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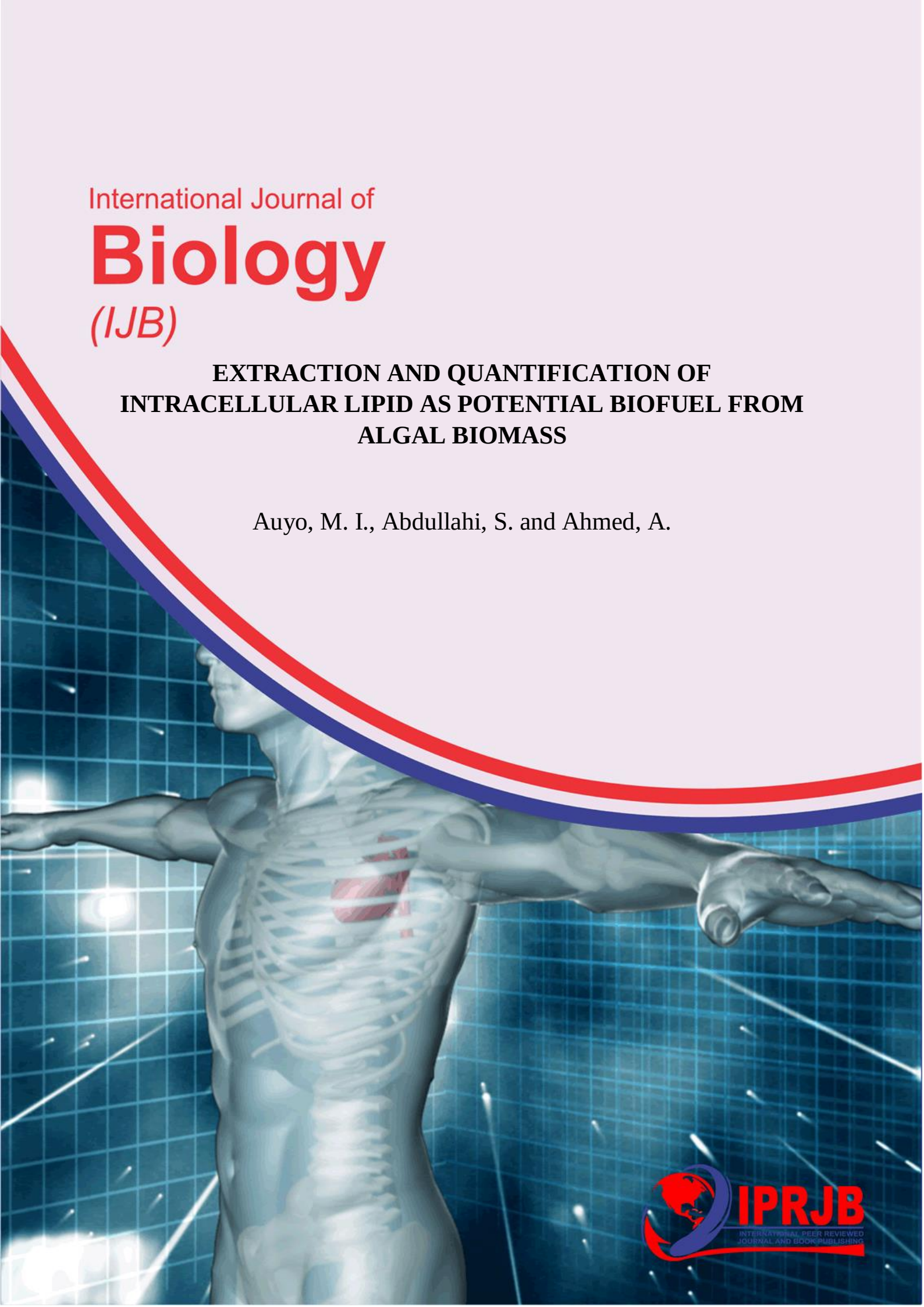

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EXTRACTION AND QUANTIFICATION OF INTRACELLULAR LIPID AS POTENTIAL BIOFUEL FROM ALGAL BIOMASS

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Abstract

Purpose: Fossil fuels occuring as ancient algae deposit is the major source of energy that we energise our engines today. It is a limited non-renewable resource that will eventually run out. Algae have been reported to generate renewable fuels known as biofuel. It is in this regards that this work was carried out to determine, extract and quantify intracellular lipid in algal biomass for subsequent use as biofuel.

Methodology: Four average sized ponds within Federal University Dutse Campus, Nigeria (11.00˚N to 13.00˚N and longitude 8.00˚E to 10.15˚E and altitude 465.5m). were randomly selected and 20ml pond water were collected from top, middle and bottom of each pond. 60ml pond water was taken to laboratory and were processed by first preparing the media. 3ml NPK gel fertilizer was mixed with distilled water and autoclave at 121°C for 15 min. Bold's Basal Medium (BBM) consisting stock solution and BBM consisting of trace elements were separately mixed with distilled water. 10 ml of the stock solution and 1.0 ml of the trace elements were autoclave at 121°C for 15 min. The media (BBM and fertilizer) were cooled at room temperature, antibiotics (penicillin G, di-hydro-streptomycin sulfate and gentamycin sulfate) were added and pH adjusted to 7.5 and the media were kept ready for used. Isolations of species were carried out by serial dilution using BBM and under microscope sterile syringe and needles were used to isolate some target cells from the samples. Thus, four different algal species; Euglena, Spirogyra, Selenastrum and Chlorella were isolated and identified. Culturing of the microalgae isolates was carried out by transferring into 50 ml growth chamber containing BBM and NPK fertilizer media separately and cultured using sunlight for 10 days under controlled pH. Two successfully grown species; Spirogyra and Selenastrum were transferred aseptically into four constructed photobioreactors containing 400 ml liquid media; two containing BBM and the other fertilizer media. Nile Red was used to stain the algal wet biomass and observed under microscope and subsequently photographed. Microalgal cultures were dewatered by repeated centrifugations at 4000 rpm and the supernatants were discarded and the micoralgal biomass rinsed with deionised water to remove the residual salt and later dried using thermostatic drying oven and subsequently

grinded. Growths were determined through dry weight determination and growth percentage was obtained as: dried biomass of a species from each medium/total dried biomass of the species x 100. The dried algal biomass was extracted and evaporated and extracts were heated using vacuum rotary evaporator to separate the solvent (chlorofoam) from the extracts. Transesterification was carried out and first prepared solution was poured into a conical flask containing 5.2 g lipid of Selenastrum species and the second solution was poured into a conical flask containing 2 g lipid of Spirogyra spp. Both flasks were heated over constant temperature magnetic stirrer with condenser attached maintained at 60°C.m Phase separation was carried out along with purification in which soap and other impurities were removed and resulting solution remained flammable biodiesel.

Findings: While Euglena and Chlorella failed to grow in the initial media, both Spirogyra, and Selenastrum produced significant amount of biodiesel with Selenastrum species producing higher quantity than that obtained from Spirogyra, hence a potential source of the fuel.

Key Words: *Extraction, Intracellular lipid, Algae, Biofuel, Production.*

INTRODUCTION

Fossil fuel is one of the major sources of energy that we energise our engines. It is actually ancient algae deposit sequestered under the ground (Mayfield, 2010a), generated by earth via natural process (Drennan, 2016), and is a limited resource that will eventually run out or become very difficult to recover (Batan et al., 2010). The utilization of fossil fuel emits green house gas into the atmosphere, which is a great threat to our lives. For each gallon of gasoline burned in an automobile, 19.4 lb of CO_2 emitted into the atmosphere (Highina, Bugaje, & Umar, 2014). However, the source of fossil fuel is non renewable, which means if we continue pulling it out of the ground, one day we will be shortage of energy. Nigeria is one of the fossil fuel (petroleum) producers in the world but will soon become shortage of it. Daily Trust on Facebook (2012), reported that, Nigeria's crude oil reserve might dry up in the next 41 years unless new wells are been discovered. The world total energy reserves is around approximately 2194–2434 Terra Watts (TW) (Mayfield, 2010b), while the world proven reserves of oil total is 1.4 billion barrels (Mayfield, 2010b). To replace fossil fuels, various biomass feedstocks, including both terrestrial plants and aquatic algae have been discovered to generate renewable fuels known as biofuel (Bahadar & Khan, 2013). Biofuel is a fuel derived from living materials (Drennan, 2016). Increasing attention is being focused on the production of biofuels as the alternatives that will contribute to global reduction in green house gas emissions (Oniemola & Sanusi, 2009). In Nigeria, the use of biofuels is anticipated to make significant impact on petroleum products quality enhancement in view of the current limitations of the fossil-based fuels, which have not kept pace with the increasing demand for environmentally friendly fuel (Sokan-Adeaga, 2015).

Microalgae are the only known renewable source of biofuel that can replace the use of fossil fuels (Achara, 2012). Like other plants, microalgae use CO_2 and sunlight to produce oils but they do so more efficiently than crop plants (Palmer et al., 2004). The productivity of oil of many microalgae greatly exceeds the productivity of oil of the best producing oil crops (Chisti, 2007). Triacylglycerol (TAGs) are the known intracellular lipid also found in algae that consist of glycerol backbone with three fatty acids esterifies to it. TAGs can be evaluated using Nile red stain. Nile red is a lipid-soluble fluorescent dye that has been frequently employed to

evaluate lipid content in many cells such as mammalian, bacterial, yeasts, zooplankton, and microalgal cells (Chen et al., 2009). TAGs can be extracted and transesterified into biodiesel.

Biodiesel is a light to dark yellow liquid immiscible with water, with high boiling point and low vapour pressure. It also refers to a diesel equivalent processed fuel derived from biodiesel sources (such as vegetable oils), which can be used in unmodified diesel-engine vehicles (Sokan-Adeaga, 2015). It is also biodegradable, non-toxic, and typically produces about 60% less net carbon dioxide emissions than petroleum-based diesel.

This work aimed at determination, extraction and quantification of intracellular lipid in algal biomass for industrial applications. It is focused on isolation and culturing microalgal species in photobioreactors in order to evaluate their intracellular lipids using Nile red stain, to determine their relative biomass lipid contents after extraction and to demonstrate how the lipid content can be use in industries such as refineries following its transesterification into biodiesel.

MATERIALS AND METHOD

Four average sized ponds including Males' Hostel, Faculty of Sciences, University Library Building and Old Site Mosque within Federal University Dutse (FUD) Campus**,** Jigawa State, Nigeria (gps) were randomly selected and 20ml pond water were collected from top, middle and bottom of each pond. The 60ml sample pond water was taken to FUD Microbiology laboratory.

For the Media Preparation, NPK Gel Fertilizer gel fertilizer was first used composing of 28.0% w/v Total nitrogen, 3.8% w/v Ammoniac nitrogen, 24.2% w/v Ureic nitrogen, 11.0% w/v Phosphorous pentoxide, 14.1% w/v Potassium oxide (K_2O), 0.03% w/v Boron water soluble, 0.04% w/v Iron (fe) EDTA chelated, 0.03% w/v Copper (Cu) chelated, 0.03% w/v Manganese EDTA chelated, 0.01% w/v Zink (Zn) EDTA chelated and 0.03% w/v Molybdenum water soluble. 3ml of the fertilizer was added into four different 1L conical flasks, each containing 400ml distilled water and autoclaved at 121°C for 15 min. Bold's Basal Medium (BBM) was thed used composing of (a) Stock solution: 5.0 g $NaNO_3$, 1.5 g $MgSO_4$ 7H2O, 0.5 g NaCl, 1.5 K_2HPO_4, 3.5 g KH_2PO_4, 0.5 g $CaCl_2$ $2H_2O$ were added into a conical flask of 200 ml distilled water, **(b) Trace elements:** 0.72 g MnCl. $4H_2O$, 0.35 g MoO_3, 0.9 g $CuSO_4$. $5H_2O$, 0.25 g $Co(NO_3)$ $6H_2O$, 5.71 g H_3BO_3, 15.5 g KOH, $FeSO_4$. $7H_2O$ 2.4 g, 25 g EDTA, 0.5 ml H_2SO (Con.), 4.41 g $ZnSO_4$. $7H_2O$, were added into a conical flask of 500 ml distilled water a method developed by Bischoff & Bold (1963).

10 ml of the stock solution and 1.0 ml of the trace elements were added into four different 1L conical flasks, each containing 400 ml distilled water and autoclaved at 121°C for 15 min, as recommended by Andersen (2005). After the preparations, media (BBM and fertilizer) were cooled at room temperature. Antibiotics including penicillin G, di-hydro-streptomycin sulfate and gentamycin sulfate were added as according to Guillard, (2005) and pH was adjusted to 7.5 after which the media were kept ready for used.

Isolation of Species was carried out by serially dilution and species were isolated using 0.2 ml sterilized BBM added onto two different places, close to each other on a clean glass slide and the 0.1 ml water sample was added onto another different place on the same slide. While observing under microscope (Nikon, Model: XSZ-107BN, Manufacturer: China), sterile syringe and needles were used to move some target cells from the sample drops onto the slide to the nearby BBM drops repeatedly and four different algal species; Euglena, Spirogyra, Selenastrum and Chlorella were isolated, a method recommended by Hill (2015). The species

were identified by comparing with those provided by Imase et al., (2008); Janse et al. (2006) and Bellinger and Sigie (2010).

The pure isolates were transferred into 50 ml growth chamber containing BBM and NPK fertilizer media, where they were cultured for 10 days. Two species Chlorella and Euglena failed to grow while the two successful species Spirogyra and Selenastrum were transferred aseptically into four constructed photobioreactors; two containing BBM and two containing fertilizer media. The organisms were cultured in the photobioreactors with natural sunlight under controlled pH (7.50 ± 0.40) by addition of HCl and NaOH and temperature range of 24 ± 4°C for 28 days. Growths were determined through dry weight determination and growth percentage was obtained as: "dried biomass of one spp. (from one medium)/total dried biomass of that spp. (from two media) x 100.

Nile Red stain (9-diethylamino-5H-benzo[α]phenoxazine-5-one, $C_{20}H_{18}N_2O_2$) working solution was prepared by dissolving 2.5 mg of Nile red into 10ml of acetone as prepared by Lee et al. (1998) and stored chilled and protected from light. Prior to centrifugation, the wet algal biomass was stained and the algal wet biomass (0.2 ml) was placed onto a glass slide. The slide was allowed to air dried for 15 min. The slide was fixed over flame (for 3 passing steps). Ethanol, approximately 0.4 ml was added to the slide and oven dried at 60°C for 2 min. Aliquot of Nile red solution (0.6 ml) was added onto the slide and oven died for 4 min, at 60^0C.

After staining, the slide was completely rinsed and observed under microscope (Nikon, Model: XSZ-107BN, Manufacturer: China), using 10x objective lens. LED UV flashlight (model: SK68, Light colour: purple violet and Light intensity: 395 nm) which has the ability to fluoresce at 935 nm (together with the light of the microscope) was used to send bright purple violet transmission light (from under the microscope's stage) and the accumulated lipid bodies appeared as yellow colour. The images were compared with those provided by Storms et al., (2014).

The microalgal cultures were dewatered using centrifuge the supernatants were discarded and the micoralgal biomass were rinsed with deionised water to remove the residual salt as similarly done by Halim & Webley (2015). The procedure was repeated and the dried algal biomasses were grinded into powder,The dried algal biomass (45.14 g of Selenastrum spp. and 54.20 g of Spirogyra spp.) were placed into two different thimbles and placed into brosilicate glass of soxlet extractors as a method similarly done by Konga et al.(2016) and the extraction began, using chlorofoam as a solvent. For Spirogyra spp., the extraction was carried out for 90 minutes with 18 refluxes at 3:1 ratio of chlorofoam to sample as recommended by Chisti (2007). While in the case of Selenastrum spp., the extraction was carried out for 75 minutes with 15 refluxes at 3:1 chlorofoam to sample ratio as demonstrated by Chisti (2007). The extracts were heated using vacuum rotary evaporator (Model: RE52-3, Manufacturer: China) to separate the solvent (chlorofoam) from the extracts as done by Khola & Ghazala (2012). i.e. solvent from extracting oils is separated using rotary evaporator. KOH (0.052 g) was mixed with 16 ml CH_3OH and another 0.02 g KOH was mixed with 6 ml CH_3OH and stirred properly for 20 minutes as done by Hossain et al. (2008); Khola & Ghazala (2012). i.e., NaOH was mixed properly with CH_3OH and stirred for 20 min. The first prepared solution was poured into a conical flask containing the lipid of Selenastrum spp. (5.2 g) and the second solution was poured into a conical flask containing the lipid of Spirogyra spp. (2 g). Both flasks were heated over constant temperature magnetic stirrer (Model: 78HW-1, Manufacturer: China) with condenser attached at the top, at 60°C with medium stirring for 90 minutes as demonstrated by Chisti (2007); Bajhaiya et al. (2010); Pati & Meti (2014). After the heating, the mixtures were centrifuge for 10 min, at 4000

rpm and the supernatants were carefully transferred to clean conical flasks. Magnesium sulphate by 3% of oil volume was added to the flasks and the heating repeated at 60°C for 30 min. The mixtures were separated by centrifugation for 10 min, at 4000 rpm as a method recommended by Chisti (2007). The results were "tested flammable biodiesel".

RESULTS AND DISCUSSION

Four different microalgal spp. (Selenastrum, Chlorella, Euglena and Spirogyra) were isolated with different number of cells as shown in Ttable 1. The isolated species were primarily cultured in 50ml growth chamber media in which two of the species; Chlorella and Euglena failed to grow while Spirogyra and Selenastrum grew successfully as shown in the table.

Table 1: Relative Number of Cells Grown in BBM and Fertilizer Media Cultures.

ISOLATED SPECIES	NO. OF INITIAL CELL ISOLATED	MEDIUM	GROWTH
Euglena	5-6	BBM	-Ve
Euglena	4	NPK	-Ve
Selenastrum	8	BBM	+Ve
Selenastrum	7	NPK	+Ve
Spirogyra	5	BBM	+Ve
Spirogyra	3	NPK	+Ve
Chlorella	4	BBM	-Ve
Chlorella	6	NPK	-Ve

The successful species Spirogyra and Selenastrum undergone secondary culture in photobioreactors containing BBM and NPK fertilizer and they succeed in pure culture. After culturing, the algal biomasses were dewatered by centrifugation and dried in hot air oven and the Spirogyra dried biomass cultured using NPK was higher than that of Selenastrum using same medium as shown in Ttable 2. The dried biomass of Selenastrum cultured using BBM was higher compare to that of Spirogyra using same medium as shown in table. The total dried biomass of Spirogyra cultured using two different media (BBM+NPK) was higher compare to that of Selenastrum. However, the percentages of Selenastrum growth using NPK and BBM were higher than those of Spirogyra. Nazry et al. (2017) isolated Chlorella from coal-fired power plant in Malaysian Peninsular. Dakhal et al. (2014) isolated different algal species from different locations. In this research work, species of microalgae (Selenastrum, Chlorella, Euglena and Spirogyra) were also isolated.

Different media can support the growth of microalgae. In this research work, BBM and NPK gel fertilizer were used as media of choice and each was found to be effective as supported by the below findings. Munir et al. (2015) cultured Spirogyra, Oedogonium and Chlorella using

BBM where Spirogyra showed better growth in the medium likewise in this work Spirogyra also showed better growth in BBM than Selenastrum species. Gartner et al. (2015) cultured Chlorella using BBM where the medium was found to be effective. Dakhal et al. (2014) also found BBM as an effective medium in the culture of spp. of microalgae. In this research work, both the Spirogyra and Selenastrum showed better growth using BBM. Microalgae can also be cultured using crop fertilizer. Eze et al. (2017) cultured Euglena using NPK crop fertilizer and BG11 where the NPK was found to be less effective than the BG11. In the case of media effectivity used in this work, NPK was also found to be less effective for both Selenastrum and Spirogyra when compared with BBM. However, as microalgae are plants, therefore, they can also be cultured using crop fertilizer.

Table 2: Relative Biomass of Selenastrum and Spirogyra Species Grown Using BBM and NPK Media

SPECIES	NPK GROWTH (g)	BBM GROWTH (g)	TOTAL DRIED BIOMASS (g)	NPK GROWTH (%)	BBM GROWTH (%)
Spirogyra	21.36	32.84	54.20	39.41	60.59
Selenastrum	18.71	35.43	45.14	41.44	78.48

The lipid extracted from Selenastrum was found to be higher than that from Spirogyra as shows in figure 1. The percentage of lipid extracted from Selenastrum was higher than that of Spirogyra.

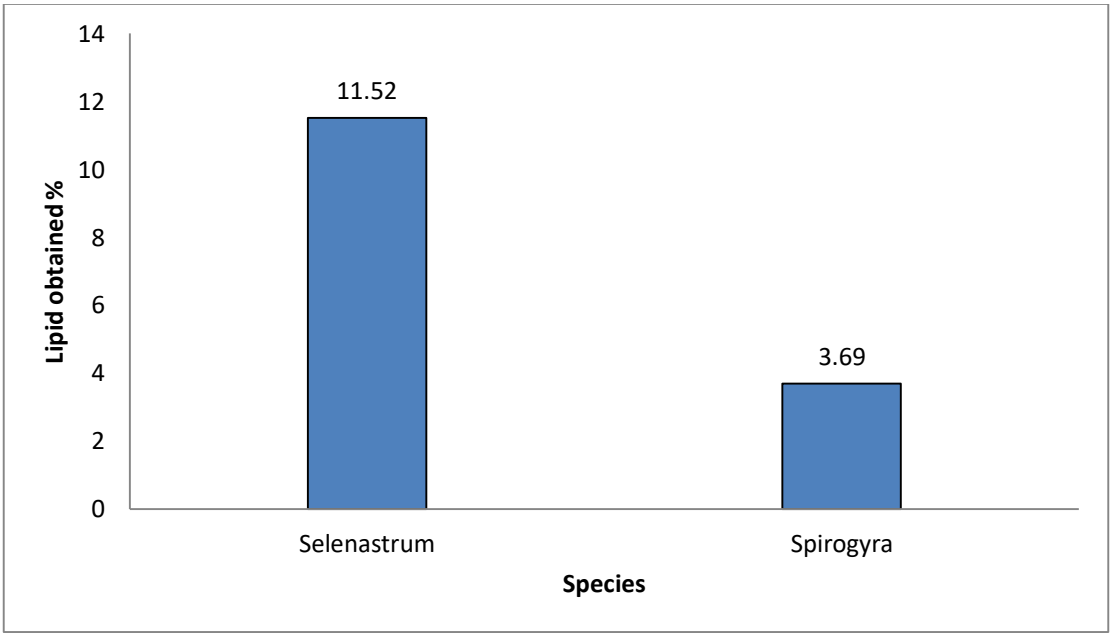

Figure.1: Percentage of Lipid Extracted from Selenastrum and Spirogyra

The biodiesel obtained from Selenastrum spp. was higher when compared with that from Spirogyra as shown in Table 3 likewise the percentage of biodiesel obtained from Selenastrum was also higher than that from Spirogyra

Table 3: Total Biomass, Relative Lipid Content Extracted and Biodiesel Obtained From Selenastrum and Spirogyra Species Grown Using BBM and NPK Media

SPECIES	TOTAL DRIED BIOMASS (g)	TOTAL LIPID EXTRACTED (g)	TOTAL BIODIESEL OBTAINED (ml)
Spirogyra	54.2	2	4.5
Selenastrum	45.14	5.2	14

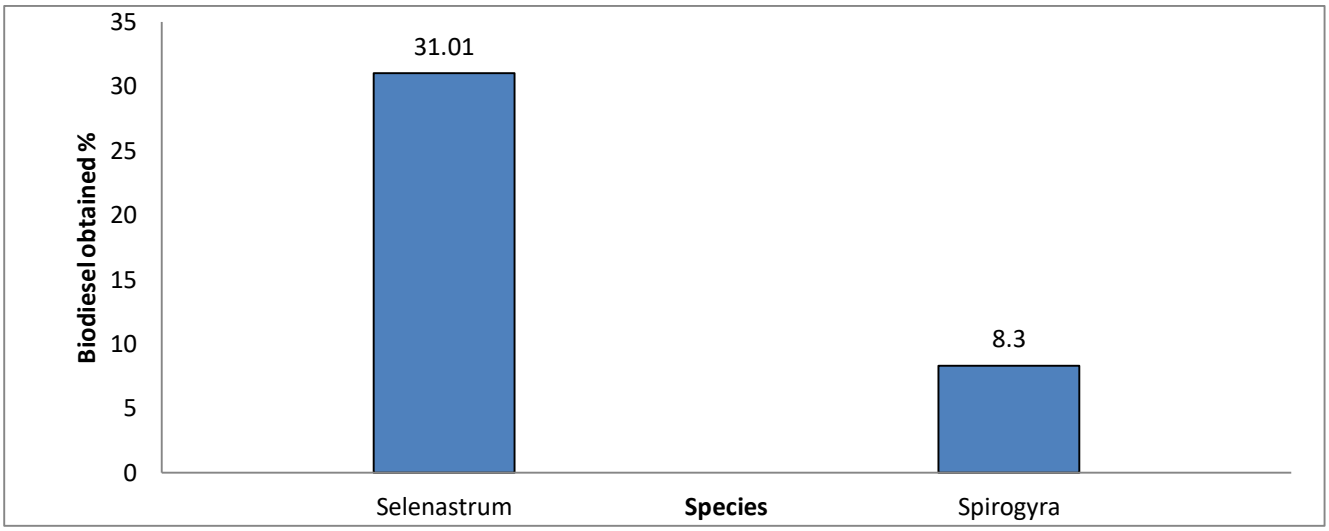

Figure 2: Percentage of Biodiesel Extracted From Selenastrum and Spirogyra

The result of this work shows that the isolation method employed during the work was effective as four different algal spp. (Selenastrum, Euglena, Spirogyra and Chlorella) were successfully isolated and it was found that during primary culture, two of the isolated spp. (Chlorella and Euglena) failed to grow. This is an indication that the two spp. are less tolerant than the two successful ones. Therefore, primary culture of these algae (Chlorella and Euglena) has to start with many numbers of cells. To obtain pure culture of microalgae, the use of photobioreactor is required as it was employed during this work. It was also found that microalgae could be cultured using plant fertilizer. The result of this work also shows that the new Nile red staining method could be adapted because it was effective as the cells were stained perfectly. Even in the absence of fluorescence microscope, we can improvise using the above LED UV flashlight. Selenastrum has high lipid content and produce much biodiesel than spirogyra.

CONCLUSIONS

It can be concluded that is possible to isolate and culture microalgal species in photobioreactors, and relative biomass lipid contents can be determined and the microalgal spp have high potential for biodiesel production and though Selenastrum, Euglena, Spirogyra and Chlorella are abundant in our environment , Selenastrum and Spirogyra with with their high lipid accumulation would be the best for biodiesel generation.

RECOMMENDATIONS

Further researches should be explored to find out other species that accumulate high amount of lipid and there should be collaboration among the microbiologists, botanists, biochemists, biotechnologists and the chemical engineers to carry the research further in order to have sustainable biofuel in as much as the government and other non-governmental organizations should intervene because the research it still at its toe-stage and requires a lot of funding.

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