International Journal of Chemical Studies

P-ISSN: 2349–8528
E-ISSN: 2321–4902
IJCS 2018; 6(6): 550-554

Received: 16-09-2018
Accepted: 17-10-2018

Bolthajira Siddartha Manoj
Department of Biochemistry, Sher-E-Kashmir University of Agricultural Science and Technology, Jammu, Jammu and Kashmir, India

Sushma Ahlawat
Department of Biochemistry and Biochemical Engineering, JIBB, SHUATS, Allahabad, Uttar Pradesh, India

Mohan Chavan
Department of Biotechnology, University of Agricultural Sciences, Bangalore, Karnataka, India

Arti Karosiya
Department of Biotechnology and Crop Improvement, College of Horticulture, UHS, Bagalkot, Bangalore, Karnataka, India

Correspondence
Bolthajira Siddartha Manoj
Department of Biochemistry, Sher-E-Kashmir University of Agricultural Science and Technology, Jammu, Jammu and Kashmir, India

Successive production of biodiesel and bioethanol feedstock from the *Cosmarium* sp

Bolthajira Siddartha Manoj, Sushma Ahlawat, Mohan Chavan and Arti Karosiya

https://doi.org/10.22271/chemi.2018.v6.i6j.14

Abstract
Cosmarium sp is unicellular microalga which was grown in bold basal medium with the controlled condition and investigated biochemical characterizations for biodiesel production and evaluated standardization of ethanol production. Once microalgae were reaches the proper growth stage total lipid was analyzed and these lipids were used for the production of biodiesel by transesterification process using Calcium oxide (0.66%) as a catalyst. Bioethanol production was done by using the defatted residues of *Cosmarium* sp. with *Saccharomyces cerevisiae* (10%). In view of the above-mentioned facts, the present study has been designed to enhance the production of biodiesel and bioethanol.

Keywords: biodiesel, bioethanol, *Cosmarium* sp.; *Saccharomyces cerevisiae*; transesterification

Introduction
Microalgae had a wide range of active substances in response to the ecological pressure because of their biochemical and physiological characteristics (Flores-Moya *et al*. 2005) [1]. The *Cosmarium* sp. is a unicellular freshwater alga (Felisberto and Rodrigues 2004) [2]. The commercial use of microalgal biomass is cost-effective harvesting and which can be done by flotation, sedimentation, filtration, and centrifugation (Guschina and Harwood 2006) [3]. An alga is known as the third generation biofuel feedstock and which has an increasing role in the sustainable energy (Patil *et al*. 2008) [4]. Microalgae can be considered as major feedstock for biodiesel production because (1) The biodiesel production from microalgae succeeded due to its rapid growth rate, high lipid accumulation and ability to act as carbon neutralizer enhances the microalgal contribution towards less emission of greenhouse gasses and air pollutants (Pandit *et al*. 2017) [5]; (2) it not required high-quality agricultural land to grow the biomass (Scott *et al*. 2010) [6] and (3) Algae as 200 times more productive per hectare than a land-based crop (Shraddha *et al*. 2016) [7]. Microalgae are one of the important sources of nutrition rich in vitamins, minerals, protein and in some region their concentrations greater than traditional plant and animal protein sources (Bleakley and Hayes 2017) [8].

Materials and Methods
Cosmarium sp. sample was collected from the storage water tank in agriculture field of Agricultural College Hassan Campus, Hassan district the village lies between latitude 12.9772108 °N and longitude 76.2584164 °E. A microalga purified strain was identified based on microscopic morphological traits (Felisberto and Rodrigues 2004; Brook and Johnson 2002; Croasdale and Flint 1998) [2, 9, 10]. Algae sample was identified by using the compound optical microscope attached with a camera for digital imaging. Structures of microalgae were captured under the scanning electronic microscope (Quanta FEG 200) by using a dehydrated sample with 80% ethanol and coated with gold particles then mounted it on a specimen stub with the help of carbon tape and were examined. *Cosmarium* sp. was grown in flasks containing a Bold's growth medium (Bischoff and Bold 1963) [11]. After 18th days of inoculation, the samples were subcultured. *Cosmarium* sp. culture was kept in the orbital shaker at the rate of 120 rpm (Brennan and Owende 2010; Guerrero-Cabrera *et al*. 2014) [12, 13]. The optical density was measured at the interval of 5 days at 600 nm using spectrophotometer

(CECIL CE 7400) (Stanbury *et al*. 2013) [14]. Collected algae sample were centrifuged at 10000 rpm for 10 min and the pellet was collected for further study. The drying was performed on blotting towels under shade in laboratories (Brennan and Owende 2010) [12].

A dry weight of 10 g of shade-dried algae was taken in a Thimble placed it in the Soxhlet extractor and connected a dry pre-weighed solvent flask ('a' gram) beneath the apparatus and added 200 ml of extraction solvent (Petroleum ether). Adjusted the heating rate of the apparatus to 65 ℃ and allowed to siphoning till at least 18 times (it takes 3-4 hr). Removed the thimble and extraction solvent mixture was recovered from the apparatus by distillation method. An excess amount of extraction solvent from the solvent flask was evaporated by using a hot water bath. Cooled the flask and weighed ('b' gram) (Sadasivam and Manickam 1992) [15].

Crude oil content in sample (%) = [(b-a)/ Weight of the sample]*100

Where 'a' is a pre-weighed dried round bottom flask (g); 'b' is a flask with oil/lipid (g)

Biodiesel extraction and quality estimation

In transesterification, triglycerides are converted to diglyceride, then diglycerides are converted to monoglycerides and monoglycerides are then converted to fatty acid esters (biodiesel) and glycerol as a byproduct (Saifuddin *et al*. 2015) [16]. In the present study transesterification of a sample was done using Calcium oxide (CaO) as an alkali catalyst. CaO was activated by pretreatment with methanol, a small amount of CaO gets converted into $Ca(OCH_3)_2$ that acts as an initiating reagent for the transesterification reaction (Kawashima *et al*. 2009) [17]. During transesterification the standardized amount of methanol 26 g and calcium oxide 0.66 g was placed in a 250 ml three-necked flask connected with a condenser and stirred for 30 minutes at 30 ℃ for CaO activation. Added 100 g of algal oil to the 250 ml three-necked flasks; the mixture was kept it on the magnetic stirrer heating mantle and subsequently heated at 60 °C for 150 rpm for 3 hr. As for the activation by glycerol, CaO was mixed with glycerol for a few minutes at 60 °C; at the same time, the solid phase of the reaction mixture was formed by two layers. After 3 hrs separate the upper layer which contains biodiesel using separating flask and washed the biodiesel with warm water (60℃) five times to remove the impurities.

Biodiesel quality was analyzed by using the following test, *viz.* clarity test, pH, flash point, density, cloud point test, density, viscosity and acid value (Brennan and Owende 2010; Indhumathi *et al*. 2014; Tyson 2009) [12, 18, 19]. Clarity test was done by washing the biodiesel with hot water. Flashpoint, cloud point, kinematic viscosity, and density was measured using standard protocols *viz.*, ASTM D 93, ASTM D 2500, ASTM D 445 and EN 14214, respectively (Indhumathi *et al*. 2014; Tyson 2009) [18, 19]. The acid value was analyzed by weighing 5g of biodiesel in 25 ml of Isopropyl alcohol in 250 ml conical flask. 2 drops of Phenolphthalein indicator was added and mixed the content thoroughly. It was titrated with 0.1 N KOH with shaking constantly until pink color persists for 15 sec. The acid value is calculated by using given formula (Rajan 2011) [20].

Acid value (mg KOH/g) = [(titer value × Normality of KOH × 56.1) / weight of a sample (g)].

Bioethanol production by fermentation

Substrate preparation was done by hydrolyzed the defatted algal biomass. Hydrolysis was done by ground the dried *Cosmarium* sp. sample to a fine powder and treated with HCl (2.5 N) for 3 hrs, which converts polysaccharides into monosaccharides, then neutralized with a pinch of Na_2CO_3 (Harun *et al*. 2014; Chaudhary *et al*. 2017) [21, 22]. *Saccharomyces cerevisiae* is known for the production of Ethanol (Markou *et al*. 2012) [23]. Defatted 20 g of hydrolyzed algae were inoculated with 10% *S. cerevisiae* and 1% glucose was used as a starter for this experiment. 180 ml of sterilized distilled water was added to 500 ml conical flasks and 5 ml of samples were taken for initial analysis that was considered as the 0^{th}-day sample before airtight. Conical flasks were airtight by sterilized rubber cork fixed with 2 silicon pipes among two one silicon pipes was immersed to a test tube with water to ensure anaerobic condition. Another pipe connected to stopper and syringe to analyzing the sample. The stoppers fixed checks the air entry to the anaerobic condition. This experiment was conducted with three replications R_1, R_2 and R_3 to minimize errors.

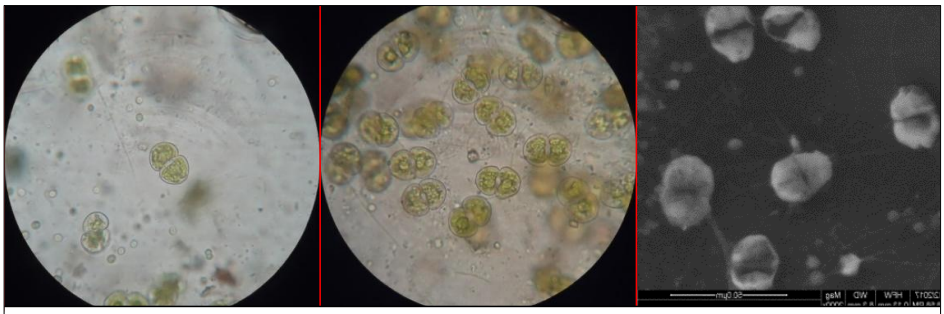
The biochemical and physical characteristics of the sample during fermentation

For estimation of the total soluble sugar (TSS), 100 µl of fermented sample was diluted to 100 ml with distilled water and centrifuged at 10000 rpm for 10 min. Collect the supernatant and used 0.2 ml of aliquot made up the volume to 1 ml with distilled water. Added 4 ml of freshly prepared anthrone reagent and the colour was developed by keeping it on boiling water bath for 8 min, cooled absorbance was read at 630 nm (Hedge and Hofreiter 1962) [24]. Total reducing sugar (TRS) of the fermented sample was estimated by dinitrosalicylic acid (DNS) method by measuring the absorbance at 510 nm using the spectrophotometer (Sadasivam and Manickam 1992; Chaudhary *et al*. 2017; Miller 1959) [15, 22, 25]. Total soluble protein was extracted from fermented 1 ml of *Cosmarium* sp. sample with 10 ml of phosphate buffer (0.1 M, pH 7.4) in a pestle and mortar; added pinch of polyvinylpyrrolidone and 2 µl β mercaptoethanol. After that sample centrifuged at 6000 rpm for 10 min. Added equal amount of chilled acetone to the collected supernatant and kept at -20 ℃ for overnight then the sample was centrifuged at 12000 rpm for 15min and discarded the supernatant. Washed the pellet with 75% ethanol and dissolved in 1ml of phosphate buffer (0.1 M, pH 7.4) and used it for protein estimation. Total protein content was estimated according to Lowry *et al*., 1951 [26]. pH of the fermented sample was measured by calibrated digital pH meter (Systronic Pvt. Ltd). The ºBrix analysis was done using refractro-meter for estimation of total sugar content during the fermentation period (Neto *et al*. 2006) [27]. Ethyl alcohol estimation was done using potassium dichromate (Stanbury *et al*. 2013; Williams and Laurens 2010) [14, 28], where 1 ml of anaerobic maintained algae sample was taken into 250 ml round bottom distillation flask connected with a condenser and diluted with 30 ml of distilled water. The sample was distilled at 75 ℃. The distillate was collected in 25 ml flask containing $K_2Cr_2O_7$ (0.23 N) reagents kept at receiving end. The alcohol was collected till the total volume obtained 45 ml. Similarly, standard (20-100 mg ethanol) was carried out and distillate the sample. Standards were heated in a water bath at 60 ℃ for 20 minutes and cooled. The volume made up to 50 ml with distilled water and absorbance was measured at

600 nm using spectrophotometer (Caputi *et al*. 1968) [29]. 2 $Cr_2O_7^{2-}$ + 3 C_2H_5OH + 16 H^+ → 4 Cr^{3+} + 3 CH_3COOH + 11 H_2O

Result and Discussion

Algae sample was identified by using the compound microscope (40X and 100X) and scanning electronic microscope (Fig 1) based on the reference of Prescott (1970)[30]. A microalga was identified as *Cosmarium* sp. based on microscopic morphological characteristics (Felisberto and Rodrigues 2004; Brook and Johnson 2002; Croasdale and Flint 1998) [2, 9, 10]. *Cosmarium* sp. belongs to Plantae kingdom, Charophyta division, and family Desmidiaceae. It is a unicellular species with bi-lobed appearance, in the middle which holds the nucleus. The two semi-cells were round when viewed from the front and flattened, oval, or elliptic when viewed from the side. *Cosmarium* sp. was found in freshwater bodies of little acidic pH 6.5 (Brook A. J. Johnson L.R. 2002; Prescott and Prescott 1978) [9, 30]. Using petroleum ether, extracted 31.50 ± 1.09 % lipid from *Cosmarium* sp. Microalgal biomass is a potential feedstock for biofuel production (Medipally *et al*. 2015) [31]. According to (Peterson and Scarrah 1984) [32], CaO is the well-researched heterogeneous catalysts which have higher basicity, lower solubility, lower cost, and is easier to handle than KOH. Use of methanol makes the purification process easier as the mixture produced break without difficulty to form a lower glycerol as well as an upper methyl ester rich layer (Haq *et al*. 2014) [33]. *Cosmarium* sp. recovered 72.00 ± 1.80% of biodiesel and 20.00 ± 0.80 % of glycerol during transesterification process from the lipid 31.50 ± 1.09% (Table 1).



40X resolution 100X resolution SEM (2000X)

Fig 1: Microscopic identified *Cosmarium sp.* collected from water tank of Agriculture College, Hassan Campus

Table 1: Beneficial product obtained from *Cosmarium* sp

Sl. No.	Properties	Contents
01	Lipid (%)	31.50 ± 1.09
02	Biodiesel (%)	72.00 ± 1.80
03	Glycerol (%)	20.00 ± 0.80
04	Ethanol (mg/ml)	118.5 ± 1.12
05	Total soluble sugar (%)	34.79 ± 0.12
06	Total reducing sugar (%)	25.30 ± 0.60
07	Total protein content (mg/g)	13.08 ± 0.05

Biodiesel quality and combustion tests were done according to Tyson (2009) [19]; Brennan and Owende (2010) [12]; (Kumar *et al*. 2011) [34]; (Indhumathi *et al*. 2014) [18]. Biodiesel formed clear zone with water after washed with hot water (60℃) repeatedly, indicating that it has very less content CaO and Glycerol. CaO solubilizes in glycerol, not in methanol it reduced the CaO compound in biodiesel (Williams 2013) [35]. The density of biodiesel sample matched the density ranges of a biofuel. According to EN 14214 and ISO 15607, the density range of biodiesel is 0.86 to 0.90 g/cm^3 (Tyson 2009; Vijayaraghavan and Hemanathan 2009) [19, 36], current study it as 0.88 ± 0.005 g/cm^3. The viscosity range in present results is compatible with by EN 14214 and ISO 15607 standards. Density and viscosity property of present studied biodiesel samples were compatible with (Kumar *et al*. 2011)[34]. Other important fuel properties such as Flashpoint ranges given by ASTM D93 was (100 to 170 ℃), Pour point ranges given by ASTM D2500 (-15 to 10 ℃), Cloud point ranges given by ASTM 2500 (-3 to 10 ºC), pH (7.0) and Acid values ranges given by ASTM D664 (0.8 max. mg KOH/g) were found matching with the ranges of a biofuels given by (Tyson 2009); (Kumar *et al*. 2011) and (Indhumathi *et al*. 2014) [19, 34, 18]. Biodiesel obtained from current studied species was comparable to ASTM were shown in Table 2.

Table 2: Analysis of a biodiesel and its comparison with international standards

Sl. No.	Properties	Biodiesel from *Cosmarium* sp.	Regular Diesel*	Standard value of Biodiesel**
01	Density at 40 ºC (g/cm^3)	0.88 ± 0.005	0.86 to 0.92	-
02	Viscosity at 40 ºC (mm^2/sec)	4.46 ± 0.17	3.5 to 5.0	1.9 to 6.0
03	Flash Point (ºC)	126 ± 1.73	60 to 80	>130
04	Pour Point (ºC)	-14 ± 1.00	-5 to 10	-15 to 10
05	Cloud Point (ºC)	-5	-5	-3 to 12
06	pH	6.8 ± 0.1	7	7
07	Acid value (mg KOH/g)	0.5 ± 0.17	0.8	0.8max

*Regular diesel properties details where collected from the source: Indhumathi *et al*., (2014) [18] and Tyson (2009) [19]. Standard** (ASTM D-6751 ~ 02 Standard).

Physio-biochemical characters changes during Bioethanol production by fermentation

In the current study standardized the ethanol production on the basis of biochemical changes *viz.* TSS, TRS, protein, pH and ethanol content. The anaerobic fermentation setup of present studied species in the duration of 21 days showed in Table 3. The results of total soluble sugars were estimated from fermentation setup for every 3 days of an interval from the 0th day to 21st days. In the present study the total soluble sugar decreased, the decreased percentage difference between

the 0th and 9th day was 49.98%. It was mainly because *Saccharomyces cerevisiae* which breaks down the sugar content during the fermentation period to ethanol. But after 12th to 18th days, TSS percentage was increased because of releasing sugar content from algal biomass inside fermentation set up and it is known as secondary fermentation (Robinson 2006) [38]. *Saccharomyces cerevisiae* utilized this sugar content so on 21st days total soluble sugar content were decreased and decreased percentage difference between the 0th and 21st day was 58.82%. This carbohydrate saccharified to mono-sugars by treated with sulfuric acid and these monosaccharides served as good substrates for the fermentative production (Sung-Soo Jang 2012) [39]. These total soluble sugars are feedstocks for ethanol production by fermentation after hydrolysis (Chaudhary *et al*. 2017; Manoj *et al*. 2018) [22, 40]. Total reducing sugar of samples was decreased and the decreased percentage difference between the 0th and 21st day of current studied samples was 34.37%. The sugar content decreased gradually from the 0th to 21st days due to the utilization of sugar by the *Saccharomyces cerevisiae* (Manoj *et al*. 2018; Yang and Wiegand 1949) [40, 41] which break down the sugar content during the fermentation period and produced the ethanol. The results of TRS in the present study are in accordance with the similar study done by (Chaudhary *et al*. 2017) [22], who also observed decreased sugar content during fermentation on different species of microalgae.

Table 3: Physio-biochemical analysis during fermentation period

Period	TSS (%)	TRS (%)	TSP (mg/g)	pH	Ethanol (mg/ml)
0th Day	33.15 ± 1.64	25.31 ± 0.10	1.15 ± 0.02	6.98 ± 0.09	34.80 ± 1.30
3rd Day	32.53 ± 1.88	24.17 ± 0.06	1.32 ± 0.08	6.89 ± 0.12	42.81 ± 0.65
6th Day	17.67 ± 0.70	23.41 ± 0.42	1.32 ± 0.02	6.53 ± 0.15	88.15 ± 0.57
9th Day	16.58 ± 0.36	20.83 ± 0.25	1.35 ± 0.06	6.26 ± 0.05	89.05 ± 2.70
12th Day	20.38 ± 0.73	19.47 ± 0.10	1.38 ± 0.12	6.38 ± 0.99	86.43 ± 1.30
15th Day	20.38 ± 1.03	20.93 ± 0.55	1.38 ± 0.07	6.54 ± 0.05	83.49 ± 0.75
18th Day	20.94 ± 1.42	17.34 ± 0.60	1.59 ± 0.04	6.66 ± 0.19	95.28 ± 1.26
21th Day	13.65 ± 0.59	16.61 ± 0.15	1.68 ± 0.09	6.66 ± 0.22	118.52 ± 1.12

Footnote: Total soluble sugar (TSS), total reducing sugar (TRS) and total soluble protein (TSP)

The total proteins of the sample are gradually increased and the increased percentage difference between the 0th and 21st day of present studied samples was 46.08%. During fermentation, protein content was increased due to decreased sugar content. Protein, nitrogen, and ash in the hydrolysate might be favorable ingredients supporting fermentation (Sung-Soo Jang 2012) [39]. The result of the present study is in accordance with the similar study done by (Williams and Laurens 2010) [28], according to whom higher protein content increases production of other byproducts like enzymes, nucleic acids and organic acids which helps production of ethanol. The pH was decreased, the decreased percentage difference between the 0th and 9th day was 10.31%. But after the 12th to 18th day's pH percentages were increased, it because of increasing TSS inside anaerobic fermentation set up. The rate of ethanol production maximum at pH 6.66 was observed. pH during fermentation period act as an important role in the growth and adaptation of microorganism (Chaudhary *et al*. 2017) [22], in their study microorganism growth, increased below pH 7 and maximum ethanol produced at pH 6.

The alcohol contents were high on the 6th and 21st day of anaerobic fermentation. The alcohol quantity observed on the 6th day was 88.15 ± 0.57 mg/ml. The maximum alcohol quantity was observed on the 21st day of the present studied sample was 118.52 ± 1.12 mg/ml. During the period of fermentation of the 6th and 21st-day carbohydrates content in fermentation setup decreases and microbial load increases, it is also one reason for the increased percentage of alcohol on the 6th and 21st day. But after the 12th to 18th-day alcohol percentage was decreases, because of decreased microbial load, due to increase in pH inside fermentation set up At the period of bio-ethanol fermentation, the concentration of the reducing sugar decreased with respect to days and the concentration of bio-ethanol further increased gradually, clearly indicating that the present studied sample was used as fermentation sugars.

Conclusion

The biochemical composition of the *Cosmarium* sp. is played the major role in the production of biodiesel and bioethanol. Current findings showed that the carbohydrate was the major compound that's break down by *Saccharomyces cerevisiae* and produced bioethanol from the defatted residues after extracting lipid. The experiments conclude that the maximum carbohydrates, protein, and microbial load help in the production of an efficient quantity of bioethanol.

Acknowledgment

The authors are thankful to the Department of Biochemistry and Biochemical Engineering, SHUATS, Allahabad and Agricultural College Hassan for providing good laboratory facilities. And heartily thanks to Mamatha P and Yashoda for their cooperation to conduct this experiment.

References

1. Flores-Moya A, Costas E, Bañares-España E, García-Villada L, Altamirano M, López-Rodas V. Adaptation of Spirogyra insignis (Chlorophyta) to an extreme natural environment (sulphureous waters) through Preselective mutations. New Phytologist. 2005; 166:655-661.
2. Felisberto SA, Rodrigues L. Periphytic Desmids in Corumbá Reservoir, Goiás, Brazil: Genus Cosmarium Corda. Brazilian Journal of Biology. 2004; 64:141-150.
3. Guschina IA, Harwood JL. Lipids and lipid metabolism in eukaryotic algae. Progress in Lipid Research. 2006; 45:160-186.
4. Patil V, Tran KQ, Giselrød HR. Towards sustainable production of biofuels from microalgae. International Journal of Molecular Sciences. 2008; 9:1188-1195.
5. Pandit PR, Fulekar MH, Karuna MSL. Effect of salinity stress on growth, lipid productivity, fatty acid composition, and biodiesel properties in Acutodesmus obliquus and Chlorella vulgaris. Environmental Science and Pollution Research. 2017; 24:13437-13451.

6. Scott SA, Davey MP, Dennis JS, *et al*., Biodiesel from algae: Challenges and prospects. Current Opinion in Biotechnology. 2010; 21:277-286.
7. Shraddha G, Harshal T, Prashant B, Ganesh D. Biofuels: an emerging technology. International Journal of Institutional Pharmacy and Life Sciences. 2016; 6:372-380.
8. Bleakley S, Hayes M. Algal Proteins: Extraction, Application, and Challenges Concerning Production. Foods. 2017; 6:33.
9. Brook AJ, Johnsonm LR. Order Zygnematales. In The freshwater algal flora of the British Isles : an identification guide to freshwater and terrestrial algae (ed. Johan DM. Whitton BA. Brook Alan J.), Cambridge University Press, 2002, 49-593.
10. Croasdale H, Flint EA. Flora of New Zealand: Freshwater algae Chlorophyta, Desmids Manaaki Whenua Press, Christchurch, New Zealand, 1998.
11. Bischoff HW, Bold HC. Phycological Studies IV. Some soil algae from Enchanted Rock and related algal species, 1963.
12. Brennan L, Owende P. Biofuels from microalgae-A review of technologies for production, processing, and extractions of biofuels and co-products. Renewable and Sustainable Energy Reviews. 2010; 14:557-577.
13. Guerrero-Cabrera L, Rueda JA, García-Lozano H, Navarro AK. Cultivation of Monoraphidium sp., Chlorella sp. and Scenedesmus sp. algae in Batch culture using Nile tilapia effluent. Bio resource Technology. 2014; 161:455-460.
14. Stanbury PF, Whitaker A, Hall SJ. Principles of fermentation technology. Journal of Chemical Information and Modeling. 2013; 53:1689-1699.
15. Sadasivam S, Manickam A. Biochemical Methods for Agricultural Sciences Wiley Eastern Ltd, New Delhi, 1992.
16. Saifuddin N, Kumaran P. A Review on Processing Technology for Biodiesel Production. Trends in Applied Sciences Research, 2015, 1-37.
17. Kawashima A, Matsubara K, Honda K. Acceleration of catalytic activity of calcium oxide for biodiesel production. Bioresource Technology. 2009; 100:696-700.
18. Indhumathi P, Syed Shabudeen PS, Shoba US. A method for production and characterization of biodiesel from green micro algae. International Journal of Bio-Science and Bio-Technology. 2014; 6:111-122.
19. Tyson KS. Biodiesel handling and use Guide Laboratory_National_Renewable_Energy, 2009.
20. Rajan K. Determination of acid value of fats and oil. In Analytical Techniques in Biochemistry and Molecular Biology Springer Science & Business Media, New York, 2011, 80-81.
21. Harun R, Danquah MK, Thiruvenkadam S. Particulate size of microalgal biomass affects hydrolysate properties and bioethanol concentration. Bio Med Research International, 2014.
22. Chaudhary L, Pradhan P, Soni NK, Tiwari A. Proudyogiki Vishwavidyalaya RG. Dunaliella tertiolecta Microalgae as biological feedstock, for bioethanol production. Helix. 2017; 7:834-842.
23. Markou G, Angelidaki I, Georgakakis D. Microalgal carbohydrates: an overview of the factors influencing carbohydrates production, and of main bioconversion technologies for production of biofuels. Applied Microbiology and Biotechnology. 2012; 96:631-645.
24. Hedge JE, Hofreiter BT. *Carbohydrate chemistry* (ed. Whistler RL. Be Miller JN.), Academic Press, New York, 1962.
25. Miller GL. Use of Dinitrosalicylic Acid Reagent for Determination of Reducing Sugar. Analytical Chemistry. 1959; 31:426-428.
26. Lowry OH, Rosebrough NJ, Farr AL, Randall R. Protein measurement with the Folin phenol reagent. Journal of Biological Chemistry. 1951; 193:265-275.
27. Neto ABT, Da Silva ME, Silva WB, Swarnakar R, Da Silva FLH. Cinética e caracterização físico-química do fermentado do pseudofruto do caju (*Anacardium occidentale* L.). Quimica Nova. 2006; 29:489-492.
28. Williams PJ le B, Laurens LML. Microalgae as biodiesel & amp; biomass feedstocks: Review & analysis of the biochemistry, energetics & economics. Energy & Environmental Science. 2010; 3:554.
29. Caputi A, Ueda M, Thomas Brown. Spectrophotometric Determination of Ethanol in Wine. American Journal of Enology and Viticulture. 1968; 19:160-165.
30. Prescott W, Prescott GW. The fresh-water algae. How To Know, 1978, 224.
31. Medipally SR, Yusoff FM, Banerjee S, Shariff M. Microalgae as sustainable renewable energy feedstock for biofuel production. Bio Med Research International, 2015.
32. Peterson GR, Scarrah WP. Rapeseed oil transesterification by heterogeneous catalysis. Journal of the American Oil Chemists' Society. 1984; 61:1593-1597.
33. Haq I, Muhammad A, Hameed U. Comparative assessment of Cladophora, Spirogyra and Oedogonium biomass for the production of fatty acid methyl esters. Applied Biochemistry and Microbiology. 2014; 50:69-72.
34. Kumar P, Suseela MR, Toppo K. Physico-Chemical Characterization of Algal oil: a Potential Biofuel. Asian Journal of Experimental Biological Sciences. 2011; 2:493-497.
35. Williams M. The Merck Index: An Encyclopedia of Chemicals, Drugs, and Biologicals. In Drug Development Research (ed. M.J.O'Neil), 15th ed. Royal Society of Chemistry, Cambridge, UK, 2013, 339-339.
36. Vijayaraghavan K, Hemanathan K. Biodiesel Production from Freshwater Algae. Energy & Fuels. 2009; 23:5448-5453.
37. Bowyer JR, Leegood RC. Photosynthesis. In Plant Biochemistry (ed. Dey, P. M., Harbome, J. B.), 1st ed. Academic press, New York, 1997, 49.
38. Robinson J. The Oxford Companion to Wine 3rd ed. Oxford University Press, 2006.
39. Sung-Soo Jang. Production of mono sugar from acid hydrolysis of seaweed. African Journal of Biotechnology. 2012; 11:1953-1963.
40. Manoj BS, Sushma, Chavan M, Karosiya A. Western Ghats terrestrial microalgae serve as a source of amylase and antioxidants enzymes. Journal of Pharmacognosy and Phytochemistry. 2018; 7:1555-1560.
41. Yang HY, Wiegand EH. Production of Fruit wines in the Pacific North West. Fruit Production Journal. 1949; 29:8-12.