CULTIVATION OF *Chroococcus species* IN PHOTOBIOREACTOR FOR PRODUCTION OF BIOFUEL

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Abstract - Microalgae have been suggested as valuable feedstocks for biofuel production because of their simple cellular structure and ability to control cell composition without decreasing productivity, high photosynthesis yields, i.e., about 3 – 8% of solar energy can be converted to biomass as compared to terrestrial plants where it is approximately 0.5% and fast growth of algae compared to other energy crops and it can utilize carbon dioxide from flue gases from industries. Algae can accumulate more lipids per dry cell weight than other sources and produces more oil than oleaginous seeds and vegetable oils such as jatropha...The biofuel production using microalgae belongs to the third generation of biofuel which can be cultivated in either an open tank or photobioreactor. For obtaining high biomass of algae, a large-scale production system is necessary. Through the transesterification process, monoalkyl esters generated from organic oils, either plant or animal-based, are converted into biodiesel, a type of biofuel. In comparison to petroleum diesel, it is also biodegradable, non-toxic, and has low emission levels. In reality, producing enough car fuel to replace current gasoline use may only be possible with biodiesel made from algae. 7 to 31 times more oil is produced by algae than by palm trees. Microalgae would be the ideal type of algae for biodiesel. Microalgae grow far more quickly and easily than macroalgae, and they produce a lot more oil. The objective of this project was the cultivation of *Chroococcus sp*. in the tubular photobioreactor. For obtaining high biomass of *Chroococcus sp* the effect of dissolved oxygen, pH, air flow rate, and electrical conductivity were measured daily. The dissolved oxygen levels around 2.95, 3.90, and 4.43 mg/mL in air flow rate was maintained as 0.5, 1.0, and 1.5 liter/minute and measured on daily basis. The airflow rate should be maintained at 1.0 when the biomass was better compared to other flow rates for the growth of *Chroococcus sp*. Both pH and conductivity were measured daily and the results indicate that the increase in pH was gradual and after the 6th day, it was around 9.90. Regarding the electrical conductivity, it was around 3.95 µs cm^{-1} throughout the study. The lipid productivity was found to be 358 mg/L/day, 492 mg/L/day, and 268 mg/L/day for *Chroococcus sp*. respectively with a different air flow rate in the cultivation of algae. The lipid algal cells were identified by Thin Layer Chromatography and extracted algal biomass was analyzed by FT-IR.

Index Terms – Photobioreactor (PBR), Fourier-transform infrared (FT-IR), Extended Kalman filter (EKF),Thin Layer Chromatography(TLC), Central Food Technological Research Institute(CFTRI) Medium ,Ultra-Violet (UV) Spectroscopy.

I. INTRODUCTION

Energy needs are always rising as a result of increased industry and population growth. The basic sources of energy are fossil fuels (petroleum, coal and natural gas), hydro, and nuclear energy however, fossil sources are limited and will be exhausted by the near future Through the transesterification process, monoalkyl esters generated from organic oils, either plant or animal-based, are converted into biodiesel, a type of biofuel. It is also biodegradable, non-toxic and has a low emission profile as compared to petroleum diesel. **Shay (1993)** reported that algae are one of the best sources of biodiesel[1]. In fact, algae are the highest-yielding feedstock for biodiesel. It is capable of producing 250 times as much oil per acre as soybeans. In fact, biodiesel from algae may be the only way to produce enough automobile fuels to replace current gasoline usage . Algae would produce about 7 to 31 times greater oil content than palm oil. The best algae for biodiesel would be microalgae. Microalgae have much more oil than macroalgae and it is much faster and easier to grow and harvest[2]**.**

Microalgae have been suggested as valuable feedstocks for biofuel production because of their simple cellular structure and ability to control cell composition without decreasing productivity, high photosynthesis yields, i.e. about 3 – 8% of solar energy can be converted to biomass as compared to terrestrial plants where it is approximately 0.5% and fast growth of algae compared to other energy crops [3,4,5**].** The algae have the unique ability to grow under various environmental conditions and it can utilize carbon dioxide from flue gases from industries. Algae can accumulate more lipids per dry cell weight thanother sources and produces more oil than oleaginous seeds and vegetable oils such as jatropha...The biofuel production using microalgae belongs to the third generation of biofuel which can be cultivated in either open tank or photobioreactor. For obtaining high biomass of algae, largescale production system is necessary.

Algae are cultivated in a large scale for a variety of applications ranging from pharmaceuticals to nutraceuticals. They are the source of well-known antioxidants [6] such as carotenoids, and leutin etc., Leutin prevents the age-related macular degeneration [7].They are also used in the production of cosmetics and food additives. The polysaccharides secreted by brown algae are well known for their medicinal properties. Biomass itself serves as a good feedstock for both human beings and animals. The lipid which is extracted from the biomass can be transesterified into biodiesel. High lipid yield can be achieved with high biomass by microalgae. Thus, it avoids the usage of feedstock such as sugar cane, cereals, oil seeds [8], and lignocellulosic crops [9**]** in the production of biofuels.

Large-scale algal cultivation can be achieved in two ways: open ponds and photobioreactors (PBR). Open ponds can be divided into two categories: naturally occurring waterways (lakes, lagoons, and ponds) and man-made ponds or containers. Systems like shallow huge ponds, tanks, circular ponds, and raceway ponds are the most frequently employed ones. Open ponds have the benefit of being simpler to build and maintain than most closed systems, which is one of its main advantages. A few thousand tonnes of microalgae biomass are generated annually, primarily in open ponds. Closed photo-bioreactors can only produce a few hundred tonnes. But open

ponds have some limitations, including contamination that can cause problems like salinity and pH imbalances inhibiting algae growth. There are also other limitations to algal growth in colder and hot, humid climates. For the generation of low-value biofuels, biomass has attracted overwhelmingly positive attention. The limitation of open ponds for large-scale cultivation of algae has to be overcome by a photobioreactor.

PHOTOBIOREACTOR:

Photobioreactors are used for culturing photosynthetic microorganisms such as microalgae, cyanobacteria, plant cells, and photosynthetic bacteria for various biotechnology applications [10]. It is a closed equipment that provides a controlled environment and enables high productivity of algae. As it is a closed system, all growth requirements of algae are introduced into the system and the parameters are controlled according to the requirements. PBRs make it easier to control things like the amount of carbon dioxide, water, optimal temperature, effective light exposure, culture density, pH levels, gas supply rate, and mixing regime in the culture environment and dissolved oxygen level.

Chroococcus species:

Chroococcus, a unicellular organism that is a genus of cyanobacteria, is blue-green in colour and microscopic colony mounded. Tiny colonies with blurry trichomes can be found within the outer sheath. Chroococcus are typically found in colonies consisting of two, four, or eight cells and covered in a transparent sheath of photosynthetic pigments. Chroococcus, like all cyanobacteria, is a prokaryote without the membrane-forming organelles of eukaryotes because of this. Chroococcus prefers the sludge of lake and river bottoms as its home because it is known for its underwater habitat.

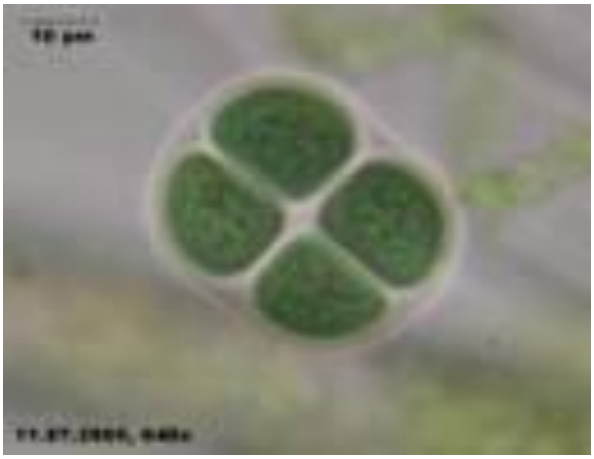

Fig2. *Chroococcus species*

Chroococcus, like other cyanobacteria, has significant ecological implications as a productive microbe. Chroococcus uses a lot of carbon in the air for photosynthetic processes, which make free oxygen in the air. Chroococcus also belongs to the first genus that used water to get electrons and hydrogen for photosynthesis, which also makes more free oxygen for other organisms to use.

Effect of Dissolved Oxygen, Conductivity, and ph:

Algae act both as a source and sink of dissolved oxygen. Algal photosynthesis frequently results in dissolved oxygen levels that are higher than saturation, or super-saturation, when oxygen production exceeds algal demands (respiration) during daylight hours. During nighttime periods when photosynthesis is absent, algal respiration may reduce dissolved oxygen levels significantly.

Another physicochemical parameter analyzed in the current investigation was electrolyticconductivity, *i.e.*, the total concentration of ionized compounds. Electrolytic conductivity mainly depends on ions (Ca_2+, $Mg^{2+,}$ Na^+, HCO_3^- SO_4^{2-} and Cl^-). Therefore, when electrolytic conductivity increases, the range of changes in the percentages of ions is low. The most significant correlation was found between total amounts of dissolved substances and HCO_3— andCa_2+, which affect phytoplankton development and are the principal ions in most freshwater bodies[11**]**.

The electrolytic conductivity of water might be an indirect indicator of system fertility. The numbers of blue-green algae in Lake Jeziorak Mały were affected to a higher degree by water temperature than electrolytic conductivity. Furthermore, the highest electrolytic conductivity of water was recorded in temperature ranges at which the numbers of the alga species examined were relatively low [12].

pH is also an important parameter for the cultivation of *Chroococcus sp*. As the culture medium moves along a photobioreactor tube, pH increases because of the consumption of carbon dioxide [13]

In this present study, different parameters like pH, Dissolved oxygen, and electrical conductivity were monitored for obtaining high growth of *chroococcus sp* in the photobioreactor.

Transesterification of algal oil:

Any substance with a considerable amount of fatty acids is a potential feedstock for biodiesel conversion through transesterification. Triglycerides (TAGs), three fatty acids molecules esterified with a molecule of glycerol suitable for biodiesel conversion, come from a variety of sources; animal fats, raw vegetable oils from oil seed plants, used vegetable oils from restaurants, and algae [14]Biodiesel is a mixture of fatty acid alkyl esters obtained by transesterification (ester exchange reaction) of algal oil. Algae are composed by 90-

98% (weight) of triglycerides and small amounts of mono and diglycerides, free fatty acids (1- 5%), and residual amounts of phospholipids, phosphatides, carotenes, tocopherols, sulfur compounds, and traces of water [15].

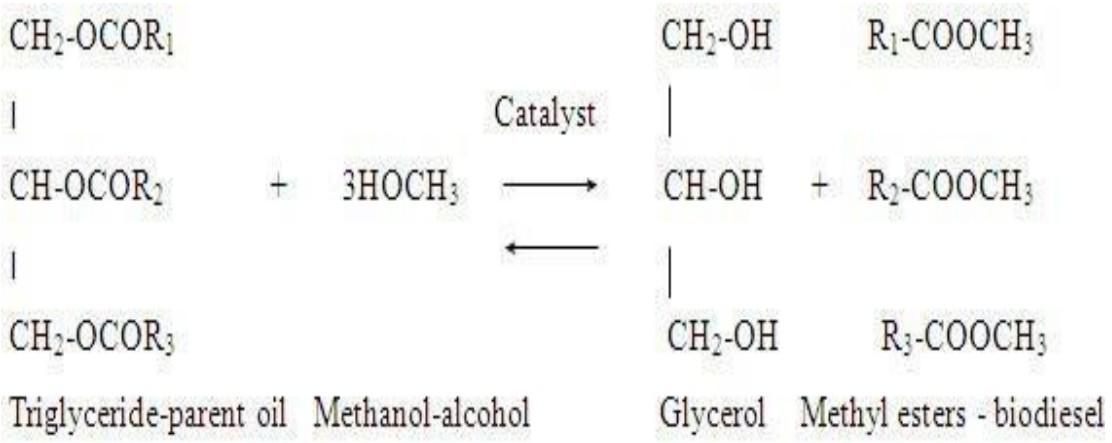

Fig3.Transesterification process

Fourier-transform infrared (FT-IR):

Automated Fourier-transform infrared (FT-IR) sampling methods that were initially developed for pharmaceutical high-throughput screening systems can be used to effectively characterize the chemical makeup of biological systems like algae lipids. This is important because algae might be able to provide the biomass that is needed to make a lot of biofuels. The use of Fourier transform infrared (FT-IR) spectroscopy in algal biomass analysis has been useful in monitoring biochemical changes [16]. Furthermore, the use of chemometrics in combination with FTIR spectroscopy has been shown to be useful for the discrimination of cyanobacterial strains [17]not needed

Objective:

i. To monitor different parameters like
   - pH
   - Dissolved oxygen
   - Electrical conductivity for obtaining high growth of *chroococcus sp* in photobioreactor

ii. To extract oil from *Chroococcus sp*.

II LITERATURE SURVEY:

MICROALGAE-BASED BIODIESEL PRODUCTION:

Microalgae have the potential to be used in the production of the third generation of biofuels because they can reduce CO2 emissions and produce oil at high productivity. The key technologies for producing microalgal biofuels include the identification of preferable culture conditions for high oil productivity, the development of effective and economical microalgae cultivation systems, as well as separation and harvesting of microalgal biomass and oil. The effects of different microalgal metabolisms (i.e., phototrophic, heterotrophic, mixotrophic, and photo heterotrophic growth), cultivation systems (emphasizing the effect of light sources), and biomass harvesting methods (chemical/physical methods) on Oil production and microalgal biomass are compared and critically analyzed. This review aims to provide useful information to help the future development of efficient and commercially viable technology for microalgae-based biodiesel production [18].

Stirred- tanks Photobioreactor for the cultivation of *Dunaliella salina:*

Sensing in a photobioreactor presents unique difficulties not encountered in conventional fermenters. Since photosynthesis is the primary driver of photobioreactor processes, conventional fermentors fail to take into account a number of photobioreactor-specific parameters. Furthermore, the utilization of intricate sensing hardware is out of the question because photobioreactor processes are typically associated with stringent cost constraints. This necessitates creative solutions to sensing issues in photobioreactors. Jian Li and others, 2003 accounted for the improvement of a powerful model-based assessor that is equipped for following key culture states in mixed tank microalgal photobioreactor frameworks. This study used Dunaliella salina, a marine microalga, as a model organism. Based on a dynamic process model and online dissolved oxygen measurement, the extended Kalman filter (EKF) was used to provide the best estimates of photobioreactor states. This state estimation system offers a financially savvy implication for observing the cycle elements of microalgal photobioreactor societies on the web, through which the efficiency of such an interaction could be improved.

Photobioreactors that work well are needed to make use of photosynthetic cells to make useful metabolites. Although numerous laboratory-scale photobioreactors have been described, the majority are extremely challenging to scale up. Moreover, the utilization of open lakes and outside rounded photobioreactors is restricted by the prerequisite for enormous spaces and the trouble in keeping up with sterile circumstances. In light of this, we designed and built a stirred tank photobioreactor with internal illumination. Similar to conventional stirred tank bioreactors, the photobioreactor is straightforward, heat-sterilizable, and mechanically agitated. In addition, it is simple to scale up while maintaining the productivity and light supply coefficient. Solar light was collected and distributed inside the reactor using optical fibers by a new installation. It had a light-tracking sensor so that the lenses could rotate with the sun's position. This makes it possible to use solar light for photosynthetic cell cultivation in indoor photobioreactors. As a solution to the problems of night biomass loss and low productivity on cloudy days, an artificial light source was coupled with the solar light collecting device. The

artificial light is automatically turned on or off in response to the solar light intensity, which is monitored by a light intensity sensor. In this way, continuous light supply to the reactor is achieved by using solar light during the sunny period and artificial light at night and on cloudy days has been reported by [19].

SMALL LED-BASED PBR:

The algal biotechnological potential has been restricted by a lack of high-density photobioreactors (PBR).Biotechnological potential of algae. Late improvements of profoundly productive light-discharging diodes (Drove utilizing gallium aluminum arsenide chips) have made the improvement of a little Driven based PBR conceivable. In order to support high-density algal cultures using the 680 nm monochromatic red light from an LED as a light source, we have calculated theoretical values for the requirements for light intensity and gas mass transfer. On the basis of these calculations, a prototype PBR has been designed. A cell concentration of more than 2 x 10^9 cells/mL (more than 6.6% v/v), cell doubling times as low as 12 h, and an oxygen production rate as high as 10 mmol oxygen/L culture/h were achieved using on-line ultra filtration to periodically provide freshmedium were reported by **(**Lee *et al*.,1994)[20]**.**

Flat-cuvette photobioreactor:

Small-scale photobioreactors for the cultivation of photoautotrophic microbes are required for the precise characterization of growth parameters of wild-type and engineered strains of these organisms for their screening and for optimization of culture conditions. They described the design and use of a flat-cuvette photobioreactor that allows accurate control of culture irradiance, temperature, pH and gas composition combined with real-time monitoring bya built-in fluorometer and densitometer. The user-created protocols instruct the high-power LED light source to produce precise irradiance levels.The irradiance, temperature, and gas composition may be static or dynamically modulated, while optical density and pH may be stabilized in turbidostat and pH-stat modes, respectively **(**Ladislav Nedbal *et al.*,2008) has been demonstrated that the instrument is able to detect minute variations of growth caused, for example, by sudden dilution or by circadian rhythms. The instrument has enough sensitivity to keep an eye on suspension optical density as low as 10-2. The research and application of photoautotrophic microbes in systems biology and biotechnology can greatly benefit from this newly developed photobioreactor[21].

Helical-tubular photobioreactor for cultivation of *Nannochloropsis sp:*

Nannochloropsis sp. can be controlled and continuously produced in an experimental helical-tubular photobioreactor. The main benefits it offers are: 1) the combination of the optimized light penetration depth and the high ratio of culture volume to surface area; 2) the ease with which contaminants and temperature can be controlled; 3) the efficient spatial distribution of CO2 and fresh air; 4) improved CO2 transfer through the extensive interface surface between the culture-liquid medium and fresh air; and 5) a novel automated flow-through sensor that provides continuous cell concentration monitoring. sp. Nannochloropsis With a mix of natural and artificial light and rather high temperatures, population density reached its highest level.An average daily increase of30×10^6 cells ml-1 was obtained at population densities above 350×10^6 cells ml-1 allowing daily harvesting rates of at least 10% the total volume. Measured cellular density productivity data and estimated volumetric productivity range of g L-1 day -1.10–3.03 g L-1 day-1, areamong the highest *Nannochloropsis sp*. productivities reported by (D. Briassoulis *et al*., 2010)[22**].**

Hydrocarbon, carbohydrate, fatty acid, and carotenoids were influenced by salinity:

Different salinity levels affected the growth of Botryococcus braunii (race "A") and its production of hydrocarbon, carbohydrate, fatty acid, and carotenoids.Under salinity at 34 mM and 85 mM, 1.7–2.25-fold increase in the relative proportion ofpalmitic acid and two fold increases in oleic acid were observed. A twofold increase in carotenoid content was noticed at 85 mM salinity with lutein (75% of total carotenoid) as the major carotenoid followed by β-carotene. The organism's adaptability to the tested salinity levels (17 mM to 85 mM) was shown by the rise in biomass yields and changes in other components.were reported by (A. Ranga Rao *et al*., 2007)[23].

Hybrid types of reactors:

As a means of mitigating the effects of rising atmospheric CO2, cyanobacteria and green algae are receiving increased attention. They can produce renewable energy carriers like carbon-free hydrogen, bioethanol, biodiesel, and other valuable biomolecules in addition to CO2 capture. The characteristics of the microbial strains, as well as their tolerance to temperature and the CO2 in the flue gas, which includes SOx and NOx, have a significant impact on the biological fixation of CO2. However, the CO2 sequestration process will be significantly impacted by additional factors such as the availability of light, pH, O2 removal, appropriate photobioreactor design, culture density, and reactor agitation. Kanhaiya Kumar and others, 2011) describe the various photobioreactor geometry options for producing biomass. In addition, it focuses on hybrid reactors, which combine two reactors to circumvent the limitations of a single photobioreactor [24].

TUBULAR PHOTOBIOREACTORS:

Design and scale-up of tubular photobioreactors were discussed by (E. Molina Grima *et al*., 1999) for the outdoor culture of microalgae. The availability of light always affects cultural productivity, especially as the scale of operations grows. As a result, details of a method for calculating the internal culture illumination levels in outdoor systems are included in the light regime analysis. Algal culture's supply of carbon dioxide is also mentioned as an important feature. Finally, potential scale-up approaches are outlined including promising novel concepts based on fundamentals of the unavoidable light–dark cycling of the culture[25].

HELICOIDAL PHOTOBIOREACTOR:

In order to determine the maximum productivity of Arthrospira platensis cultivated in a helicoidal photobioreactor up to the attainment of pseudo-steady-state conditions, semicontinuous cultures were carried out at various dilution rates (D) and light intensities (I). Maximum cell concentration (Xm = 5772 113 mg L-1) and productivity (PXS = 1319 25 mg L-1 d-1) at the lowest (D = 0.1 day-1) and highest (D = 0.3 day-1) dilution rates, respectively, were maintained in the semicontinuous regime at I = 108 lmol photons m-2 s-1.The cell productivity and the product of light intensity to dilution rate (ID), which were found to have a combined effect on this parameter, were shown to be influenced by a kinetic model derived from Monod's. This result put into evidence that pseudo steady-

state conditions could be modified according to circumstances, conveniently varying one or otherof the two independent variables were demonstrated by (Raquel Pedrosa Bezerra *et al*., 2010)[26].

BIODIESEL FROM MICROALGAE:

Sunlight powers the cell factories of microalgae and cyanobacteria, which use carbon dioxide to make food, feed, and high-value bioactives, as well as biofuels. Additionally, these microorganisms can be utilized as nitrogen-fixing biofertilizers and in a variety of bioremediation processes. (Yusuf Chisti et al., 2006) has been reported that the industrial and environmental applications of microalgae and the production of algae in photobioreactors. Photobioreactors with the right design are much more productive than open ponds and "raceways" that have traditionally been used to cultivate microalgae.o Furthermore, photobioreactors allow monoseptic culture of many more algae than can be grown in open systems In order to design photobioreactors, it is necessary to be able to accurately estimate the level of photosynthetic irradiance; provide the required quantity of carbon dioxide; remove the photosynthesis-produced inhibitory oxygen; ensure prompt light–dark cycling of cells for enhanced productivity[27].

High Rate Algal (HRA) pond for cultivation of *Chlorella vulgaris*

In recent years, in addition to being utilized as single-cell proteins, microalgae have been proposed as living-cell factories for the production of biofuels and a variety of beneficial biochemicals that are utilized in food, aquaculture, poultry and pharmaceutical industries. Chlorella vulgaris, a green microalga isolated from industrial effluents, was the focus of this study, which used a suitable growth medium to cultivate it in a large-scale HRA pond. During cultivation, biomolecules like total protein, total carbohydrates, and total lipid, in addition to the pigments chlorophyll and beta-carotene, were analyzed on a regular basis.In addition, the total bacterial cell numbers were enumerated during the study and their influence on algal growth was studied by (N. Mohan *et al*., 2009). The algal biomass was harvested by low-cost methods such as settling using flocculants and auto-flocculation[28].

Kinetic studies on oil extraction:

Ulva lactuca, a marine macroalga, was the subject of kinetic studies on oil extraction. Scanning electron microscopy and Fourier transform infrared spectroscopy were used to examine the biomass of the algal cells. In order to determine the most effective method for maximizing oil extraction, six distinct pretreatment procedures were tested. At 5% moisture content, 0.12 mm particle size 500 rpm stirrer speed, 55 °C temperature, 140 minutes, and a 6:1 solvent-to-solid ratio in a n-hexane solvent mixture with 1% diethyl-ether and 10% methylene chloride, high oil yield was achieved. From 30 grams of algal biomass, 10.88% (g/g) of oil extraction yield was achieved following optimization. The differential method was used to determine the rate constant for the first-order kinetic study. It was determined that the activation energy (Ea) was 63.031 kJ/mol. The investigation's findings indicate that U. lactuca biomass is an appropriate source for the production of biodiesel [29].

Effect of pH, pO2, optical density, temperature, agitation and light intensity:

Under anaerobic conditions, the green alga Chlamydomonas reinhardtii can photosynthesize and produce molecular hydrogen. It provides a biological means of producing carbon-neutral, renewable hydrogen from water and sunlight. In order to develop suitable system parameters and design photobioreactors, it is necessary to have a better comprehension of the parameters that influence the kinetics of algal growth and hydrogen production. For effective hydrogen production, it is necessary to grow algal biomass in an effective and cost-effective manner and to attain high cell densities. The process conditions and nutrient requirements that encourage the growth of dense, healthy algal cultures were investigated. Sulfur deprivation, which necessitates the exchange of the algal growth medium via centrifugation, dilution, or ultrafiltration, imposed anaerobic conditions. Commissioned was a tubular-flow photobioreactor with a high surface-to-volume ratio and excellent light penetration. The hydrogen production process's most important parameters, such as pH, pO2, optical density, temperature, agitation, and light intensity, were monitored and controlled with it .Analytical techniques are being developed to characterize the reactor products, including dissolved hydrogen measurement by membrane inlet mass spectrometry. An overall H_2 yield of 3.1±0.3ml/l was measured in the tubular-flow photobioreactor were studied by (Bojan Tamburic *et al*., 2009)[30].

EFFECT OF NITROGEN SOURCES, LIGHT INTENSITIES, AERATION RATES, AND VITAMINS:

(Tolga Goksan *et al*., 2011**)** has been reported that the effects of various inorganic nitrogen sources, light intensities, aeration rates, and especially vitamins were investigated in vegetative *Haematococcus Pluvialis* Flotow cultures. NaNO3 (1.0 g/L) and KNO3 (0.5 g/L) had the highest cell densities, achieving 25.3 and 26.3x104 cells/ml, respectively, and were the best nitrogen sources for growth. In vitamin trials, the concentrations of 0.1 (0.3 M), 1.0 (4 M), and 0.1 mg/L (0.75 M) of thiamin, biotin, and B12, respectively, produced the highest cell numbers. Additionally, it was discovered that thiamin alone was sufficient in place of the vitamin blend. Cell growth was significantly higher in 75 and 150 µmol photon m-2 s-1 illuminations compared to 20 and 40 µmol photon m-2 s-1. In terms of aeration, the optimal rate of airflow was 1.0 lpm, while supra-optimum levels halted growth and increased cell diameter and pigment content. Therefore, it may act as a secondary stressor for H. Therefore, it may act as a secondary stressor for H. *Pluvialis* cells in the reddening stage[31].

OPTIMIZATION OF PH, PHOTOPERIOD, NITRATE CONCENTRATION AND LIGHT INTENSITY FOR THREE STRAINS:

Increasing concentrations of CO2 in the atmosphere are causing severe environmental destruction to the earth. Microalgal sequestration is increasingly being explored to capture excess CO2. Producing a large number of value-added products, such as pigments, carotenoids, carbohydrates, lipids, and others, this method has the potential to become a lucrative industry that would also contribute to the reduction of global warming. In this regard, three strains of microalgae, namely, sp. Calothrix, The growth characteristics of Chlorella minutissima and Spirulina platensis were investigated.. Based upon thekinetic parameters, possible by-products, tolerance to CO2, etc., the best strain was selected for further studies. The, photoperiod, optimum pH , light intensity and nitrate concentration for this strain were experimentally determined. Also this studies had been conducted with and without baffles,with varying aeration rates and with two different impellers and i.e., marine propeller and disc turbine impeller, in the presence of air alone and a mixture containing air with 15% CO2. C. minutissima was found to be the best strain for further research out of the three that were examined.It produces the most biomass at pH 6, has a 14-hour photoperiod, and 10 hours of darkness, 5 g/l nitrate, and a light intensity of 6000 lx. The biomass of runs

with baffles was higher. Marine propeller gave better biomass yields in the two runs i.e., with and without extra CO2. Also, the optimum aeration rate was determined to be 1 lpm. The carbohydrate, lipid, protein, chlorophyll and carotenoid content of the biomass were estimated by **(**Vani Sankar *et al*., 2011)[32].

DIFFERENT HETEROTROPHIC GROWTH CONDITIONS:

Biomass and lipid productivities of Chlorella vulgaris under various development conditions were explored. Although autotrophic growth yielded a cellular lipid content that was 38% higher than that of heterotrophic growth, the lipid productivity was significantly lower when compared to that of acetate, glucose, or glycerol. At 1% (w/v) glucose, optimal cell growth (2g l-1) and lipid productivity (54 mg l-1) were achieved, whereas higher concentrations were inhibitory. The dose effects of C. vulgaris growth on glycerol were comparable to those of glucose.Overall, *C.vulgaris* is mixotrophic has been reported by (Yanna Liang *et al* 2009)[33].

Algal oil extraction and lipid content:

Chad Share Weldy *et al*., used Bligh Dyer method of lipid extraction which uses a solvent system of chloroform, methanol and water. (Govindarajan *et al*., 2009) found that magnetic stirrer based agitation was cost effective when compared to extraction methods like super critical extraction, nano-assisted extraction which are widely under research. Apart from this, solvent extraction using hexane and soxhlet apparatus was also carried out according to UNE-EN 734-1 (2006). (Lusia Gouveia *et al*., 2009) extracted oil from *Neochloris oleabundans*by soxhlet apparatus using n-hexane as solvent with sample pre-treatment (propanol) after cell disruption by sonication during 20 minutes.(Govindarajan *et al*., 2009) found that *C.vulgaris* had a lipid content of 14-56% of the dry cell weight. **(**Harris *et al*., 1965) characterized the fatty acid composition of *C.vulgaris*. He found that of the total lipids, 18% of hexanoic acid (C16:0), 5% of C16:w7,12% of C18:2, 2% of C16:3, 9.2 % of C18:1w9, 43% of C18:2w6, 10% of C18:3w3. According to (Borowitzka,1988), the majority of the algae examined contain predominantly saturated and mono-unsaturated fatty acids.S pecifically, the major fatty acids are C16:0 and C18:1 in the Chlorophyceae (Cobelas and Lechado, 1989).The major PUFAs are C18:2 and C18:3w3 in green algae (Basova 2005, Cobelas and Lechado, 1989).The extracted yield of solvent-extracted oil of *Cuminum cyminum* was 18.7%. The oil was classified into hydrocarbon 1%, wax esters 1%, sterol ester 25%, triglycerides 55%, 1,3diglycerides 1%, 1,2 diglycerides 1%, monoglycerides 2%, free fatty acid 10%, phosphatidyl- ethanolamines 2.0%, phospatidylcholine1.2%, lysophosphatidyl eth-anolamines 0.6% and phosphatidylinositol 0.2 Utilizing thin layer and gas liquid chromatography, the fatty acid composition of all neutral and polar lipid classes was analyzed. The oil is considered as a decent wellspring of petroselinic corrosive (51.7%) in the unsaturated fat structure. The scope of unsaturated fat was found from C10 to C20[34,35,36,37,38,39,40].

III.MATERIALS & METHODS:

MATERIALS:

ORGANISM:

CHROOCOCCUS SPECIES

EQUIPMENTS:

- Photobioreactor with white fluorescent light
- Haemocytometer (Neubauer chamber, improved)
- UV-Visible Spectrophotometer (UV- Pharmaspec 1700, Shimadzu, Japan)
- Centrifuge (Remi), Orion star digital Meter (Thermal electrical co-operation)
- Digital Lux Meter
- Air controller (Eureka)
- Laminar Air Flow
- Hot Air Oven
- Test tubes
- Screw cap bottle
- Silicon tubes
- Aquarium air pump
- Glass tubes
- FT-IR
- Separating funnel
- TLC plate

PREPARATION OF CHEMICALS:

Composition of CFTRI Medium:

Chemical Composition	g/L
Sodium bicarbonate	4.5
Dipotassium hydrogen phosphate	0.5
Sodium nitrate	1.5
Potassium sulphate	1.0
Sodium chloride	1.0
Magnesium sulphate hepta hydrate	0.2
Calcium chloride	0.04
Ferrous sulphate	0.01

Stock solutions of 100X were prepared, autoclaved and stored in a sterile condition.When required, the medium was prepared from sterile stock solutions. For the preparation (1X Working solution) of 2000mL CFTRI Medium, 1840mL of water was sterilized initially. To this required amount of the chemicals from the stock solution was added. The pH was adjusted to 10.0.

METHODOLOGY:

Cultivation of *Chroococcus sp:*

The *Chroococcus sp.* was kept in a light chamber of light intensity 2000 Lux and temperature at 30.8°C. 100mL of the culture was added to 2000mL of the CFTRI medium. The light chamber was used to give 13.7/10.3 light/dark cycle and the optical density was measuredat 540nm.

CELL COUNTING BY HAEMOCYTOMETER:

This was performed before inoculating cells into the medium and also used to monitor the growth on a daily basis. The cells were suitably diluted (1:100). The cell number was calculated by counting the cells in all grids and multiplying it with the dilution factor (10^2) and with a conversion factor 10^4. The cells were inoculated in such a way that the final concentration was 10^6 cells/mL. To attain the above concentration in CFTRI medium, 100mL from the *Chroococcus sp.* culture were inoculated. The doubling time (d.t) and Specific growth rate (µ) and were calculated as in following equation:

$$\mu = \ln X2 - \ln X1/t2 - t1$$

$$d.t = \ln 2 / \mu$$

LIGHT SOURCE:

As for photosynthesis the light spectra used by white fluorescent light CFL which has thelight intensity (24 µmoles m^{2-} sec^{1-}) that is sufficient for the photosynthesis of *Chroococcus sp*. CFL was small enough to fit in photobioreactor.

MEASUREMENTS OF DISSOLVED OXYGEN:

The dissolved oxygen concentration in the *chroococcus sp.* growth was measured on daily basis with Orion star digital DO electrode (Thermal electrical co-operation).Dissolved oxygen was measured in a routine basis and that the dissolved oxygen levels.

Air flow rate:

Air flow rate can also be correlated to the media flow rate because both will control the turbulence inside the phototubes. Algae will settle on the bottom of still media and will compressand kill any algae that may be on the bottom of the settlement. Therefore continuous agitation is required to ensure proper mixing and suspension of the algae cells. In order to increase the CO2 and substrate suspension, proper mixing is also very important. When turbulence increases so does the probability that an algae cell can come into contact with substrate or CO2 and thus increasing thenutrient uptake rate. This will result in better growth. Another benefit in keeping the turbulence as high as possible without damaging the cells is that the light-dark cycling would be increased with higher turbulence. This would give the cells better efficiency in photosynthesis and thus have better growth rates. The air flow rate was mentioned in different levels like 0.5, 1.0 and 1.5 liter/minutes.

MEASUREMENTS OF ELECTRICAL CONDUCTIVITY AND PH:

Electrical conductivity and pH levels were measured by Orion star digital conductivity and pH electrode (Thermal electrical co-operation) were used to monitor the growth of *chroococcus sp.* Both pH and electrical conductivity were measured daily.

PHOTOBIOREACTOR DESIGN:

A novel 2 liter Tubular PBR (Photobioreactor) has been designed for cultivation of *Chroococcus sp.* in large scale to obtain high amount of oil. Coherent, non-heating illumination was provided by a panel of white fluorescent light CFL.The reactor consists of three glass tubes in between this tubes the two white fluorescent lights were placed in equal distance (15cm) from each tube. Here, three glass tubes were connected together by silicon tubes. In 1st tube contained 4 holes in which 2 filter hole for gas in and culture medium was poured into other 1 hole and other 1 for air flow connected through aquarium pump. Similarly 3rd tube contained 4 holes in which 2 filter hole for gas out and other 1 hole for CO_2 analyzer and other 1 used for collected sample. The reactor was fully autoclavable and molecular nature of the reactor allowed efficient cleaning and maintenance.

Fig 4.Photobioreactor design .

Qualitative analysis by FT-IR:

Extracted algal biomass were suspended in 10 mL chloroform (2mg), thoroughly mixed with KBr and dried at 100 °C for 4 hours. Using a Fourier transform IR spectrometer, the dried sample received an FT-IR spectrum.

Lipid extraction:

Extraction of lipid was done by following the protocol of modified Bligh and dyer (1959).The cells were harvested by centrifugation at 10,000rpm for 15minutes at 4 °C. The supernatant was discarded and pellet was subjected to wet weight estimation and then dried in oven for 2 hours at 80 °C. Chloroform and methanol was added in equal ratio 1:1 and transfer to separating panel and leave it for 24hours at room temperature. After 24 hours the layers were separated and lower organic layer containing lipids was transferred to a clean pre-weighed vial (W_1). Evaporation was carried out in hot air oven at 80 °C for 40 minutes. The weight of the vial had been again recorded (W_2). Lipid content was calculated by subtracting W_1 from W_2 and was expressed as % dry cell weight[41].

$$\text{Lipid content} = (W_2 - W_1) \times 100$$

The lipid productivity was calculated by the equation given below[42]:

$$P_{Liquid}\ gL^{-1}day^{-1} = \frac{C_{Lipid}\,(g/g) \times DCW\ (g/L)}{\text{Time (day)}}$$

Where, P_{Liquid} is lipid productivity, C_{Lipid} is lipid content of cells, DCW is dry cell weight andTime is the cultivation period in days.

Analytical Methods:

Estimation of Carbohydrates by Anthrone Method

Table, Standard for Glucose:

Concentrationof glucose	Volume ofAnthrone	Incubationtime	Absorbanceat 630nm
0.2	4	Heat for 8minutes	0.126
0.4	4		0.528
0.6	4		0.631
0.8	4		1.189
1.0	4		1.875
Ultra-sonication sample	4		0.214
Supernatant sample	4		0.092

CALCULATION:

µg of glucose × 100Volume of test sample

Estimation of Protein by Lowry's MethodTable: Standard for BSA:

Concentrationof BSA	Volume of Alkaline copper sulphate	Volume ofFolin-ciocalteau	Incubation Time	Absorbanceat 540nm
0.2	5	0.5	Kept dark conditions For 30minutes at room temperature	0.025
0.4	5	0.5		0.073
0.6	5	0.5		0.101
0.8	5	0.5		0.124
Ultra-sonication sample	5	0.5		0.864
Supernatant sample	5	0.5		0.915

Calculation:

µg of BSA × 100Volume of test sample

HETEROTROPHIC CULTURE:

For the heterotrophic cultivation of algae, carbon source viz glucose was used. 1% of thesterilized solutions were added to the 6-day old culture and the biomass was estimated on 9th day.

THIN LAYER CHROMATOGRAPHY:

The oil was fractionated qualitatively and quantitatively on 0.25mm and 0.5mm thick silica gel chromatoplates. Thin layer chromatograms of 0.25mm thickness were prepared by using 25 gm silica gel and 50 ml water. For two hours these plates were activated . A known weight of oil (10% solution in chloroform) was loaded in a straight line about 3cm above the lower edge of chromatogram. The developing media for neutral and polar lipids were isopropyl ester: acetic acid (24:1) 0.5 % ethanolic rhodamine B was used for the identification of lipids. Appearance of yellow/blue violet spots on a pink background TLC plate when kept at 100°C for 10 min., confirmed the presence of these compounds. The reagent molybdenum blue dragendorff and ninhydrin were also used for the identification of phospholipids, phosphatidylcholine and lysophosphatidylethanolamine which showed blue, strand orange and red violet spot, respectively on thin layer chromatography **[**43].

IV. RESULTS AND DISCUSSION:

PREPARATION OF SEED INOCULUM:

Chroococcus sp. was cultivated in screw cap bottle containing 2 liter of CFTRI medium. The cultures were maintained at a temperature of 30.8°C.The light-dark cycle was maintained as 13.7:10.3 hours for 6 day till the cell density reaches to 0.1.this culture of *Chroococcus sp*. was used as seed culture for tubular photobioreactor.

Cultivation of *Chroococcus sp*. in tubular photobioreactor:

A novel 2 liter Tubular PBR (Photobioreactor) has been designed for cultivation of *Chroococcus sp.* in large scale to obtain high amount of oil. The seed inoculum was poured in a photobioreactor through peristaltic pump and the air flow was given by aquarium pump for their proper mixing. The air flow rate was controlled by air controller and it was adjusted to 0.5 -1.5 lit/minutes. The light intensity should be maintained as 2000 lux. The light-dark cycle was maintained as 13.7:10.3 hours at ambient temperature. The samples were collected periodically at an interval of 24hrs and the cell numbers were counted by using Haemocytometer (Neubauer chamber, improved) and the optical density was measured at 540 nm. The dissolved oxygen, electrical conductivity and pH were measured daily basis by Orion star digital DO, conductivity and pH electrode (Thermal electric co-operation).

EFFECTS OF DISSOLVED OXYGEN, AIR FLOW RATE, PH AND ELECTRICAL CONDUCTIVITY:

Effects of dissolved oxygen on *Chroococcus sp*. growth was measured by Orion star digital DO electrode. Dissolved oxygen was measured routine basis and the result indicates that the dissolved oxygen levels around 2.95, 3.90 and 4.43 mg/mL in air flow rate were maintained as 0.5, 1.0 and 1.5 liter/minutes. In photosynthesis, it generates oxygen, dissolved oxygen levels much greater than air saturation values inhibit photosynthesis. Air flow was adjusted to 0.5 -1.5 lit/minutes. Air flow should be maintained 1.0, when the biomass was better compared to another flow rate.Vani Sankar *et al*., 2011 has been demonstrated that *C. minutissima* are small eukaryoticorganisms and are susceptible to high shear rates. High aeration rates were found to be detrimental to the growth of algae. At 2 lpm, the biomass yield was only 0.56785 g/l at the end of120 hour cultivation. On the other hand, culture at very low flow rate of 0.5 lp resulted in even lower productivities. In this case, the cells were observed to sediment at the bottom of the reactor. The biomass was as low as 0.4123 g/l. The best productivity was obtained with 1 lpm flow rate. The biomass in this case was 0.97794 g/l and was

considerably higher than the other obtained biomass yields. In our present study, Chroococcus sp. was small eukaryotic organisms and was susceptible to high shear rates. High aeration rates were found to be detrimental to the growth of algae. At 1.5 liter/minutes the biomass yield was only 0.714 g/L at the end of 144 hours cultivation (Fig. 2). On the other hand, culture at very low flow rate of 0.5 liter/minutes resulted in even lower productivities. In this case, the cells were observed to sediment to the bottom of the reactor and there was no proper mixing in the culture medium. The biomass was as low as 0.824 g/l. The best productivity was obtained with 1.0 liter/minutes flow rate and the biomass yield was 1.15g/L. Vani Sankar *et al*., 2011 may justify the high flow rate in cultivation of algae inhibit photosynthesis and the cells were observed to sediment at the bottom of the reactor and there was no proper mixing in the culture medium in low flow rate[43].

Both pH and conductivity were measured on daily and the results indicate that the pH level rise from 9.0 on day 0 to 9.86 on day 6. For all the 6 days, the increase in pH was gradual and after the 6th day, it was around 9.90. Regarding the electrical conductivity, it was around3.95 µs cm^{-1} throughout the study.Various studies were conducted on *Chroococcus sp.* during their growth. The growth curve pattern of *Chroococcus sp.* showed similar patterns depicting log phase, lag phase, stationary phase and decline phase. The growth of *Chroococcus sp.* was maximum on the 25th day whereas from the cell numbers obtained, the growth rate /day was calculated for *Chlorella vulgaris* had a growth rate of 0.16 divisions/day. In case of pH and conductivity study, showed as steady increase in pH reaching to around 9 at the end of the study. Electrical conductivity was also measured since this is an indirect indication of total dissolved solids. In *Chlorella sp.,* remained almost the same throughout the study were reported by N. Mohan et al., 2009.This may justify the pH level increases because of consumption of carbon dioxide **(**Camacho Rubio *et al*., 1999**)** and electrical conductivity same through out study[44,45].Tabl**e** reported routine cycles of *Chroococcus sp.* growth till 6th day. Each cycle startswith 13.7 hours light cycle of white fluorescent light and 10.3 hours dark cycle which was optimized previously. The air flow rate was given as 0.5 liter/minutes.24 hours once the optical density was measured at 540nm using UV-Visible Spectrophotometer (UV- Pharmaspec 1700, Shimadzu, Japan).The cell number was counted by using Haemocytometer and biomass was calculated from optical density.

Growth of *Chroococcus sp*. in different air flow rate:

S.NO	Time (Days)	Biomass (g/L)		
		Air flow rate (liter/minutes)		
		0.5	1.0	1.5
1	0	0.098	0.108	0.099
2	1	0.124	0.216	0.166
3	2	0.229	0.347	0.291
4	3	0.421	0.469	0.443
5	4	0.679	0.635	0.537
6	5	0.741	0.765	0.624
7	6	0.824	1.15	0.716

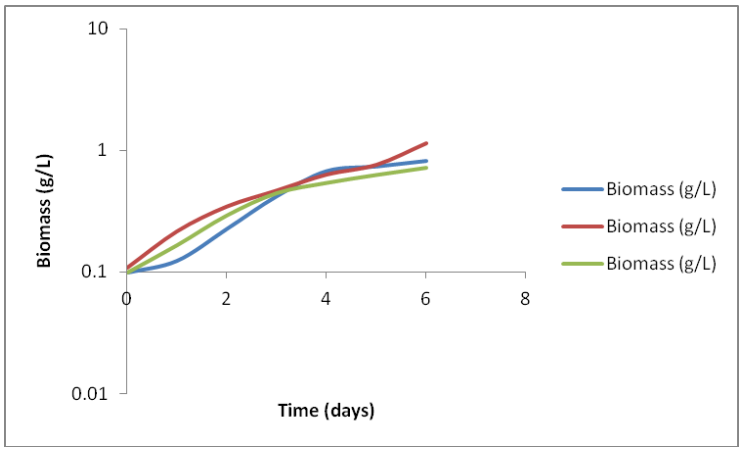

Fig .5 Growth curve of *Chroococcus sp*. in different air flow rate

HETEROTROPHIC CULTIVATION:

Microalgae *Chroococcus sp*. can grow heterotrophically with glucose as the carbonsource and accumulate high proportion of lipids (Chunfang Gsao, 2010). Heterotrophic cultivation was attempted by adding sterile exogenous carbon source viz glucose to the culture of*Chroococcus sp*. on 6th day of phototrophic cultivation. On 6th day of phototrophic cultivation thedry cell biomass of Chroococcus sp.was 0.824 g/L however the biomass increased to 4.29g/L on 9th day during heterotrophic cultivation. It was observed that the biomass was increased though there was not much increase in cell number. Extraneous carbon sources can be incorporated into the medium in order to increase the biomass addition of 1% glucose. It also affects the growth rate. During heterotrophic cultivation loss of green colour was which may be due to accumulation of lipid in cell has been reported by Yanna Liang *et al*., 2009 that the lipid accumulation results indissoluble of *Chroococcus sp*. Wang *et al*., 2002 reported that productionof reactive oxygen (e.g. O_2 and O_2^-) during the process of degradation of plant cells[46,47,48

Growth curve of *Chroococcus sp*. in Heterotrophic cultivation:

Time (Days)	Biomass (g/L)
0	0.098
1	0.124
2	0.229
3	0.421
4	0.679
5	0.741
6	0.824
9	4.29

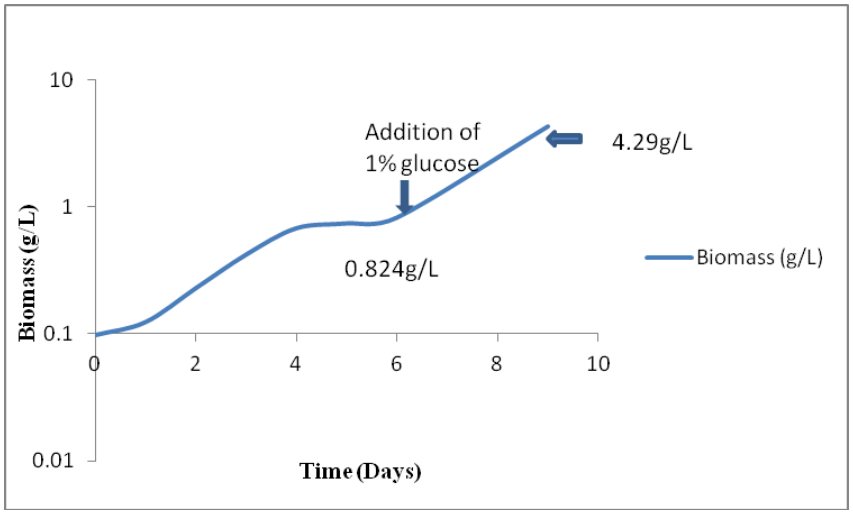

Figure 6.Growth curve of *Chroococcus sp*. in Heterotrophic cultivation

The carbohydrate was estimated from 100mL of 6 days old culture of Chroococcus sp. The cells were separated by centrifugation and the pellets were ultra sonicated to extract intracellular compounds. This was subjected to carbohydrate estimation by modified Anthrone method. The results shows that the carbohydrate content in ultrsonication sample and supernatantwere 28.44 µg/100 ml and 20.75 µg/100 ml. The total protein according to Lowry *et al*. (1951**)** and the results show that the protein content in ultrsonication sample and supernatant were 497.00 µg/100 ml and 526.78 µg/100 ml. Compared to supernatant the ultrsonication sample shows high carbohydrate and protein content in cell density. This may be due to release of intracellular polysaccharides during ultrsonication[49].The total amount of lipids, as determined by Bligh and Dyer (1959). From 100 mL of the culture of Chroococcus sp. 4.35 g, 5.2g and 3.81g of dry cell weight were obtained in cultivation of algae in different air flow rate[50].The lipid productivity was calculated using the dry cell weight. It was found to be 358 mg/L/day, 492 mg/L/day and 268 mg/L/day for Chroococcus sp. respectively with different air flow rate in cultivation of algae.Gouveia and Oliveira, 2009 found that the 43.2 % lipid in dry cell weight (DCW) in C.vulgaris as 14-56%. In this case, it was found to be Illman *et al*., 2000 found the lipid productivity of C.vulgaris as 14.9 (mg/L/day).In this case, for Chroococcus sp. lipid productivity was calculated as 358 mg/L/day, 492 mg/L/day and 268 mg/L/day respectively with different air flow rate in cultivation of algae[51,52].

EFFECT OF AIR FLOW RATE IN LIPID PRODUCTIVITY:

Air flow rate (Liter/minutes)	Lipid productivity (mg/L/day)
0.5	358
1.0	492
1.5	268

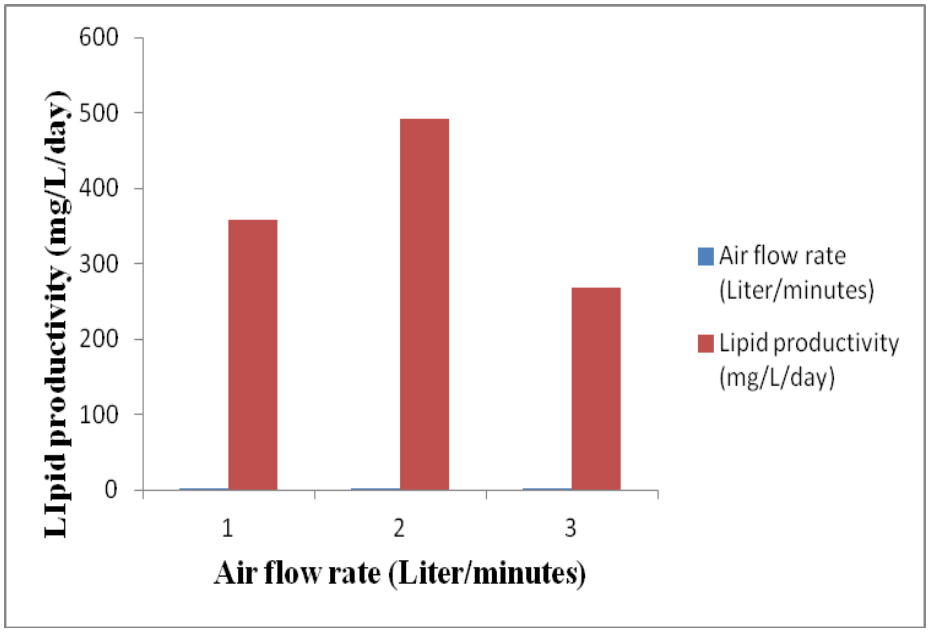

Figure 7 Effect of air flow rate in lipid productivity

THIN LAYER CHROMATOGRAPHY:

Thin layer chromatography (TLC) was performed for the esterified oil samples and lipid extracted from *Chroococcus sp.* The oil was fractionated qualitatively and quantitatively on 0.25mm and 0.5mm thick silica gel chromatoplates. Thin layer chromatograms of 0.25mm thickness were prepared by using 25 gm silica gel and 50 ml water. For two hours these plates were activated . A known weight of oil (10% solution in chloroform) was loaded in a straight line about 3cm above the lower edge of chromatogram. The developing media for neutral and polar lipids were isopropyl ester: acetic acid (24:1) 0.5 % ethanolic rhodamine B wasused for the identification of lipids. Appearance of yellow violet spots on a pink background TLC plate when kept at 100°C for 10 min., confirmed the presence of these compounds. The reagent molybdenum blue dragendorff and ninhydrin were also used for the identification of phospholipids, phosphatidylcholine and lysophosphatidylethanolamine which showed blue, strand orange and red violet spot, respectively on thin layer chromatography were reported by Lowsenstein, 1969)[53].Patil and others, determined the triglyceride Rf values (Rf. Methyl ester (Rf. TG) and ME) as 0.645 and 0.564. The Rf value of the lipid that was extracted from Chroococcus sp. was 0.547[54].

FT-IR ANALYSIS:

The extracted algal biomass were suspended in 10 mL chloroform (2mg), thoroughly mixed with KBr and dried at 100 °C for 4 hour. Using a Fourier transform IR spectrometer, the dried sample received an FT-IR spectrum.The result showed **C═O:** The main characteristic of the IR spectra of carbonylic compounds (aldehydes, acids, etc.) is the strong C═O stretching absorption band in the region of 1870–1540 cm-1. On account of esters, this band shows up in the 1750-1735 cm-1. C─O─C: equivalent to ethers. A strong band is produced by these stretching vibrations in the 1200–900 cm-1 range. C─H: absorption bands, such as 2924 and 2854 cm-1, which represent the asymmetric and symmetric vibrational modes of methyl groups, are examples of absorption bands that are associated with the vibrations of C-H bonds.**CO_2:** they produce strong bands in between 2854-2000 cm^{-1} as well as in 700 cm-1 region. **H_2O:** the adsorption bands of water can be observed in the range of 1750-2000 cm^{-1}. As numerous algal species have been found to develop quickly and produce significant measures of TAG or oil and are in this way alluded to as oleaginous green growth.

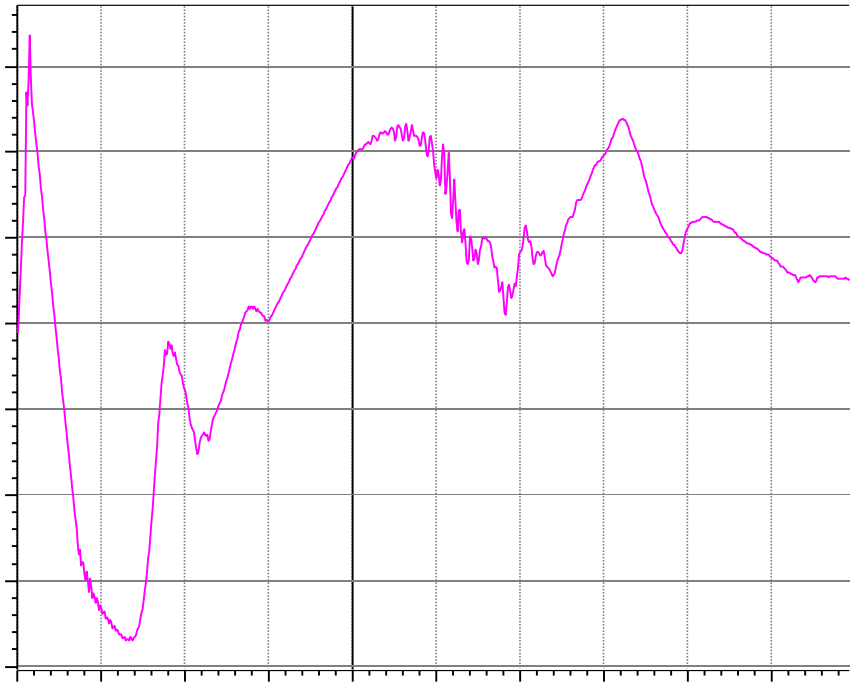

Fig 8.FTIR Analysis

V. CONCLUSIONS:

Laboratory scale to pilot scale was necessary to obtained high amount of oil. A novel 2 liter Tubular PBR (Photobioreactor) has been designed for cultivation of *Chroococcus sp.* in large scale to obtain high amount of oil. The effects of dissolved oxygen, air flow rate, pH and electrical conductivity were summarized as follows:

- Dissolved oxygen was measured routine basis and the result indicates that the dissolved oxygen levels around 2.95, 3.90 and 4.43 mg/mL in air flow rate were maintained as 0.5,
1.0 and 1.5 liter/minutes.

- The air flow rate should be maintained 1.0, when the biomass was better compared to other flow rate for growth of *Chroococcus sp*

- Both pH and conductivity were measured on daily and the results indicate that the pH level rise from 9.0 on day 0 to 9.86 on day 6. For all the 6 days, the increase in pH was gradual and after the 6th day, it was around 9.90. Regarding the electrical conductivity,it was around 3.95 µs cm^{-1} throughout the study.

- The lipid productivity was calculated using the dry cell weight. It was found to be 358 mg/L/day, 492 mg/L/day and 268 mg/L/day for *Chroococcus sp*. respectively withdifferent air flow rate in cultivation of algae.

- The lipid algal cells were identified by Thin Layer Chromatography and extracted algal biomass was analyzed by FT-IR.

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