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Microalgal lipid augmentation of *Chlorella* sp. and algal biodiesel production using CaO as catalyst-A green outlook

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Abstract

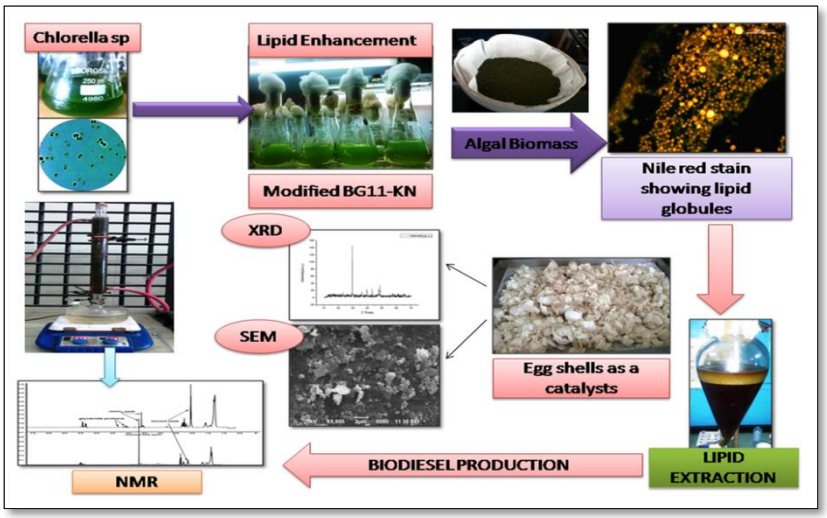
The present work has been undertaken to enhance the biomass and lipid content of microalga *Chlorella* sp. Here, KNO_3 in microalgae growth media BG11 has been used as a growth promoting agent for increased lipid production. The produced lipid has been converted into biodiesel using CaO as a catalyst. CaO catalyst has been prepared using waste egg shells. The produced catalyst has been characterized through XRD and SEM techniques. The production of biodiesel has been confirmed through ^{1}HNMR. It has been found that 17mM KNO_3 concentration in BG11 media is best for fastest growth of *Chlorella* sp and enhanced production of lipid.

Keywords: *Chlorella* sp., KNO_3, Lipid, CaO catalyst, biodiesel, ^{1}H NMR

1.Introduction

Global fossil fuel reserves are likely to deplete by the next 50–60 years that is a present day big concern for the future fuel supply stability [1]. Thus it is important to search out an alternative fuel for the sustainable development of the civilization globally and compensate the depletion of fossil resources. In the course of last 10 years many researchers have been enormously engaged in the production of liquid biofuels, like biodiesel, bioethanol, butanol and green diesel [2]. Among all these biofuels only bio diesel, which has been synthesized either via catalytic transformation of fats or triglycerides to fatty acid methyl esters shows the same fuel properties as conventional diesel, since it has an alike composition it can be readily utilized to replace the fossil fuels [3]. Biodiesel, can easily produced from a variety of feed stocks (i.e., vegetable oil and animal fat, microalgae)But the problem of using vegetable oil and animal fats is that they lead to the challenges of food Vs fuel dilemma and requires a big cultivable land [4]. Thus whole of the attention is been drawn to the use of microalgae which is a potential feedstock for the biodiesel production. However, the viability of this technology as a potential source of renewable energy at a global scale requires considerable increases to be achieved in production of algal feedstock in order to meet up the increasing demands [5]. Selection of the potential strain whose biofuel properties can be harnessed solely depends on the lipid content of the species. More the amount of lipid content more will be the production of biodiesel. But, at the first step we can say that both biomass and lipid content is species dependent [6]. However, growth factors such as nitrogen content of the media, substrate type, elemental composition, illumination or light intensity, cultivation factors, and photo bioreactor designs influences a lot for the final productivities of the biomass and lipid to higher extent. Efforts are being made by many researchers to enhance the biomass content and lipid content of the algal species [7]. Now, prime focus is on the use of microalgae *Chlorella*, which is unicellular eukaryotic green algae belonging to the phylum chlorophyta. This species is known for having higher energy in the form of TAG (triacylglyceride) of appropriate quantity 30 to 50 wt% in photoautotrophic conditions and more than 50 wt% in mixotrophic conditions. Studies have shown that lipid content of *Chlorella protothecoides* is increased from 15% to 55% due to glucose supply of 10g/L [8]. *C. vulgaris* achieved biomass productivity of 254 mg/L.d and lipid content upto 38% in growth conditions of using three different carbon sources in the media with light illuminations in it that is the growth in the mixotrophic conditions [9]. Another work reported higher cell densities in *C. vulgaris* was when it was grown heterotrophically and then was subjected to a light mediated condition for photo induction. After 12 h of illumination, biomass content reached a level of 3333 mg/L.d and lipid productivity was 85 mg/L.d while lipid content of 25% [10]. Although it is now clear

that the heterotrophic conditions improved the biomass productivities and lipid content significantly, this cultivation method was not feasible and cost effectiveness has been bothering the algal feedstock supplying industries [5]. After the proper growth regulating conditions it is necessary to harvest the algae and utilization of proper drying methods is required, followed by that algae extraction is important, many extraction methods using solvents had already been reported by quite a good number of researchers [11]. However the focus is now primarily given on how much lipid can be extracted from the biomass so that it can be used for biodiesel production. A study reported that nanomaterials are applied for the extraction of lipid [12].Since these nano materials are good carriers due to its higher surface area, and by the filtration and centrifugation process these solid nanomaterials can be easily recovered from liquid phase. A study recently revealed that implication of nano sphere silica helped in the extraction of lipid from the microalgae during the time of cultivation itself [17]. After the lipid has been extracted these, algal lipid is now transesterified to biodiesel. Algae lipid transesterification has been already reported by a few authors. Alkali catalyzed transesterification results 95% FAME yields within 1 hour of the reaction using KOH as a catalyst [18]. However NaOH is being utilized for catalytic transformation of lipid to biodiesel of algal species like Chlorella and Nannochloropsis [13].In-situ transesterification of Chlorella sp was done using acid H_2SO_4 and HCl as a catalysts; however, the conversion of TGA to FAME was found to be low after 4hrs of transesterification reaction and temperature between 60° to 90° [13].The FAME yield was found to be 60-70 wt %.And moreover use of acid catalysts is not regarded hazardous. Due to the presence of oxygen, biodiesel is not compatible with diesel motors as it can lead to corrosion of the engines and incomplete combustion[13,16].Thus the lipid can be catalytically deoxygenated to form the green diesel or renewable diesel [19].Using of basic renewable heteregenous catalysts can lead to high conversion efficiency of biodiesel along with that these catalysts are reusable and environmental benign in nature [20]. Lithium doped calcium oxide catalysts is used for transesterification of non-edible vegetable oil extracted from *Mesua ferrea* Linn) seeds [21].The aforementioned summary has clearly stated that cultivation and growth of algae is not a easy task, if we look for biomass productivity then the lipid content is detoriated, and if we focus on enhancement of the lipid than, biomass yield is detoriated. Many workers are working in this field however proper techniques are yet to be explored and implemented. This work is basically focused on using BG11 media which is modified little bit and nitrogen is added as nutrient source with optimized amount for the biomass yield and increase in lipid content of microalgae *Chlorella* sp. After providing the various growth regulating conditions, the biomass is harvested and lyophilized to extract the lipid. Proper adoptions of extraction methods have lead to more amount of lipid. This lipid is further more catalytically transformed to biodiesel using calcium oxide nano particles derived from waste egg shells which gives a clean and green fuel.



Graphical Abstract

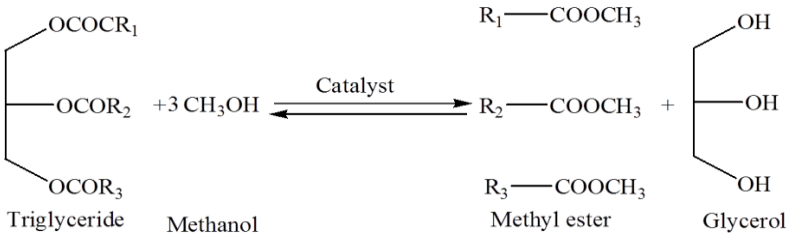


Fig: 1 Transesterification of Microalgal lipid to biodiesel

2.Experimental

2.1 *Strain collection*:

It is very important to select the proper strain before conducting any studies. The selection of the species came into our consideration to use the *Chlorella* sp as the particular species for biofuel production because of its higher fatty acid content in it, which can be transesterified to FAME [22].The freshwater unicellular green microalgae *Chlorella sp* strain was collected from Department of Biotechnology, Gauhati University. Under the study with labomed compound microscope in 10x resolution the details gathered is that the species cell is spherical, non-motile, and minute with 4.5-5.0µ diameter with a single cup-shaped chloroplast.

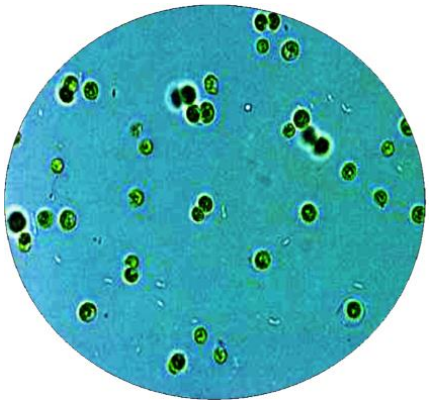


Fig 2: Microscopic image of Chlorella sp. seen under 40x resolution

2.2 *Light condition*:

To conduct our experiments fluorescent lamps were used as a source of light and intensity was adjusted to 2000~2500 lux for all the culture flasks and 14hrs of light and 10 hrs of dark cycles were repeated for growth of all the cultures. The temperature was adjusted at 23 ^{o}C for all the flasks.

2.3 *Growth stimulating Condition:*

To facilitate photo-autotrophic cultivation a media called as BG-11 was modified as follows (ing/L):$NaNO_3$(1.5g),KH_2PO_4(0.004g),$MgSO_4.7H_2O$(0.075g),$CaCl_2.2H_2O$(0.027g),EDTA.Na_2(0.001g),Citric acid(0.006g),Ammonium Ferric Citrate(0.006g),Na_2CO_3(0.02g) and micro element solution of 1mL.The pH of the medium was adjusted to 7 before autoclaving at 121^{o}C for 20min.Pure strain microalgae of *Chlorella sp.* was cultivated in four different nitrogen concentration where nitrogen source is from $NaNO_3$,KNO_3,NH_4NO_3,Urea (BGNN,BGKN,BGAN,BGU).The microalgae was cultured in a 500mL Erlenmeyer flask containing 250 working volume with initial cell 1×10^6 cells/mL (10%, v/v inoculums). The flasks were left at room temperature (25±3^{o}C), aeration and mixing were achieved by air pump with a flow rate 5L/min. In addition, the light was supply by cool white fluorescent lamp with light intensity of 2,000 lux using interval periods of 12h: 12h in the dark, respectively.

Fig 3: The growth stimulating conditions provided inside the laboratory.

2.4 *Analytical methods*:
The microscopic cell count was performed directly by Neubour haemocytometer using optical microscope (Labomed). Optical densities of microalgae cultures were studied at a regular interval of time (24Hrs) by taking the absorbance at 680nm with the help of spectrophotometer (Systronics) in four replicates and average value was recorded. The spectrophotometer was blanked was done every time with each of the medium.

2.5 *Determination of Specific Growth Rate*
Specific Growth Rate is a measure of number of generations (the number of doublings) that occur per unit of time in an exponentially growing culture. The exponential (straight line) phase of growth was carefully determined and specific growth rate was obtained using following equation [23].
μ= ln (*Nt/No*)/*Tt-To*
Nt= No of cells at the end of log phase.
No= No of cells at the start of log phase
Tt= Final day of log phase
To= Starting day of log phase
If T expressed in days from the growth rate (μ) can be converted to division or doublings per
Day (k) by dividing (μ) by the natural log of 2(0.6931). K=μ/0.6931
The time required to achieve a doubling of the no of viable cells is termed as doubling time (Tt) Which is calculated by the following formula- Tt= 0.6931/μ

2.6 *Harvesting of Algal biomass*:
The algal cells of *Chlorella* sp were harvested from the culture medium by auto-flocculation method following up by centrifugation at 5000rpm for 4 min or by filtration technique through preweighted GF/C filter paper. Before doing the harvesting process it is necessary to adjust the pH at optimum level. Since the pH of the culture was pH 10±3 by adding of 1M KOH solution. However the initial culture pH at the time harvesting was diagnosed as 8.Then after the adjustment of the pH, the culture was stirred manually at 200 rpm (approximately) for 10 min using a clean glass rod, and kept for 60–120 min at room temperature (25 ± 2◦C) to facilitate the settlement of the cells. The cells were finally harvested from the bottom of the culture vessels by centrifugation at 5000rpm for 4 min. Harvested cells were then washed with double distilled water followed by centrifugation and lyophilized for 48h at -180◦C using a freeze drier (SCANVAC Cool safe, ALPHA 1-4, Germany) [23]
.
2.7 *Determination of total protein*:
Determination of total protein content in the algal biomass was done by Lowry's method with slight modification. Appropriate volume of the protein standard stock containing 0, 20, 40, 60, 80, 100, 120 and 140 *μ*g of BSA were used to prepare the standard curve by taking the optical density of the blue colored complex developed at 660nm (Markwell, 1978).

$$\text{Protein yeild}(\text{mg/gm}) = \frac{\text{protein value from standard curve}}{\text{volume of test sample}} \times 100$$

2.8 *Determination of carbohydrate content*:

The carbohydrate content was estimated following the method provided by Hedge and colleague with slight modification. The optical density of the green colored complex was measured at 630nm.A calibration curve has to be prepared using glucose as standard concentration of 0.002mg-0.01mg [25].

$$\text{Carbohydrate yeild mg/gm} = \frac{\text{Carbohydrate values from standard curve}}{\text{volume of the test sample}} \times 100$$

2.9 *Staining of Microalgae Cells with Nile Red Fluorescence Dye*:

When the early stationary phase is achieved then it is the period for accumulation of neutral lipids. So, in this stage biomass is collected and harvested for tits study. The cells then were harvested and centrifuged at 5000 rpm for 10min.The algal samples cultivated in the media were washed three times with double distilled water. Then, algal cells 30mL were mixed with 600mL of 25% DMSO. The mixture was pretreated using a microwave oven for 3min. After the addition of 30mL of Nile red solution (300mg/mL in acetone),the mixture was placed in a microwave oven for 1min, and stained in the dark for 10min.Next viewed under a fluorescent microscope (Olympus, BH-2,RFCA) with 100× objective lens were used to visualize the fluorescent yellow-gold neutral lipid globules in microalgal cells [26].

2.10 *Determination of total lipid:*

Microalgal lipid extraction was done by Bligh and Dyer method. The lipid content was determined gravimetrically and expressed as dry weight % after evaporating the chloroform [27].

2.11 *Catalyst preparation*:

The egg shells catalyst was prepared by calcination method. The collected eggshells were cleaned properly at first with tap water to remove the organic impurities adhered to its inner surface and then rinsed with double distilled water for 6-8 times. Then it was dried in oven overnight at 200°C.Then the crushed eggshells were sieved (100-200 mesh) and subjected to heat treatment at 700°C for 4hrs in the muffle furnace [21].

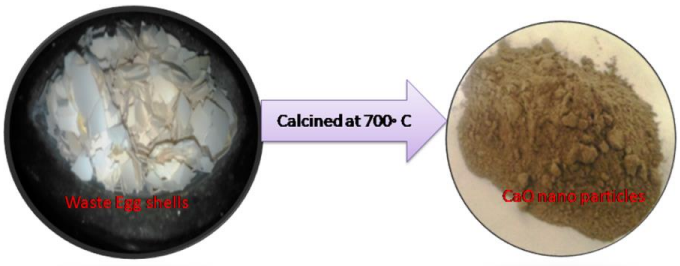


Fig.4. Egg shells as catalysts calcined at 700◦ C as a source of calcium oxide

2.12 *Determination of the fatty acids present in the lipid by NMR (spectroscopy)*

All the ^{1}H NMR spectra were recorded on a Bruker 500 MHz NMR (11.7 T) spectrometer equipped with broadband probe(BB) and inverse broadband probe (BBI).The solutions were prepared by dissolving approximately 2 to 7 mg of microalgae extracts in choloroform.And later the NMR spectra are analyzed [28].

2.*13 Biodiesel Production and Fatty Acid Analysis*

One step in-situ transesterification process was used to transform the algal biomass to biodiesel. The dried algae samples (200mg) were weighed into 25mL screw-top dried glass vial, 5mL of the mixture of methanol and concentrated sulfuric acid (4:1) and 7mL of hexane were added. The chemical reaction was carried out at 60°C with magnetic stirring for 4hrs. Then placed at room temperature for 30 mins, then distilled water 2mL and 10 % Na_2SO_4 1mL were added to it. Afterwards complete mixing and by centrifugation method, the methanol and sulfuric acid was separated with the water in the upper phase, while FAMEs, TAG, and other lipids got separated with hexane in the lower organic phase. The residues formed a layer the between these two phases. The hexane phase was removed with a gas tight syringe to a 10mL glass vial, then the solvent was evaporated and finally FAMEs were collected. However

another method was also implemented using a basic renewable heterogeneous catalysts obtained from egg shells. This initiative is taken to replace the use of acid, as use of acid is hazardous so to minimize the use of acid, CaO basic catalysts is used for biodiesel production. Fatty acids and FAME obtained were analyzed by Nuclear Magnetic Resonance (NMR spectroscopy) [29-32].

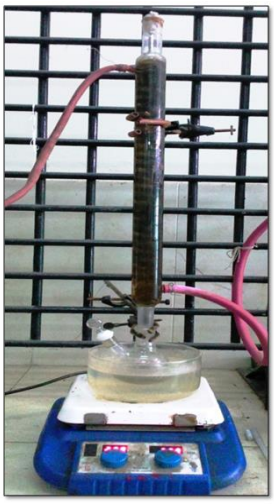


Fig 5: Biodiesel production using heterogenous catalyst.

3. Results and discussions

3.1 *To find out the effect of four different modified BG11 media:*
Accumulation of lipids is an important factor for the production of biodiesel in Microalgae. And this work highlights mainly in providing growth modifying conditions to increase the biomass along with enhancement of lipid. The fresh culture of microalgae *Chlorella* sp. was taken and grown in a photo autotrophic medium BG11 media with slight modifications. In that media four different nitrogen sources are used with trace (5mM in 150ml) amount was used to see the growth responses and to screen the best nitrogen source which can be used for mass production of the algae. The N_2 sources used are KNO_3, $NaNO_3$, and NH_4NO_3, Urea (BGKN, BGNN, BGAN, BGU).The study was conducted for 10 days taking two replicates of all the four sources and during the study the cell count and optical densities were check on regular mode. The *Chlorella* sp. result showed comparatively best results in BGKN media. The growth responses seen are illustrated below in Figure 6.

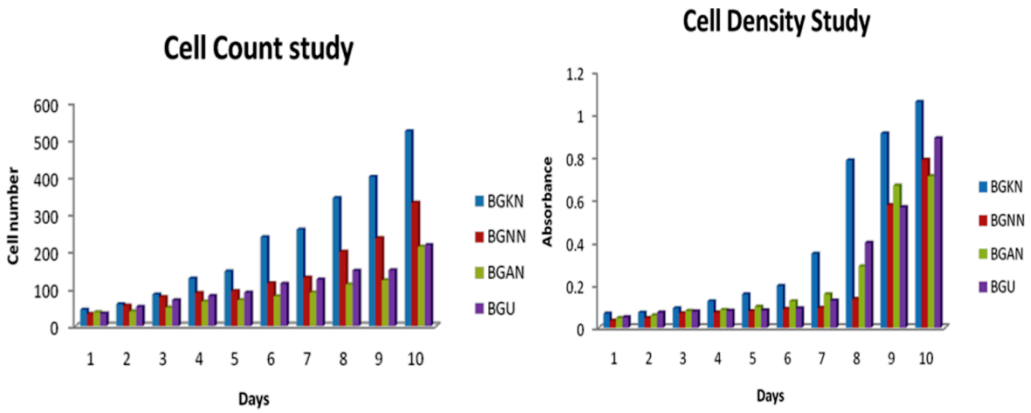


Fig: 6 Cell count and Cell density studies of *Chlorella* sp in different nitrogen sources.

The nitrogen source as a supplement is essential for the growth of microalgal species. Now since the best nitrogen source among the four sources has been found. Now it is important to find the proper concentration of KNO_3 for the optimum growth of the algae so the biomass yield can be enhanced. It is already discussed above that to boost the lipid content simultaneous increase of biomass is also

essential. A study was conducted for 10 days to select the appropriate concentration of KNO_3 in BG11.Different concentrations of KNO_3 starting from 3mM to 21mM was taken and *Chlorella* sp. was cultivate in it. During this study both cell number and optical densities (measured at 680nm) was observed in a regular mode. The concentrations were named (3BGKN, 5BGKN, 7BGKN, 9BGKN, 11BGKN, 13BGKN, 15BGKN, 17BGKN, 19BGKN, 21BGKN).The studied revealed that 17mM concentration was showing a good yield compared to other concentrations. Thus this concentration was used for mass production of biomass to extract out the biofuel potentiality of the species. The graphs (Figure 7) below will illustrate the result.

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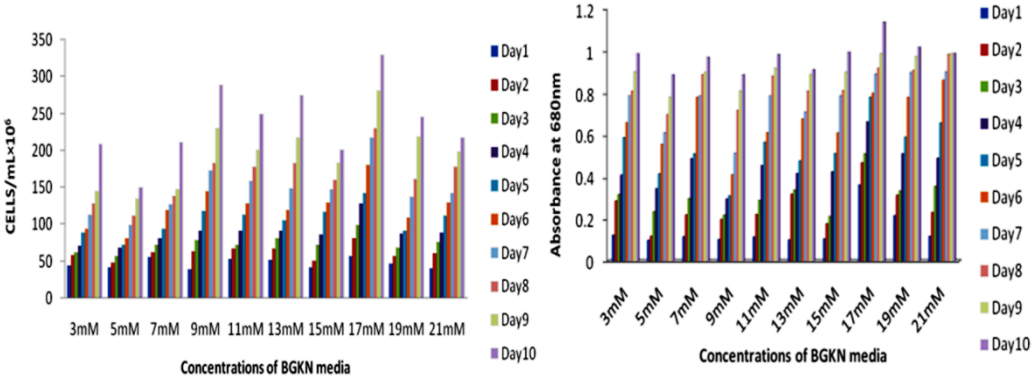

Fig: 7 Cell count and Cell density study of *Chlorella* sp in different concentrations of KNO_3

3.2 *Total carbohydrate and protein content*

The total cellular carbohydrate and protein was studied by harvesting the algal broth and freeze dried biomass on 10^{th}, 20^{th}, 30^{th}, 40^{th} day after inoculation. During this study the highest content of carbohydrate was found on 30^{th} day is 25±5 and it started decreasing as the days goes on. Exactly the same result was seen in for the protein content of the aforementioned species which is 45.2±0.33 and gradual decrease after that. The result is shown on the Table 1.This result infers that biomass yield is high in 30^{th} day of inoculum because during this stage the *Chlorella* sp. shows stationary phase, during which exponential growth takes place, but after that day slowly the growth deterioration occurs so harvesting should be done prior to that day if the mass production is done at laboratory scale.

3.3 *Total lipid (TL) content*

There was variation seen in the total lipid content of the cultures of *Chlorella* sp.Under the investigation it is found that 40.3±2.8 of its dry cellular weight harvested from the culture age of 30 days after inoculation. During this period the growth rate is also at the peak which is inferred from the specific growth calculated and optical densities analyzed. Thus it has been reported that oil can also be extracted from the fractions of proteins and carbohydrates even after extraction of the lipid by acting on the biomass [23].And in our study we have also seen a good increase of all the three biochemical parameters by focusing on elevating the biomass yield. Moreover the total lipid content is found comparatively higher than the reported studies.

Table 1: Biochemical contents of the *Chlorella* sp.

Days	Total lipid	Total Carbohydrate	Total Protein
10	12.7±1.9	9.36±0.25	20.66±1.15
20	33.2±4.1	12.3±6.4	32.51±1.38
30	54.3±2.8	25±5	45.2±0.33
40	40.3±5.6	12.1±2.66	22.4 ±2.2

3.*4 Fluorescence Characteristics of Nile red stained microalgae*

To study the effect of Nitrogen source BG17KN on enhancement of the lipid of the *Chlorella* sp. the fluorescent technique is implemented for two different culture stages. One study was done in the initial inoculum stage and second was done in the final growth stage before harvesting. The accumulations of lipid bodies were examined when the species was stained with Nile Red Stain. The characteristics of the microalgal species were observed under the fluorescent microscope after staining it. During the study done 30days after inoculation it revealed that the lipid globules were glowed yellow, whereas there was no such visible change seen on the study done in the 10th day after inoculation.

3.5 *The XRD analysis of the catalysts derived from egg-shells*

The XRD spectra of calcined eggshell sample were obtained with Cu radiation (λ=0.154178 nm) at 40 kV,30 mA, a scan speed of 0.1 ˚/ s, and a scan range of 0-60.Image 6 shows the XRD of egg shell calcined at 950˚C.For the uncalcined eggshell catalyst, the main peak at 2θ=28.468˚and other peaks were at 2θ=48.801˚,50.805˚, 59.579˚,and 66.168˚. These peaks were characteristics of calcium carbonate. The peaks for the calcined catalyst appeared at 2θ=32.208˚, 39.455˚, and 54.930˚,which were characteristics of calcium oxide.XRD patterns of the egg shell-derived catalyst showed clear and sharp peaks identically. Thus, it can be inferred that the catalysts is calcium oxide derived and hence can be implemented as a basic renewable catalysts for catalytic transformation of algal lipid to biodiesel [21].

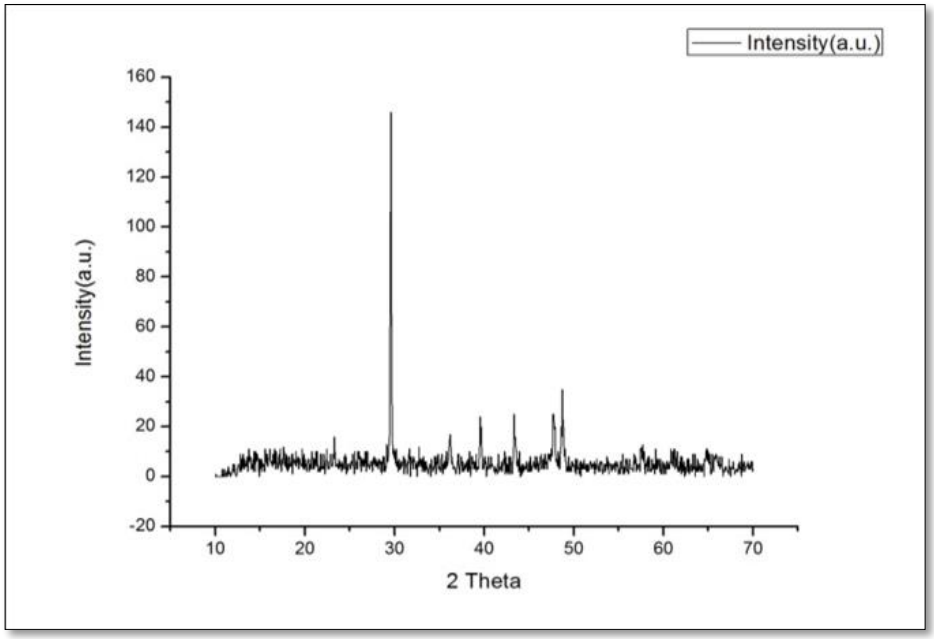

Fig.8. XRD image of the CaO catalysts derived from eggshells calcined at 700˚C

3.6 *Scanning Electron Microscope analysis of the catalysts derived from egg shells*

The SEM analysis of the calcined waste eggshell typically reveals some irregular shape of particles with various sizes, and shapes. Because of the smaller sizes of the grains and aggregates could provide higher specific surface areas so as increases the effective sites for accelerating catalytic activity.

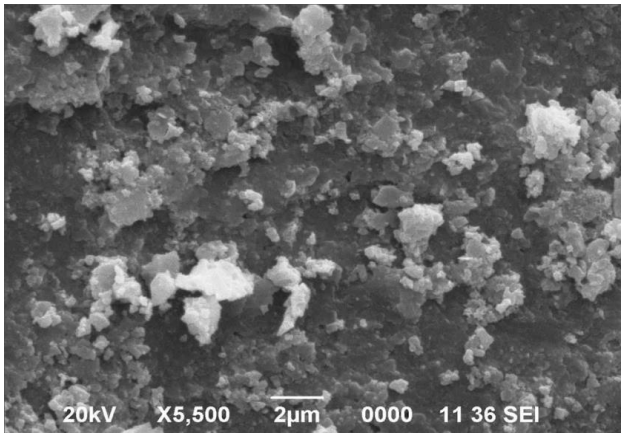

Fig.9.SEM image of CaO catalyst derived from eggshells calcined at 950◦C

3.7 *Analysis of the produced biodiesel using [1]HNMR:*

The [1]HNMR spectra have been given in the Fig.12. Herein, the formation of methyl ester has been confirmed by the prominent peak at 3.6 ppm and 2.3 ppm which denotes the formation of methoxy protons and α-methylene protons. This study confirms that CaO catalyst produced from waste egg shell can be a useful heterogeneous catalyst for transformation of algal lipid to biodiesel. The use of renewable catalyst should be encouraged to maintain environmental balance and restoring renewable nature of the whole biodiesel production process.

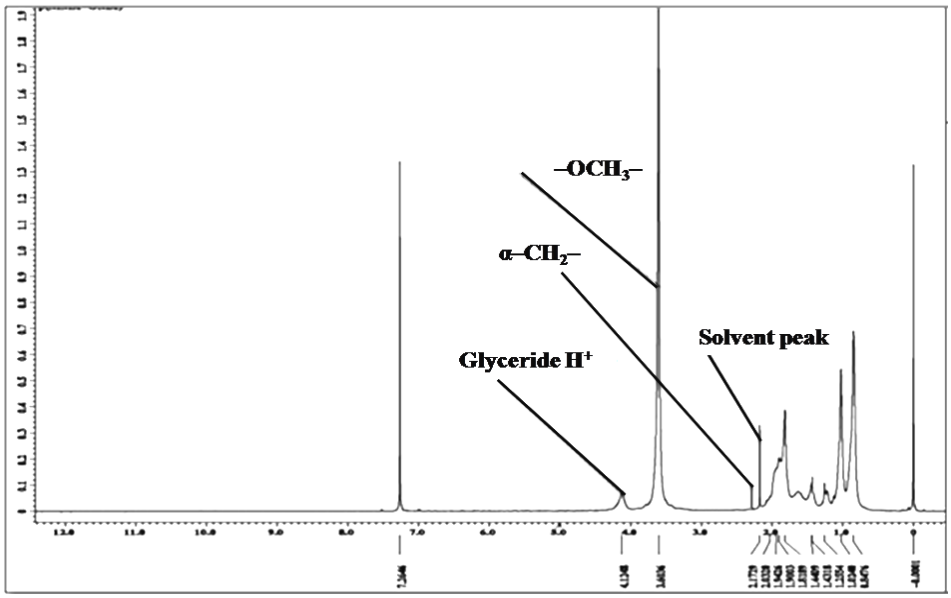

Fig.10 NMR spectroscopy of Microalgal FAME (biodiesel) using CaO particles as catalysts.

4. Conclusion

The growth of Chlorella sp. in modified BGKN17 media leads to substantial enhancement of biomass as well as the lipid and Thus, conclusion can be drawn that potassium nitrate in 17mm concentration increases both biomass and algal lipid. This lipid enhancement was due to the increase in cell number and cell densities of the algal species. The biochemical compounds were also seen to be increased, which indicated the better yield of algal biomass. The study also revealed calcium oxide derived from calcined waste egg shells at 950◦C can be a potent renewable heteregenous catalyst which can be utilized for transformation of Algal lipid to biodiesel. Further works are encouraged for more feasibility and conversion efficiency using CaO as catalyst in algal biodiesel production and also to utilize other renewable sources which can be utilized for enhancement of lipid along with tailoring biodiesel production in simpler and easy way.

5. Acknowledgement

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6. References

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