Optimization Study of *Chlorella vulgaris* for Biomass, Lipid and Protein

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**Abstract:** The micro-alga chosen was *Chlorella vulgaris* in this work and it was cultivated under a variety of environmental conditions in various growth media to evaluate and optimize growth rate and biomass productivity along with chlorophyll content and Protein concentration. The pains of this work investigated the growth rates of *Chlorella* in different media namely Bold Basal Medium, F-Si, Half strength chu10, Chu10 and modified Zarrouk’s medium. The chlorophyll (chl a and chl b) and protein content was estimated for all the media cultures and compared.

Higher growth rates of 1.619 mg/ml for F-Si medium culture was obtained in a week under lab conditions. The chlorophyll concentration was higher at about 16.1 mg/ml for seven days observation. Similarly the protein contents were observed at the tip of about 2.750 g/100ml in Half Strength chu10 medium culture.

The comparison has given a clear result on the efficient protein production in half strength Chu 10 medium. However there has observed declining growth rate of *Chlorella* in modified Zarrouk’s medium, which can be interpreted to be some toxicity due to the nutrients or the environment conditions at growth phase.

The greatest growth, protein and chlorophyll concentration observed in the culture grown in five different media, indicate that this particular alga grown in the specific efficient medium may be appropriate for the respective production of metabolites, both primary and secondary metabolite. This study has proved that the best medium for growth and lipid concentration of *Chlorella vulgaris* is F-Si medium, Half Strength Chu10 and BBM. For better protein concentration it is proved apt to use Half Strength Chu10 medium.

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www.ijamb2002.com
Keywords: Microalgae, *Chlorella vulgaris*, Biomass, Protein isolation, Chlorophyll, Lowry’s method

**Introduction**

Microalgae are microscopic algae, capable to convert solar energy to chemical energy via photosynthesis (Whitton, 2011). They are typically found in freshwater and marine systems. They contain numerous bioactive compounds that can be harnessed for commercial use. Some of the most biotechnologically relevant microalgae are the green algae (*Chlorophyceae*) *Chlorella vulgaris*, *Haematococcus pluvialis*, *Dunaliella salina* and the Cyanobacteria *Spirulina maxima* which are widely commercialized. Essential nutrients also have exhibited in some microalgal biomasses (Becker, 2007). In addition, Tokuşoğlu and Ünal (2003) have shown that edible microalgae, have been potential for food supplements and food additives after being cultured. From their nutritional compositions, a very high-protein content (avg. 63.0%) (p<0.01) was in *S. platensis*. Microalgae are recently seen feasibility studies for profitable biofuel production (Wijffels and Barbosa, 2010; Williams and Laurens, 2010). Microalgae proteins are considered as an alternative protein source in foods (Spolaore et al., 2006).

To produce high quality biomass culture status was maintained. The large-scale production of *Chlorella* biomass depends on many factors and the most important of which is nutrient availability. Green microalgae are known to contain a very high amount of nutrients, proteins in particular. Under optimal growth, the relative content of the various nutrients is fairly similar among species (Hu, 2004). The potential of microalgal photosynthesis for the production of valuable compounds or for energetic use is widely recognized due to their more efficient utilization of sunlight energy as compared with higher plants. Growth rates give an index of algal physiological condition in the cultures, since it reflects algal metabolism, as a response to all of its cellular cycles (Lombardi and Maldonado, 2011).

*Chlorella vulgaris* is a photosynthetic microorganisms with a fast growth rate (Phukan et al., 2011) and are industrially produced for its high protein contents (Becker, 2007; Lubitz, 1961; Morris et al., 2009; Seyfabadi et al., 2011).

In this work optimization of the growth media for Chlorella is done and the estimation of biomass concentration using spectrophotometer, chlorophyll estimation, cell counting for five consequent week. Five different media used for this study are namely F-Si (Guillard, R.R., Ryther, J.H., 1962), BBM (Bischoff HW and Bold HC, 1963), Chu10 (Chu and Nalewajiko, 1942, Stein, 1973), Half strength chu10 and Zarrouk medium (Zarrouk,1966).

Observations were carried out over a period of five weeks after initial readings. Growth was followed through optical density, cell count and chlorophyll content. Optical density was recorded by using UV visible spectrophotometer at 670 nm and cell count examination was performed using Haemocytometer. UV visible spectrophotometer was used for pigment estimation in different cultures of *C. vulgaris* at 645 nm and 663 nm. The chlorophyll-a and chlorophyll-b content of the samples were estimated. The protein content was also estimated for the grown culture in the five media and its concentration was compared.
Methods
The chemicals purchased from Merck or Hi Media were used for the preparation of algal media namely F-Si, BBM, Chu10, Half strength chu10 and Zarrouk medium. Microalgae strains namely *Chlorella vulgaris* was obtained from Fermentation technology laboratory, Department Of Microbiology, Periyar University, Salem.

**a) Preparation of algal biomass**
The obtained strains were cultivated in 100ml of Chu 10 medium, Bold Basal Medium, Fe-Si medium, modified Zarrouks medium (Zarrouk, 1966) and Half Strength Chu medium in 250ml Erlenmayer flask each to be served as inoculum. The flasks were kept at 25°C under white fluorescent light 2000lux illumination and followed light and dark condition. Aeration was provided and agitated at interval. This was later cultured into 250 ml flasks and then to 500ml flasks and so on.

**b) Determination of Algal growth concentration**
The algal samples were collected from the culture medium and algal growth was determined using spectrophotometer at 680nm. The sample was collected at interval of 7 days and various parameters described below were studied.

**c) Microscopic observation**
Microscopic observations were performed frequently to monitor growth of the different algal species, and to check that cultures were free of any contaminants. It is crucial in studies of changes in morphology. All microscopic observations were performed frequently to monitor growth of the different algal species.

**d) Cell Counting using Hemocytometer ((Neubauer improved))**
1ml of sample was taken in an eppendorf and spinned at 10rpm for 1 minute. The supernatant was discarded and to the wet pellet 10 µl of Lugol’s iodine was added and mixed well. This was allowed to stand for few minutes. Hemocytometer was washed, cleaned, sterilized with ethanol and dried. To this about 10 µl of the sample was spread into the cover slide on the hemocytometer. It was focused concurrently before drying under microscope at 40X and the cells were counted in center 25 squares of ruling area. All squares have the same area (known) and by multiplying with a conversion factor a number of cells per milliliter are obtained. After each sample counting the hemocytometer was washed and dried.

The following calculation was done:

\[
\text{Total count} \times 10^4 = \text{Number of cells} \times 10^4 \\
\text{Cell count} = \frac{\text{Total count x final volume}}{\text{Initial volume}}
\]
e) Acquiring the Biomass  
Sterilized centrifuge tubes were taken and to each cultured algal sample were taken. This was centrifuged at 3000rpm for 10 minutes or 4000rpm for 5 minutes. The algal biomass obtained was used for Chlorophyll and Protein extraction.

f) Chlorophyll estimation  
The spectrophotometric definition of photosynthetic pigments was first determined by Stokes in 1864. This was defined as to cause light energy to turn into chemical energy in all photosynthetic organisms. The chlorophyll estimation was estimated by Lichtentater and Well Burn method (1985,1987) of chlorophyll estimation. 100% acetone was added to the biomass obtained in the ratio of 50ml per gram of biomass. This was homogenized at 1000 rpm for one minute and kept for some time. It was then filtered through cheese cloth and centrifuged to remove any excess residue (2500 rpm for 10 minutes). The supernatant was separated and the absorbance was read at 645nm and 663 nm respectively using UV spectrophotometer.

The following calculation was done to obtain the total chlorophyll, chlorophyll a and chlorophyll b concentration:

Total chlorophyll (µg/ml) : 20.2 (absorbance 645nm) + 8.02 (absorbance 663nm) Chlorophyll a (µg/ml) : 12.7 (absorbance 663nm) – 2.69 (absorbance 645nm) Chlorophyll b (µg/ml) : 22.9 (absorbance 645nm) – 4.68 (absorbance 663nm)


g) Protein Hydrolysate Extraction  
The cell lysis techniques use mechanical actions like (high pressure homogenisers, bead mills), ultrasounds, enzymatic or chemical treatments, thermal or osmotic shocks (repeated freezing/thawing) (Doucha and Livansky, 2008; Hopkins, 1991; Middelberg, 1995; Sari et al., 2013). The protein hydrolysate was extracted using chemical treatment method in this study. Mother solutions were prepared with approximately 500 mL of distilled water and 2 N NaOH was added to adjust the solution to pH 12 with help of pH meter. This is for maximum protein solubility. A sample of 0.5 g of freeze-dried biomass was added to 25 mL of mother solution and mixed well. The mixture was then stirred for 2 h at 40 °C. The temperature is brought by using a water bath and a thermometer to keep in check the temperature. The separation of the supernatant from the pellet was conducted by centrifugation at 10,000 g for 10 min at 20 °C. The supernatant was then taken into a new tube and adjusted to pH 3 with 0.1 M HCl in order to precipitate the proteins. The protein isolate was collected a pellet after further centrifugation at 10,000 g for 10 min at 20 °C and the pellet was neutralized to pH7 with 0.01 M NaOH. These samples were later taken for protein analysis (Chronakis, et al; 2000).

h) Protein analysis

The protein concentration was quantified using Lowry’s method of Protein estimation (Lowry et al., 1951 protocol). The reagents 0.1N NaOH, alkaline copper reagent, folin’s reagent were prepared. Bovine serum albumin was weighed and made upto 50ml with distilled water. This
served as stock. From the above stock 5ml was taken and made upto 50ml with distilled water. From the working standard volumes from 0.2 to 1.0ml was taken in a series of test tubes. The algal protein sample each 0.2ml was taken in another series of test tubes. To each of the above test tubes 5.0ml alkaline copper solution was added and allowed to stand for 10 minutes in room temperature. Then 0.5ml of Folins-Ciocalteau or phenol reagent was added and mixed well. It was allowed to stand for 30 minutes. The absorbance was observed then at 660nm in UV Spectrophotometer. The graph was drawn with the working standard OD values and the sample values were marked and the protein concentration was calculated using the following steps:

Optical density $x$ obtained corresponds to the $y$ amount of protein. Concentration of protein in 5ml of test solution was calculated using: $[(y \div 0.2) \times 5] = z \text{ mg.}$ the 5ml of test solution contain 0.2ml of sample and hence the concentration of protein in 0.2ml of sample $= z \text{ mg.}$

Therefore, the amount of protein in 100ml of the sample culture: $(z \div 0.2) \times 100 = m \text{ mg/100ml.}$

i) Lipid Analysis

The cells were washed three times with distilled water and dried at 80°C for 1 day and then weighed to obtain the biomass concentration. The measurement of the lipid content used a modified Bligh and Dyer method (1959). Gas Chromatography was performed to quantify the lipid content. The volume lipid productivity was calculated according to the equation (Eq. (1)):

$$\text{Volume lipid productivity} = \left(\frac{\text{mg} \cdot \text{d}^{-1}}{\text{T}}\right)$$

where $T$ (unit: d) was the culture time, $C_t$ (unit: g/1) and $L_t$ (unit: %) were the biomass concentration and lipid content at $T$, $C_0$ (unit: g/1) and $L_0$ (unit: %) were the initial biomass concentration and initial lipid content

Result and discussion

Determination of Algal growth and Biomass concentration

The growth (OD) was taken for Chlorella culture at the interval of 7 days for 5 weeks and it was found that the growth was highest in F-Si (1.619 mg/ml) at the end of fifth week.

<table>
<thead>
<tr>
<th>Week</th>
<th>Chu 10 (mg/ml)</th>
<th>BBM (mg/ml)</th>
<th>Half Strength Chu10 (mg/ml)</th>
<th>F-Si(mg/ml)</th>
<th>Modified Zarrouk(mg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Initial</td>
<td>0.100</td>
<td>0.100</td>
<td>0.100</td>
<td>0.100</td>
<td>0.100</td>
</tr>
<tr>
<td>I</td>
<td>0.180</td>
<td>0.164</td>
<td>0.147</td>
<td>0.206</td>
<td>0.280</td>
</tr>
<tr>
<td>II</td>
<td>0.234</td>
<td>0.390</td>
<td>0.470</td>
<td>0.480</td>
<td>0.205</td>
</tr>
<tr>
<td>III</td>
<td>0.260</td>
<td>0.421</td>
<td>0.584</td>
<td>1.024</td>
<td>0.150</td>
</tr>
<tr>
<td>IV</td>
<td>0.286</td>
<td>0.589</td>
<td>0.594</td>
<td>1.400</td>
<td>0.100</td>
</tr>
<tr>
<td>V</td>
<td>0.300</td>
<td>0.591</td>
<td>0.600</td>
<td>1.619</td>
<td>0.091</td>
</tr>
</tbody>
</table>
Chlorophyll estimation

The chlorophyll content of the culture in each medium was calculated with the OD obtained at 645 and 663 nanometers.

It was found that the total chlorophyll content was higher in Half Strength Chu10 medium for *Chlorella* at about 4.8 mg/ml in second week to 16.1 mg/ml in the fifth week. Zarrouk’s medium showed a senescence stage for *Chlorella*, the chlorophyll content and growth were in a decline stage. This can be due to the nutrient variations or the conditions maintained.

<table>
<thead>
<tr>
<th></th>
<th>Half Strength Chu10 (Mg/ml)</th>
<th>F-Si (Mg/ml)</th>
<th>Modified Zarrouk’s (Mg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>II</td>
<td>III</td>
<td>IV</td>
</tr>
<tr>
<td>Total Chl</td>
<td>4.8</td>
<td>9.5</td>
<td>14.1</td>
</tr>
<tr>
<td>Chl a</td>
<td>2.7</td>
<td>3.9</td>
<td>44.95</td>
</tr>
<tr>
<td>Chl b</td>
<td>2.1</td>
<td>5.5</td>
<td>9.1</td>
</tr>
<tr>
<td>BBM (Mg/ml)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total Chlorophyl</td>
<td>1.02</td>
<td>1.09</td>
<td>2.32</td>
</tr>
<tr>
<td>Chlorophyll a</td>
<td>0.36</td>
<td>0.35</td>
<td>1.18</td>
</tr>
<tr>
<td>Chlorophyll b</td>
<td>0.66</td>
<td>0.75</td>
<td>1.14</td>
</tr>
</tbody>
</table>

Protein extraction and Estimation

The protein hydrolysate was extracted using chemical treatment method from culture maintained in different media namely BBM, F-Si, Half Strength Chu 10 media, Chu10 and Zarrouk medium.
The protein was extracted in the first week and fifth week and the protein was estimated using Lowry method of protein estimation. The OD was obtained through Colorimeter at 660nm.

**OD VALUES AT 660nm**

<table>
<thead>
<tr>
<th></th>
<th>BBM</th>
<th>Chu10</th>
<th>F-Si</th>
<th>Half strength Chu10</th>
<th>Modified Zarrouk</th>
</tr>
</thead>
<tbody>
<tr>
<td>1st week</td>
<td>0.198</td>
<td>0.137</td>
<td>0.173</td>
<td>0.190</td>
<td>0.089</td>
</tr>
<tr>
<td>5th week</td>
<td>0.288</td>
<td>0.17</td>
<td>0.196</td>
<td>0.324</td>
<td>0.099</td>
</tr>
</tbody>
</table>

**CONCENTRATION IN G/100ML**

<table>
<thead>
<tr>
<th>Incubation time</th>
<th>BBM</th>
<th>Chu10</th>
<th>F-Si</th>
<th>Half strength Chu10</th>
<th>Modified Zarrouk</th>
</tr>
</thead>
<tbody>
<tr>
<td>1st week</td>
<td>1.7375</td>
<td>1.000</td>
<td>1.4375</td>
<td>1.625</td>
<td>0.500</td>
</tr>
<tr>
<td>5th week</td>
<td>2.375</td>
<td>1.375</td>
<td>1.6875</td>
<td>2.750</td>
<td>0.625</td>
</tr>
</tbody>
</table>

Half strength Chu 10 media (1.7375g/100ml in first week, 2.375 g/100ml) in fifth week and Chu 10 (1g/100ml in first week, 1.375g/100ml) were found to have high protein content on estimation by Lowry method.

*Chlorella* cells on hemocytometer under microscope 40X
Lipid analysis
The volume lipid productivity was calculated according to the equation (Eq. (1): Volume lipid productivity (mg l⁻¹ d⁻¹)

\[
\text{Volume lipid productivity} = (G_t \times L_t - C_0 \times L_0) \times 1000/T
\]

It was found that F-Si medium was suited best for lipid production followed by BBM, Half strength chu10 medium. The least was in chu10 and zarrouk’s medium.

<table>
<thead>
<tr>
<th>Sl.No</th>
<th>Medium for Chlorella</th>
<th>Concentration of lipid (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>BBM</td>
<td>44.2</td>
</tr>
<tr>
<td>2</td>
<td>F-Si</td>
<td>47.3</td>
</tr>
<tr>
<td>3</td>
<td>Half Strength Chu 10</td>
<td>39.8</td>
</tr>
<tr>
<td>4</td>
<td>Chu10</td>
<td>22.6</td>
</tr>
<tr>
<td>5</td>
<td>Zarruok</td>
<td>5.3</td>
</tr>
</tbody>
</table>

Conclusion
The comparison has given a clear result on the efficient protein production in half strength Chu 10 medium. However there has been observed declining growth rate of Chlorella in modified...
Zarrouk’s medium, which can be interpreted to be some toxicity due to the nutrients or the environment conditions at growth phase.

The greatest growth, protein, lipid and chlorophyll concentration observed in the culture grown in five different media, indicate that this particular alga grown in the specific efficient medium may be appropriate for the respective production of metabolites, both primary and secondary metabolite.

Acknowledgement

Author gratefully acknowledges Fermentation Laboratory, Periyar University for the help and guidance. Also I gratefully acknowledge Dr.R Dhandapani, Hemalatha for their help during this study.

References

12. Lombardi AT and Wangersky PJ. 1991. Influence of phosphorus and silicon on lipid class production by the marine diatom