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Cultivation of *Spirulina* species in different liquid media

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ABSTRACT:

Abellon CleanEnergy Ltd (www.abelloncleanenergy.com) is working on algae cultivation for fuel and other by products through utilization of various water resources. *Spirulina* is one of the most explored cyanobacteria. Since ancient time it is being used as source of protein *Spirulina* sp. NCIM – 5421 was cultivated in different liquid medium like; synthetic medium (SM), fertilizer medium (FM) and seawater medium (SM). Dry weight and pH were monitored for 30 days on daily basis. pH was found in range from 9.1 to 10.4 in SM, 9.0 to 10.1 in FM and 8.51 to 8.55 in SM. Gradually increase in dry weight (dw) was noticed along with the age of culture, 1.84 dw/L & 1.81 dw/L was achieved in SM and FM respectively. *Spirulina* inoculated in SM was survived but growth was not flourished, achieving maximum dry weight of 0.28 dw/L on 18th day of cultivation. Natural seawater fortified with different amount of $NaHCO_3$ and $NaNO_3$ did not shown significant impact on *Spirulina* growth. However results of present investigation could be consider for commercial cultivation of *Spirulina* using seawater.

KEY WORDS: Cyanobacteria, Fertilizer, Seawater, *Spirulina* – NCIM 5412

INTRODUCTION:

Since centuries cyanobacteria have been receiving increasing interest due to their potential to produce a diverse range of chemicals and biologically active compounds, such as vitamins, carotenoid pigments, proteins, lipids and polysaccharides (Zhang *et al*., 1999). For exploration of these potentials of cyanobacteria it should be cultivated in commercial way. Globally researcher2 are trying to produce microalgae/cyanobacteria commercially (Belay 1997; Ben-Amotz 2004). Yet very little or primary information is available on detailed design criteria, location selection, scaling considerations, or constrains involved in large scale cultivation.

Spirulina is a planktonic photosynthetic filamentous cyanobacterium that forms massive populations in tropical and subtropical bodies of water which have high levels of carbonate

and bicarbonate and alkaline pH values of up to 11. *Spirulina* from Chad Lake in Africa and Texcoco Lake in Mexico have been harvested as a source of food (Vonshak, 1997). *Spirulina* has been studied for single cell protein (SPC) (Anupama, 2000), vitamins, minerals, proteins and polyunsaturated fatty acids (gamma-linolenic acid) (Miranda et al., 1998), therapeutic properties (Belay et al., 1993), antioxidant activity (Estrada et al., 2001). Several cultivation methods like; open ponds (Lee YK, 1997), tubular photobioreactors (Torzillo et al., 1986), inclined glass panels (Hu Q, et al., 1996) have been tried. Cost and composition of cultivation media along with growth rate of the algae we challenging factors for commercially viable production. Different media have been tried for cultivation of spirulina such as Zarrouk's media (Zarrouk, C. 1966), Rao's media (Singh, S. 2006), CFTIR media (Venkataraman *et al*., 1995), OFERR media (Singh, S. 2006), Revised media(6) (Raoof *et al.,* 2006) and Bangladesh medium (Khatum *et al*., 1994).

The present report aimed to study three objectives: (1) Cultivation of *Spirulina* sp. NCIM – 5412 in FM, SM & SW media. (2) Effect on growth behavior of *Spirulina* in seawater enriched with $NaHCO_3$ and $NaNO_3$. (3) Adaptation of *Spirulina* in seawater medium.

MATERIALS AND METHODS:

Strain procurement, culture development & maintenance :

Spirulina sp. NCIM – 5412 (on solid media) was procured from National Collection of Industrial Microorganisms (NCIM) laboratory, Pune- India. Procured strain was previously maintained on Zarrouk's agar media slants at 4°C. Loop full of *Spirulina* culture was inoculated in 50 ml flask containing 10 ml sterile SM medium (Modified Zarrouk's Medium) under sterile condition. All the reagents used were of analytical grade, obtained from the Rankam Chemical Co. Sodium carbonate was added after autoclaving and pH was adjusted to 8.8 - 9.0. Growth and maintenance of the culture was done in an illuminated (4500 lux) growth room at 30 ± 2 °C under 12/12 hour light-dark cycles. Manual shaking of cultures was done 3 times daily.

Cultivation:

Spirulina sp. was inoculated in three media *viz*; SM, FM & SW as mention in Table. 1. Natural seawater was collected freshly from the bay of Khambhat (Latitude: 22° 13' 60 N, Longitude: 72° 47' 60 E). Total 30 flask of 50 ml capacity containing 20 ml of each medium were inoculated with same amount of inoculums. All flask were kept at room temperature under shadow condition, every day during day time lux and temperature were recorded. Manual shaking of cultures was done 3 times daily.

Table 1. Ingredients of synthetic medium (SM), Fertilizer medium (FM and seawater (SW)

Sr. no	Ingredient	SM	FM	SW
		Amount (g/L)		
1	$NaHCO_3$	16.8	8.0	0
2	$NaNO_3$	2.5	2.5	0
3	NaCl	1.0	0.5	0
4	$K2SO_4$	1.0	0	0
5	K_2HPO_4	0.5	0	0
6	$MgSO_4.7H_2O$	0.2	0.15	0
7	$FeSO_4.7H_2O$	0.01	0	0
8	$CaCl_2.2H_2O$	0.04	0.04	0
9	EDTA	0.08	0	0
10	Single supar phospate	0	1.25	0
11	Muriate of potash	0	0.98	0
12	H_3BO_4	0.00286	0	0
13	$MnCl_2.4H_2O$	0.00181	0	0
14	$ZnSO_4.7H_2O$	0.00022	0	0
15	MoO_3	0.00001	0	0
16	$CuSO_4.5H_2O$	0.00008	0	0
17	Distilled water	1000 ml	1000 ml	0
18	Natural seawater	0	0	1000 ml

Cultivation enriched seawater:

Seawater collected from Khambhat was enriched with different carbonate ($NaHCO_3$) and nitrate ($NaNO_3$) salt at concentration mention in Table–2. All flask containing different salt concentration were inoculated ated flask were maintained as mention above.

with same amount of inoculum *Spirulina* cell mass was filtered by filter paper and washed with buffer solution (pH-7) and resuspended in seawater by cyaclomixture for making homogenized mixture. Homogenized culture was used for inoculum. Inocul

Table : 2 Results of pH and dry weight of Spirulina (NCIM 5421) cultivation in seawater fortified with sodium bicarbonate ($NaHCO_3$)and sodium nitrate ($NaNO_3$

Filtration & washing:

Every day one flask from set of 30 was

Medium Ingredient	Amount (g/l)	0 Day		15th Day		30th Day	
		pH	dw/l	pH	dw/l	pH	dw/l
Sea water	0	8.51	0.09	8.55	0.28	8.47	0.15
$NaHCO_3$	1	8.26	0.09	8.53	0.11	8.64	0.05
	2	8.15	0.09	8.80	0.16	8.93	0.07
	5	8.12	0.09	9.03	0.24	9.12	0.18
	10	7.92	0.09	9.33	0.27	9.41	0.17
$NaNO_3$	0.5	8.52	0.09	8.53	0.13	8.65	0.09
	1	8.53	0.09	8.66	0.19	8.46	0.04
	1.5	8.48	0.09	8.27	0.24	8.43	0.27
	2.5	8.46	0.09	8.50	0.16	8.63	0.10
$NaHCO_3$ + $NaNO_3$	1 + 0.5	8.22	0.09	8.57	0.13	8.65	0.08
	2 + 1.5	8.06	0.09	8.84	0.24	8.88	0.07
	5 +1.5	7.80	0.09	9.17	0.28	9.12	0.26
	10 + 2.5	7.78	0.09	9.40	0.37	9.31	0.25

harvested for dw determination. Cells were collected by filtration using whatman no 1 filter paper. Collected cells were wash with dilute HCL (0.0001 N) to remove any excess salt and dust attached to cell surface.

Determination of dry weight:

After filtration and washing filter paper was dried in oven at 100°C for 16 hr. Kept desiccator and cool to room temperature. Weight carefully up to 0.0001 g level by weigh balance. Cells were dried in oven at 100°C for 16 hrs, placed in desiccator & cool to room temperature and weight the mass using analytical balance.

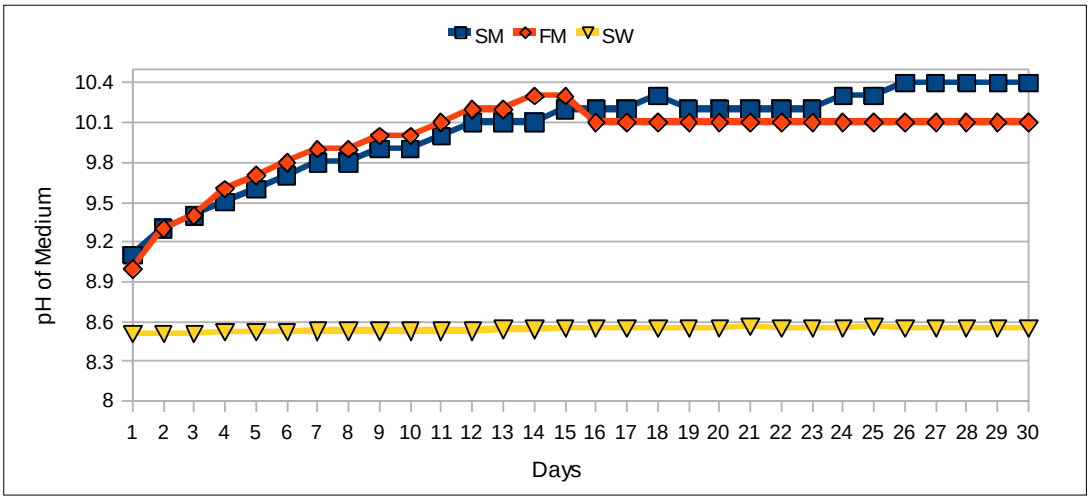
Monitoring experiments:

Before filtration culture of each flask was monitored for pH and microscopic examination. For microscopic examination, 100µl sample was drawn after proper shaking by micro pipettes and observed under microscope at 40 X . All experiments were performed in triplicate and results were expressed as mean value of respective parameter.

RESULTS AND DISCUSSION:

Spirulina sp. NCIM – 5412 was successfully cultured in SM liquid form from solid media slant. *Spirulina* sp. was cultured in SM, FM and SW for thirty days. pH (Fig. 1), microscopic & dry weight (Table 3) were determined on daily basis. *Spirulina* sp. grows well in both SM and FM culture. In SM and FM, pH of medium became more basic as culture became older. Appearance of culture also shifted from light green to dark green in proportion to the increasing cell mass. While cultivation of *Spirulina* in SW, both pH and appearance dose not changed as compared to cultivation in SM and FM medium. In FM medium pH of culture became more basic (pH 10) as compared to SM medium (Fig.1). Microscopic & visual observation revealed culture was grown healthy and morphology of *Spirulina* filament also maintain its colour and shape as reported by FAO (Fisheries and Aquaculture Circular No. 1034) (FAO, 2008).

Figure-1: The pH of medium during cultivation of Spirulina (NCIM 5421) in Synthetic medium (SM), Fertilizer medium (FM) and Seawater medium (SM).



Culturing *Spirulina* in conical flask has its limitation in providing complete information related to growth, development and production of value added chemicals (Capone, *et al.*, 1997), however it would give preliminary information for further demo or commercial level of cultivation. Behavior of *Spirulina* in SW was found totally different compared to SM and FM. Sea water as medium did not show any significant enhancement into the growth of *Spirulina*. Maximum 0.28 g/l dry mass was found. One distinct observation observed was behavior of pH value when cultured *Spirulina* in SW. The pH value did not much changed from initial 8.51 to 8.55 (Table 3) on 30th day of experimentation. Like pH value, dry weight of biomass was also not increased significantly (Table -3).

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Figure-2: Temperature behavior during experimentation of cultivation of Spirulina (NCIM 5421) in Synthetic medium (SM), Fertilizer medium (FM) and Seawater medium (SM), (values are average 60 days).

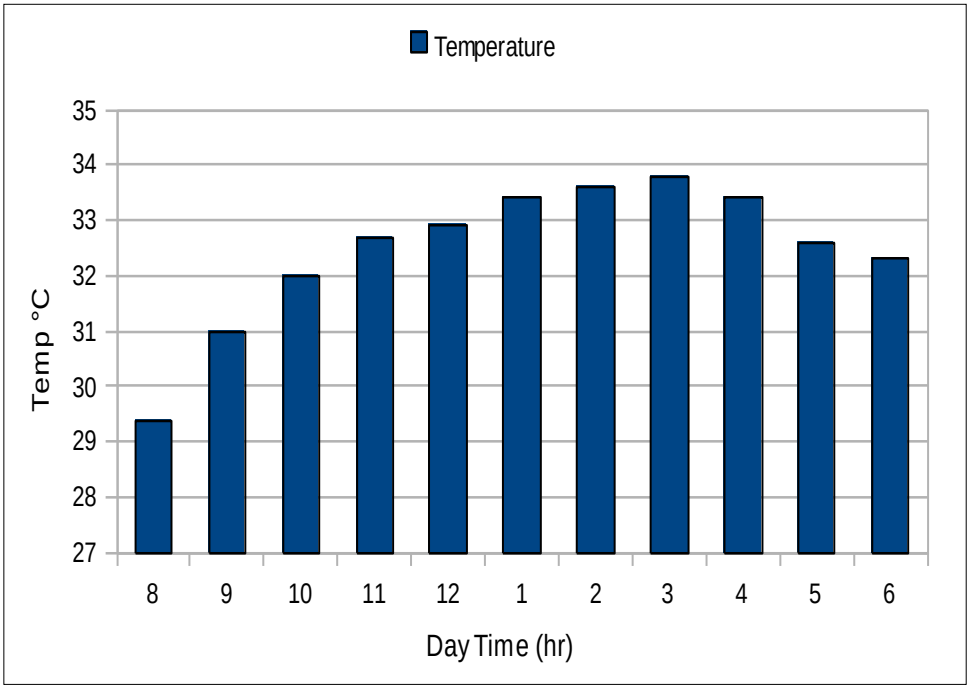
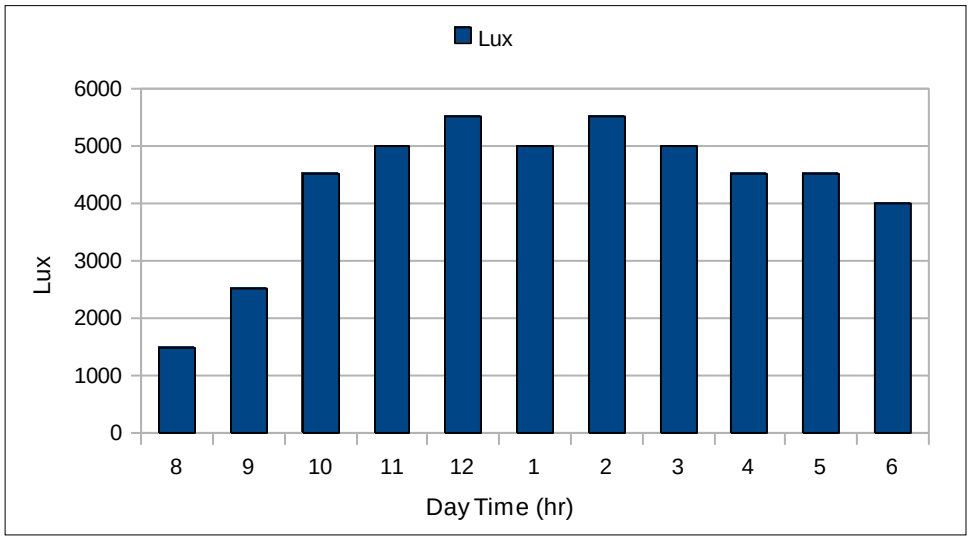


Figure- 3: Irradiance lux behavior during experimentation of cultivation of Spirulina (NCIM 5421) in Synthetic medium (SM), Fertilizer medium (FM) and Seawater medium (SM), (values are average 60 days).



Environmental factors particularity irradiance flux and temperature are important evolution of biomass production and their general characterization. *Spirulina* sp. growth is maximum at 30-35°C while high alkalinity is mandatory for growth of *Spirulina* (Belkin & Boussiba, 1971). In present investigation temperature and irradiance lux was found as mentioned in Fig 2 and Fig 3 respectively. Growth behavior and dry yield of *Spirulina* sp. NCIM – 5412 cultured in FM and SM clearly indicate that environmental factor as mentioned in Fig 2 & 3 were supporting growth. Results of *Spirulina* cultivation in similar condition in SW indicated that sea water composition is not supportive to growth, but *Spirulina* sp. survived in SW medium indicated that gradually exposure to SW and further enrichment of SW would favour the growth of *Spirulina*

Table: 3 Daily observation of *Spirulina* (NCIM 5421) cultivation in Synthetic medium (SM), Fertilizer medium (FM) and Seawater medium (SM).

Days	SM		FM		SW	
	dw/L	Colour	dw/L	Colour	dw/L	Colour
1	0.04	Culture light green, cells free flowing, no contamination	0.04	Culture light green, cells free flowing, no contamination	0.04	Culture light green, cells free flowing, no contamination
2	0.08		0.08		0.04	
3	0.15		0.13	Culture green, few clumps were observed, no contamination	0.06	Culture light green, clumps were formed with white precipitation on surface, no contamination
4	0.21		0.2		0.06	
5	0.27	Culture green, cells free flowing, no contamination	0.3	Culture green, few clumps were observed, no contamination	0.08	Culture light green, clumps were formed with white precipitation on surface, no contamination
6	0.33		0.48		0.08	
7	0.46		0.64		0.08	
8	0.53		0.75	Culture dark green, thick & clumpy, few clumps were become white , no contamination	0.12	Culture light green, clumps were formed disappear white precipitation on surface, no contamination
9	0.55		0.76		0.14	
10	0.63	Culture dark green, thick, few clumps were observed , no contamination	0.87	Culture dark green, thick & clumpy, few clumps were become white , no contamination	0.18	Culture green, clumpy, no contamination
11	0.76		0.97		0.18	
12	0.81		1.07		0.24	Culture green, clumps become dark green, no contamination
13	0.82		1.12		0.25	
14	0.88		1.12		0.25	
15	0.88		1.15		0.26	
16	0.89	Culture dark green, thick, few clumps were observed, thin film of cells on flask wall, no contamination	1.15	Culture dark green, thick & clumpy, attachment of clump to flask wall was noticed, no contamination	0.26	Culture dark green and clumpy, few clumps stick at bottom.
17	0.91		1.17		0.26	
18	0.91		1.19		0.28	
19	0.93		1.22		0.28	

20	0.95		1.34		0.28	
21	1.08		1.49		0.28	
22	1.32		1.54		0.24	Culture lose its green color and become light yellowish, contamination observed (micro algae and protozoa)
23	1.56		1.63		0.21	
24	1.64		1.65		0.20	
25	1.79		1.79		0.20	
26	1.78		1.79		0.20	Culture become more yellowish with similar contamination status
27	1.79		1.80		0.17	
28	1.84	Culture dark green, thick, few clumps were observed, thin film of cells on flask wall and part toward surface become light yellowish green, no contamination	1.80	Culture dark green, thick & clumpy, attachment of clump to flask wall, few clump change dark green to yellowish green, no contamination	0.17	Culture become more yellowish, contamination percentage relatively high
29	1.84		1.80		0.17	
30	1.84		1.80		0.15	

In present investigation SW enriched with $NaNO_3$ and $NaHCO_3$ were explored alone as well as in combination at different concentration for *Spirulina* cultivation. *Spirulina* sp. cultured in fortified SW were observed for dry weight and microscopy for 30 days as mention in table Table 3. Total three results (o day, 15^{th} day and 30^{th} day) for each combination were considered (Table -3). It is depicted from Table 3, that $NaHCO_3$ and $NaHCO_3$ has some influence in *Spirulina* cultivation in SW. Sea water fortified with $NaNO_3$ was more suitable as compared to $NaHCO_3$. $NaNO_3$ alone showed good results in *Spirulina* growth while in combination with $NaHCO_3$ no significant enhancement in *Spirulina* growth was observed. Maximum 0.27 g/l dry weight on 30^{th} day was achieved in SW fortified with $NaNO_3$ (1.5 g/l) which was comparatively high when compared with only SW as medium (0.15 g/l). Dry weight of *Spirulina* in SW medium was not good enough to scale it for commercialization but it would consider as simulation study. During cultivation pH of SW medium was not significantly change will indicate very poor or suppressive growth in respective salt concentration (Table 3). Behavior of coiled filament of *Spirulina* is good

indicator to study effect of light, temp, cultivation vessels, medium composition and other biotic& abiotic components (Parvin *et al*., 2008). Daily microscopic observations were taken from each combination (Table 2). Fig 4 and 5 are representing filament behavior in natural and fortified SW at 15th day of cultivation and 30th day of cultivation respectively. It was clearly predicated from both figures that initially coiled filament (Fig. 4) of *Spirulina* became straight (Fig. 5) as culture became old. During the period of this study, having many cloudy and rainy days, sunlight seemed also to play a significant role apart from the medium combination.

CONCLUSION:

The present study indicates that, natural seawater has potential to grow *Spirulina* sp. NCIM 5421. Further investigations are required for ascertaining this supposition of seawater as cultivation medium.

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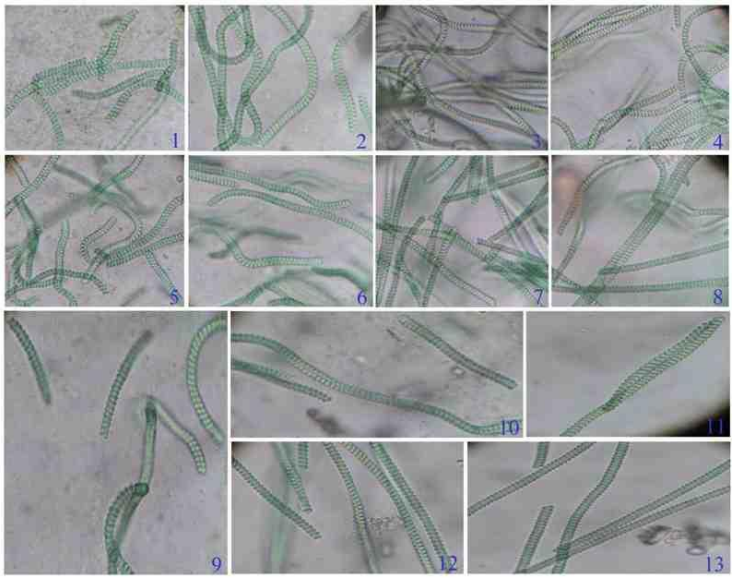

Figure- 4: Microscopic view of *Spirulina* filament cultured in natural sea water after 15th day fortified with $NaHCO_3$ and $NaNO_3$.

The images captured at 40X magnification. 1- natural sea water; 2, 3, 4, 5 – sea water enriched with NaHCO3 at 1 g/l, 2 g/l, 5 g/l and 10 g/l respectively; 6, 7, 8, 9 - sea water enriched with NaNO3 at 0.5 g/l, 1 g/l, 1.5 g/l and 2.5 g/l respectively; 10, 11, 12, 13 - sea water enriched with NaHCO3 + NaNO3 at 1+0.5 g/l, 2 + 1.5 g/l, 5 + 1.5 g/l and 10 + 2.5 g/l respectively.

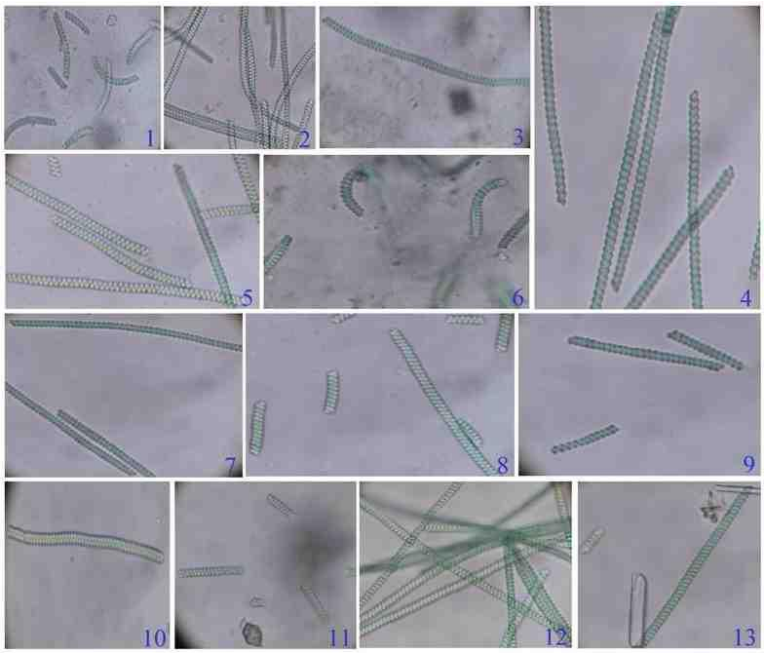

Figure-5: Microscopic view of Spirulina filament cultured in natural sea water after 30th day fortified with $NaHCO_3$ and $NaNO_3$.

The images captured at 40X magnification. 1- natural sea water; 2, 3, 4, 5 – sea water enriched with NaHCO3 at 1 g/l, 2 g/l, 5 g/l and 10 g/l respectively; 6, 7, 8, 9 - sea water enriched with NaNO3 at 0.5 g/l, 1 g/l, 1.5 g/l and 2.5 g/l respectively; 10, 11, 12, 13 - sea water enriched with NaHCO3 + NaNO3 at 1+0.5 g/l, 2 + 1.5 g/l, 5 + 1.5 g/l and 10 + 2.5 g/l respectively