	11.2 ± 1.15	10.3 ± 4.61	2.9 ± 0.07	8.1
15	1.2 ± 2.46	1.9 ± 0.92	3.2 ± 0.12	2.1
16	4.8 ± 0.46	9.8 ± 1.23	4.2 ± 0.004	6.3
17	11.3 ± 1.42	9.8 ± 0.39	1.2 ± 0.01	7.4
18	12.4 ± 0.70	2.4 ± 2.24	1.8 ± 0.06	5.5
19	2.9 ± 0.61	6.9 ± 0.66	0.7 ± 0.03	3.5
20	5.1 ± 1.53	5.7 ± 1.29	1.3 ± 0.02	4.0
21	2.9 ± 0.11	8.1 ± 0.27	0.3 ± 0.003	3.8
22	0.9 ± 0.45	1.2 ± 0.35	0.5 ± 0.01	0.9
23	8.7 ± 0.64	10.3 ± 0.12	3.1 ± 0.03	7.4
24	1.1 ± 0.14	1.4 ± 0.08	0.4 ± 0.01	1.0
25	2.9 ± 0.84	4.2 ± 0.79	3.1 ± 0.08	3.4
Mean	7.0	7.9	3.3	
SE(±)	6.65	1.31	0.90	2.95
CD ($p < 0.05$)	13.30	2.62	0.18	5.3

SE, Standard error of the mean; CD, Critical difference of the mean and $p < 0.05$.

sp., and *Nannochloropsis* have reported varied proteins (7.4–42.5%), carbohydrates (9.5–52.3%), and lipids (6.8–53.0%) on a dry wt. basis (Laurens & Wolfrum 2013).

The results have clearly indicated that microalgae have a potential in nutritional value of food and feed and can be effectively used in the aquaculture as well as fish feeding. Out of 24 microalgal strains isolated from selected aquatic sites, five strains were subjected to axenization using triple antibiotic solution. Based on the best performing strains, five microalgal genera were used for 18S rRNA gene amplification and sequencing. The microscopy-based identification was supported with BLAST analysis of 18S ribosomal DNA using specific primers (Duff *et al.* 2008) for five isolates (MCC 3, MCC 9, MCC 10, MCC 12, MCC 19). Based upon results, identification was authenticated as *Scenedesmus* sp. MCC 3; *Scenedesmus* sp. MCC 9; *Dictyosphaerium* sp. MCC 10; *Dictyosphaerium* sp. MCC 12 and *Chlorella* sp. MCC 19. These were given the accession numbers as KT 808247-KT808248 (*Scenedesmus* sp.), KT 808249-KT808250 (*Dictyosphaerium* sp.), KT 808251 (*Chlorella* sp.) (NCBI database). Identification of *Scenedesmus* sp. was in accordance with the morphological characters. *Scenedesmus* strains are known to form colonies of 2–8 cells called coenobia (Hegewald *et al.* 2013). This morphological feature was authenticated through 18S rRNA sequences which showed a similarity of >95% with other *Scenedesmus* sp. deposited in Genbank. The cells in *Scenedesmus* can be linearly or laterally arranged surrounded by mucilage. Cells are elongate and cylindrical or ovoid or rounded and the walls can be smooth, toothed or granular.

18S rRNA gene sequence analysis identified MCC 10 and MCC 12 as *Dictyosphaerium* sp. The morphophyte of *Dictyosphaerium* is characterised by spherical or oval cells connected by gelatinised strands to microscopic colonies and according to molecular studies *Dictyosphaerium* belongs to Chlorellaceae (Krienitz *et al.* 2004). Two strains of *Dictyosphaerium* sp. belonging to different clusters were the isolates of Khurda lake and Nandankanan lake. The molecular phylogenetic studies of *Dictyosphaerium* morphophytes indicates close relationship to the members of *Chlorella* and polyphyletic origin of *Dictyosphaerium* morphophytes has been reported (Luo *et al.* 2010; Krienitz & Bock 2012).

The strain MCC 19 was identified microscopically as *Chlorella* sp. *Chlorella* comprises unicellular green algae belonging to the family Chlorellaceae and the cells are solitary, spherical or globular in shape without flagella having cup-shaped chloroplast with a single pyrenoid surrounded within a thin wall (Tale *et al.* 2014). The identification of *Chlorella* is one of the most difficult and a large number of species have been isolated from different ecosystems (Krienitz *et al.* 2004). Based on sequence homology with >95% similarity, the strain MCC 19 was identified as *Chlorella* sp. (KT808251).

The evolutionary history was inferred using neighbor-joining method (Saitou & Nei 1987). The tree is drawn to scale (Figure 2), with branch lengths (0.53579240) which can be used to infer the evolutionary distances in the phylogenetic tree. The evolutionary distances were computed using the Maximum Composite Likelihood method (Tamura *et al.* 2004) and are in the units of the number of

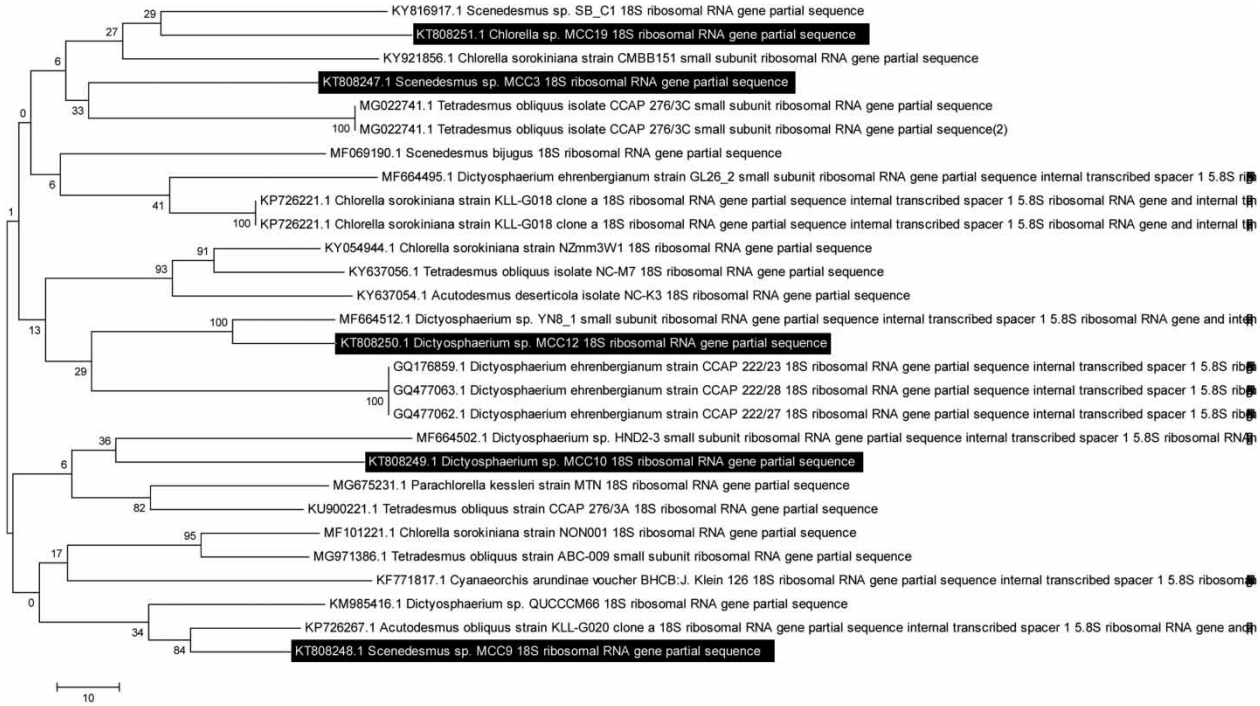

Figure 2 | Phylogenetic tree of selected five green microalgae generated using 18 S rRNA gene sequences and available NCBI sequences, constructed by neighbor-joining method. Highlighted genera indicate sequenced green microalgal strains from this study.

base substitutions per site. The analysis involved six nucleotide sequences. Codon positions included were 1st + 2nd + 3rd + Noncoding. All ambiguous positions were removed for each sequence pair. There was a total of 973 positions in the final dataset. Evolutionary analyses were conducted in MEGA5 (Tamura *et al.* 2011).

Statistical analysis

One-way analysis of variance (ANOVA) was used to evaluate the differences among different strains under study. The statistical analysis using one-way ANOVA indicated an average of total soluble proteins, carbohydrates and lipids with the degrees of freedom between groups and within groups (Table 5). Linear regression curve plotted amongst carbohydrates vs lipids and proteins vs lipids of 25 microalgal strains is depicted in Figure 3. Fitted curves can be used as an aid for data visualization, which can infer values of a function where no data are available, and such information can also help to summarize the relationships among different variables. Curve estimation is shown to be appropriate when the relationship between the dependent variable(s) and the independent variable is not necessarily linear. Based on the regression analyses, it could be inferred that the carbohydrates, proteins and lipids exhibited random distribution

Table 5 | Single factor ANOVA for significance of regression

Groups	Count	Sum	Average	Variance		
Proteins	25	7,358.8	294.35	5,319.66		
Lipids	25	153.3	6.13	7.05477		
Carbohydrates	25	3,799.7	151.99	3,426.70		
ANOVA						
Source of variation	**SS**	**df**	**MS**	**F**	***P*-value**	**F crit**
Between groups	1,038,435.41	2	519,217.70	177.948	1.37E − 28	3.123
Within groups	210,082.06	72	2,917.80			
Total	1,248,517.47	74				

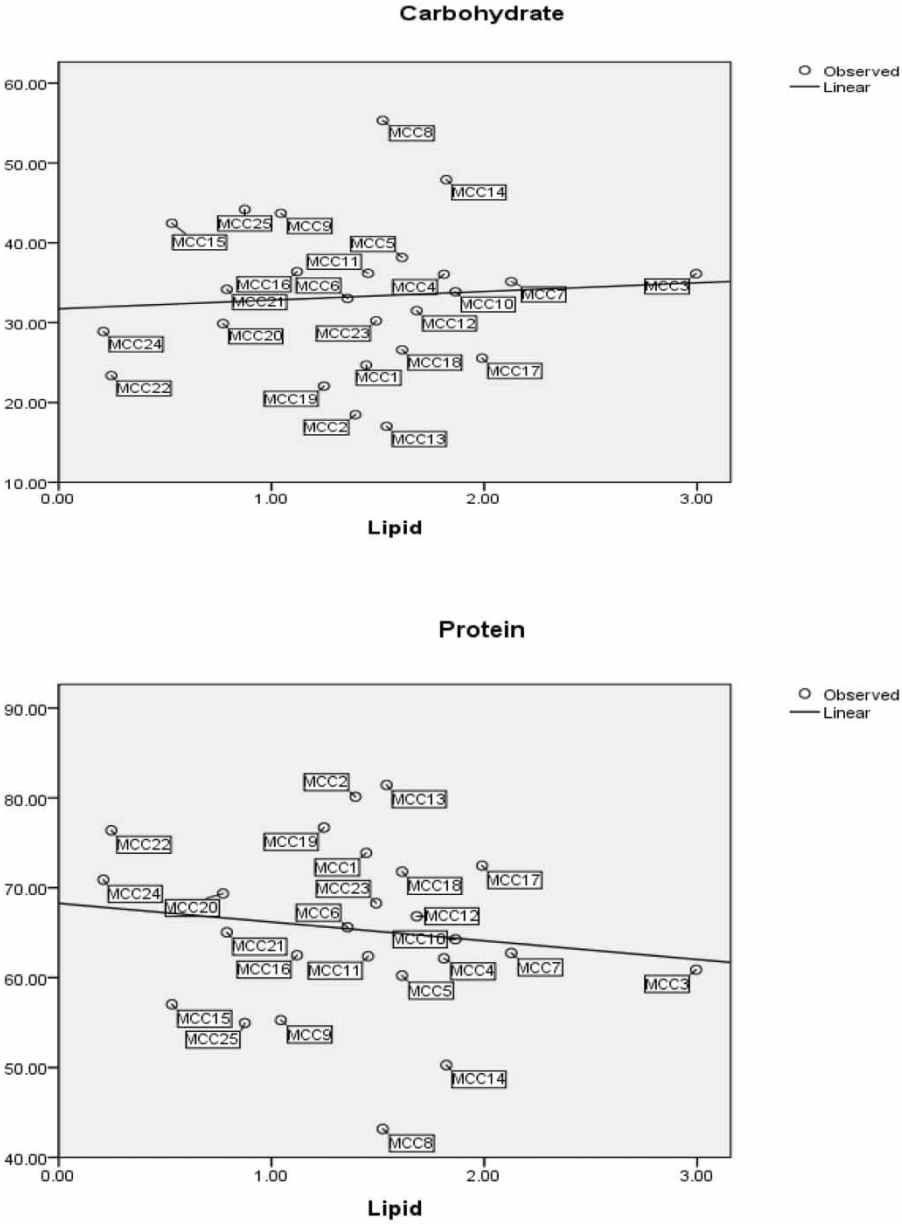

Figure 3 | Linear regression curve of total soluble proteins, carbohydrates and lipids of 25 microalgal strains.

amongst green microalgal strains at different stages of growth. The correlation matrix showed positive correlation between carbohydrates and lipids, while negative correlation was seen between proteins and carbohydrates and between proteins and lipids.

CONCLUSIONS

Aquatic sites of Odisha represent a microalgal biodiversity hotspot as a large number of green algae were isolated from such habitats. *Dictyosphaerium* sp., *Scenedesmus* sp. *and Chlorella* sp. were the most dominant and representative genera. Some of them recorded a high concentration of proteins and carbohydrates; hence, they exhibit important and valuable germplasm for further utilization in the area of food or feed.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

ACKNOWLEDGEMENT

The authors are grateful to the Director, ICAR-IARI, New Delhi for the necessary facilities and the Department of Biotechnology for financial support under Indo-Denmark Collaborative Project, GOI, India.

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First received 25 January 2020; accepted in revised form 14 April 2020. Available online 24 April 2020