Journal of Basic and Applied Engineering Research
p-ISSN: 2350-0077; e-ISSN: 2350-0255; Volume 4, Issue 2; January-March, 2017, pp. 97-102

http://www.krishisanskriti.org/Publication.html

Lipid Enhancement of Microalgae *Scenedesmus sp* and Catalytic Transformation of the Lipid to Biodiesel using CaO as a Catalyst

Velentina Das[1*], Anuchaya Devi[2], ManashJyoti Bora[3] and Dhanapati Deka[4]

[1,2,3,4]Biomass Conversion Laboratory, Department of Energy,Tezpur University,India.
E-mail: [1]velen.mani09@gmail.com, [2]anuchaya.tezu13@gmail.com

Abstract—*Oleaginous microalgae Scenedesmus sp. has a tremendous potentiality for the production of biodiesel. The lipid content of the species can be enhanced using different growth promoting sources. Hence, in this work we have prepared four BG11 media adding four different nitrogen sources namely BGNN ($NaNO_3$), BGKN (KNO_3), BGAN (NH_4NO_3), and BGU (Urea). The growth of the species has been monitored using Neubour haemocytometer and Optical densities with the help of spectrophotometer. Out of these four different media BGKN (KNO_3) has shown best growth for the Scenedesmus sp. Therefore, an optimization for different concentrations of KNO_3 has been done and found that 17mM KNO_3 concentration is best as a source of nitrogen for fastest growth of Scenedesmus sp and for production of lipid. After the extraction of Lipid by chloroform extraction method we have applied CaO catalyst which has been prepared from calcined waste egg shells for in-situ catalytic transformation of lipid to biodiesel. The catalyst has been characterized through XRD and SEM techniques. The production of biodiesel from the algal lipid has been confirmed through ^{1}HNMR spectra. This study reports a novel use of nitrogen source KNO_3 as a growth promoting agent rather than stress producing agent and biodiesel production from the algal lipid using CaO as catalyst adds one more dimension in the field of biodiesel production from algal oil using renewable catalyst.*

Keywords*: Scenedesmus sp., KNO_3 Nitrogen source, Lipid, Eggshell, catalysts, biodiesel, ^{1}H NMR.*

1. INTRODUCTION

The elevation of global demand for energy is going to be 59% by 2030[1].For this there is a need for environmentally benign and innocuous source of energy produced from oil crops, waste cooking oil, algae oil, animal fat or recycled oils and fats via a relatively simple transesterification process which can easily displace fossil fuels [2, 3, 4]. Oil productivity, which can be denoted as the mass of oil produced per unit volume of the microalgal broth per day, rely on the algal growth rate and the oil content of the biomass. Microalgae with high oil productivities are desired for producing biodiesel [5]. When appropriate culture condition are provided some microalgal species are able to accumulate up to 50–70% of oil/lipid per dry weight, And thus we can say that this photosynthetically active species of algae has a proficient fatty acid profile in the algal oil which is suitable for the transformation of biodiesel [6].Under certain unfavorable environmental or stress conditions such as phosphate limitation, salinity trials which can be both high content or low content, iron concentration and nitrogen sources can leads to enhancement of the lipid content in the microalgal cells whereas its depletion can lead to degradation of the biomass [7], [8].Generally a nitrogen-deficient or nutrient deficient condition was incorporated to increase the lipid content in microalgae. Thus, nitrogen is necessary for the growth of the microalgal cells and protein synthesis in it. In the absence of the nitrogen, overabundance of carbon from photosynthesis appears to accumulate in the storage cells of the microalgae, in the form of starch and TAG. Under such kind of stressful conditions synthesis of neutral lipids has been found to be defensive mechanism for cells to thrive the stress conditions whatsoever it is and it results, in elevation of the lipid at the expense of lowering the biomass productivity. Thus, we cannot say that the lipid content is the sole factor which determines the oil producing ability of microalgae. Instead, both lipid content and biomass production need to be regarded concurrently. Hence, lipid efficiency, which represents the merge effects of oil content and biomass production, is a more appropriate interpreting index to indicate the ability and performance of microalgae with relation to oil production depletion. *Scenedesmus* is one of the most common freshwater algae genera belonging to class Chlorophyceae.The different species which are generally used for study purpose are *S.dimorphus, S.quadricauda, S.obliquus*.Enhancement of the lipid using various solvents is absolutely necessary for the people associated with microalgal cultivation and biodiesel production in order to sustain knowledge about drawing judgmental statements on appropriate lipid yield, conversion techniques to produce biodiesel..Quality enhancement can be done in three stages of the biodiesel production; one is at selection of the proper feedstock and concentrating more on biomass yield. Secondly on application of different catalysts to make the conversion process a sustainable one and thirdly at

the product level where the biodiesel quality can be enhanced by restoring its quality and enhancing it [7].The objectives of this study are to provide various culture conditions in the first stage to increase the yield of biomass so as to intensify the lipid content extracted thereby and later to characterize the quantification of intracellular neutral lipids in microalgae which can be transformed in biodiesel production. In the present study the extracted lipid is studied with Nile Red fluorescent technique and later ^{1}H NMR spectroscopic techniques were being implemented to generate detailed compositional and quantitative information of polar lipids, neutral lipids and unsaturated fatty acid profile of microalgae biomass samples produced on a laboratory scale. The *Scenedesmus* species which was cultivated and grown under BG11 media with different nitrogen sources. And later lipid was extracted by chloroform extraction method. In-situ transesterification process was carried out using egg shell catalysts to replace acid as a catalysts and ^{1}H NMR spectroscopic technique is used for the analysis purpose.

2. MATERIALS AND METHODS

2.1Microalgae Strain selection and its morphology study:

It is very important to select the proper strain before conducting any studies. After review of certain literatures mentioned in the reference [4] the selection of the species came into our consideration to use the *Scenedesmus* sp as the particular species for biofuel production. The freshwater unicellular green microalgae Scenedesmus *sp* strain was collected from a nearby pond inside the Tezpur University Campus. and isolated for the study purpose. Under the study with labomed compound microscope in 10x resolution the details gathered is that the species cell fusiformis, colonies are composed of basically 2 to 6 cells in the form of structure called as coenobia ,intermediate cells are more likely with convex sides, terminal cells are concave in nature. Apices of the cells are apiculate in nature and cell wall is smooth ,cells are 11.6µ-17.5 µ and diameter is 3.3 µ to 5.5 µ.

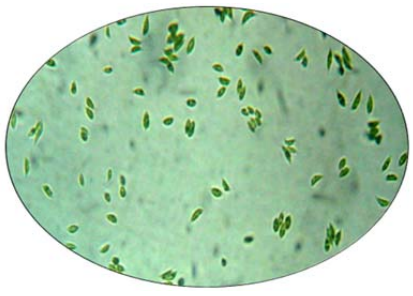

Fig. 1: Microscopic image of Scenedesmus sp. seen under 40x resolution

2.2 Growth stimulating Conditions

Microalgae are also referred to as the light factories for using sunlight to produce energy. Since here the experimental setup was done in the laboratory scale so light source was provided artificially. 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 oC for all the flasks. To facilitate photo-autotrophic cultivation a media called as BG-11 was modified .The pH of the medium was adjusted to 7 before autoclaving at 121oC for 20min.Pure strain microalgae of Scenedesmus sp. was cultivated in four different nitrogen concentration (nitrogen from NaNO3, KNO3, NH4NO3,Urea) in BG-11 medium. The flasks were left at room temperature (25±3oC), aeration and mixing were achieved by air pump with a flow rate 5L/min.

Fig. 2: The growth stimulating conditions provided inside the laboratory.

2.3 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.

2.4 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 [8].

$$\mu = \ln (Nt/No)/Tt-To$$

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- $Tt = 0.6931/\mu$

2.5 Harvesting of Algal biomass

The harvesting of algal biomass is done in an easy way by centrifugation and filtration since it is very time consuming job to harvest the free floating microalgal colonies of the species. The algal cells 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. Harvested cells were then washed with double distilled water followed by centrifugation and lyophilized for 48h at180°C using a freeze drier (SCANVAC Cool safe, ALPHA 1-4, Germany) [7].

2.6 Determination of total protein

Determination of total protein content in the algal biomass was done by Lowry's method with slight modification.

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

2.7 Determination of carbohydrate content:

The carbohydrate content was estimated following the (Hedge *et al*, 1962) with slight modification.

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

2.8 Staining of Microalgae Cells with Nile Red Fluorescence Dye:

Microalgae cells were photo-autotrophically cultivated in the laboratory as discussed earlier until the early stationary phase is achieved for accumulation of neutral lipids. 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[1].

2.9 Determination of total lipid:

Microalgal lipid extraction was done by Bligh and Dyer method. [22].

2.10 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 180°C.Then the crushed eggshells were sieved (100-200 mesh) and subjected to heat treatment at 900°C for 4hrs in the muffle furnace [28].

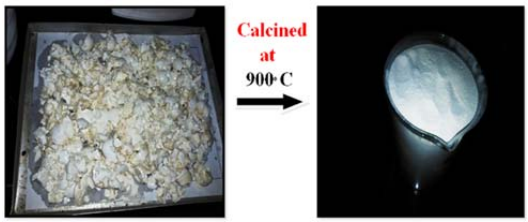

Fig. 3: Egg shells as catalysts calcined at 900° C as a source of calcium oxide.

2.11 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 [3].

2.12 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) [1].

3. RESULTS AND DISCUSSION

3.1 Microalgae growth

Accumulation of lipids is an important factor for the production of biodiesel in Microalgae. And present work focuses basically in providing growth stimulating factors so as to increase the biomass along with increase of lipid. The fresh

culture of microalgae *Scenedesmus* sp. was taken and grown in BG11 media with slight modifications with four different nitrogen sources 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 Scenedesmus sp. result showed comparatively best results in BGKN media. The growth responses seen are illustrated below in Fig. 4 and Fig. 5 respectively.

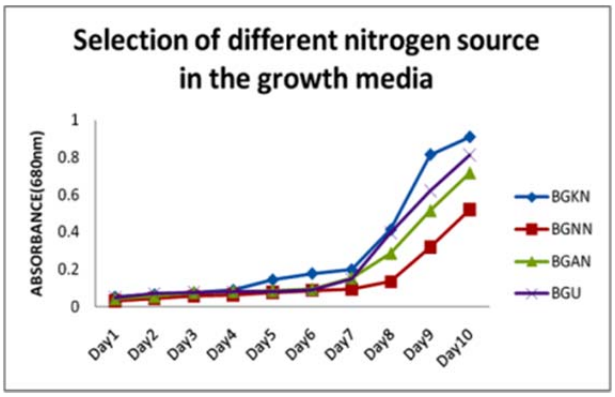

Fig. 4: Optical density of the *Scenedesmus* sp

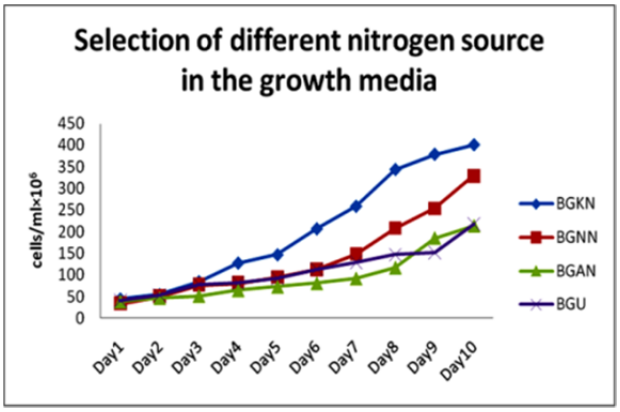

Fig. 5: Cell count of the *Scenedesmus* sp.

The nitrogen source as a supplement is essential for the growth of microalgal species [5].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 *Scenedesmus* 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 (Fig. 6 and 7) below will illustrate the result.

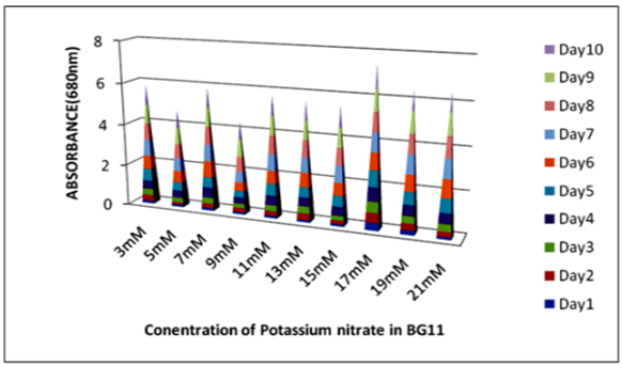

Fig. 6: Optical density of *Scenedesmus* sp. in KNO_3

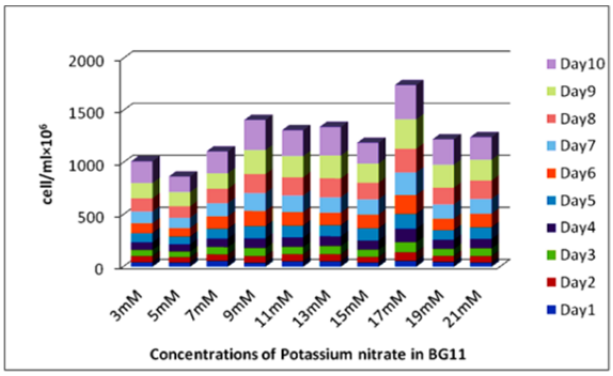

Fig. 7: Cell count of *Scenedesmus* sp. in KNO_3

Total biochemical Contents

The total biochemical contents like lipid protein and carbohydrate in the species in during the study period is tabulated below in Table1.

Table1: Biochemical contents of the *Scenedesmus* sp.

Culture Age(days)	Cellular content(%DCW)		
	Total lipid	Total Carbohydrate	Total Protein
10	23.7±1.9	9.36±0.25	10.66±1.15
20	40.2±4.1	29±6.4	22.51±1.38
30	55.3±2.8	38±5	40.23±0.33
40	40.3±5.6	26.1±2.66	22.4 ±2.2

Fluorescence Characteristics of Nile red stained microalgae

To study the effect of Nitrogen source BG17KN on enhancement of the lipid of the *Scenedesmus* sp. the fluorescent technique is implemented for two different culture stages. 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. The figure8 and 9 gives the idea about the changes that can be seen.

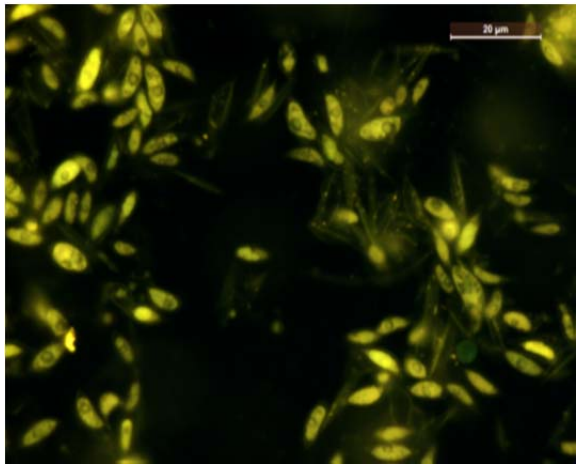

Fig. 8: Stained at 10th day of inoculation.

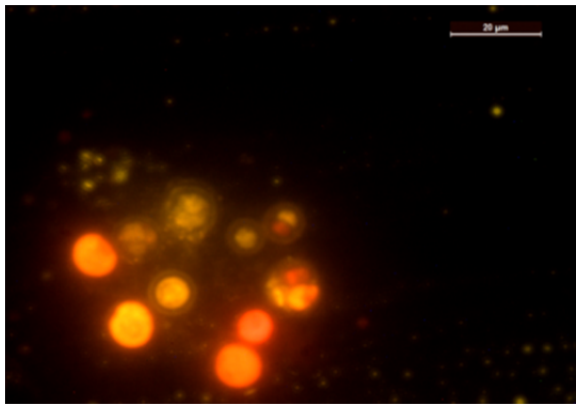

Fig. 9: Stained at 30th day of inoculation.

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 900˚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 biodiesel [8].

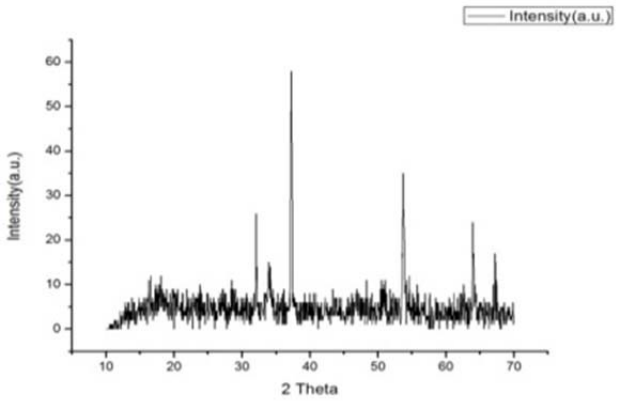

Fig. 10: XRD image of the CaO catalysts derived from eggshells calcined at 900°C

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.

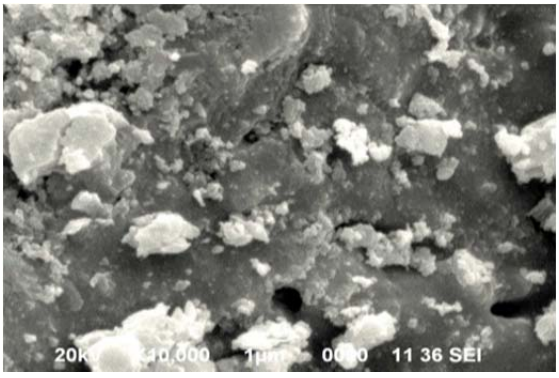

Fig. 11: SEM image of CaO catalyst derived from eggshells calcined at 900°C

Analysis of the produced biodiesel using ^{1}HNMR:

The ^{1}HNMR spectra have been given in the Fig.8. where the formation of methyl ester has been confirmed by the prominent peak at 3.6ppm. 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.[8]

cause of ific surface areas. s image102102102102102102102102102102102102102102102 102102102102102102102102102102102102102102102102 102102102102102102102102102102102102102102102102 102102102102102102102102102102102102102102102102 102102102102102102102102102102102102102102102102 102102102102102102102102102102

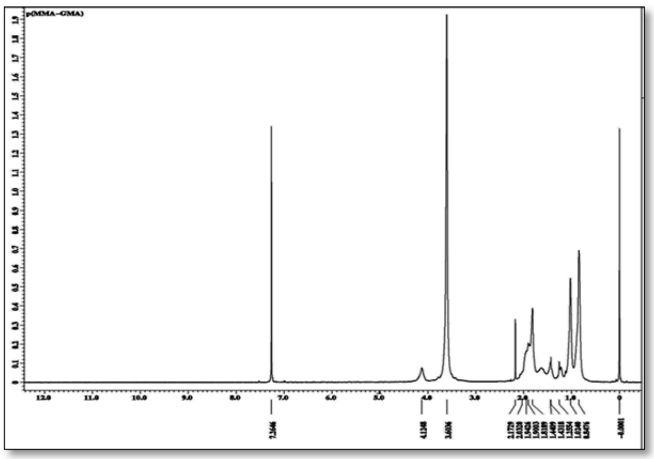

Fig. 12. NMR spectroscopy of Microalgal FAME (biodiesel) using CaO particles as catalysts.

4. CONCLUSION

This aforementioned species utilized the nitrogen source for proper growth along with the total biochemical compounds were increased too. The results of Nile red stain technique showed the presence of neutral lipid in Scenedesmus species. This green algal species has the immense potentiality for biodiesel production. So, it can be concluded that if modifications is done in the growth conditions of the algae lipid yield can be obtained and along with modification of catalysts can lead to high conversion of that lipid to biodiesel. Further works are in progress 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

VD is thankful to Tezpur University for providing institutional fellowship to her. And also thankful to AICTE-NEQIP Funds for providing us the financial assistantship to attend this conference Greentech-2017 at JNU, New Delhi.

REFERENCES

[1] EIA.International Energy Outlook Early release 2012. <http: //www.iea.doe.gov.> [access 10.6.14].

[2] Pinto,A.C.;Guarieiro,L.N.,Rezende,M.J.,Ribeiro,N.M.,Torres,E. "Biodiesel: An overview".*Brazil Chemical Society.*, 16,2005,pp.1313–30.

[3] Hurley MD, Ball J. Wallington TJ, Toft A, Nielsen OJ, Bertman S."Atmospheric chemistry of a model biodiesel fuel, CH_3C $(O)O(CH_2)2OC(O)CH_3$: kinetics, mechanisms, and products of CL atom and OH radical initiated oxidation in the presence and absence of NOx" *Journal of Physical Chemistry A.*111, 2007, pp.2547–54.

[4] Encinar JM, Gonza´lez, J F, Rodrı´guez-Reinares A. Ethanolysis of used frying oil-Biodiesel preparation and characterization. *Fuel Processing Technology*,88, 2007, pp.513–22.

[5] Chisti Y."Research review paper Biodiesel from microalgae". *BiotechnologyAdvances*, 25, 2007, pp.294–306

[6] Chen W, Sommerfeld M, and Hu Q. "Microwave-assisted Nile red method for in vivo quantification of neutral lipids in microalgae", *Bioresource Technology*,102,2011,pp.135-141.

[7] Ho S, Chen H, Chang WM."Scenedesmus obliquus CNW-N as a potential candidate for CO_2 mitigation and biodiesel production" .*Bioresource Technology,*101,2010,pp,8725-8730.

[8] Lin Q, Lin J. Effects of nitrogen source and concentration on biomass and oil production of a Scenedesmus rubescens like micro alga. *Bioresource Technology,*102,2011,pp.1615-1621.