Indian Journal of Experimental Biology
Vol. 57, September 2019, pp. 700-707

Isolation and biochemical characterization of microalgae from waterlogged areas of Southwest Punjab

Dalveer Kaur[1], Monica Sachdeva Taggar[2]*, Anu Kalia[3] & Iqbal Singh[2]

[1]Department of Biochemistry; [2] Department of Renewable Energy Engineering; [3]Department of Soil Science, Punjab Agricultural University, Ludhiana-141 004, Punjab, India

Received 05 December 2016; *revised 18 November 2018*

Microalgae are considered as a potential feedstock for producing sustainable biofuel. The high growth rate and ability to rapidly improve the strains for high lipid and carbohydrate content without competing for arable land, makes microalgae a viable option for sustainable fuel production. Five microalgae designated as MA-1, MA-2, MA-3, MA-4 and MA-5 were isolated from the waterlogged areas of villages Theri, Ghagga and Gurusar of district Muktsar, Punjab. On the basis of optical and scanning electron microscopy, these microalgal isolates were found to be round in shape, green coloured and unicellular in structure and were tentatively identified as *Chlorella* sp. *Chlorella* sp. MA-1 was found to be fast growing with maximum absorbance (A_{600nm}), viable cell count and chlorophyll content of 3.26, 369.4×10^5 cfu/mL and 24.31 mg/g dry wt., respectively at 5 days after culturing (DAC) However, significantly high total soluble protein content of 11.42% was found in this isolate at 5 days after culturing. *Chlorella* sp. MA-3 showed significantly high total carbohydrate content of 5.10 percent at 5 days after culturing (DAC), whereas the total lipid content was found to be significantly high in *Chlorella* sp. MA- 2 (4.16%) at 7 DAC. Alteration in the environmental and culturing conditions can further improve the chemical constituents of these microalgal isolates to improve biofuel production.

Keywords: Biofuel, *Chlorella*, Total carbohydrates, Total lipids, Total soluble protein, Total chlorophyll

Rapid growth of human population and technological advancements have led to increased energy demands, which in future is projected to increase by 50% or more[1]. In the light of depleting fossil-fuel reserves, dwindling resources, geopolitical conflict and climate change with increased atmospheric CO_2 concentration, clean and green energy has become one of most overwhelming challenge in the overall world[2,3]. Biofuels can be produced from starch, vegetable oils, animal fats, waste biomass or algal biomasses, which are nontoxic, biodegradable and renewable[4]. Unfortunately, the present biofuel projections are mostly based on feedstocks that are also food commodities as well as resources suitable for conventional agriculture[5]. The cultivation of edible crops for first generation biofuels can have detrimental effects on food security, induce deforestation and show poor energy balances[6]. They also require fresh water, fertilizers and a vast area of arable land area for their cultivation. This can even result in a further increase of net carbon emissions[7]. The substitution of diesel by biodiesel requires the utilization of lands meant to produce food and the fiscal incentives by governments are thus decreasing the availability of these lands for food production[8].

Microalgal biofuels are promising to replace fossil fuels in the light of microalga's intrinsic efficiency to convert solar energy into chemical energy[9]. Depending upon the species and cultivation conditions, they can produce useful quantities of polysaccharides (sugars) and triacylglycerides (fats) which are the raw materials for producing bioethanol and biodiesel transport fuels. Microalgae also produce proteins that can be used as a source of animal feed and commercially valuable compounds such as pigments and pharmaceuticals[10]. Their metabolic and ecological diversity allows selection of taxa that are adapted for growth in locally available aquifers[1]. In the southwestern regions of Punjab, about 70% of the area is under severe waterlogging. The water bodies of these regions have been identified as rich pool of many halophytic plants that can be exploited for biofuel production. These water bodies due to their high salt content cannot be utilised for conventional agriculture practices[11].

Hence, there is a need to isolate different microalgae from the waterlogged area of Southwest

*Correspondence:
E-mail: monicasachdeva@pau.edu

Punjab and biochemically characterize these species in terms of carbohydrate, lipid, chlorophyll and protein content to assess their potential for biofuel production. In the present study, we have made an attempt for isolation and biochemical characterization of different microalgal species from the waterlogged areas of different villages located in Southwest Punjab to find their potential application in biofuel production.

Materials and Methods

Culture collection

The algal cultures were collected from waterlogged areas of villages Theri (N 30° 12.435′, E 0.74° 34.915), Ghagga (N 30° 14.320′, E 0.74°34.814′) and Gurusar (N 30° 14.108′, E 0.74° 34.821′) in district Muktsar (Punjab). The algal cultures were collected in sterilized wide mouth bottles. The cultures were brought to the laboratory for isolation, purification and identification of microalgae. The standard culture *Chlorella sorokiniana* NCIM 5561 was procured from National Collection of Industrial Microorganisms (NCIM), CSIR-National Chemical Laboratory (NCL), Pune, Maharashtra, India.

Media composition

The basal culture media by Wu *et al.*[12] with some modification was used for enrichment and growth of microalgal cultures. The composition of culture medium was as follows : KH_2PO_4 0.50 g L^{-1}, K_2HPO_4 0.20 g L^{-1}, $MgSO_4.7H_2O$ 0.20 g L^{-1}, $FeSO_4.7H_2O$ 3 mg L^{-1}, Glycine 5.00 g L^{-1}, Vitamin B1 0.05 mg L^{-1}, and A5 trace mineral solution 1 mL L^{-1}. The nutrient media was prepared in double distilled water and pH was maintained in the range of 6-7. The culture media was sterilized by autoclaving at 121°C, 15 psi for 15 min in 250 mL flask containing 125 mL of culture medium. The solid culture media was prepared by adding 2% (w/v) bacteriological agar to the liquid broth.

Isolation and purification of microalgae

The isolation of microalgae was performed from enrichment culture developed by adding a small portion of sample in liquid broth[13]. The culture was incubated in an orbital shaking incubator at 100 rpm under light (6000 lux) at 28±2°C for 7 days. Isolation was done by serial dilutions of the enrichment culture and further spread plating on solidified growth medium. The plates were again incubated in an orbital shaking incubator at 28±2°C under 6000 lux irradiance for 7 days in algal culture room and observed for appearance of microalgal colonies on the surface of media. Single, well distinct algal colonies were picked by sterilized loop and transferred to freshly prepare liquid culture medium. After 7-8 days of confluent growth, a loopful of culture was streak plated on solid algal nutrient medium and incubated as described earlier. Distinct healthy algal colonies were again picked up and inoculated into fresh liquid medium contained in 250 mL flasks. The procedure was repeated till pure culture was established. Isolated and purified microalgae were propagated and maintained on agar slants inside the incubator.

Morphological characterization of microalgal isolates

Morphological characterization of different microalgal isolates was carried out by optical research microscopy using both bright field (primary staining) and phase contrast condenser (unstained condenser). A drop of algal suspension was spotted on the glass slide, covered with cover slip and observed under microscope with bright field and phase contrast condenser (40X). The scanning electron microscopy (SEM) (S-3400 N, Hitachi Science Limited, Japan) of microalgal isolates and standard culture *C. sorokiniana* was also performed as per standard protocol of Bozzola & Russell[14]. The microalgal cells were pelleted by centrifugation at 10000 rpm for 10 min. The primary fixation of cultures was carried out by overnight incubation in 2.5% glutaraldehyde solution. The cultures were later washed thrice with 0.1 M sodium cacodylate buffer. The cultures were, then, post fixed with 1 per cent osmium tetraoxide for 2 h at 4°C and washed thrice with the same buffer. The fixed cultures were gradually dehydrated using graded alcohol series (30-100%). The dehydrated cultures were mounted with double-sided sticky carbon conductive tape on circular specimen holder. The stubbed cultures were sputter coated in gold ion sputter coater prior to imaging under SEM at 500 and 2000X magnifications.

Microalgal growth measurement

The growth of microalgal isolates was monitored by measuring absorbance as well as colony forming units (CFU) of respective cultures. Appropriate amount of exponentially growing algal culture was inoculated in 125 mL of sterilized liquid medium so as to obtain initial absorbance of 0.1 and incubated at 28±2°C under 6000 lux irradiance in an orbital shaking incubator. Each isolate was cultivated for

7 days. At the end of each day, aliquots were withdrawn for 7 days and absorbance of culture was measured at 600 nm using visible spectrophotometer. The number of viable cells was determined using serial dilution spread plate count method[15]. Serially diluted microalgal inoculum was spread over solidified agar plate with the help of sterilized glass spreader. The plates were, then, incubated for 5 days at 28±2°C under 6000 lux irradiance in an orbital shaking incubator. The number of colonies were counted and expressed as CFU/mL using following formula[16]:

$$\text{CFU /mL} = \frac{\text{Number of colonies} \times \text{Dilution factor}}{\text{Size of inoculum}}$$

Biochemical composition of microalgal isolates

The biochemical composition of microalgal isolates, *viz*. total carbohydrates, lipids, total soluble protein and total chlorophyll content was determined at different days after culturing (DAC), i.e 5, 7 and 9 DAC.

Total carbohydrates

The total carbohydrates were extracted from dried algal biomass by the method of Margarites & Costa[17]. For this, 25 mL of HCl (1.5 N) was added to the known amount of dried algal biomass and resulting algal suspension was autoclaved at 121°C, 15 psi for 15 min. The mixture was then cooled to room temperature (28±2°C), neutralized with 40% NaOH solution and total volume was made to 50 mL with distilled water. After this, 1 mL each of 15% potassium ferrocyanide and 30% zinc acetate were added and the resulting solution was allowed to stand for 30 min. Further, 0.2 mL of sodium tungstate (12%) was added to the solution. The mixture was subsequently filtered through Whatmann no. 1 filter paper and the total sugars were then estimated from the filtrate by the method of Dubois *et al*.[18].

Lipids

The estimation of lipids was carried out by the method of Mishra *et al.*[19]. The fresh microalgal biomass was collected from the liquid culture medium by centrifugation at 10000 rpm for 10 min. To the known amount of dried algal biomass, 100 µL of distilled water and 2 mL of concentrated sulphuric acid was added. The reaction mixture was heated for 10 min at 100°C. The tubes were cooled in ice bath for 5 min. Five mL of freshly prepared phospho-vanillin reagent (0.6 g vanillin dissolved in 10 mL ethanol and diluted to 100 mL with deionized water followed by addition of 400 mL of concentrated phosphoric acid) was added to the mixture and incubated at 37°C in water bath for 15 min. The absorbance of pink colour, thus developed, was read at 530 nm. The amount of total lipids in the microalgal isolates were calculated from the standard curve prepared using olive oil (10 to 100 µg) as standard.

Total soluble proteins

The extraction of total soluble proteins was carried out by the method of Kong *et al.*[20] with some modifications. The dried microalgal biomass (50 mg) was homogenized and extracted in 20 mL of 90% ethanol for 1 h. The homogenate was centrifuged at 10000 rpm for 10 min. The obtained pellet was mixed with 10 mL of 20% trichloroacetic acid and incubated at 95°C in water bath for 15 min and then recentrifuged. The pellet was repeatedly washed with 95% ethanol for removal of trichloroacetic acid. It was further dissolved in 10 mL of 0.1 N NaOH and extracted twice for 1 h at 60°C in a water bath. The soluble protein content was estimated by method of Lowry *et al.*[21].

Total chlorophyll content

The total chlorophyll content of microalgal isolates was estimated by the method of Arnon[22] with some modifications. The dried algal biomass (50 mg) was grinded using pestle mortar and suspended in 8 mL of 90% acetone to extract the pigments. The tubes were kept in water bath at 65°C for 2 h. The algal suspension was shaken vigorously after every 15 min during 2 h incubation. The tubes were then allowed to stand in dark for 24 h. Acetone was added to compensate for any evaporation and the culture was centrifuged at 10000 rpm for 5 min. The absorbance of resulting supernatant was measured at 663 and 645 nm using visible spectrophotometer.

The following equation was used to calculate the concentration of total chlorophyll[22]:

$$\text{Total chlorophyll (mg/g)} = \frac{20.2\,OD_{645} + 8.02\,OD_{663}}{W \times 1000} \times \text{Volume}$$

V= volume of solvent; W= dry weight of algal biomass

Statistical analysis

Statistical analysis of the data was done using CPCS1 software. Data on biochemical parameters were analysed by using Completely Randomised Designs (CRD) in CPCS1.

Results and Discussion

Isolation of microalgae

The water samples containing microalgae were collected from waterlogged areas of villages Theri,

Ghagga and Gurusar of district Muktsar (Punjab). Five microalgae, designated as MA-1, MA-2, MA-3, MA-4 and MA-5 were isolated and purified by streak plate method (Fig. 1). Individual colonies of each isolate appearing at the tail end of streak were picked up and repeatedly subcultured. The purity of the culture was ensured by repeated plating and regular observation under microscope.

Morphological characterization of microalgal isolates

Morphological characterization of five microalgal isolates using bright field condenser (primary staining technique) and phase contrast condenser (unstained cultures) was carried out on the basis of cell shape, cell size, colour and cellular structure, i.e. unicellular or multicellular. All the five isolates (MA-1, MA-2, MA-3, MA-4 and MA-5) were observed to be round in shape, green coloured and unicellular in structure with different cell size when observed under bright field condenser (Fig. 2). Moreover, under the phase contrast condenser anisotropic dense structures such as nuclei were observed in microalgal isolates in various quantity and sizes (Fig. 2). On the basis of morphological characterization, the microalgal isolates were tentatively identified to be *Chlorella* sp.

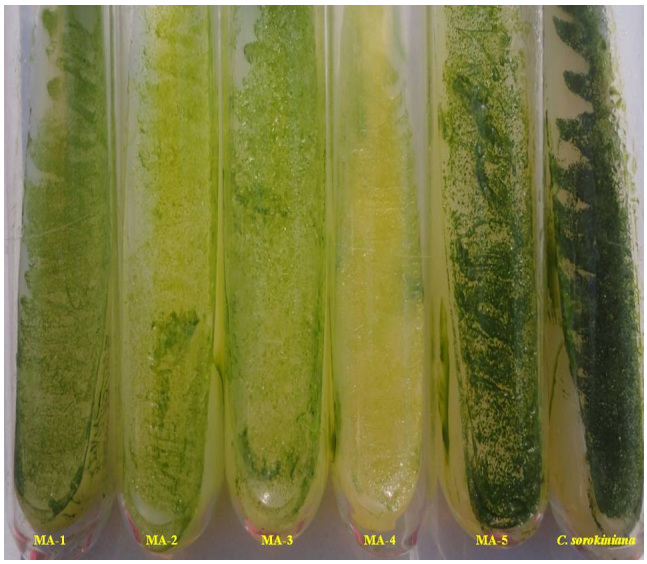

Fig. 1—Purified microalgal isolates on agar slants

Morphometric analysis of microalgal isolates using scanning electron microscope (SEM) was carried out for morphological characterization. The microalgal isolates MA-1, MA-2, MA-3, MA-4, MA-5 possessed cells with an average size diameter of 3.62, 3.43, 3.57, 3.01 and 3.24 µm, respectively and were spherical with smooth surface (Fig. 3). The individual cells of isolates exhibited aggregation to form larger oblong shaped aggregates. However, standard culture of *C. sorokiniana* possessed cells with average diameter of 3.86 µm. Ponnuswamy *et al*.[23] isolated *Chlorella vulgaris* from water samples collected from Bhavanilake at Erode, Tamil Nadu, India. The morphological characterisation using light microscope and scanning electron microscopy revealed that cells were green coloured, unicellular, spherical in shape and individual cells of the colonies were in range of 10 µm. Elumalai *et al*.[24] isolated algae from Kabaleeshwarar temple tank, India and reported that cells were unicellular, spherical about 5-10 µm in size and surrounded by a cell wall consisting of two

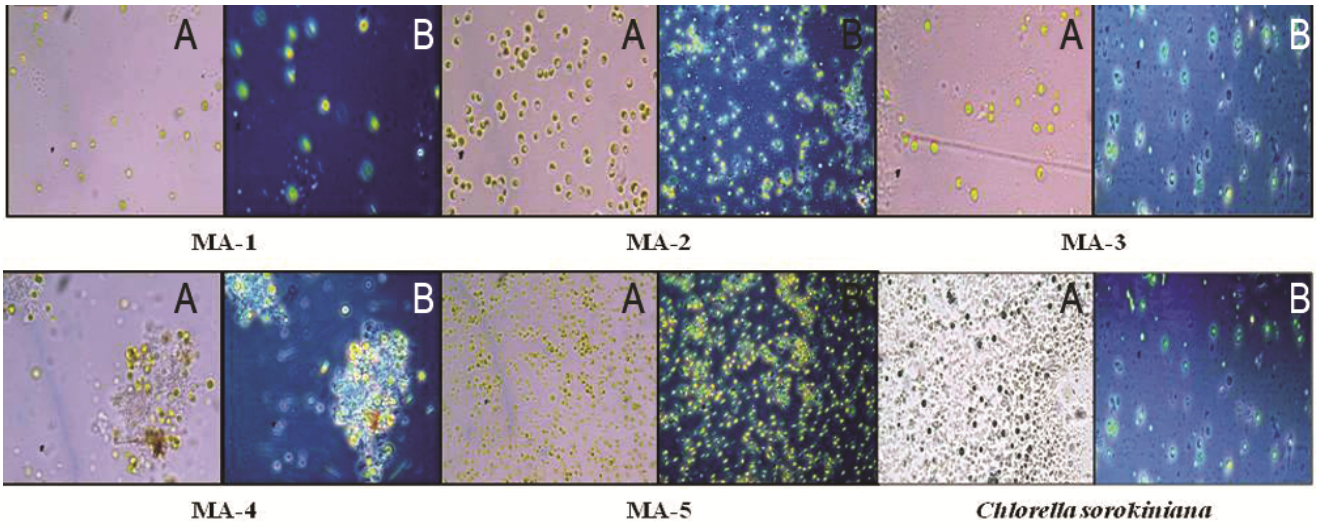

Fig. 2— Morphological view of different microalgal isolates using bright field condenser (A); and phase contrast condenser (B) viewed at 40X

regions, an outer thin layer and an inner bulky microfibrillar layer. The cells were identified as were identified as *Chlorella vulgaris.*

Growth and Viable cell count

The study of growth of five microalgal isolates and standard culture *C. sorokiniana* in terms of absorbance at wavelength 600 nm at different days after culturing (Fig. 4) revealed that in all the microalgal isolates, growth was observed to be maximum at 5 days after culturing and then, declined thereafter. The maximum growth was observed in isolate MA-1 (3.69) followed by MA-2 (3.42) at 5 days after culturing. However, the standard culture *C. sorokiniana* showed maximum growth in terms of absorbance of 2.07 at 5 days after culturing. Wong *et al.*[25] studied the effect of various growth medium compositions on the growth of *Chlorella vulgaris*. Thirteen different media in the culture tubes were tested for the growth of alga. The optical density of the batch cultures in different culture media ranged from 0.101 to 3.389. The maximum absorbance of culture medium was recorded for the bold basal medium (OD_{683}: 3.389 ±0.023) with highest biomass productivity (114.208± 0.850 mg L^{-1} day^{-1}) and specific growth rate (0.279± 0.001 d^{-1}) at 12th day. Similarly, Verma *et al*[26] studied the cultivation of *Chlorella pyrenoidosa* in six different media and modified CHU-11 media (containing urea) was found to be the best on the basis of optical density (5.7) and pigment content in algal cells. The viable cell count of different microalgal isolates ranged from 211.8 to 369.4×10^5 cfu mL^{-1}. The highest viable cell count of 369.4×10^{5}cfu mL^{-1} was observed in isolate MA-1, followed by cell count of 287.9×10^{5}cfu mL^{-1} in isolate MA-2 (Fig. 5). However, standard culture of *C. sorokiniana* was observed to have viable cell count 226.8×10^5 cfu mL^{-1}. Farhani *et al.*[27] isolated microalga from Tiab estuary and the identified strain belonged to genus *Chlorella*. The maximum viable cell count was found to be 6.25×10^4 cfu mL^{-1} with maximum specific growth rate at 72 h and biomass productivity of 852 mg L^{-1}.

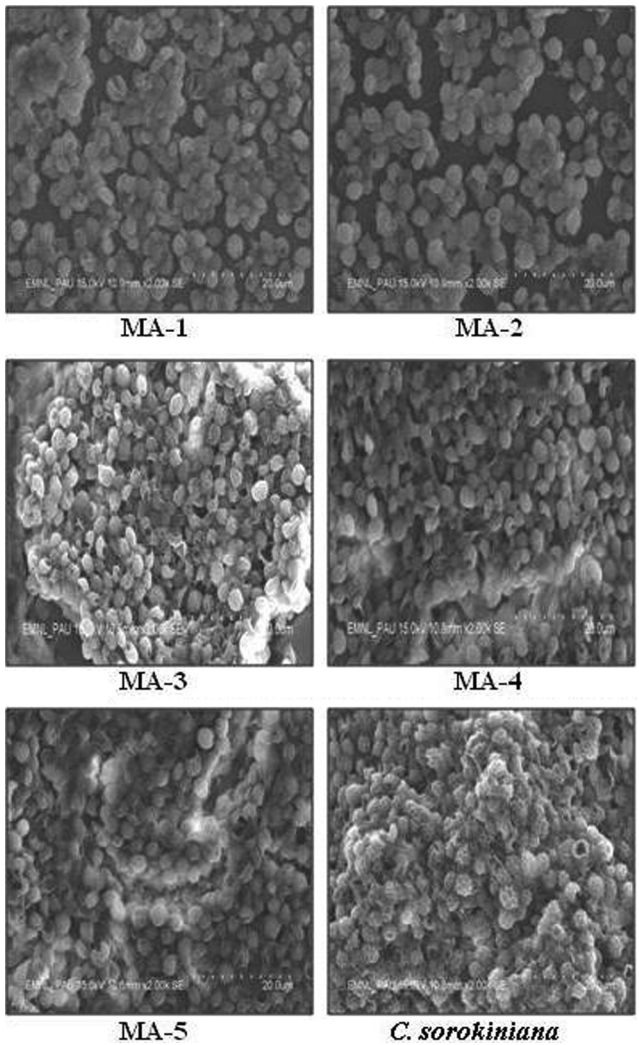

Fig. 3—Scanning electron micrographs of microalgal isolate (2000X)

Biochemical components of microalgal isolates

Carbohydrates of different algael species can be found in the form of starch, cellulose, and other polysaccharides, as storage products or cell wall components. In eukaryotic green algae *Chlorella* sp. carbohydrates are accumulated in the form of starch and cell wall constituents such as cellulose and hemicellulose[28]. The total carbohydrate content of different microalgal isolates was reported to be maximum at 5 days after culturing and declined thereafter (Table 1). Among different isolates, significantly high total carbohydrate content of 5.10% was recorded in *Chlorella* sp. MA-3 followed by

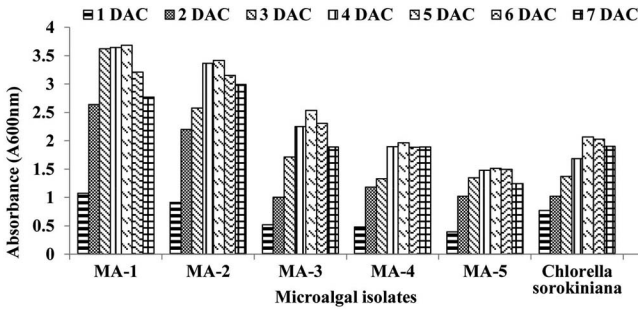

Fig. 4—Growth of different microalgal isolates at different days after culturing

Chlorella sp. isolate MA-4 (4.19%) at 5 days after culturing. The total carbohydrate content of standard culture of *C. sorokiniana* was observed to be 5.70% at 5 DAC. The carbohydrate content in these species varied significantly, and even for the same species, it was growth-stage dependent. Similarly, Dong *et al*.[29] studied the composition of different microalgae species viz., *Scenedesmus acutus*, *C. vulgaris* and *Nannochloropsis granulate* at different growth stages (early, middle and late). The carbohydrate content in *N. granulata* and *S. acutus* increased from early to middle growth stage, while decreased from middle to late stage For *C. vulgaris*, carbohydrate content increased with the nutrient depletion time. The total carbohydrate content of *S. acutus*, *C. vulgaris* and *N. granulata* at middle stage was found to be 49.7, 35.7 and 11.1%, respectively. The compositional characterisation of these isolates further helped in optimising the pretreatment conditions to maximize the valorization of algal biomass conversion for biofuels and bioproducts. Guccione *et al*.[30] evaluated the performance in terms of protein, carbohydrate and lipid content and productivity of selected *Chlorella* strains grown under controlled conditions to increase the sustainability of the cultivation process. Total carbohydrates varied from a minimum of 24.2% for microalgal strain F&M-M49 to a maximum of 35.6% for BdR3 strain. Under nitrogen deprivation, six microalgal strains accumulated carbohydrates up to 50%. Extraction of the target product (for example, protein or lipid) from both the nutrient sufficient and nitrogen starved algal biomasses could find application as biomaterials or energy feedstocks.

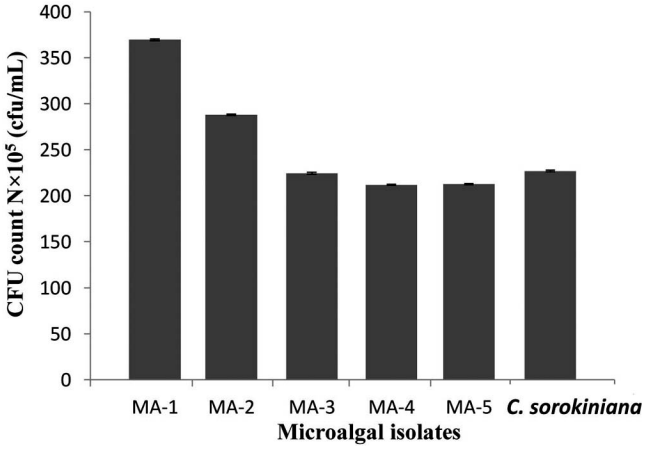

Fig. 5—Viable cell count of different microalgal isolates

The lipid content of different microalgal isolates was reported to be maximum at 7 days after culturing and declined, thereafter (Table 1). Significantly high total lipid content of 4.16% was observed in *Chlorella* sp. MA-2 followed by *Chlorella* sp. MA-5 (3.89%) which was statistically at par with *Chlorella* sp. MA-1 (3.85%) at 7 days after culturing. The total lipid content of standard culture of *C. sorokiniana* was observed to be 3.46 per cent at 7 DAC. Feller *et al*.[31] similarly reported that the lipid content of different algal biomasses varied from 7.4 to 12.5% with high lipid content in green microalgae *C. vulgaris* (12.5%) and *Scenedesmus* sp. (12.1%). *Phaeodactylum tricornutum* presented the lowest lipid content of 7.4%.

The total soluble protein content of different microalgal isolates was reported to be maximum at 7 days after culturing (Table 1). Among different isolates, significantly high total soluble protein content of 11.42% was recorded in *Chlorella* sp. MA-1 followed by *Chlorella* sp. MA-2 (10.76%) at 7 days after culturing. However, standard culture of *C. sorokiniana* was observed to have total soluble protein content of 10.01% at 7 DAC. The chlorophyll content of all the microalgal isolates decreased from 5 to 9 DAC with maximum chlorophyll content at 5 days after culturing (Table 1). Significantly high chlorophyll content of

Table 1—Mean biochemical contents (% dry wt. basis) of different microalgal isolates at different days after culturing (DAC)

Microalgal isolates	Total carbohydrate content (% dry wt. basis)				Total lipid content (% dry wt. basis)				Total soluble protein content (% dry wt. basis)				Total chlorophyll content (mg/g dry wt.)			
	5 DAC	7 DAC	9 DAC	Mean	5 DAC	7 DAC	9 DAC	Mean	5 DAC	7 DAC	9 DAC	Mean	5 DAC	7 DAC	9 DAC	Mean
MA1	3.15	2.87	2.50	2.84	2.60	3.85	1.81	2.75	10.12	11.42	5.43	8.99	24.31	22.81	20.42	22.52
MA2	3.50	2.83	2.80	3.04	2.10	4.16	3.10	3.12	8.25	10.76	3.75	7.58	21.65	20.25	18.60	20.16
MA3	5.10	4.21	2.76	4.02	2.14	3.24	1.11	2.16	7.18	9.99	3.85	7.00	19.58	18.12	16.35	18.02
MA4	4.19	2.53	1.81	2.84	2.28	3.52	1.83	2.54	5.95	7.68	4.72	6.11	18.85	16.68	14.43	16.65
MA5	4.08	2.42	0.98	2.49	2.07	3.89	1.64	2.53	5.18	6.92	3.39	5.16	18.33	16.49	13.39	16.07
Chlorella sorokiniana	5.70	4.22	2.35	4.09	1.72	2.14	1.02	1.63	7.40	10.01	3.86	7.09	20.92	18.31	16.60	18.60
Mean	4.28	3.18	2.20		2.15	3.46	1.75		7.34	9.46	4.16		20.60	18.78	16.63	
C.D. (5%)	DAC: 0.03 Isolates: 0.05 DAC×Isolates: 0.09				DAC: 0.18 Isolates: 0.26 DAC×Isolates: 0.46				DAC: 0.42 Isolates: 0.59 DAC×Isolates: 1.03				DAC: 0.06 Isolates: 0.09 DAC×Isolates: 0.16			

24.31% was observed in *Chlorella* sp. MA-1 followed by MA-2 (21.65%) at 5 days after culturing. The decrease in chlorophyll content with days may be due to exhaustion of nutrients (nitrogen) in the aqueous media[32]. *Idrissi et al.*[33] studied nineteen microalgal species from different reservoirs in Fez region (northern Morocco). Protein, carbohydrate and lipid content varied between taxa and within genera. The total carbohydrate, lipids and protein content of *Chlorella* sp. ranged from 4.13 to 9.76, 9.80 to 18.36 and 9.55 to 18.47%, respectively. The lipid content in some species such as *Lyngbya bergei*, *Oscillatoria amphibian* and *Polytoma papillatum* was found to be as high as 40%. Such species with high lipid production capacity could play an alternative role in energy sources.

Conclusion

The present study revealed that waterlogged area of Southwest Punjab is a rich pool of diverse microalgal strains. The microalgal strains isolated from this region were found to have different growth patterns, total carbohydrate, lipid, total soluble protein and chlorophyll content. *Chlorella* sp. MA-2 and MA-3 were reported to be most promising in terms of high lipid and carbohydrate content. Further, multitude of environmental and nutritional factors impact microalgal growth and its metabolic regulation. These factors must be considered for the promotion of microalgal culture system to maximise the production chemical constituents required for harnessing bio energy.

Conflict of Interest

The authors have no conflict of interests to declare.

References

1 Shuba ES & Kifle D, Microalgae to biofuels: 'Promising' alternative and renewable energy, review. *Renew Sust Energ Rev*, 81 (2018) 743.

2 Rittmann BE, Opportunities for renewable bioenergy using microorganisms. *Biotechnol Bioeng*, 100 (2008) 203.

3 Medipally SR, Yusoff FM, Banerjee S & Shariff M, Microalgae as sustainable renewable energy feedstock for biofuel production. BioMed Res Int, 2015 (2015) 519.

4 Aro E M, From first generation biofuels to advanced solar biofuels. *Ambio*, 45 (2016) 24.

5 Hoogeveen J, Faures J & Giessen N, Increased biofuel production in the coming decade: to what extent will it affect global freshwater resources? *Irrig Drain*, 58 (2009) 148.

6 Lü J, Sheahan C & Fu P, Metabolic engineering of algae for fourth generation biofuels production. *Energ Environ Sci*, 4 (2011) 2451.

7 González LE, Díaz GC, Aranda DAG, Cruz YR & Fortes MM, Biodiesel production based in microalgae: a biorefinery approach. *Nat Sci*, 7 (2015) 358.

8 Satyanarayana G, Mariano A & Vargas J, Microalgae, a versatile source for sustainable energy and materials. *Int J Energy Res*, 35 (2011) 291.

9 Taggar MS, Singh I & Sooch SS, Lipid accumulation in microalgae and its induction under different stress conditions for biodiesel production. In: Impending Power Demand and Innovative Energy Paths, (Ed. GC Mishra; Excellent Publishing House, New Delhi, India), 2014, 221.

10 Slade R & Bauen A, Microalgae cultivation for biofuels: cost, energy balance, environmental impacts and future prospects. *Biomass and Bioenerg*, 53 (2013) 29.

11 Planning Commission, Report of the high level expert group on waterlogging in Punjab. (Govt. of India), 2013, 95.

12 Wu QY, Yin S, Sheng GY & Fu JM, A comparative study of gases generated from simulant thermal degradation of autotrophic and heterotrophic *Chlorella*. *Prog Nat Sci*, 3 (1992) 435.

13 Thomas D J, Minj N, Mohan N & Rao P H, Cultivation of microalgae in domestic wastewater for biofuel applications-An upstream approach. *J Algal Biomass Utln*, 7 (2016) 62.

14 Bozzola JJ & Russel LD, *Electron Microscopy: Principles and Techniques for Biologist*. (Jones & Barlett Publishers, Inc Sudbury, MA, USA), 1999, 670.

15 Bashir KMI & Cho MG, The effect of kanamycin and tetracycline on growth and photosynthetic activity of two chlorophyte algae. *Biomed Res Int*, (2016) 1.

16 Aneja KR, *Experiments in Microbiology, Plant Pathology and Biotechnology* (New Age International (P) Limited, New Delhi, India), 2005, 71.

17 Margarites ACF & Costa JAV, Increment of carbohydrate concentration of *Chlorella minutissina* microalgae for bioethanol production. *Int J Eng Res Appl*, 4 (2014) 80.

18 Dubois M, Gilles KA, Hamilton K, Rebers PA & Smith F, Colorimetric method for determination of sugars and related substances, *Anal Chem*, 28 (1956) 350.

19 Mishra SK, Suh WL, Farooq W, Moon M, Shrivastav A, Park MS & Yang JW, Rapid quantification of microalgal lipids in aqueous medium by simple colorimetric method. *Bioresour Technol*, 155 (2014) 330.

20 Kong WB, Yang H, Cao YT, Song H, Hua SF & Xia CG, Effect of glycerol and glucose on the enhancement of biomass, lipid and soluble carbohydrate production by *Chlorella vulgaris* in mixotrophic culture. *Food Technol Biotechnol*, 51 (2013) 62.

21 Lowry OH, Rosenbrough NL, Farr AL & Radall RJ, Protein measurement with folin phenol reagent. *J Biol Chem*, 193 (1951) 265.

22 Arnon DI, Copper enzymes in isolated chloroplasts polyphenol oxidase in *Beta vulgaris*. *Plant Physiol*, 24 (1949) 1.

23 Ponnuswamy I, Madhavan S & Shabudeen S, Isolation and characterization of green microalgae for carbon sequestration, waste water treatment and bio-fuel production. *Int J Biol Sci Biotechnol*, 5 (2013) 17.

24 Elumalai S, Prakasam V & Selvarajan R, Optimization of abiotic conditions suitable for the production of biodiesel from *Chlorella vulgaris*. *Indian J Sci Technol*, 4 (2011) 91.

25 Wong Y K, Ho YH, Ho KC, Leung HM &Yung KKL, Growth medium screening for *Chlorella vulgaris* growth and lipid production. *J Aquac Mar Biol*, 6 (2017) 00143.

26 Verma R, Fulekar MH & Pathak B, Effect of different culture media formulations on growth and biodiesel production potential of *Chlorella pyrenoidosa*. *J Algal Biomass Utln*, 6 (2015) 7.

27 Farahani F, Ahmadi AL, Farmohammadi SA & Golkhoo S, Isolation of new isolate of microalgae *Chorella* sp. Al-25 from Tiab Estuary of Iran. *Pak J Biol Sci*, 9 (2006) 448.

28 Batista AP, Gouveia, L, Bandarra NM, Franco JM & Raymundo A, Comparison of microalgal biomass profiles as novel functional ingredient for food products. *Algal Res*, 2 (2013) 164.

29 Dong T, Wychen SV, Nagle N, Pienkos PT & Laurens LML, Impact of biochemical composition on susceptibility of algal biomass to acid-catalyzed pretreatment for sugar and lipid recovery. *Algal Res*, 18 (2016) 69.

30 Guccione A, Biondi N, Sampietro G, Rodolfi L, Bassi N & Tredici MR, *Chlorella* for protein and biofuels: from strain selection to outdoor cultivation in a green wall panel photobioreactor. *Biotechnol Biofuels*, 7 (2014) 84.

31 Feller R, Matos AP, Moecke EHS, Carvalho RM, Lopes RG, Camargo CPA, Sant'anna ES, Derner RB, Oliveira JV & Furigo JA, Comparative study of biochemical composition of five microalgae for biodiesel/bioproducts application. In: *Proceedings of the XX Brazilian Congress of Chemical Engineering, Florianopolis* (Edgard Blucher, Sao Paulo, Brazil), 2014, 1499.

32 Kiran B, Pathak K, Kumar R , Deshmukh D & Rani N, Influence of varying nitrogen levels on lipid accumulation in *Chlorella* sp. *Int J Environ Sci Technol*, 13 (2016) 1823.

33 Idrissi Abdelkhalek ELA, Mohamed B, Mohammed AM & Lofti A, Growth performance and biochemical composition of nineteen microalgae collected from different Moroccan reservoirs. *Mediterr Mar* Sci, 17 (2016) 323.