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Cultivation of *Chlorella protothecoides* (ISIBES –101) under autotrophic and heterotrophic conditions for biofuel production.

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Abstract:

The present working paper describes autotrophic and heterotrophic cultivation of *Chlorella protothecoides* (ISIBES – 101) as a part of algae biofuel project. Heterotrophic and autotrophic cultivation were performed with different carbon and nitrogen source respectively. Achieved highest cell density in heterotrophic condition was 17.18 g/l and in autotrophic cultivation it was 0.871 g/l. Maximum biomass productivity (P_{max}) in heterotrophic cultivation (1.562 g/l/day) was higher then autotrophic cultivation (0.061 g/l/day). Lipids and carbohydrate were estimated higher in heterotrophic cultivation, while protein was expressed in grater amount in autotrophic cultivation. Dry algal powder was used in oil extraction by chloroform-methanol solvent mixture. Fatty acid methyl ester (FAME) was prepared by methanolysis and subjected to fatty acid profiling by Gas Chromatography method. Extracted algal oil was dominated by Linoleic acid ($C_{18.2}$), irrespective of cultivation type. Results indicate ISIBES-101 is potential candidate for biodiesel production. With respect to cultivation of ISIBES-101 at commercial scale heterotrophic cultivation is more promising then conventional autotrophic cultivation.

Key words: *Chlorella protohecoides;* autotrophic; heterotrophic; biomass productivity

1. Introduction:

Micro algae was used as part of food & medicine in human life since centuries (Benemann, et al., 1987), but in last few decades these tiny organisms were explored as various types of fuels like; methane (Spolaore, et al., 2006), biodiesel (Sheehan, et al., 1998, Sawayama, et al., 1995), biohydrogen (Melis, 2002; Kapadan and Kargi, 2006). Utilizing the petroleum sourced fossil fuels is now widely recognized as unsustainable because of depleting supplies, escalating price and the contribution of these fuels to the accumulation of carbon dioxide in the environment (Dwi, et al., 2009). To address this problem many new technologies were developed to produce various types of alternative fuel. Biodiesel was one of them, many feed stocks are being used as source for biodiesel production such as; oil crop, waste vegetable oil, algae oil, animal fats, etc., (Barnwal and Sharma, 2005). Biodiesel has some superior quality then petroleum diesel in many ways like; biodegradability, decreased emission levels of polycyclic aromatic hydrocarbons (PAH) and nitrated polycyclic aromatic hydrocarbons (nPAH), non-toxic, renewable, less in sulfur content and carbon neutral (Delucchi, 2003;Vicente, et al., 2004).

Many research articles and reports were explained advantages of using microalgae for biodiesel production verses other feedstock (Li, et al., 2008; Chisti, 2007). Biodiesel production from micro algae required large-scale cultivation of algae. Micro algae cultivation can be done in high rate ponds (HRP), open raceway ponds and photobioreactors (PBR) (Chisti, 2007). Few micro algae also grow in heterotrophic condition with different organic chemical as carbon sources (Chen and Chen 2006). Heterotrophically cultivation of micro algae is being explored in many laboratories for biodiesel production.

Chlorella protothecoides is a green micro algae belonging to the phylum chlorophyta. It has capacity to grow in autotrophic and heterotrophic condition. Several attempts have been made for cultivation of *C.protothecoides* under heterotrophic condition with different organic substance as carbon source (Wei, and Liu, 2008; Xu et al., 2006). Optimization of this cultivation technology with dawn stream processing has promising way to produce high cell density cultivation of micro algae. Present investigation was a part of biofuel project in which isolated *C.protothecoides* (ISIBES-101) was evaluate for cultivation under autotrophic and heterotrophic condition, with different nitrogen and organic carbon sources respectively. Both cultivation types were evaluated for biomass productivity, lipid production, cell composition, and fatty acid profile. Schematic presentation of present work with future work is shown in Fig–1.

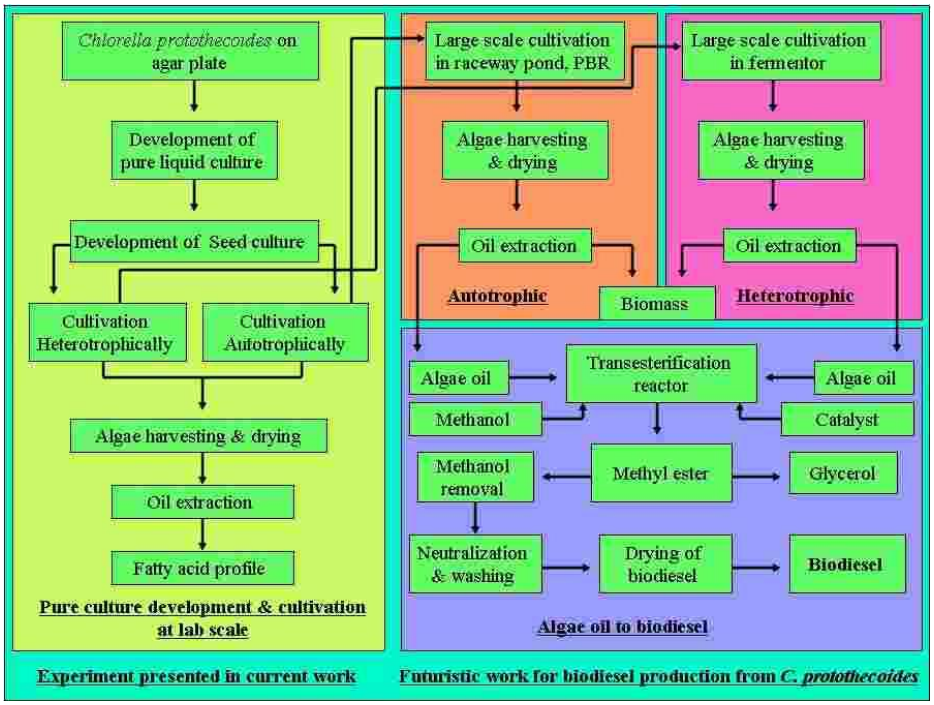

Fig 1 Schematic process flow diagram for biofuel production from *Chlorella protothecoides* (ISIBES- 101).

2. Materials and methods:

2.1 Algae strain:

Solid agar plate of *Chlorella protothecoides* (ISIBES - 101) was collected from Algal Biotechnology Laboratory, Department of Biosciences, Sardar Patel University, Anand – Gujarat. Liquid culture was developed from isolated colony as method describe by Senger. (Senger, et al., 1976).

2.2 Autotrophic cultivation:

Stock culture was developed aseptically from isolated algae colony by in to 50 ml flask containing 10 ml of sterile A1 medium (Table -1). Under exponential phase stock culture at 15 % v/v was further inoculated in to 500 ml flak containing 255 ml of A1 medium, same culture was used as seed culture. Two different autotrophic medium A1 and A2 were prepared as per ingredient mention Table 1. Medium was autoclaved at 121 ^{o} C, 15 psi for 30 min, and pH of medium was set to 6.5. The rotation speed and cultivation temperature were 125 rpm (REMI, India) and 29°C respectively. Flasks were kept (10 cm away) under, 3500 lux by daylight type fluorescent tubes using a 12 h light/dark photoperiod. The cultures were aerated with 0.45 l/min of air supplemental with CO_2 (50 %) from a cylinder, for five min in every hour during illuminated period of photoperiod. Mouth of flask was covered with cotton & aluminum foil to avoid any external contamination. Above mention cultivation condition was kept constant during complete experiment.

2.3 Heterotrophic cultivation:

As described in autotrophic cultivation, stock culture & seed culture were developed in H1 (Table -1) medium for heterotrophic cultivation. Two different heterotrophic medium H1 and H2 were prepared as per ingredient mention in Table 1. Analytical grade glucose was used in H1 medium (99.5 % pure), while maize- glucose a residue from maize processing unit was used as carbon source in H2 medium, it contained 85 % glucose. Two nitrogen source NH_4Cl (26 % nitrogen) in H1 medium and urea (46.6 % nitrogen) in H2 medium were used. Flasks were covered with cotton & aluminum foil and kept in dark at 29°C, air flow without CO_2 was passed for two min in every five hour interval. Initially all flasks were rotted at 125 rpm but during exponential phase flasks were rotted at 150 rpm. At regular interval both medium H1 and H2 were fed with glucose and maize-glucose respectively. Glucose level of medium was analyzed by method of Miller (1959).

2.4 Growth measurement:

Cultivation lasted for up to 20 days, with samples being taken, aseptically every 24 h. Sample was centrifuged at 4000 rpm (REMI, India) at 4 °C, algae pellet was wash with 0.01 N HCl followed by sterile water wash. Salt free biomass was used for dry weight determination (Fan, et al., 2000) and Maximum productivity (P_{max}) determination (Schmidell, et al., 2001). P_{max} was calculated according to the equation $P_{max} = (X_t - X_0)/(t - t_0)$, where X_0 is the initial biomass concentration g/l at time t_0, X_t is the biomass concentration in g/l at any time t subsequent to t_0. After 20 day total biomass was harvested by centrifugation and washed as mention above. Harvested biomass was dried in oven at 105 °C for 12 h, and used for measurement of cell composition analysis; such as total lipid (Bligh & Dyer, 1959), total carbohydrate (Dubois, et al., 1959) and total protein (Hartree, 1972). Ash and moisture of same algae biomass were determined by loss on drying (LOD) method.

2.5 Analytical methods:

Total lipids were extracted from dried algae biomass using a modified method of Bligh & Dyer (1959). Chloroform-methanol (2:1 v/v) was used as solvent for lipid extraction, phase was then separated into chloroform and aqueous methanol layer by addition of methanol and water to give a final solvent ratio of chloroform:methanol:water of 1:1:0.9. The chloroform layer was washed with 20 ml of 0.5 % NaCl solution, and evaporated to dryness. Total lipids were measured gravimetrically.

The fatty acids of oil were analyzed using the method of Lepage and Roy (1984). FAME was prepared by fifty-milligram sample saponified with a saturated KOH-methanol (1ml) solution at 75°C in water bath (Komal Scientific India) for 10 min, followed by addition of 5 % HCl in methanol at 75°C for methanolysis. Cool mixture at room temperature, and 2 ml sterile distilled water was added to separate fatty acid containing phase. Blowing hot air further

dried fatty acid containing phase and analyzed by gas chromatography. Comparing their retention time and fragmentation patterns with those standards identified the peaks in chromatogram.

Table 1: Media composition of autotrophic & heterotrophic cultivation of *C.protothecoides* (ISIBES – 101).

Ingredients	Medium for autotrophic cultivation		Medium for heterotrophic cultivation	
	A1	**A 2**	**H1**	**H 2**
K_2HPO_4 (g/l)	1.0	1.0	1.0	1.0
KH2PO4 (g/l)	1.0	1.0	1.0	1.0
$FeSO_4$ (mg/l)	5.0	5 .0	5.0	5.0
Thiamine HCl (µg/l)	10.0	10.0	10.0	10.0
NH4 Cl (g/l)	0.1	0.0	0.1	0.0
Urea (g/l)	0.0	0.1	0.0	0.1
Maize – Glucose (g/l)	0.0	0.0	0.0	12.0
Glucose (g/l)	0.0	0.0	12.0	0.0
A5 Liquid (ml/l) (2.86 g/l – H_3BO_3; 1.81 g/l – $MnCl_2 \cdot 4H_2O$; 0.222g/l -$ZnSO_4 \cdot 7H_2O$; 0.391 g/l-$Na_2MO_4 \cdot 2H_2O$; 0.079g/l – $CuSO_4 \cdot 5H_2O$).	1 .0	1.0	1.0	1.0

3. Results and discussion:

3.1 Axenic culture

Axenic cultures are required for lab scale experiment to understand clear-cut phenomenon of experimentation. And especially in heterotrophic cultivation axenic culture is must other wise resulted batch will be dominated by contaminant. Pure and isolated colony of *C. protothecoides* (ISIBES-101) was developed from collected solid agar plate which was contaminated with bacterial colony (Fig .2 – A). Serial plating under aseptic condition on fresh agar plate developed contamination free colony. Axenic culture of *C. protothecoides* (ISIBES-101) was developed from pure isolated colony by serial plating and was used further in experimentation.

3.2 Autotrophic cultivation

Under autotrophic condition *C. protothecoides* (ISIBES-101) (Fig 2 – B) was cultured with two medium A1 & A2 with different nitrogen sources ammonium chloride and urea respectively. Maximum dry weight 0.871 g/l was observed in A2 medium, while in A1 medium it was 0.812 g/l. As shown in Fig - 3, no significant difference was observed in dry weight yield with urea and ammonium chloride at 0.1 g/l concentration. Maximum productivity (P_{max}) of *C. protothecoides* (ISIBES-101) as a function of time is as shown in Table 2. P_{max} values were similar for *C. protothecoides* (ISIBES-101) when cultured in A1 and A2 medium with 0.1 % nitrogen source. One keen observation notice during experiment was continues growth of *C. protothecoides* (ISIBES-101) in A1 and A2 medium. Thus selected combination of medium would be used for long-term culture maintenance of *C. protothecoides* (ISIBES-101).

3.3 Heterotrophic cultivation

Heterotrophic cultivation of *C. protothecoides* (ISIBES-101) (Fig.2 – C) was performed with two different carbon and nitrogen sources, as mentioned in Table 1. Carbon source, in H2 medium was named as maize–glucose, the residual

waste discarded by Anil Starch Ltd - a maize starch processing company. Utilization pattern of glucose and maize-glucose in H1 and H2 medium respectively are shown in Fig – 3. Pure glucose in H1 medium easily uptake by *C. protothecoides* (ISIBES-101) as compared to maize-glucose. The cell density reached maximum value (17.180 g/l) after 11th day of cultivation in H1 medium, while the maximum value 17.140 g/l was achieved in H2 medium. Cultivation of *C. protothecoides* (ISIBES-101) was started with 12 g/l of substrate as carbon source, and cultivation batch was fed with fresh substrate at fifth and ninth day of cultivation. Utilization of substrate was slow during last days of cultivation, which has accordance with the steady productivity of dry weight as shown in Fig 4. Dry weight yield started to reduce in both H1 & H2 medium from twelfth day of cultivation, white precipitation was observed at the bottom of flask. May be due to some cultural response against metabolites secreted by *C. protothecoides* (ISIBES-101) during growth phase. Maximum P_{max} value 1.562 g/l/day (Table 2) was observed in H1 medium, while in H2 medium P_{max} value was 1.559 g/l/day (Table 2). The results of biomass yield and P_{max} value for *C. protothecoides* (ISIBES-101) cultivation with irrespective of substrate suggest that short cultivation duration will obtain maximum biomass.

3.4 Cell composition analysis

Dry biomass of autotrophically (Fig 2 –D) and heterotrophically (Fig 3 – E) cultured *C. protothecoides* (ISIBES-101) was used for cell composition analysis, and results were recorded in table 3. Dry biomass of *C. protothecoides* (ISIBES-101) was dominated with protein when grow autotrophically, while if grow heterotrophically biomass was contained high amount of lipid. The maximum protein content 55.23 □ 0.17 % was with A1 medium, while with urea as nitrogen source in A2 medium protein content was 49.61 □ 0.49 %. Lipid and carbohydrate content were also higher in *C. protothecoides* (ISIBES-101) cultivated in A2 medium as compared to C. *protothecoides* (ISIBES-101) cultured in A1 medium. Heterotrophic cultivation of *C. protothecoides* (ISIBES-101) with substrate maize-glucose (40.82 □ 0.12) resulted superior for lipid production to substrate glucose (38.57 □ 0.05). Several studies states *C. protothecoides* (ISIBES-101) under heterotrophic cultivation produce lipid up to 57.4 % (Wu and Miao, 2004) and 55.20 % (Xu, et al., 2006). While in present study average lipid percentage of *C. protothecoides* (ISIBES-101) was 39.69 %, which is lesser than the reported one. But again heterotrophic production of lipid by microalgae is dependent on many factors, such as culture age, media component, and environmental factors like pH, temperature, and salinity (Wen and Chen, 2003).

3.5 Fatty acid composition of oil

Table 4 shows the fatty acid composition of *C. protothecoides* (ISIBES-101) cultured under autotrophic and hetrotrophic cultivation in media containing urea and ammonium chloride as nitrogen source. It is predicted from Table 4 that irrespective of cultivation condition, oil extracted from *C. prototecoieds* (ISIBES-101) was dominated by long carbon chain fatty acid, over 94 % of total fatty acid (TFA) was consist of C_{16} (28.76 %) and C_{18} (65.07 %). The nitrogen source and concentration in the culture media has significant role in fatty acid composition of micro algae oil (Chen and Johns, 1991). Percentages of fatty acid were varied as the nitrogen source and cultivation methods differed. In general much higher biomass and lipid production can be achieved with fed-batch culture (Wei and Liu, 2008), same results were obtained in present study also. Present studies indicate, composition of oil affected by nitrogen sources rather the type of cultivation. In heterotrophically cultivation of *C.ptotothecoieds* (ISIBES-101), $C_{16:1}$ and $C_{16:2}$ were dramatically reduced and $C_{18:0}$ and $C_{18:1}$ were found higher in H2 medium as compared to H1 medium. For biodiesel production oil containing $C_{18:0}$ and $C_{18:1}$ is prime target of interest because they are responsible for industrial grade biodiesel quality (Meng, et al., 2009). Urea as nitrogen source was superior to ammonium chloride in heterotrophic cultivation, but still concentration of urea and feeding of maize-glucose can improve the biomass productivity and high quality lipid content.

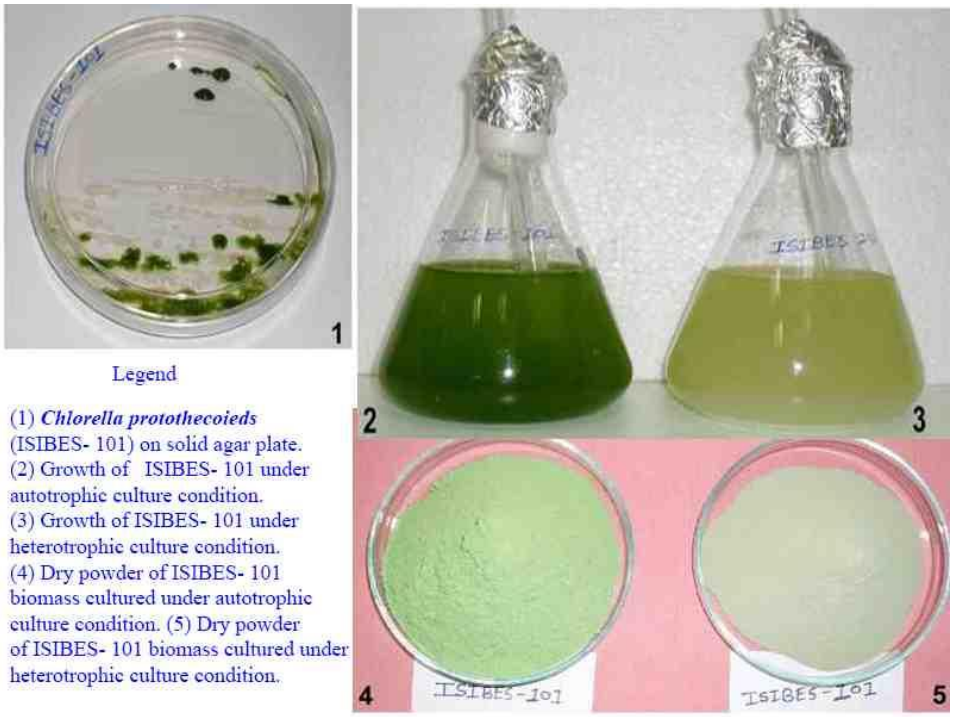

Fig 2. (1) *Chlorella protothecoides* (ISIBES- 101) on solid agar plate. (2) Growth of ISIBES- 101 under autotrophic culture condition. (3) Growth of ISIBES- 101 under heterotrophic culture condition. (4) Dry powder of ISIBES- 101 biomass cultured under autotrophic culture condition. (5) Dry powder of ISIBES- 101 biomass cultured under heterotrophic culture condition.

Table 2: Maximum Biomass Productivity (P_{max} (g/L/day) of *C.protothecoides* (ISIBES – 101) under autotrophic & heterotrophic cultivation for 14 days. Data are expressed as mean value of three replicate.

Time (days)	Maximum Biomass Productivity (dry weight) P_{max} (g/L/day)			
	Autotrophic cultivation		Heterotrophic cultivation	
	A 1	A 2	H 1	H 2
1	0.000	0.000	0.000	0.000
2	0.003	0.003	0.007	0.004
3	0.006	0.006	0.107	0.107
4	0.007	0.007	0.367	0.379
5	0.012	0.012	0.433	0.473
6	0.015	0.015	0.719	0.727
7	0.018	0.018	0.964	0.954
8	0.02	0.02	1.137	1.156
9	0.022	0.022	1.461	1.458
10	0.039	0.039	1.517	1.514
11	0.044	0.044	1.562	1.559
12	0.042	0.042	1.417	1.417
13	0.052	0.052	1.288	1.285
14	0.061	0.061	1.144	1.148

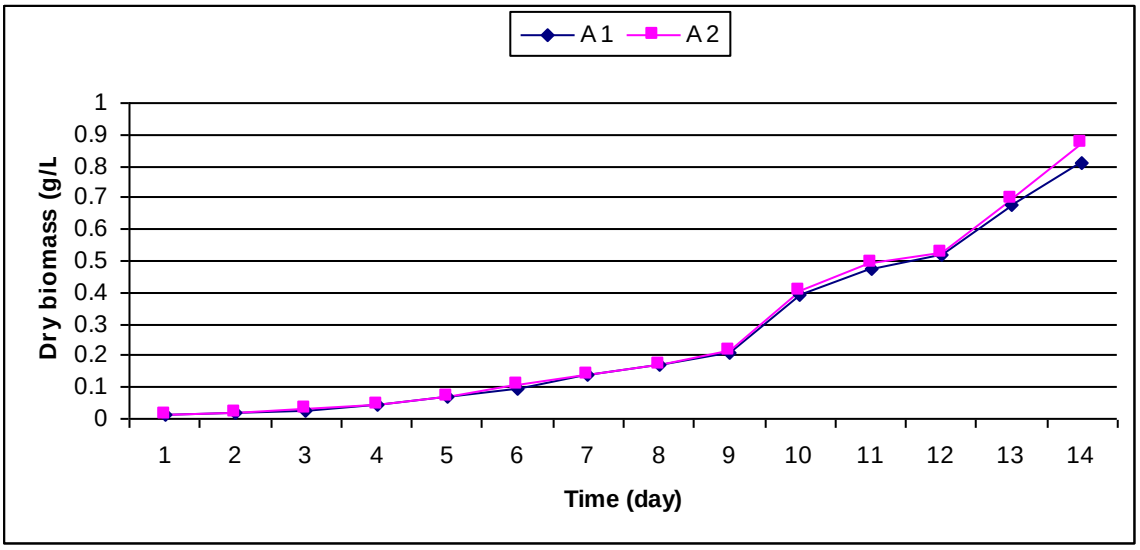

Fig 3. Growth curve of *Chlorella protothecoides* (ISIBES- 101) cultured autotrophically in A1 and A2 medium.

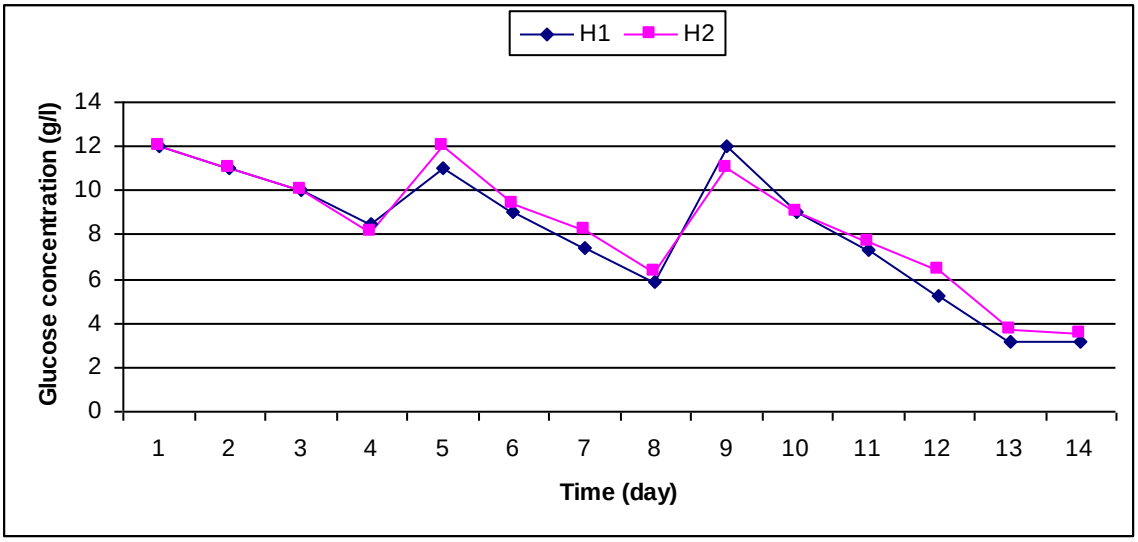

Fig 4. Glucose consumption curve of *Chlorella protothecoides* (ISIBES- 101) cultured heterotrophically in H1 and H2 medium.

Table 3: Cell composition of dry *C.protothecoides* (ISIBES – 101) cultivated under autotrophic & heterotrophic condition for 14 days. Data are expressed as mean ± SD of five replicate.

Cell component	Autotrophic cultivation		Hetrotrophic cultivation	
	A 1	A 2	H 1	H 2
Protein %	55.23 ± 0.17	49.61 ± 0.49	18.17 0.44	17.78 ± 0.08
Lipid %	10.00 ± 0.34	12.17 ± 0.38	38.57 ± 0.05	40.82 ± 0.12
Carbohydrate %	10.50 ± 0.27	11.83 ± 0.41	15.73 ± 0.83	15.18 ± 0.32
Ash %	07.50 ± 0.51	7.13 ± 0.68	08.61 ± 0.46	08.02 ± 0.03
Moisture %	08.60 ± 0.21	08.00 ± 0.28	08.50 ± 0.29	08.23 ± 0.17
Others %	08.17 ± 0.33	11.26 ± 0.17	10.42 ± 0.16	09.97 ± 0.21

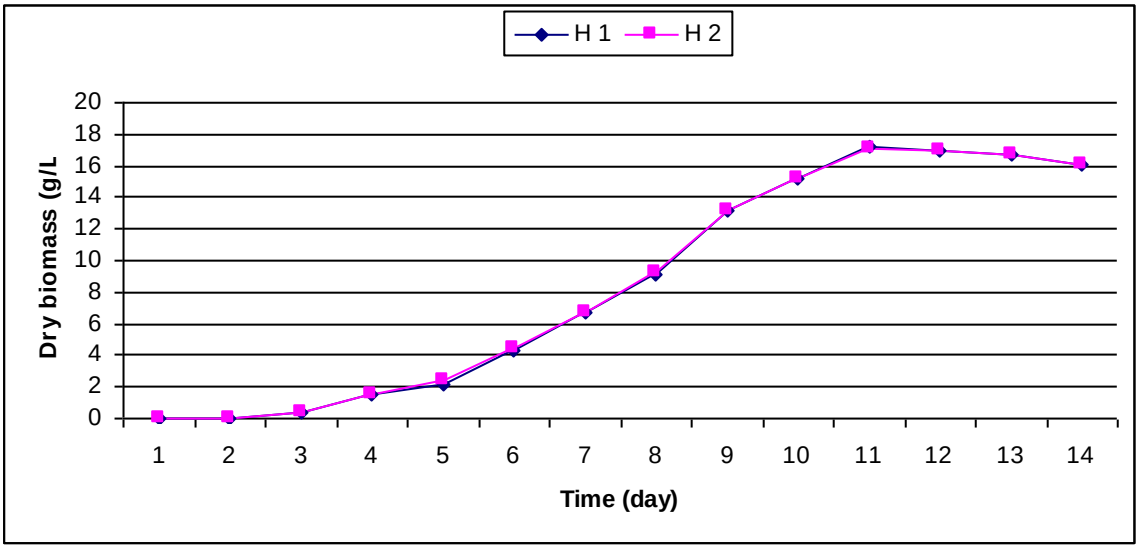

Fig. 5 Growth curve of *Chlorella protothecoides* (ISIBES- 101) cultured heterotrophically in H1 and H2 medium.

Table 4: Fatty acid composition of *C. protothecoides* (ISIBES – 101) cultured under autotrophic & heterotrophic condition for 14 days. Data are expressed as mean value of three replicate.

Fatty acid	Relative content %			
	Autographic cultivation		Hetrotrophic cultivation	
	A 1	A 2	H 1	H 2
$C_{15.0}$	1.07	1.27	0.81	1.23
$C_{16.0}$	15.36	13.32	14.78	12.70
$C_{16.1}$	5.08	6.76	7.06	2.18
$C_{16.2}$	7.81	6.23	7.21	2.33
$C_{16.3}$	0.51	1.10	1.21	2.10
$C_{18.0}$	12.01	12.78	11.53	13.03
$C_{18.1}$	35.3	37.1	29.2	39.11
$C_{18.2}$	15.57	14.1	19.21	18.10
$C_{18.3}$	2.19	3.11	3.91	3.26
$C_{19.0}$	2.45	2.38	3.48	3.77
$C_{20.0}$	0.31	0.37	0.32	0.48
$C_{21.0}$	0.22	0.31	0.27	0.05
Others	2.12	1.17	1.01	1.66

4. Conclusion:

Chlorella protothecoides (ISIBES – 101) can be cultivated in both autotrophic and heterotrophic condition. A cultivation type has no significant role in fatty acid composition of algae oil. Heterotrophic cultivation with fed-batch culture was more suitable for high cell density cultivation with high lipid content. Low price carbon source like maize-glucose with urea produce lipid with fatty acid composition, which is more suitable for high quality biodiesel production. Heterotophic cultivation is recommended to further cultivation in fed-batch mode.

5. Acknowledgement:

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