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Screening of micro algae for Growth and lipid accumulation properties

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Abstract:

Selection of appropriate native species/strains of micro algae that work well in local conditions is imperative for a viable algae-based biofuel production system. We have identified and isolated native predominant algal species of Himachal Pradesh India, which can serve as starting material for strain development. *Chlorella sp.* was dominant in most of the sample collections followed by *Scenedesmus quadricauda* and *Scenedesmus dimorphus.* The uni-algal culture were established and characterized for growth and lipid production potential. Overall biomass yield and biomass productivity of all the species was less when cultured in BG-11 medium compared to BG11+0.1%urea indicating urea can be used as an alternative nitrogen source for commercial-scale cultivation which is cheaper and easily available. *Scenedesmus dimorphus* produced highest lipid content while *Scenedesmus quadricauda* was less productive. Different isolates of *Chlorella sp.* were characterized for growth and lipid accumulation potential under normal growth conditions to understand variation for these traits so that *Chlorella sp.* Isolates with the best combination of lipid content, biomass and lipid productivity can be identified.

Keywords: biofuel; micro algae; native stains/isolates; biomass and lipid productivity

Introduction

Photosynthetic organisms are thought to provide an alternative renewable energy resource as these can effectively harvest and convert solar energy to a variety of energy dense biofuels. Recently micro algae are increasingly being recognized as a promising feedstock for biofuel production, due to higher oil productivity and non-competition with agricultural resources unlike oil producing crops. Compared with oil crops, micro algae have higher photosynthetic efficiency, faster growth, synthesize and accumulate larger quantities of lipids (Chisti et al. 2006, Chisti et al. 2007, Hu et al. 2008, Demirba et al. 2011). Algae as biofuel feedstock are attractive in India for several reasons. The tropical climate is conducive to grow various species of micro-algae. Most parts of the country experience clear sunny weather for 250 to 300 days per year with the sunshine hours ranging between 2,300 and 3,200 per year. It has at least 20 major river systems and huge costal line where water resources and waste land can be dedicated for algae cultivation. A large number of stationary carbon dioxide sources like thermal power plants, steel plants, cement plants, fertilizer plants, refineries, sugar/ethanol plants and petrochemical plants can provide opportunities for co-locating algae farms and recycling the CO_2. Huge livestock population and abundant domestic and industrial wastewater sources can provide renewable nutrients for algae farming. So the adoption of large-scale biodiesel production and consumption can potentially lower India's dependence on imports. Nevertheless, several issues need to be addressed for the development of biofuel industry based on micro algae as a feedstock. The production of biodiesel from algal oil is not yet competitive with conventional diesel and productivity does not satisfy the market needs. Much of the on-going research work is focused on a small number of fast-growing micro-algal species which have been found to accumulate substantial quantities of lipids, though under specific conditions. The success of large scale algae-based biodiesel production will also depend on the interaction of the production systems with the environment, besides the technological advancements. Thus the selection of appropriate micro algae amongst native species/strains is imperative. So the first step should be selection of promising strains that work well in local conditions which require exploration, collection, isolation identification and characterization of species/strains on the basis of their growth and lipid accumulation potential. Efforts also need to be focused to optimize production from selected stains and use them as starting point for genetic improvement. North-Western hilly states of India, including Himachal Pradesh have not been explored as yet for the occurrence and distribution of micro algal species, so as to identify dominant species with a potential to be used as a feedstock in biodiesel production. This is the first report on collection, isolation, identification and characterization of micro algae species from North western Himalayan state of India.

Materials and Methods

Collection of micro algae

The algal samples were collected from different water bodies such as streams, ponds and lakes from different geographical regions of Himachal Pradesh (Table 1 Fig. 1). At the time of sampling, 5-10 ml of water samples was enriched with BG-11 medium. Each sample was also preserved by adding 10% formaldehyde to 5 ml of sample. The samples were sub cultured in fresh BG-11 medium in the laboratory and allowed to grow for 15-20 days. The preserved samples were observed under the microscope to conferm identity of the algal flora present.

Medium and culture conditions

The algal samples were cultured in liquid and solidified, modified BG-11 medium, for, identification and isolation of uni-algal cultures. The culture flasks and plates were incubated at 25+_2^{0}C under 3000 Lux irradiance with 16:8 hours light and dark cycle. Three predominant species observed in the most of the samples were isolated and characterized for growth and lipid production in two media, BG11 and BG11 supplemented with 0.1g urea l^{-1} instead of $NaNO_3$.

Assessment of predominant species at different sites

This experiment was conducted to assess the relative prevalence of different micro algae species in the collected samples. The samples of liquid BG-11 medium were allowed to grow for 15-20 days in the laboratory. These were then diluted 10^4 times and were plated on BG-11 medium with 1.5% agar. The algal colonies appeared after two weeks, observed under microscope to record the prevalence of micro algae species in the collected samples. Thirty colonies were examined randomly from each sample and presence of different micro algae was recorded and converted into a percentage of colonies in which specie was observed.

Isolation of uni-algal cultures

The algal culture grown in the laboratory from each set of collection was serially diluted in 10^4 to 10^6 times and was plated on BG-11 medium with 1.5% agar. After 2-3 weeks, isolated colonies appeared. The individual colonies were carefully picked up from the agar plate and transferred onto a drop of BG-11 medium to a glass slide and cover slip was placed on (both alcohol wiped) and observed under the microscope for morphological identification. The uni-algal colony of target species observed so was transferred to 5 ml of BG11 medium after removing the cover slip with a micropipette. The colonies of non-target species and containing more than one species were discarded after examination. After 15-18 days, the 5ml of uni-algal culture were transferred to, 100 ml of sterile BG-11 medium and allowed to grow at 25^{0}C. The cultures were regularly observed for the purity of species.

Characterization of isolated species for growth and lipid content

This experiment was conducted to characterize and compare the growth and lipid accumulation by three algal species viz. *Scenedesmus quadricauda*, *Scenedesmus dimorphs* and *Chlorella sp.* cultured on BG11 and BG11 medium with 0.1g urea l^{-1} instead of $NaNO_3$ as a nitrogen source. In this experiment, six culture flasks containing 800 ml medium were inoculated with 80 ml of full grown (stationary phase) inoculum of each species (10%v/v) and observations for growth parameters were taken on every 4th day. A direct microscopic cell count (cells/ml^{-1}) was performed on micro algal suspension cultures using a haemocytometer. Optical densities of the micro algal suspension cultures were measured as absorbance at 730 nm with the help of *ELICO SL-159* UV-VIS spectrophotometer. At the end of the experiment when the stationary phase of growth reached, cultures were centrifuged to harvest cell biomass, which was freeze dried to determine dry biomass content. Lipid extraction from micro algae was done using Bligh and Dyer method with minor modifications (Bligh and Dyer 1959) and total lipid content quantified in term of %age of dry biomass. The known amount of freeze dried biomass were vortexed with chloroform: methanol 1: 2, sonicated in an ultrasonic bath for 5 minutes and kept on shaker for overnight. Next day an equal volume of chloroform : distilled water (1:1) was added, vortexed and then centrifuged at 6000x g for 10 minutes. Lipids being soluble in the chloroform form a dense layer at the bottom of the centrifuge tube, methanol and water create a uniform top layer, while cell debris creates a middle layer. The chloroform lipid was taken with the help of micropipette by applying gentle positive pressure. The lipid collected was passed through a 2.5 cm thick layer of anhydrous sodium sulfate using whatman filter paper in a funnel into a pre-weighed container suitable for a rotary evaporation. The solvent was removed using a rotary evaporator under reduced pressure at 60˚C and weight of remaining lipids was recorded. Following parameters were calculated from the observed data to assess the growth characteristics

- **Specific Growth rate (μ)** was measured with the help of equation $\boldsymbol{\mu = ln\ (N_t/N_0)\ /T_t\text{-}T_0}$ where $\boldsymbol{N_t}$ is the number of cells at the end of log phase, $\boldsymbol{N_0}$ is the number of cells at the start of log phase, $\boldsymbol{T_t}$ is the final day of log phase and $\boldsymbol{T_0}$is starting day of log phase.
- **No. of divisions per day:** Growth rate (***μ***) can be converted to division or doublings per day (K) by dividing (***μ***) by the natural log of 2 i.e. **K= μ/0.6931**
- **Doubling Time: Tt = 0.6931/μ**
- **Biomass productivity (Pdwt)** : as the dry biomass produced (in grams per liter per day)

- **Total lipids content (Lc):** as percentage of the total biomass (in % dwt)
- **Lipid productivity (Lp):** Calculated according to the equation Lp=Pdwt×Lc×1000/100 and expressed as milligrams per liter per day.

Characterization of Chlorella sp.isolates of for growth and lipid content

This experiment was conducted to figure out variation for growth and lipid production among isolates of *Chlorella sp*. The isolates of *Chlorella sp.* were cultured in triplicate in one liter flasks containing 800 ml BG11 medium and 80 ml of full grown (stationary phase) inoculum (10%v/v). The observation for growth parameters as described above were taken on every 3rd day. At the end of the experiment when the stationary phase of growth reached, dry biomass and lipid content were estimated as discussed above by Bligh and Dyer method.

Results and Discussion

Collection and assessment of predominant of micro algae Species

Fourteen, out of 21 samples collected from different locations responded well and green algal growth could be seen within 10-15 days after inoculation in BG11 medium. The samples having negligible initial inoculums might have failed to respond to growth. *Chlorella sp.*, *Scenedesmus quadricauda*, *Scenedesmus dimorphus*, *Microcystis*, *Closterium* and *Anabena*, and diatoms were detected in most of the preserved samples. One of the goals of this survey was to identify and isolate native dominant algal species to serve as starting material for strain development. *Chlorella sp*. was the dominant in nine out of fourteen samples and the percentage of colonies in which this species was observed ranged from 8.33 to 90.62 in different samples (Table 1). *Scenedesmus quadricauda* and *Scenedesmus dimorphus* showed dominance in three and two samples respectively but followed *Chlorella sp.* in the rest of the samples with presence in 11.81-95.83 and 9.37-65.62 percent of the observed algal colonies grown from the samples under observation. Elumalai et al. (2011) also found *C. Vulgaris* as a predominant micro algae in many samples isolated from different locations of Tamil Nadu having different environmental conditions like temple tanks, rock ponds, forest lagoons and inland lakes. Freshwater *Chlorella sp.* have been grown for over 30 years in the health food market. It is also an important alga in some aquaculture feeds and wastewater treatment and has a great potential as a resource for biodiesel production due to faster growth, and already available cultivation technology. Recently there are many reports in scientific literature on standardization and refinement of cultivation of *Chlorella sp.* for increased lipid accumulation and large scale biodiesel production (Makareiciene et al. 2011, Wijanarko et al. 2008, Leesing et al. 2011). Many workers have reported *Scenedesmus sp.* as potential source of algal oil in term of productivity and oil quality (Nascimento et al. 2013, Prabakaran and Ravindram 2012, Makareiciene et al. 2011, Goswami and kalita 2011) concluded *Scenedesmus sp.* best in terms of biomass and lipid productivity among freshwater alga they tested. So it will be prudent to explore strain diversity of these micro algae prevalent in North-Western hilly states of India for traits influencing lipid productivity.

Table 1 Prevalence of dominant micro algae species in different sample collections

Sites Codes	**Sites**	**Percentage of colonies in which micro algae species were observed**		
		S.quadricauda	*S.dimorphus*	*Chlorella sp.*
01	Chamkhari pull (Shala), Bilaspur (H. P.)	25.80	22.58	**87.09**
02	NalwarJukhala Farm, Small bridge, Bilaspur (H. P.)	29.59	39.64	**43.35**
03	Fish Breeding Farm (Deoli), Bilaspur (H. P.)	**95.83**	33.33	08.33
04	Seer Khad (Ghumarwin), Bilaspur (H. P.)	39.25	32.56	**41.52**
05	Rewal Tal (Dehra), Bilaspur (H. P.)	37.50	09.37	**90.62**
11	Kuhan Khad Rangus, Hamirpur (H. P.)	**51.25**	38.86	47.56
12	Deharian Kuan, Kangra (H.P.)	42.25	40.50	**52.12**
14	Baner Khad, Kangra (H.P.)	11.81	**62.50**	43.75

15	Kapur Sagar (Kangra Fort), Kangra (H.P.)	37.50	**65.62**	37.50
16	Fish Breeding Farm (Gupt Ganga), Kangra (H.P.)	28.12	09.37	**62.50**
18	Ratti Khad, Mandi (H.P.)	48.38	45.16	**51.61**
19	Rewalshar Lake, Mandi (H.P.)	**95.83**	33.33	8.33
20	Waknaghat, Solan (H.P.)	42.50	31.25	**45.20**
21	Gambhar Khad, Solan (H.P.)	50.00	40.62	**62.50**

Fig. 1 A-D A few locations from where samples were collected; Kapur Sagar. B) Fish Breeding Farm Kangra C) Rewalshar Lake D) Baner Khad

Isolation of uni-algal cultures

Uni-algal cultures of three micro algae viz. *Chlorella sp.*, *Scenedesmus quadricauda* and *Scenedesmus dimorphus*, from all the samples were established. These isolates have also been cryopreserved using 10% methanol (Liu et al. 2008) and 10% DMSO as cryoprotectant and kept at -80 ^{0}C for future use.

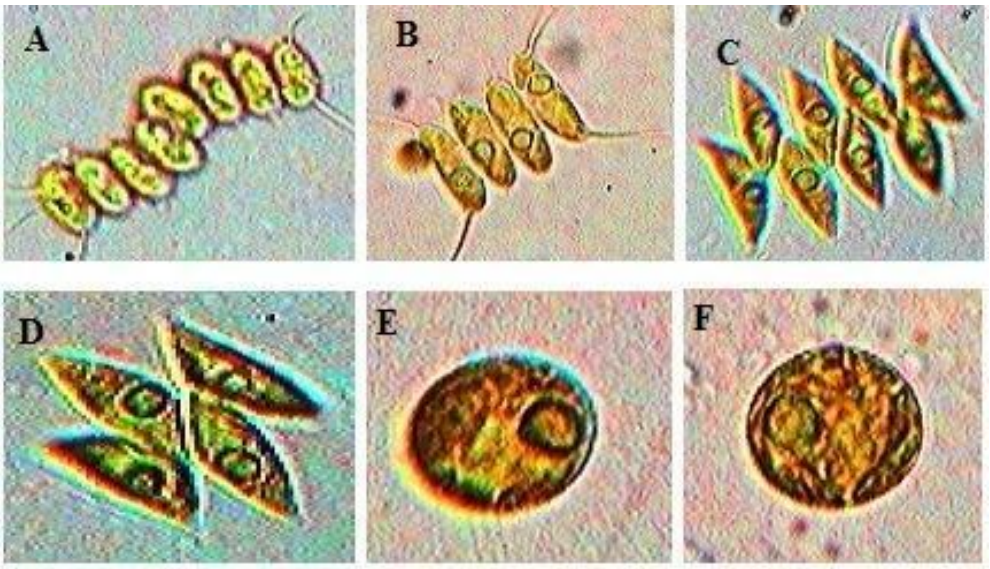

Fig. 2 Cell morphology of Micro algal species: A & B) *Scenedesmus quadricauda,* C & D) *Scenedesmus dimorphus,* E & F)

Chlorella sp.

Characterization of isolated species for growth and lipid content

Fig. 3 shows the progress of growth of three micro algae species measured as optical density (OD) of cultures at 730 nm, cultured in BG11+0.1% urea. It is evident that all the species grew exponentially devoid of lag phase, followed by a stationary and decline phase. Micro algae species picked up rapid growth with no lag phase as sufficient amount of inoculums were taken from exponentially growing cultures under similar growth conditions of light, temperature, pH and medium with little variation. The optical density of the culture of all three species reached a maximum on the sixteenth day of cultivation on BG11+0.1% urea, as compared to nineteenth day on BG11 medium indicating that urea as a nitrogen source derive better growth of all the three species under experimentation. Highest optical density (OD) was observed for *Scenedesmus quadricauda* followed by *Scenedesmus dimorphus* and least for *Chlorella sp*. A similar trend of growth was observed when growth was measured as the number of cells per ml of cultures in both the media as depicted in Fig. 4. The results of growth kinetics parameters, specific growth rate and growth rate constant K and doubling time are presented in Table 3. By comparing these parameters, it can be concluded that all the micro algae species grew faster in BG11+0.1% urea as compared to BG11 medium. Doubling time of three species ranged from 5.096 to 6.19 days. *Chlorella sp.* and *Scenedesmus quadricauda* recorded least and highest on BG11+0.1% urea and BG11 medium respectively. Nitrogen source is one of the major factors which affect lipid accumulation in algae. Urea is used as a nitrogen fertilizer, so it can be a good nitrogen source for commercial-scale cultivation of micro algal biomass due to its cheaper costs and availability.

The Micro algal biomass were harvested at the end of an experiment and used to determine dry biomass yield, biomass productivity, lipid content and lipid productivity (Table 2). Highest biomass yield and productivity was observed in *Scenedesmus dimorphus* cultured on BG11+0.1% urea followed by *Chlorella sp*. Overall biomass yield and biomass productivity of all the species was less when cultured in BG-11 medium compared to BG11+0.1% urea.

The lipid production potential of the micro algae was evaluated under normal growth conditions in order to reveal their genetic potential to produce and accumulate lipids. *Scenedesmus dimorphus* produced highest lipid content of 27.58% and 25 % in BG11+0.1% urea and BG11 media, followed by *Chlorella sp.* showing lipid content of 18.42% and 17.14% in the same media respectively. The lipid content of the species under investigation is in agreement with that of observed by Rodolfi et al. 2009, but the biomass productivity of these native species was low as compared to the earlier reports.

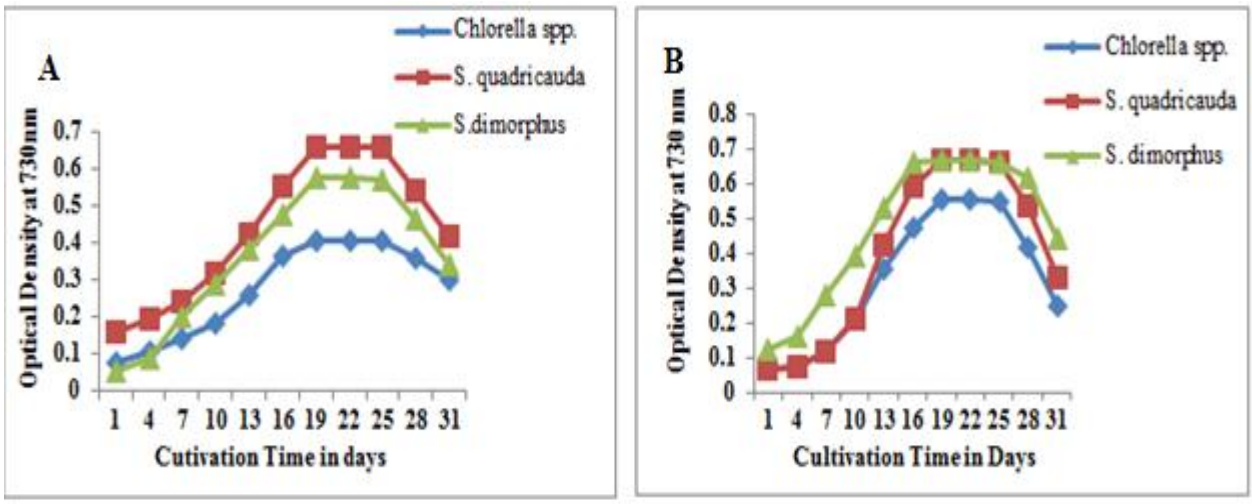

Fig. 3 Growth response of *Chlorella sp.*, *Scenedesmus quadricauda* and *Scenedesmus dimorphus,* measured as optical density of cultures at 730nm; A) Cultured in BG11+0.1 % urea in place of $NaNO_3$. B) Cultured in BG11 media

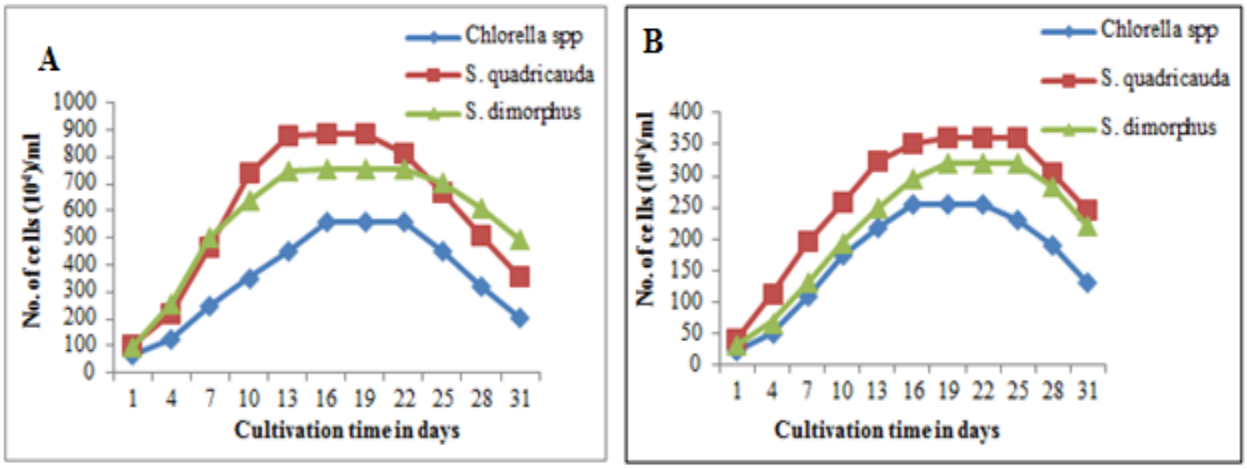

Fig. 4 Growth response of *Chlorella sp.*, *Scenedesmus quadricauda* and *Scenedesmus dimorphus,* measured as number of cells per ml of cultures; A) Cultured in BG11+0.1 % urea in place of $NaNO_3$ B) Cultured in BG11 media.

Table 2 Growth kinetics, lipid content, and lipid productivity of micro algae

Parameters / **Species/Media**	**SGR** (μ)	**DPD** (K)	**Tt**	**TB** ($g\ l^{-1}$)	**Pdwt** ($gl^{-1}\ day^{-1}$)	**Tc** (%)	**Lp** ($mg\ l^{-1}\ day^{-1}$)
S.quadricauda (BG11+0.1 Urea in place of $NaNO_3$)	0.135	0.195	5.134	0.16	0.008	10.25	0.82
S.dimorphus (BG11+0.1 Urea in place of $NaNO_3$)	0.130	0.188	5.331	0.20	0.01	27.58	2.75
Chlorella sp (BG11+0.1 Urea in place of $NaNO_3$)	0.136	0.196	5.096	0.16	0.01	18.42	1.84
S.quadricauda (BG-11)	0.112	0.162	6.190	0.18	0.009	7.08	0.57
S.dimorphus (BG-11)	0.121	0.175	5.728	0.20	0.01	25	2.5
Chlorella sp (BG-11)	0.130	0.188	5.331	0.16	0.008	17.14	1.37

SGR= Specific Growth Rate, DPD= Doubling Per Day, Tt= Doubling Time, TB= Total Biomass, Pdwt = Biomass Productivity, Tc= Total Lipid and Lp= Lipid Productivity.

Characterization of isolates of Chlorella sp. for growth and lipid content

The data pertaining to characterization of different isolates of *Chlorella sp.* measured as growth parameters, biomass and lipid productivity are presented in Table 4. There was lot of variability among isolates of *Chlorella* with respect to the growth parameters, biomass and lipid productivity. Specific growth rate (U) and Doubling Time (T_t) varied between 0.126-0.183 and 3.787- 5.500 days whereas biomass productivity ranged between 0.006-0.019$gl^{-1}day^{-1}$. The lipid content and lipid productivity varied between 7.066 - 27.60 % of dry biomass and 0.748 - 5.381 mg $l^{-1}day^{-1}$, respectively among *Chlorella sp.* isolates. The data on specific growth rate, lipid content is in agreement with previous findings for the *Chlorella sp.* (Rodolfi et al. 2008, Leesing et al. 2011) although biomass and lipid productivity of *Chlorella sp.* was higher in many of earlier reports (Rodolfi et al. 2008, Leesing et al. 2011, Nascimento et al. 2013) than in the present investigation. Kong et al. 2011 studied biomass production and lipid accumulation characteristics of

Chlorella vulgaris under photoautotrophic, mixotrophic and heterotrophic nutritional modes and found mean biomass content 0.29 g l^{-1}, biomass productivity 0.05g l^{-1} day^{-1}, lipid productivity 3.331 mg l^{-1} day^{-1}under photoautotrophic mode, which are in agreement and comparable with that of many isolates in this investigation. Although, in general, productivity and lipid content in algae are inversely related (Illman et al. 2000) one of the aims of this study was to find *Chlorella sp.* isolates showing the best combination of lipid content, biomass and lipid productivity under normal growth conditions. The isolates no. 1, 3, 11, 14, 15, 16 and 20 showed relatively faster growth with specific growth rate more than 0.150 and doubling time less than 5 days, but lipid content of these isolates except for isolate no. 16 and 20 was less than 15%. This confirms the established fact that fast growth rarely correlates with high lipid content and productivity. The isolates showing the best combination of lipid content, biomass and lipid productivity were isolate no. 16 (Fish Breeding Farm Kangra), 20 (Waknaghat Solan) and 21 (Gambhar Khad Solan) with lipid content more that 20%, lipid productivity higher than 4.0 mg l^{-1} day^{-1} and doubling time less that 5 days. *Chlorella* sp. has a great potential as a resource for biodiesel production due to faster growth and easier cultivation. However, lipid content in most of *the Chlorella sp.*strainsunder general growth conditions is up to ~20% by weight of dry biomass which cannot meet the standard industrial requirements (Illman et al. 2000, Spoiler et al. 2006). The literature on the characterization of native isolates of algae for biomass growth and lipid production is very scarce, most of the work relates to the testing of micro algae belonging to different genera or species or a few strains/isolate of a species taken from the culture collections. The hypothesis of this study was that genetic variation could exist in native isolates of an algae species for various traits of importance to be useful as feedstock. The isolates with the best combination of such traits selected so can be improved upon later by genetic engineering approaches and or by standardization of appropriate production methods. In this endeavor some promising candidates of *Chlorella sp.* native isolates have been identified.

Table 3 Growth kinetics, lipid content and lipid productivity of *Chlorella sp.* Isolates

Isolates	**SGR** (μ)	**DPD** (K)	**Tt**	**TB** (g l^{-1})	**Pdwt** (gl^{-1} day^{-1})	**Tc** (% w/v)	**Lp** (mg l^{-1} day^{-1})
1	0.153	0.220	4.530	0.086	0.006	14.97	0.748
2	0.131	0.189	5.290	0.133	0.010	12.52	1.252
3	0.166	0.239	4.175	0.200	0.015	13.25	1.988
4	0.126	0.181	5.500	0.230	0.018	8.69	1.625
5	0.174	0.251	5.211	0.246	0.019	9.50	1.805
11	0.183	0.267	3.787	0.230	0.016	12.46	1.994
12	0.147	0.212	4.714	0.190	0.015	8.62	1.293
14	0.178	0.256	3.893	0.186	0.014	7.06	0.989
15	0.172	0.248	4.029	0.200	0.015	8.09	1.214
16	0.173	0249	4.006	0.290	0.015	27.60	4.140
18	0.144	0.203	4.813	0.170	0.017	13.92	2.366
19	0.135	0.194	5.134	0.189	0.015	10.18	1.527
20	0.182	0.262	3.808	0.266	0.024	22.42	5.381
21	0.148	0.213	4.683	0.233	0.018	25.66	4.189

SGR= Specific Growth Rate, DPD= Doubling Per Day, Tt= Doubling Time, TB= Total Biomass, Pdwt = Biomass Productivity, Tc= Total Lipid and Lp= Lipid Productivity.

Conclusions

Chlorella sp. followed by *Scenedesmus quadricauda* and *Scenedesmus dimorphus* were the dominant micro algae species of the region sampled. All the isolated micro algae species grew better when urea was used as nitrogen source compared to $NaNO_3$ in BG11 media. *Scenedesmus dimorphus* produced highest lipid content while *Scenedesmus quadricauda* being least productive. A few promising native isolates of *Chlorella sp.* showing the best combination of lipid content, biomass and lipid productivity under normal growth conditions were identified.

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