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Lipid Biosynthesis and Fatty acid Characterization in Selected *Spirulina* Strains under Different Cultivation Systems

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Total lipids production and fatty acid profile in *Spirulina* strains cultivated in flasks in a culture room and in photobioreactor under optimized set of culture conditions revealed that production of total lipid was more under photobioreactor conditions (23.64%) than in flasks (17.72%). Although similar fatty acids could be detected under both cultivation conditions but their amounts varied considerably. The percentage ratio of gamma-linolenic acid (GLA) to total fatty acids (TFA) was found upto 26.83% and 28.20% under flask and photobioreactor conditions respectively and the PUFA content was enhanced upto 71.83 - 82.60% and 77.02 – 83.03% of total fatty acids for flask and photobioreactor conditions respectively.

Keywords: *Spirulina*, Photobioreactor, Fatty acids, Palmitic acid, Linoleic acid, γ -linolenic acid.

Cyanobacteria, especially *Spirulina*, have been used since ancient times as a source of food because of its high nutritional value (Dillon *et al*., 1995). *Spirulina* is found in soil, marshes, freshwater, brackish water, seawater and thermal springs. Alkaline water with high pH (8.5–11) favours good production of *Spirulina*, especially where there is a high level of solar radiation at altitude in the tropics (Sasson, 1997). *Spirulina* appears to have considerable potential for development as a small-scale crop for nutritional enhancement, livelihood development and environmental mitigation. It provides an easily digestible high protein product (60 percent) with high levels of β-carotene, vitamin B12, iron and trace minerals (Costa *et al*., 2004) and the rare essential fatty acid like γ-linolenic acid (GLA). *Spirulina platensis* and its extracts have biological properties, such as they prevent cancers, decrease blood cholesterol levels, stimulate the immunological system, reduce the nephrotoxicity of pharmaceuticals and toxic metals and provide protection against the harmful effect of radiation (Belay *et al*., 1993). These properties have been attributed to different compounds such as phenolics, phycobiliproteins, carotenoids, organic acids, and polyunsaturated fatty acids (Khan *et al*., 2005). For these reasons, *Spirulina* sp. is widely used in commercial cultivation.

The commercial culture of *Spirulina* sp. and others microalgae is carried out in either raceway type of open culture systems or in closed photobioreactors (Chisti, 2007). While *Spirulina* production occupies only a small environmental footprint, with considerable efficiencies in terms of water use, land occupation and energy consumption when compared to traditional terrestrial crops; its large scale biomass and metabolites production is influenced by various factors such as temperature, light intensity, initial

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culture pH, concentration of carbon dioxide, carbon sources, nitrogen sources, salts and the amount of inoculum etc. (Habib *et al*., 2008). A large number of studies carried out on production of *Spirulina* sp. have reported optimization of one or two factors at a time that affect the biomass production such as pH and temperature (Ogbonda, *et al.*, 2007) or pH and phosphate regime (Abuzer, *et al.*, 2009) or temperature and nitrogen (Colla *et al*., 2007) or light and temperature (Kumar *et al.*, 2011). Having studied the available published research, we found that none of the published studies have comprehensively investigated the effect of all major environment factors on the growth and lipid production by *Spirulina* sp. In our earlier studies we have reported on the optimization of growth and lipid production in *Spirulina* spp. such as temperature, light intensity (Bhakar, 2014), incubation period and salinity (Bhakar *et al*., 2013) and nutrient limitation viz. nitrogen and phosphorus (Bhakar *et al.*, 2013). Therefore, after studying the influence of most of the factors we proposed to investigate growth and lipid production of *Spirulina* spp.in flasks in a culture room and in photobioreactor under optimized set of culture conditions.

MATERIALS AND METHODS

Spirulina platensis (CCC480), *Spirulina maxima* (CCC481) and *Spirulina* sp. (S&S) were obtained from the germplasm collection of Centre for Conservation and Utilisation of Blue Green Algae (CCUBGA), Indian Agricultural Research Institute, New Delhi. India. The cultures were maintained in Zarrouk's medium (Zarrouk, 1966). The cultures were cultivated under optimized conditions in growth chamber at 25+2°C temperature and light intensity of 100 µmol photon m^{-2} s^{-1} with light/dark ratio = 16:8 supplemented with initial $NaNO_3$, K_2HPO_4 and NaCl concentration of 1.0, 0.2 and 5.0 g/L respectively as optimized previously (Bhakar, 2014). The experiments were conducted using Zarrouk's medium under two sets of cultivation systems; a) in culture room using 100 mL medium in 250 mL flasks and b) in a photobioreactor (Applikon Biotechnology BV Netherlands) of 4L working volume under agitation (200 rpm) and DO (30%). The observations for dry weight of algal biomass (Richmond and Gobbelaar, 1986), total lipids (Snyder and Stephens, 1959) were taken after 14 days and 21 days of incubation.

Fatty acid profile for each of the samples was done by preparing respective methyl esters by the method described by Ichihara and Fukubayashi (2010). Fatty acid methyl esters (FAME) were analysed by Gas Chromatography (Shimadzu GC-2010) using Flame Ionization Detector (FID). 2.5 µl of esterified sample was injected and analysed through FID (detector temperature, 280 °C; injection temperature, 270 °C; carrier gas, nitrogen at a flow rate of 1.21 mL/min) fitted with a 100 m long, (0.25 mm i.d.) SP-2560 column with a 0.20 mm film thickness and a polyethylene glycol modified nitroterephthalic acid stationary phase. Chromatography conditions used were: initial column temperature 140 °C with equilibration time of 0.5 min which was held for 5 min, a 4 °C/min rise to 240 °C, held for 15 min (total 45 min). Fatty acids were identified by comparing the retention times with FAME standards (Sigma Chemical Co., USA)) and quantified by normalization of the area under relevant peaks using Varian Star software version 4.51. The data were analysed using DMRT (Duncan Multiple Range Test) by SAS software (Little *et al*., 1991).

RESULTS AND DISCUSSION

The three *Spirulina* strains vary in terms of lipid and biomass accumulation in Zarrouk's (Z) medium under both flask and photobioreactor systems of cultivation and their comparative analysis is shown in Table 1 and Figure 1. Total lipid production in *Spirulina platensis*, *Spirulina maxima* and *Spirulina sp* was 17.72%, 13.13% and 16.33% respectively at 14 days of incubation and 15.87%, 16.34% and 16.62% respectively at 21 days of incubation under flask conditions, whereas on the other hand it was 20.26%, 16.76% and 22.89% respectively at 14 days of incubation and 23.64%, 18.33% and 18.61% respectively at 21 days of incubation under photobioreactor conditions. Lipid accumulation invariably increased from 14 to 21 days of incubation and photobioreactor condition was found superior to flask conditions for lipid biosynthesis both on quantity and percentage basis. Flask conditions however, showed higher biomass production at 14 and 21 days of incubation

Table 1. Total lipid production by *Spirulina* strains grown under flask and photobioreactor conditions in Z-medium

	Strains	14 Days			21 Days		
		Biomass (mg/ml)	Lipid (mg/ml)	Lipid (% dry wt)	Biomass (mg/ml)	Lipid (mg/ml)	Lipid (% dry wt)
Flask	*Spirulina platensis* (480)	3.58±0.12^{cd}	0.64±0.01^{c}	17.72±0.95^{c}	5.54±0.06^{a}	0.88±0.03^{c}	15.87±0.77^{c}
	Spirulina maxima (481)	4.78±0.05^{a}	0.63±0.02^{c}	13.13±0.10^{d}	5.67±0.09^{a}	0.93±0.02^{b}	16.34±0.54^{c}
	Spirulina sp (S&S)	4.16±0.09^{b}	0.68±0.03^{b}	16.33±0.37^{c}	5.11±0.12^{b}	0.85±0.03^{c}	16.62±0.76^{c}
Photo Bioreactor	*Spirulina platensis* (480)	3.41±0.11^{de}	0.69±0.02^{b}	20.26±0.37^{b}	4.27±0.10^{c}	1.01±0.02^{a}	23.64±0.51^{a}
	Spirulina maxima (481)	3.86±0.05^{c}	0.65±0.02^{c}	16.76±0.84^{c}	4.57±0.05^{c}	0.84±0.02^{cd}	18.33±0.51^{b}
	Spirulina sp (S&S)	3.28±0.05^{e}	0.75±0.01^{a}	22.89±0.93^{a}	5.05±0.08^{b}	0.94±0.03^{b}	18.61±0.68^{b}

Values are given as mean ± SD of three replicates. Means in columns with different letters are significantly different at $P < 0.05$ by Duncan's Multiple Range Test.

Table 2. Fatty acid composition of *Spirulina* strains grown under flask and photobioreactor conditions in Z-medium after 14 days of incubation

	Strains	Fatty acid concentration (%)									
		C14:0	C14:1	C16:0	C16:1	C18:0	C18:1	C18:2	C18:3	C20:0	C22:6
Flask	*Spirulina platensis* (480)	2.85	7.03	31.91	1.39	7.09	13.39	14.53	14.49	2.02	4.25
	Spirulina maxima (481)	2.94	3.85	24.83	7.67	5.34	11.81	13.81	15.67	1.01	5.71
	Spirulina sp (S&S)	1.31	1.04	38.16	1.47	2.78	13.25	15.04	14.41	7.27	1.74
Photo bioreactor	*Spirulina platensis* (480)	1.96	3.92	33.54	9.19	1.05	12.34	14.23	16.19	1.86	4.42
	Spirulina maxima (481)	3.99	1.13	31.57	1.26	2.89	18.51	11.31	17.02	2.18	4.37
	Spirulina sp (S&S)	1.92	2.48	35.79	4.05	3.99	14.51	11.02	13.91	2.53	7.74

C14:0 = Myristic acid; C14:1 = Myristoleic acid; C16:0 = Palmitic acid; C16:1 = Palmitoleic acid; C18:0 = Stearic acid; C18:1 = Oleic acid; C18:2 = Linoleic acid, C18:3 = Gamma-linolenic acid, C20:0 = Eicosanoic acid and C22:6 = Docosahexaenoic acid (nomenclature based on Sigma catalogue).

than photobioreactor conditions.

The fatty acid composition of *Spirulina* strains revealed that same fatty acids could be detected in all strains but their quantity varied in all the *Spirulina* strains (Table 2 and 3). The predominant acids in cultures grown under standard conditions were palmitic, linoleic, oleic, γ-linolenic acids and docosahexaenoic acid, which together formed 71.83 - 82.60% and 77.02 – 83.03% of total fatty acids for flask and photobioreactor conditions respectively. There were considerable differences in the relative proportions of the fatty acids i.e. palmitic, linoleic, oleic, γ-linolenic acids and docosahexaenoic acid, which ranged from 24.83 – 38.16%, 13.81 – 15.04%, 11.81 – 13.39%, 14.41 – 15.67% and 1.74 – 5.71% of total fatty acids, respectively at 14 days of incubation (Table 3) and changed to 31.11 – 32.52%, 6.12 – 8.57%, 6.88 – 8.84%, 24.15 – 24.46% and 3.85 – 8.01% respectively at 21 days of incubation under flask conditions. The content of respective fatty acids under photobioreactor conditions also changed from 31.57 – 35.79%, 11.02 – 14.23%, 12.34 – 18.51%, 13.91 – 17.02% and 4.37 – 7.74% (Table 2) at 14 days of incubation and from 31.20 – 34.58%, 7.49 – 8.85%, 10.49 – 13.38%, 23.90 – 26.84% and 1.08 – 1.78% (Table 3) at 21 days of incubation for palmitic, linoleic, oleic, γ -linolenic acids and docosahexaenoic acid respectively.

The percentage ratio of gamma-linolenic acid (GLA) to total fatty acids (TFA), unsaturated fatty acids (UFA) to TFA, GLA to oleic plus linoleic (O+L) and Docosahexaenoic acid (DHA) to total fatty acids (TFA) in the culture samples studied varied between 14.64 – 16.91%, 48.67 – 55.66%, 0.51 – 0.61 and 16.74 – 18.94% in flask conditions respectively at 14 days of incubation (Table 4) and changed to 24.94 – 26.83%, 53.87 – 57.57%, 1.39 - 1.80 and 28.86 – 35.17% respectively at 21 days of incubation (Table 5). On the other hand the percentage ratio in photobioreactor conditions varied between 14.20 – 18.06%, 54.84 – 61.08%, 0.54 – 0.61 and 20.88 – 22.70% at 14 days of incubation (Table 4) and 25.28 - 28.20%, 55.11 – 62.17%, 1.15 – 1.35 and 26.48 – 30.07% respectively at 21 days of incubation (Table 5). The GLA was found more under photobioreactor conditions (26.84%) whereas the degree of unsaturation (63.17%) and DHA (8.01%) was more under flasks conditions.

Table 3. Fatty acid composition of *Spirulina* strains grown under flask and photobioreactor conditions in Z-medium after 21 days of incubation

Strains		Fatty acid concentration (%)								
		C14:0	C14:1	C16:0	C16:1	C18:0	C18:1	C18:2	C18:3	C20:0	C22:6
Flask	*Spirulina platensis* (480)	2.82	7.38	31.49	3.54	7.56	7.49	6.12	24.46	3.37	3.85
	Spirulina maxima (481)	3.06	3.65	31.11	2.64	4.80	6.88	8.36	25.79	1.81	8.01
	Spirulina sp (S&S)	1.05	5.96	32.52	2.14	0.50	8.84	8.57	24.15	7.19	3.90
Photo bioreactor	*Spirulina platensis* (480)	1.17	4.72	31.20	9.41	1.45	10.49	8.85	25.40	2.66	1.08
	Spirulina maxima (481)	1.81	1.27	34.58	2.74	4.02	11.39	8.44	26.84	2.32	1.78
	Spirulina sp (S&S)	1.91	2.42	32.18	7.33	3.33	13.38	7.49	23.90	1.47	1.13

C14:0 = Myristic acid; C14:1 = Myristoleic acid; C16:0 = Palmitic acid; C16:1 = Palmitoleic acid; C18:0 = Stearic acid; C18:1 = Oleic acid; C18:2 = Linoleic acid, C18:3 = Gamma-linolenic acid, C20:0 = Eicosanoic acid and C22:6 = Docosahexaenoic acid (nomenclature based on Sigma catalogue)

Arthrospira (Spirulina) platensis is a filamentous cyanobacterium that has been studied and produced commercially in countries such as China, India, United States of America etc. mainly because of its great nutritional value, including high contents of proteins, fatty acids (γ-linolenic acid), and pigments (chlorophyll *a* and phycocyanin) (Pulz and Gross, 2004). Large-scale microalgal biomass productions use open-type production systems, such as artificial ponds, pools, or raceways, often placed under greenhouses, which involves extra building costs (Belay, 2002). As an alternative, closed-type systems, namely photobioreactors (PBR), have been developed from the lab-scale to the industrial-scale to produce, as a continuous production flow or in batch mode, microalgal biomasses of several strains for niche markets. Well-controlled and well-managed cultivation conditions are required for successful production to fit with the objectives of the niche markets targeted (Fernandez *et al.*, 2013). Various PBR designs have been developed: plastic bags, stirred tanks, bubble columns, airlifts, large surface area flat glass panels or thin layer reactors, horizontal glass or light transparent plastic tubes connected together, vertically or horizontally, in a closed serpentine system. To allow continued growth of the biomass, the photoperiod has to be avoided during cultivation. Sophisticated internally illuminated photobioreactors (IIPBR), equipped with optical fibres to increase permanent light availability were also developed (Pegallapati *et al.*, 2012). However, these PBRs also entail high construction costs (Wang *et al.*, 2012; Singh and Sharma, 2012).

The role of chemical environment on the biochemical profile of the *Spirulina* is of prime importance both for laboratory condition and outdoor mass culture. Although the comparative study between flask and photobioreactor system under optimized set of conditions has not been carried out to our knowledge but there are

Table 4. Fatty acid ratio in *Spirulina* strains grown under flask and photobioreactor conditions in Z-medium after 14 days of incubation

	Strains	Fatty acid ratio			
		GLA/TFA (%)	UFA/TFA (%)	GLA/O+L	GLA+DHA/TFA
Flask	*Spirulina platensis* (480)	14.64	55.66	0.52	18.94
	Spirulina maxima (481)	16.91	63.17	0.61	23.08
	Spirulina sp (S&S)	14.94	48.67	0.51	16.74
Photo	*Spirulina platensis* (480)	16.40	61.08	0.61	20.88
bioreactor	*Spirulina maxima* (481)	18.06	56.88	0.57	22.70
	Spirulina sp (S&S)	14.20	54.84	0.54	22.11

GLA = gamma-linolenic acid; TFA = total fatty acids; UFA = unsaturated fatty acids; O+L = oleic + linoleic acid and DHA = Docosahexaenoic acid

Table 5. Fatty acid ratio in *Spirulina* strains grown under flask and photobioreactor conditions in Z-medium after 21 days of incubation

	Strains	Fatty acid ratio			
		GLA/TFA (%)	UFA/TFA (%)	GLA/O+L	GLA+DHA/TFA
Flask	*Spirulina platensis* (480)	24.94	53.87	1.80	28.86
	Spirulina maxima (481)	26.83	57.57	1.69	35.17
	Spirulina sp (S&S)	25.47	56.49	1.39	29.58
Photo	*Spirulina platensis* (480)	26.34	62.17	1.31	27.46
bioreactor	*Spirulina maxima* (481)	28.20	55.11	1.35	30.07
	Spirulina sp (S&S)	25.28	58.86	1.15	26.48

GLA = gamma-linolenic acid; TFA = total fatty acids; UFA = unsaturated fatty acids; O+L = oleic + linoleic acid and DHA = Docosahexaenoic acid

numerous reports in which the photobioreactor systems were compared with open raceway ponds or static v/s continuous system of cultivations and between different type of photobioreactor. Xue *et al.*, (2013) reported higher lipid production under photobioreactor conditions than open raceways as found in our results. In photobioreactors cells are actively mixed and move between the dark and light regions. As a consequence they experience fast alternation of light and dark. Such dark/light cycles have been suggested to increase the photosynthetic efficiency in several cases (Grobbelaar, 2010; Kim *et al.*, 2006). Photobioreactor provide much greater oil yield per hectare compared with raceway ponds which is because the volumetric biomass productivity is more than 13-fold in comparison to that observed in raceway pond (Pabbi and Dhar, 2011). Control over culture conditions, minimized contamination risks, and reduced losses in water and CO_2 for closed systems allows for greater productivity (g/L/d and g/m^2/ d) over open culture systems. Simionato *et al*., (2013) suggest that very intense light can be harvested and exploited efficiently by cells growing in a photobioreactor, even if the total intensity is well beyond the saturation limit for that particular species. PBRs are more costly than raceway ponds and also present major design and operating challenges (gas exchange, overheating, fouling/ cleaning, etc.) Despite this, many, perhaps most, researchers working on algae mass production for value addition and feeds work with PBRs instead of open ponds. PBRs are viewed as more productive, more controllable, less subject to contamination, consuming less water, and achieving higher cell densities—all these advantages supposedly making up for their higher costs (Benemann, 2013).

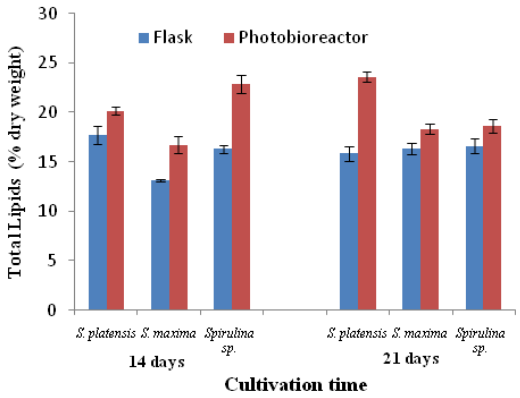

Fig. 1. Comparative lipid production by *Spirulina* strains under flask and photobioreactor conditions in Z-medium

The percentages of the major FAMEs in the *Spirulina* cultivated in this study were in accordance with previous reports by other workers (Polat and Ozogul, 2013; Hannon *et al*., 2010; Olguin *et al.*, 2001; Quoc *et al*., 1994; Cohen *et al.*, 1987), the principal fatty acids present were palmitic, γ-linolenic and linoleic acid. However, it may be possible to increase the content of γ-linolenic acid as observed here by growing *Spirulina* under most controlled conditions in a photobioreactor. The results have thus proved that the modification of the culturing conditions can tailor to the specific demands of highly productive microalgae to attain a consistently good yield of lipid and fatty acid profiles. With further understanding on the cultivation of *Spirulina* strains in photobioreactors, much greater productivity of algal lipid would be obtained. Therefore, *Spirulina* strain under investigation are good candidates for successful cultivation in static conditions in flasks in laboratory and/or in photobioreactors under different environmental condition as high value health foods, functional food and as a source of PUFA (Poly Unsaturated Fatty Acids).

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