

Mass Production of Microalgae Using Waste Water as Supplement and Extraction of Bio Oil by Transesterification

G. Krithika¹, P. Manasa Satheesh²

Student, Department of Biotechnology, Holy Cross College (Autonomous), Tiruchirappalli, Tamil Nadu, India¹

Director, Genewin Biotech, Hosur, Tamil Nadu, India²

ABSTRACT: Developments in the Biofuel production has created more awareness in Research due to the rapid increase in oil price and growing environmental issues. Microalgae have gathered attention towards them in the Biofuel production. Transesterification process converts algal oil or lipids to fatty acids. Because of their high Biomass and Lipid accumulation, the microalgae suited for the production of bio oil in industrial level. The lipid content was $28.22 \pm 1.38\%$, carbohydrate 16.8% and protein 58.2% for *Chlorella pyrenoidosa*. The amount of oil extracted was found to be 25.8 ml for (Alternate Media) AM1 and 20.6 ml for AM2. The pH was found to be 7.8 for AM1 and 7.6 for AM2. In this study, scaling up of *Chlorella pyrenoidosa* in poultry waste water was done with fertilizers and analysing parameters for its growth and production of Bio oil from *Chlorella pyrenoidosa* has been examined from the scaling up in poultry waste water.

KEYWORDS: Bio oil, Lipids, Carbohydrates, *Chlorella pyrenoidosa*, Proteins, Transesterification, Wastewater.

I. INTRODUCTION

Microalgae are sunlight-driven cells that convert carbon dioxide to potential biofuels, foods, feeds and high-value bioactives. Microalgae could provide alternatives to petroleum based fuels without competing with crops. Microalga is an economical and potential raw material of biomass energy [12].

Chlorella pyrenoidosa is a unicellular green alga that grows in fresh water. *C. pyrenoidosa* contains much more protein and chlorophyll than other plants. *Spirulina*, like any blue-green algae, can be contaminated with toxic substances called microcystins. It can also absorb heavy metals from the water where it is grown [7]. *Chlamydomonas* is the algae from genus of microscopic, unicellular green plants (algae) which live in fresh water. It has been found that they can be used to generate hydrogen from light, water, and basic nutrients. The possibility of generating large quantities of hydrogen, which is a renewable fuel, from cheap and abundant sources is produced by *Chlamydomonas* [11]. A critical growth-limiting factor is the carbon, usually supplied as CO₂ that occurs in enriched environments. Supply of pure CO₂ into algae cultivations is expensive and not an encouraged option for large scale algae production. However, through a lower cost option, algae have the ability to capture CO₂ from combustion gas and from natural gas operations and hold the opportunities to recycle CO₂ [15].

The transesterification process is the reaction of a triglyceride (fat/oil) with an alcohol to form esters and glycerol. A triglyceride has a glycerine molecule as its base with three long chain fatty acids attached [4]. Almost all biodiesel is produced in a similar chemical process using base catalysed transesterification as it is the most economical process, requiring only low temperatures and pressures while producing a 98% conversion yield. Biodiesel is, at present, the most attractive market alternative among the non-food applications of vegetable oils for transportation fuels. Bio oil can be used in pure form. Bio oil higher lubricity index compared to petroleum diesel and can contribute to longer fuel injector life. Bio oil is pollution free solvent than petroleum diesel and has been known to break down deposits of residue in the fuel lines of vehicles that have previously been run on petroleum diesel. Chemical solvents are often used in the extraction of the oils [10]. In this study the growth of *Chlorella pyrenoidosa* is scaled up

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from the results obtained by various trials and the extraction of bio oil is done successfully by transesterification process.

II. MATERIALS AND METHODS

Collection of algal strains and Waste water:
The algal strains such as *Chlorella pyrenoidosa*, *Spirulina* and *Chlamydomonas* were collected from NCIM, Pune and maintained properly in its selective medium at Genewin Biotech, Hosur. Poultry waste water was obtained from Maruthi Hatcheries, Hosur and Tannery waste water obtained from a leather industry Vanitech, Vaniyambadi.

Determination of Lipid Content in the Three Algae Species:

Extraction and determination of intracellular lipids were conducted according to the standard protocols in the previous literatures [1] and [3] by Gravimetry. Freeze-dried algal biomass (50–100 mg) were extracted three times with methanol containing 10% DMSO (volume fraction) for 50 min under stirring. Then the mixture was centrifuged (2000 g, 10 min), and the supernatant was removed. The residua were re-extracted twice with a mixture of hexane and diethyl ether (1:1, volume ratio) for 30 min. After extractions, hexane and water were added to the combined supernatants, so as to obtain a ratio of 1:1:1:1 (volume ratio) for the above four solvents. The mixture was shaken and then centrifuged for 10 min and the upper organic layer was collected. After the water phase was re-extracted twice with a mixture of hexane and diethyl ether (1:1, volume ratio), the organic phases were combined and evaporated to dryness. Then it was redissolved with a small amount of hexane. The lipid solution was transferred into a pre-weighed vial, initially evaporated in a water bath (30 °C) using a rotatory evaporator and then dried under high vacuum. The dried residua were weighed on an electronic microbalance.

Determination of Protein Content in the three Algae Species

The Lowry method was used to measure the protein content of the pre-treated biomass [2] and [16]. The absorbance of the sample was measured at a wavelength of 750 nm in a spectrophotometer [5].

Reagents

Alkaline copper sulphate solution was prepared freshly by mixing 1.0 mL of 0.5% $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$ in 1% Potassium sodium tartarate with 50.0 mL of 2% sodium carbonate in 0.1 N NaOH. Diluted Folin-phenol reagent was prepared by diluting Folin-ciocalteu reagent obtained with an equal volume of glass distilled water.

Procedure

To one millilitre of protein solution, 5.0 ml of alkaline copper sulphate solution was added and allowed to stand at room temperature for 10 minutes. Then 0.5 ml of diluted Folin-phenol reagent was added and mixed well [8]. The solution was allowed to stand for 30 minutes and absorbance was read at 500 nm using a Hitachi UV 2001 spectrophotometer. BSA (Sigma fraction V) was used as standard.

The protein content of the biomass was calculated using the following equation:

$$\text{Protein (\%, w/w)} = \text{CVD/m} \times 100$$

where C is the protein concentration (mg L^{-1}) obtained from the calibration curve,

V is the volume (L) of the lysis buffer used to resuspend the biomass,

D is the dilution factor and m is the amount of biomass (mg).

Determination of Carbohydrate Content in the Three Algae Species

Carbohydrates were extracted and estimated as the procedure given by Roe and Indian Pharmacopeia. Freshly harvested, frozen thalli were used within two days of collection. Known quantity of the alga was ground in a glass mortar and pestle with 80% ethanol and filtered through Whatman's No. 1 filter paper. The filtrate was centrifuged at 8000 X g for 20 minutes and the supernatant was made to 5.0 ml with 80% ethanol. The final volume of the extract was then made up to 10.0 ml by adding distilled water. To 1.0 ml of the above sample, 4.0 ml of anthrone reagent was added by the sides of the test tube and heated in a boiling water bath for 10 minutes. The tubes were cooled to room temperature and absorbance was read at 620 nm using UV spectrophotometer. A reagent blank was run simultaneously. Glucose was used as the standard [9].

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Anthrone reagent

Anthrone (50.0mg) and thiourea (1.0 g) were added to 100.0 ml of 72% cold sulphuric acid and stored in a dark bottle. Freshly prepared reagent was used for every estimation.

Trials done with Waste water and Fertilizers:

Two types of waste water were selected and trials were put with the Alternate media (AM1 and AM2) which are fertilizers available in the following ratios of N, P and K:

- AM1: 0:52:34 (supplementing nitrogen with Urea)
- AM2: 13:0:45 (supplementing P with super phosphate)

Various parameters necessary are measured in TWW and PWW for the growth of algae. *Chlorella pyrenoidosa* was trialled with various ratios with AM1 and AM2 fertilizers at pH 6, 7, 8. It was clearly seen that the TWW did not promote growth of *Chlorella* as the ratio of TWW was higher than the fertilizers. So the experiment was further continued with PWW. Altering the biological parameters of TWW, the *Chlorella* can be grown in the TWW with the various ratios along with the fertilizers supplementation. PWW was used for the growth of *Chlorella* once the biological parameters are analysed. Various trials such as PWW: AM1 and PWW: AM2 with the ratios 0:50, 50:0, 30:20, 20:30, 40:10, 10:40 were put using two different fertilizers for checking the suitable combination of ratio for the growth of *Chlorella pyrenoidosa*.

Estimation of Growth of *Chlorella* with CO₂:

As with all photosynthetic organisms, algae use CO₂ as a carbon source. No growth can occur in the absence of CO₂. Based on the average chemical composition of algal biomass, approximately 1.8 tonnes of CO₂ are needed to grow 1 tonne of biomass.

Method:

The CO₂ coming out from a tall chimney was collected and sent in through a motor which directly sent the CO₂ to the algae bottle. Side by side control with the aerators where kept and corresponding OD values recorded at intervals of 1 hrs. each [6].

Bio oil Production:

The collected *Chlorella* was expanded in the Fog's medium. The factors necessary for the cultivation of algae are Light, Temperature, and Nutrients. The algae which was cultivated was grown and then expanded in the 25 liters Bubble tops in order to achieve the mass production of algae. The medium used for the growth of *Chlorella* was Fog's medium. The algae was grown in the Bubble tops and the algal biomass was collected for the oil extraction. Flocculation is a reliable method for algae separation. The effect of culture pH on the flocculation efficiency was carried out by adjusting the culture pH ranging from pH 10 to pH 10.6 using either 5 M sodium hydroxide (NaOH) or 5 M potassium hydroxide (KOH). The bases were added to the culture by agitation using magnetic bar stirrer (38 mm), agitated at 200 rpm to allow for steady increase in pH [14]. Biomass biologically is the material of plants which are specifically called lignocellulosic biomass. The biomass was collected after filtration and weighed. Algae were ground with motor and pestle and the ground algae were dried for 20 min at 80°C in a incubator for releasing water. Hexane and ether solution (20 and 20 mL) were mixed with the dried ground algae to extract oil. Then the mixture was kept for 24 h for settling.

Transesterification Process:

The transesterification reaction is base catalysed. The presence of water causes undesirable base hydrolysis, so the reaction must be kept dry by transesterification process. The conical flask containing solution was shaken for 3 h by electric shaker at 300 rpm. After shaking the solution was kept for 16 h to settle the biodiesel and sediment layers clearly. The bio oil was separated from sedimentation by flask separator carefully. Biodiesel was washed by 5% water until it became clean since the washing can eliminate the unwanted impurities and removes all sorts of debris from the mixture.

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0.25 g NaOH was mixed with 24 ml methanol and stirred continuously for 20 minutes. The mixture of catalyst and methanol was poured to the algal oil in a conical flask and tranesterification process allowed to take place. The bio-oil is separated from the sedimentation by a flask separator. Bio-oil was washed with 5 % sterile water until it becomes clean. Bio-oil dried by using a dryer and kept under air for 12 hrs. Harvesting has been claimed to contribute 20–30 percent to the total cost of producing the biomass [6]. Bio oil production was measured by using measuring cylinder; pH was measured and stored for analysis.

III. RESULTS AND DISCUSSION

Determination of Lipids, Proteins and Carbohydrates

Lipids are produced during physiological experiments and biomass production processes. Here, the lipid content is estimated for three species. The lipid content was found to be high in *Chlorella pyrenoidosa* of 28.22±1.38 % followed by *Spirulina* of 24.00±4.49 % and *Chlamydomonas* 15.61±0.63%. Proteins make up a large fraction of the biomass. The Lowry method is one of the most accurate methods for quantifying proteins [5]. Bovine serum albumin (BSA) is the most commonly used standard. The protein content was found to be highest in *Chlorella pyrenoidosa* of 58.2% followed by *Spirulina* of 52.6% and *Chlamydomonas* of 48%. Determination of carbohydrate concentrations is the colorimetric method based on reaction between hydrolysed carbohydrate solution and a colouring reagent that develops colour that is detectable in the visible range of the electromagnetic spectrum. Carbohydrate content was highest in *Chlorella pyrenoidosa* of 16.8% and lowest in *Spirulina* of 12.4%.

Table 1

Algae name	Lipid content (%)	Protein content (%)	Carbohydrate content (%)
<i>Chlorella pyrenoidosa</i>	28.22±1.38	58.2%	16.8
<i>Spirulina sp.</i>	24.00±4.49	52.6	12.4
<i>Chlamydomonas</i>	15.61±0.63	48	14

In the lab scale estimation of Proteins, Carbohydrates and lipid content of the three algae species, it is clearly proved that *Chlorella pyrenoidosa* showed high level of Proteins, Carbohydrates and lipid than the other species. So the further works were carried out using *Chlorella pyrenoidosa*. According to studies available it is noticed that the bio oil extraction and lipid content is high in *Chlorella pyrenoidosa* than *Spirulina* and *Chlamydomonas*, so the work is carried out focusing only on this species.

Table 2

PARAMETERS	PWW	TWW
pH	8.23	8.13
Temperature	29 °C	29 °C
TDS	6.728 ppm	15.69 ppt
EC	1.277 mS	30.30 mS
Calcium	0.2459%	-
Magnesium	1.009%	-
Potassium	1 ppm	-
Total Phosphorous	0.011	-
Total Nitrogen	0.49	-
BOD	-	20.1 ppm
COD	-	5.376 ppm
DO	-	15.1 ppm
CHROMIUM	-	12.665 ppm

TWW did not possess any of the nutrients in it to support the growth of the *Chlorella* but the PWW possess all the nutrients required for the *Chlorella*'s survival. The parameters such as BOD, COD, DO and Chromium are the

parameters specific for the water analysis to be used and it is not related with the growth of *Chlorella*. SO, PWW was used for the further trials and Bio oil production.

CARBON DIOXIDE EXPERIMENT FOR THE ALGAL GROWTH

Carbon dioxide experiment was conducted in order to check the growth of *Chlorella* using carbon dioxide. A control was also checked without using the carbon dioxide. The absorbance was taken for a period of 5 hours to check whether Carbon dioxide is helping the *Chlorella* growth[13].

Table 3

HOURS	CONTROL	CO ₂
1	0.0050	0.0051
2	0.0350	0.0540
3	0.0553	0.0801
4	0.0560	0.0895
5	0.0583	0.0912

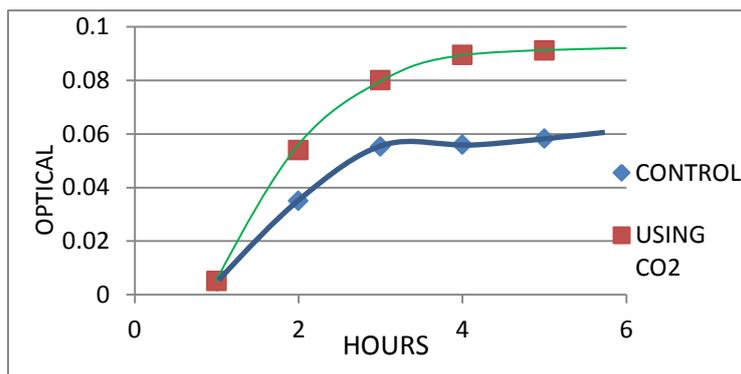


Chart 1 Graph Indicating the Growth of *Chlorella* Using Carbon dioxide

It was found that the carbon dioxide helped its growth and drastically the absorbance was increased each hour whereas the absorbance of the control was also found increasing slowly each hour. This proves that the Carbon dioxide is an important parameter for its rapid growth.

Bio oil Extraction by Transesterification and Expansion of Algae in Bubble Tops:

The *Chlorella* was expanded in 25 litres Bubble tops for the mass production of algae and the Biomass was collected for bio diesel production based on the various trials carried out. It is clearly found out that the ratio of 30:20 with AM1 and 25:25 with AM 2 proved to have fat growth of algae thus the expansion in large scale was also done with this particular ratio.



Fig - 1: EXPANSION OF CHLORELLA IN LARGE SCALE

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The expansion was done in the above ratio in the bubble tops and the growth of *Chlorella* was seen in both the ratios. It took about 1 week for the *Chlorella* to reach its maximum growth rate.

Scaling Up in Bubble Tops:

The scaling up of the PWW was done with AM1 fertilizer in the ratio 30:20 and the *Chlorella* was allowed for expansion. It took 10 days for the *Chlorella* to reach its maximum growth.

The scaling up of the PWW was done with AM2 fertilizer in the ratio 25:25 and the *Chlorella* was allowed for expansion. It took 10 days for the *Chlorella* to reach its maximum growth.

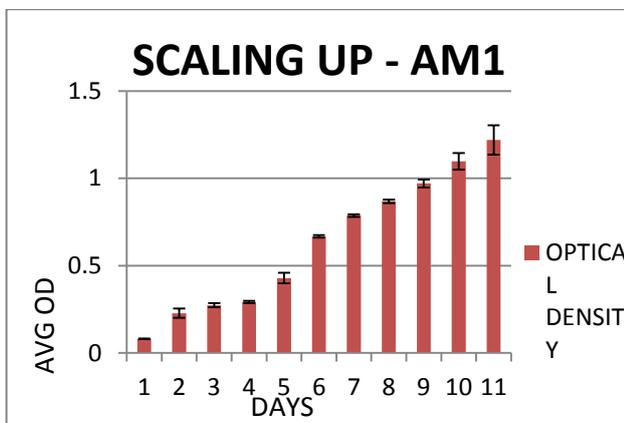


Chart 2 Graph Showing the Scaling up with AM1

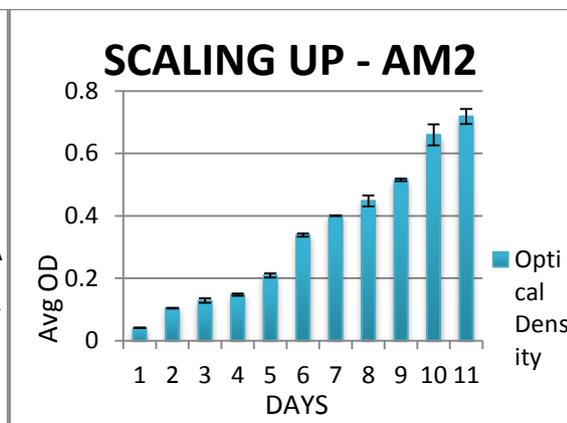


Chart 3 Graph Showing the Scaling up with AM2

It was observed that the mean absorbance was 1.463 ± 0.0039 on the 10th day which showed the efficiency of AM1 in PWW. It was observed that the mean absorbance was 0.71815 ± 0.0239 on the 10th day which showed that AM2 was not much efficient in PWW when compared to AM1.

Flocculation:

The Flocculating agent used was Aluminium Chloride for the separation of algae and the water in the bubble top for the collection of biomass. The Flocculation involved the separation of species by gravity. The various techniques used for flocculating were decreasing the pH to 4 using acid, increasing the pH to 11 using alkali, Addition of ferrous sulphate, Addition of aluminium sulphate, Addition of ferric chloride. It is found that decreasing the pH to 4 using acid seemed to help in flocculation faster followed by aluminium chloride. Trials were put and then concluded as to which method to flocculate algae in large scale production. Algae were collected and dried for 20 minutes at 80 °C in an indicator for releasing water. Hexane and ether solution (20 ml each) were mixed with the dried ground algae to extract oil. The mixture was kept for 24 hrs. for settling. The Biomass was collected after the Flocculation process and the collected biomass was weighed.



(a)

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The collected Biomass cake obtained from AM1 after drying was observed as 21 g which was higher when compared to AM2 and this was further used for carrying out Bio oil production.



(b)

Fig. 2 Biomass Collection (a) Collected Biomass – Algal cake from AM 1 (b) Collected Biomass – Algal cake from AM 2

The collected Biomass cake obtained from AM2 after drying was observed as 18g. As the quantity obtained was lesser amount, it was not used for the bio oil production.

Evaporation of oil from algae:

The collected oil was evaporated in order to eliminate the catalyst mixture from the oil. The catalyst mixture contained Sodium hydroxide and the solvent Methanol. The extracted oil was evaporated in vacuum to release hexane and other solutions using rotary evaporator.



Fig. 3 Evaporation of Oil

The evaporated solution which is free from the hexane and other solutions was then separated from the algae after the Transesterification process to obtain the Bio oil content.



Fig. 4 Separation of Bio oil

In this study, the oil was produced from the algae efficiently. The amount of oil extracted was found to be 25.8 ml for AM1 and 20.6 ml for AM2. The oil was measured using a glass measuring cylinder and pH was measured and stored in a cool dry place. The pH was found to be 7.8 for AM1 and 7.68 for AM2.

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IV. CONCLUSION

It was concluded that the Bio oil can be produced from the algal species efficiently which was environmental friendly and Pollution free. Algae species which is known for its high availability in nature, renewable energy source and low cost, it could be used to produce Bio oil in an inexpensive manner. In this study, the oil was produced from the algae efficiently by Transesterification. The amount of oil extracted was found to be 25.8 ml for AM1 and 20.6 ml for AM2. The pH was found to be 7.8 for AM1 and 7.6 for AM2. By this way, *Chlorella pyrenoidosa* can be used as renewable energy source.

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BIOGRAPHY

G. KRITHIKA



I am G. Krithika. I received my Undergraduate degree (B. Sc) in Plant Biotechnology from Holy Cross College (Autonomous), Tiruchirappalli, India, in 2006, and Master's degree in Biotechnology from the St Joseph's College, Tiruchirappalli, India, in 2008. In 2014, I joined the Department of Biotechnology, St Joseph's College of Arts and Science for women, Hosur, as a Lecturer, and currently pursuing M. Phil Biotechnology in Holy Cross College (Autonomous), Tiruchirappalli. My research interests include working with Bio-oil production of Macro and Micro algae and estimating its properties.

Mrs. MANASA SATHEESH



Degree earned: Under graduate in Bachelor of technology (Biotechnology) from Sathyabama University, Chennai, Tamil Nadu in the year 2005 – 2009. Post-graduation in Master of technology (Biotechnology) from Sathyabama University, Chennai in the year 2010 to 2012. Currently pursuing Ph.D. at VIT University, Vellore, Tamil Nadu, India
Work Experience: DIRECTOR OF GENEWIN BIOTECH (Producers of Quality Tissue Cultured plants), Hosur from 2007 till date. **Previous publication:** 1. Caral Dinesh R, Vinay P, Manasa P, Vinothkumar D and Ramesh Babu N G, Comparative study of oyster mushroom (Pleurotus ostreatus) cultivation by physical and chemical method of sterilization using two different substrates, International journal of Scientific & Engineering Research, Volume 4, issue 9, pp 898-902, September 2013. 2. E. Anitha, V. Praveena, N.G, Ramesh Babu and P. Manasa, Enumeration of Foliar Fertilizer Efficiency in India's top commercial crop – TEA, International Journal of Innovative Research in Science, Engineering and Technology, vol 2, issue 12 December 2013, ISSN:2319-8753, PP 8038-8046. **Awards:** Mrs. Satheesh was awarded the zonal winner in representing Genewin Biotech in "THINK BIG" moneycontrol.com in CNBC TV 18.