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Isolation and Culture Condition Optimization of *Chlorella vulgaris*

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Abstract
Chlorella vulgaris Beyerinck (Beijerinck) is one of the fast growing planktonic microalgae predominantly found in lentic habitats. It was isolated from water samples using BG 11 medium and identified and screened for mixotrophic growth using glucose as carbon source in the medium. The growth of this algae was also studied for autotrophic and mixotrophic regimen. It showed higher growth in a medium of pH 7 containing glucose rather than in autotrophic condition. Similarly, its growth was also studied in media containing different nitrogen sources in which $NaNO_3$ gave the best result. A pilot scale culture of *C. vulgaris* was performed in 5 l fermenter illuminated with fluorescent tube of 36 *W* maintaining 16:8 hrs of light and dark period with pH 7, temperature at 25°C, stirrer rate at 75 rpm, air flow rate at 30 L/hr. The specific growth rate was found to be 6.879 cells/ ml/ day whereas doubling time was found to be 2 hrs and 9 mins. The cells were harvested by sedimentation and centrifugation followed by drying at 50°-80°C. The dried biomass was treated with dichloromethane: methanol (2:1) v/v solution in Soxhlet extractor for 3-4 hrs followed by evaporation and extraction of non-polar lipid by hexane. The total lipid obtained was 15.53%.

Key words: biofuel, *Chlorella vulgaris*, microalgae, mixotrophy

Introduction
Chlorella vulgaris Beyerinck (Beijerinck) is a green coccoid species of single celled fresh water microalgae belonging to Chlorellaceae family. The cells are slightly ellipsoidal to spherical in shape, about 2 to 10 ì m in diameter. The cells are without flagella and have thin cell wall. It can grow prolifically doubling its biomass weight in few hr of sunlight. So, *C. vulgaris* is known as one of the fastest growing microalgae and includes a reasonable amount (14-30%) of triacylglycerol. The oil yield in microalgae can exceed 75% by weight of dry biomass under different circumstances (Christi 2007, Hu *et al.* 2008). *Chlorella* contains green photosynthetic pigments known as chlorophyll-a and -b in its chloroplasts. Through photosynthesis, it multiplies rapidly, requiring only carbon dioxide, water, sunlight, and a small amount of minerals to reproduce. *Chlorella* has much higher utilization rate (10-20%) of light energy for photosynthesis when compared to common plants (Zhang *et al.* 2000). According to Belasco (1997) and Zelitch (1971), it contains 45% protein (w/w, dry basis), 20% fat, 20% carbohydrates, 5% fibre and 10% minerals and vitamins. *Chlorella* is the richest source of chlorophyll which is widely used as a health food and feed supplement, as well as in the pharmaceutical and cosmetics industry (Sharma *et al.* 2011). It has been produced commercially in several countries for its use as neutraceutical food and medicinal purpose due to its valuable contents particularly pigments and proteins (Sharma *et al.* 2011). Similarly Â-1, 3-glucan is one of the most important substances in *Chlorella* with a good capacity for scavenging radicals and reducing blood lipids. This compound is also an immunostimulator. It has also indicated certain health benefits in gastric ulcers and wounds and prevents atherosclerosis, hypercholesterolemia and tumour effects (Spolore *et al.* 2006). Production of algal biomass can be worthwhile when considering the high-value

metabolites that can be obtained from these sources. However, lipids content in *C. vulgaris* under general growth conditions is up to ~20% by weight of dry biomass (Illman *et al.* 2000). Mixotrophic algae can grow both autotrophically and heterotrophically. It could undergo photoautotrophic, photoheterotrophic, heterotrophic as well as mixotrophic metabolism (Edberg 2010).

Compared with other superior plants, microalgae have higher photosynthetic efficiency, higher biomass productivities, faster growth rates and higher ability to fix CO_2 with an efficiency of 10-15 times more. This aspect, together with high intracellular lipid content, can potentially make a number of unicellular algae species among the most efficient producers of lipids of the planet (Scarsella *et al.* 2010). The use of algae as energy crops has potential, due to their easy adaptability to growth conditions, the possibility of growing either in fresh or marine waters and avoiding the use of land (Khola & Ghazala 2012).

Methodology

Isolation, identification and preservation

Water samples were collected from the College premises of AITM, Khumaltar, Lalitpur, Nepal and also from ponds of Godavari Lalitpur, Nepal. pH of the water samples were measured with pH meter. Samples were serial diluted and cultured in the modified BG11 medium (Geeta & Rani 2011). Instead of ferric ammonium citrate, ferric citrate and ammonium sulphate were added in the BG 11 medium. The *C. vulgaris* was identified by microscopic observation and photomicrograph. Since it is necessary to preserve the culture as a stock, cryovials filled with sterile glycerol inoculated with axenic culture of *C. vulgaris* and stored at -20°C refrigerator.

pH optimisation

The effect of pH on growth of the algae was studied using modified BG 11 media in the pH range of 4, 5, 6, 7, 8, 9 in 250 ml conical flasks. All the flasks were inoculated uniformly at 15% (v/v) inoculum and incubated at 25± 1ºC temperature and 16:8 hrs light: dark cycle. Optical density (using spectrophotometer) and cell count (using hemocytometer) were done every 24 hrs. After 10 days of culture, the cells were harvested by sedimentation, centrifugation and drying on oven and dry weight was measured (Dayananda *et al.* 2007).

Selection of growth regimen

For observing the autotrophic and mixotrophic growth condition of *C. vulgaris*, the algae were cultured in BG11 medium with and without glucose. pH of both the mediums were adjusted and sterilized. The medium with glucose was inoculated and incubated in the incubator at 25^0C without light whereas the culture flask without glucose was incubated at 25^0C with fluorescent illumination with light and dark ratio of 16:8 hours. The biomass in each culture flasks were determined by observing the optical density at 686 nm at regular interval of time i.e. 24hrs.

Selection of nitrogen source

Suitable nitrogen source for optimum algal growth was determined by subjecting the algal culture to media containing different sources of nitrogen such as $NaNO_3$, KNO_3, NH_4NO_3, urea and nitrogen deficient. Culture flasks with sterile medium were inoculated and incubated in the environmental chamber at 25°C, with fluorescent illumination. Optical density (686 nm) and cell counts were done. Dry cell weight was recorded after harvesting.

Scaling up and biomass production

The fermenter used for the experiment was biotech-5BG fermenter (Baoxing Bio-engineering equipment Co., ltd (BXBIO)) for algal culture application. In the present investigation fermenter of working volume 5.0 L was used. Basal heating system and an internal cooling coil was used to maintain 25°C. The power controller module has the controls for the temperature, agitation, rotameter and cooling water valve. Additional control module includes pH, level controller. 1N HCl acid and 1N NaOH were used to control the pH. A program was set with input of necessary parameters set values and the fermentation time was set to 6 days.

Fermenter sterilization

The fermenter vessel was filled with growth media BG11 with glucose (3L). Finally the whole fermenter vessel along with the media inside was placed inside an autoclave (YXQ-LS-SII vertical type autoclave) and sterilized. The acid and the alkali were also autoclaved.

Inoculum preparation and culture

About 15% of the algal suspension was inoculated into the fermenter vessel. All the parameters in the fermenter were set as shown in Table 1. with 16:8 hrs of light: dark period through external fluorescent tube light.

Table 1. Setting fermenter parameters

S.N	Parameters	Set value
1.	Pressure	0.05MPa
2.	Air flow rate	30 L/hr
3.	Rate of stirring	75RPM
4.	O_2 Saturation	50 %
5.	pH	7
6.	Temperature	25°C

Biomass estimation

The biomass in each culture flask as determined by measuring optical density at 686 nm, cell count and dry cell weight.

$$Biomass\ (g./ml) = \frac{Wt.\ of\ falcon\ tube\ with\ biomass\ (-)\ Wt.\ of\ empty\ falcon\ tube}{Volume\ of\ sample\ (ml)}$$

Determination of growth rate

The exponential (straight line) phase of growth was carefully determined and specific growth rate was obtained using following equation.

$$ì = \ln (N_t/N_o)/T_t - T_o$$

Where, N_t is the no of cells at the end of log phase; N_o is the no of cells at the start of log phase; T_t is the final day of log phase and T_o is the starting day of log phase. If T expressed in days from the growth rate (ì) can be converted to division or doublings per day (k) by dividing (µ) by the natural log of 2(0.6931).

$$K = \mu / 0.6931$$

Doubling time (T_t) which is calculated by the following formula.

$$T_t = 0.6931/ì$$

Lipid extraction

After 6 days of growth in the fermenter the cells were harvested and collected by centrifugation, then dried at 80°C for 24-48 hrs. The resultant cells were disrupted for lipid extraction using organic solvent (dichloromethane: methanol (2:1) v/v solution) in Soxhlet apparatus for 3-4 hours followed by evaporation of the solvents. The total lipid was determined gravimetrically and hexane with small amount of water was added to the lipid fraction, vortexed and centrifuged. The supernatant was collected and the remaining residue was again washed with hexane. All hexane fractions were mixed and evaporated. The remaining substance was weighed as non-polar liquid whereas solid residue as polar fraction (Scarsella *et al.* 2010)

Results and Discussion

Isolation and identification of *Chlorella vulgaris* from various sites

The distinct single algal colonies obtained after the spread plating of water samples were streaked on sterile BG11 and incubated. The algae obtained were characterized through microscopic observation and photomicrographs.

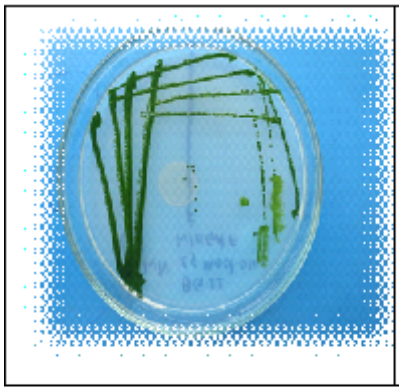

Photo 1. Streaking of single colony in BG11 Agar plate from Spread plate

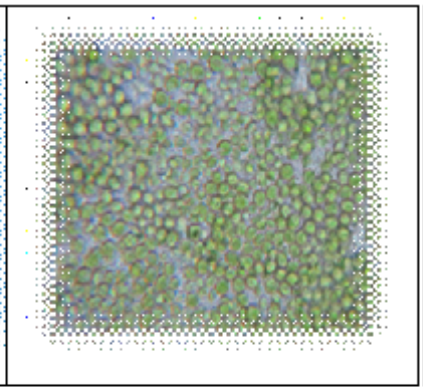

Photo 2. Microscopic observation of Cell under 40×Maginification

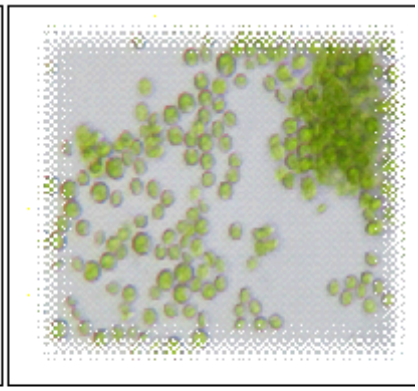

Photo 3. Photomicrograph of *Chlorella vulgaris*

pH optimization

The optimum pH for *C. vulgaris* was found to be 7 (Figures 1-3). The highest OD value was 5.100 at 686 nm for pH 7 along with highest cell count (1.227×10^8 cells/ ml) and dry weight of 3.34g/l. According to Lustigman *et al.* (1995), it is desired to strive for pH 6. However, our study showed that the optimum pH for *C. vulgaris* was pH 7. The difference in optimum pH obtained in our research and in the above mentioned research article was may be due to different strains and different culture conditions.

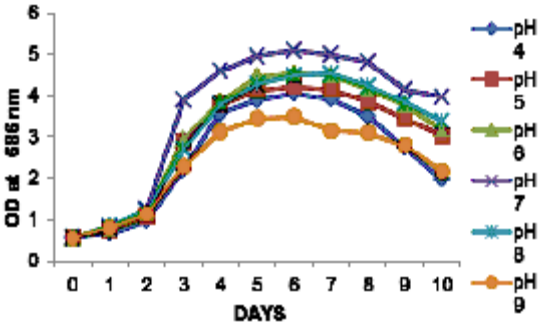

Fig. 1. Growth response of *C. vulgaris* at different pH (OD vs days)

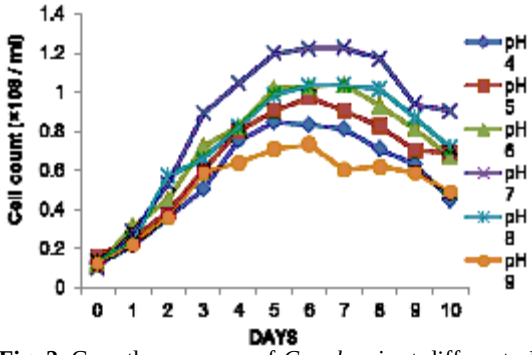

Fig. 2. Growth responses of *C. vulgaris* at different pH (Cell count vs.days

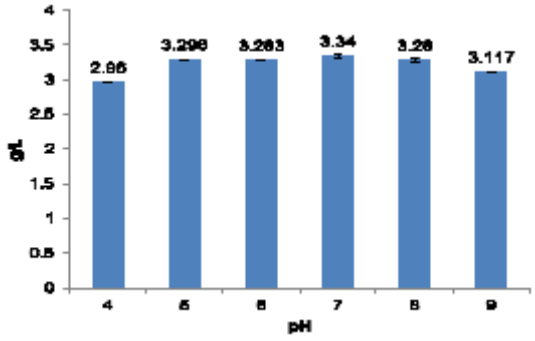

Fig. 3. Dry weight of cells in g/l vs. different pH

Selection of growth regimen

The growth in mixotrophic culture was observed to be much higher than in autotrophic culture as shown in Fig. 4). This indicates that isolated *C. vulgaris* can grow both autotrophically and mixotrophically. In mixotrophic culture the cell grew so rapidly that the log phase started within 2nd day and ended in 7th day whereas in case of autotrophic culture the cell grew so slowly that even on obtaining the decline phase of mixotrophic culture, the log phase had not attained in autotrophic culture. Thus, the *C. vulgaris* showed the best growth at mixotrophic than autotrophic conditions. Hence, for the maximum growth within short a period of time, we chose the mixotrophic culture for our further study.

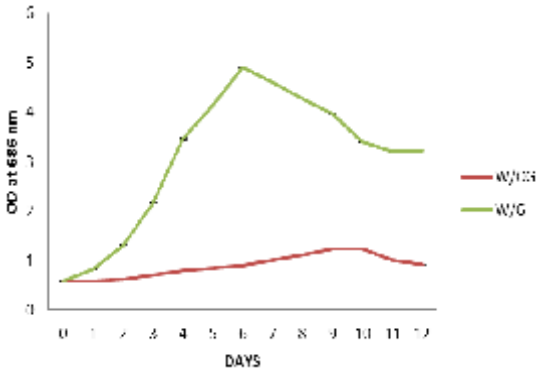

Fig. 4. Growth response of *C. vulgaris* in two different growth regimens. (OD vs days)

Selection of nitrogen sources

By observing the result of optical density (Fig. 5), cell count (Fig. 6) and dry weight (Fig. 7) in media containing different nitrogen sources, the highest growth was found in medium containing $NaNO_3$.

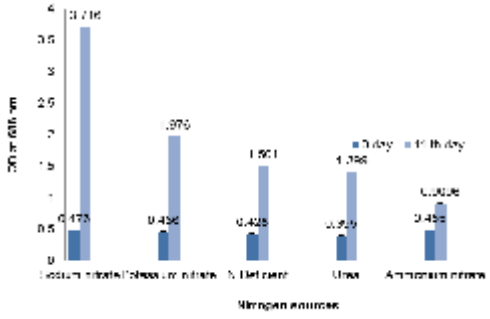

Fig. 5. Growth response of *C. vulgaris* with different Nitrogen sources (OD at 686nm vs. days).

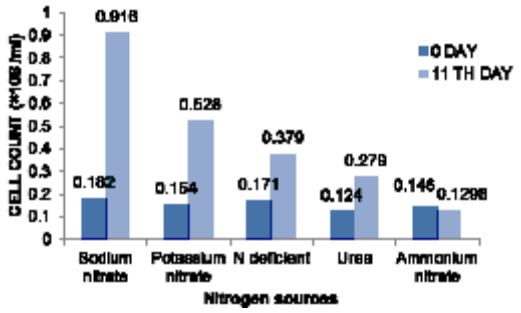

Fig. 6. Growth response of *C. vulgaris* with different Nitrogen sources (Cell count vs. days)

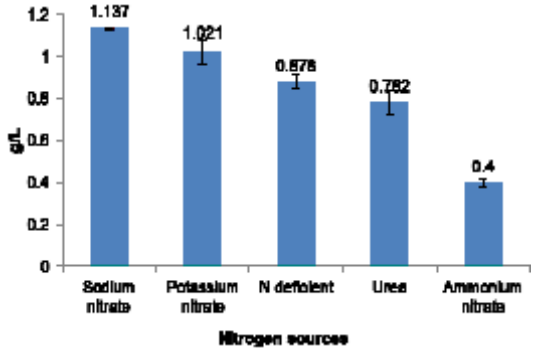

Fig. 7. Graph (with standard error) of dry weight of cells in gm. /L vs. different Nitrogen sources.

Scale up and biomass production in fermenter

The growth curve was plotted with respect to OD (Fig. 8), cell count (Fig. 9) and dry weight (Fig. 10). The growth rate was found to be 6.879 cells/ml/ day and doubling time was 2 hrs and 9 mins.

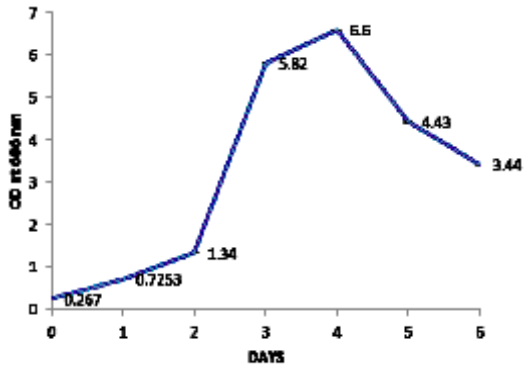

Fig. 8. Growth response of C. vulgaris in fermenter (OD vs days)

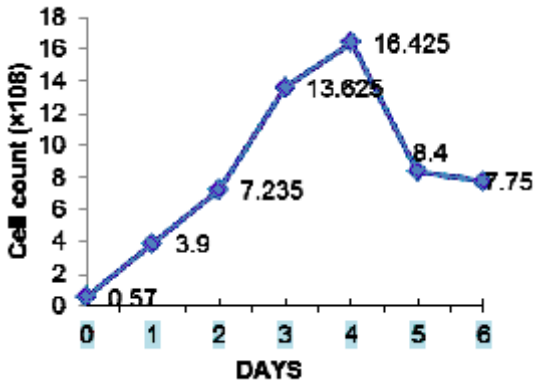

Fig. 9. Growth response of *C.vulgaris* in fermenter (Cell count vs. days)

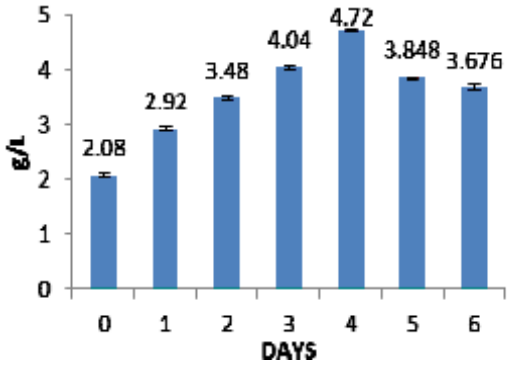

Fig. 10. Growth response of *C. vulgaris* in fermenter (Dry weight vs. days)

Determination of growth rate

After obtaining the growth kinetics of *C. vulgaris* in fermenter with controlled conditions as mentioned above, the specific growth rate and doubling time was determined by the formula given in the methodology. Then the specific growth rate and doubling time were obtained as 6.879 cells/ml/day and two hours and nine minutes respectively.

Lipid extraction

After the organic solvent extraction in Soxhlet extractor followed by evaporation of solvent, the total lipid obtained was 0.15533 g/ g dry weight of *C. vulgaris*. Total yield of total lipid obtained was 15.53%. Similarly, after mixing with hexane and evaporation the non-polar lipid obtained was 0.066933g/ g dry weight.

Direct disruption of microalgae can result in efficeint biodiesel production (Anitha & Shriman 2012). Hence

we can use chemical solvents, enzymes (lipase) or physical cell disrupter to release oil from microalgal cells.

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