Experimental Studies on the Production of Biofuel (Bioethanol) from *Spirogyra* Biomass

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ABSTRACT

The application of biofuel technology and engineering is no longer a doubted process of developing renewable alternatives to petroleum-based fuels and utilisations. This sustainable technology has increased tremendously with decreasing world crude oil reserves while the energy prices continually increasing. Bioethanol is a renewable, colourless, less toxic, and readily biodegradable form of fuel from biological sources; that can be used for heat, electricity, and fuel.

In this research study, an alternative feedstock known as algae was used instead of traditional agro-based raw materials for the production of bioethanol, as its more beneficial raw material and does not compete with food, fodder, and is available abundantly in fresh water as well as marine eco-system, and more importantly it is also renewable. The type of algae studied, specifically Spirogyra, was used for the production of bioethanol. A comparative study was also conducted using chemically pre-treated with sodium hydroxide (NaOH) and un-treated spirogyra biomass. The spirogyra biomass was subjected to saccharification process using the fungi Aspergillus Niger for hydrolysis, and for six (6) days. The process was followed by the fermentation process by using yeast (saccharomyces cerevisiae) for another six (6) days. The comparative study experimented and the results recorded showed that high-yield of ethanol was obtained from un-treated spirogyra biomass when compared to chemically pre-treated biomass.

Keywords: *Biofuel, bioethanol, renewable, sustainable, and spirogyra*

1.0 Introduction

Energy remain the prime mover of social and economic development of any the global world. The continual dependency on fossil fuel is really not a good option to the energy need of the world at large. Therefore, the need for alternatives sources of energy for both the decentralized and centralized power generation has led to the proliferation of research into alternative energy sources. Nigeria's energy sector is heavily dependent on petroleum (or fossil) fuels. The major source of energy easily available for use in homes and industries are the various forms of refined fossil fuels such as Premium Motor Spirit (PMS), Automotive Gas Oil (AGO), Dual Purpose Kerosene (DPK) etc., obtained through fractional distillation of crude oil. This is more pronounced in the transportation and electricity generation sub-sectors. Finding sustainable alternatives and/or augmentation for these sub-sectors from new and renewable energy resources is necessary for a sustainable economic development of Nigeria. Over dependence on refined

petroleum products as the main source of energy for the transportation sub-sector, sometimes leading to the importation of these products from overseas, impact negative on the nation's energy security etc. The production processes of these fuels are very expensive which subsequently affect the cost of any process utilizing it. While biofuel method could easily be processed, high environmentally friendliness and less expensive. Biofuel can therefore be considered as the best alternative of decentralized energy source for developing countries especially in this era of insecurity and unpredictability in fossil fuel supply (Bugaje *et al.*, 2008).
Bioethanol is the ethanol produced from biomass. It is a colourless alcohol produced from the fermentation of sugar substrates to ethanol by yeast. Thus, bioethanol can be produced from any biological feedstock that contains appreciable amount of sugar or materials that can be converted into sugar such as starch or cellulose. Different feedstock sources can be used for ethanol production and this research envisage algae specifically *Spirogyra* biomass; was used for the production of bioethanol by the fermentative process. A comparative study was carried out by using chemically pre-treated and untreated *Spirogyra* biomass as raw materials for the biofuel (bioethanol) production. The basic science of fermentation of this raw material (as well as product separation) has been adequately researched and can help to address local production and process development of the bioethanol technology (Fuad *et al.*, 2010).

2.0 Brief Background Studies on Biofuels

The term biofuel refers to solid, liquid, or gaseous fuels derived from renewable raw materials. Biofuels have attracted increasing interest over the last few decades. As fuels made from locally grown renewable sources, they have been proposed as an alternative to expensive fossil fuels. When first demonstrating the engine bearing his name, Rudolf Diesel ran it on peanut at the World's Fair in Paris in 1900 (Knothe, 2001).
Interest in both vegetable oils as fuels for the internal combustion engine and plant material for ethanol production for transport fuel was also reported in several countries during the 1920s and 1930s and later during World War II where there were serious fuel shortages, for example in the UK and Germany. In an interview in 1925, Henry Ford, founder of Ford Motor Company, envisaged the processing of fruit and other plant material into fuel for cars: the fuel of the future is going to come from fruit like that sumac out by the road, or from apples, weeds, sawdust - almost anything. There is fuel in every bit of vegetable matter that can be fermented. There's enough alcohol in one year's yield of an acre of potatoes to drive the machinery necessary to cultivate the fields for a hundred years (The New York Times, 1925).
Interest in biofuels was reinforced in the later decades of the 20th century by various legislative and political acts (EPA, 1970). The oil embargo by the Organization of the Petroleum Exporting Countries (OPEC) in 1973-1974, which led to a sharp increase in crude oil prices, also led to worldwide interest in alternative energy sources, including biofuels. This was also the first time that worries over dependence on oil-based fuel imports were discussed publicly in many countries in the Western world. In recent years, several major challenges to the modern world and its way of life have become a focus of public interest. By the end of the 20th century, governments and policy makers around the world faced three key issues:

- Renewed worries about energy security;
- An interest in economic development, both in the developed world and developing countries, including the creation or sustaining of jobs in agriculture; and
- The need to mitigate climate change and achieve lower greenhouse gas (GHG) emissions.

These challenges and the attempts of policy makers and other stakeholders to address them have contributed to a rapid adoption of biofuels technology. Fuels made from locally grown renewable sources were proposed as a contribution to addressing all three of these challenges, as well as providing a potentially cheap alternative to expensive fossil fuels. Moreover, they were also seen as a way of addressing some additional, important concerns at the time, including those over lead in fuel and losses of agricultural jobs and farming subsidies. Between 1978 and 1996, the Office of Fuels Development at the U.S. Department of Energy developed extensive research programs to produce renewable fuels from algae. The main objective of the program, known as The Aquatic Species Program (ASP), was to produce biodiesel from algae with a high lipid content grown in tanks that utilize CO_2 waste from coal-based power plants. After nearly two decades, many advances have been made in manipulating the metabolism of algae and the engineering of microalgae production systems (Sheehan et al,). There was also an interest in biofuels as a source of octane. From the point of view of many involved, biofuels looked like an extremely attractive option, and thus the decade 1995-2005 saw several new supportive policies for biofuels in the European Union (EU) - including the UK - and the US, as well as in many other countries around the world. These policies established markets for biofuels and acted as incentives to industry to invest in biofuels development and production. As a consequence, biofuels became available on a small but significant commercial scale, and this has remained the case. The above three drivers, which are to some degree interlinked, have become increasingly important and the motivation to develop alternatives to fossil fuels remains strong.

In the year 2008, fossil fuel accounted for 88% of the global primary energy consumption (Brennan *et al*., 2010). The current technological progress, potential reserves and increased exploitation leads to energy insecurity and climate change by increasing greenhouse gas (GHGs) emission due to consumption of energy at higher rate. The use of fossil fuels is now widely accepted as unsustainable due to depleting resources and the accumulation of GHGs in the environment that have already exceeded the "dangerously high" threshold of 450 ppm CO_2 (Schenk *et al*., 2008). With the increase in anthropogenic GHG emission and depleting fossil reserves, mainly due to large scale use of fossil fuel for transport, electricity and thermal energy generation, it has become increasingly important to develop abatement techniques and adopt policies to promote those renewable energy sources which are capable in sequestering the atmospheric CO_2 to minimize the dependency on fossil reserves and maintain environmental and economic sustainability (Brennan and Owende, 2010; Prasad *et al*., 2007a; Prasad *et al*., 2007b; Singh *et al*., 2010a; Singh *et al*., 2010b).

The biofuel that is expected to be most widely used around the globe is ethanol, which can be produced from abundant supplies of starch/cellulose biomass. The most important bioethanol production countries in the world are Brazil, US and Canada (Chiaramonti, 2007). Since biomass assimilation by algal growth utilize atmospheric carbon dioxide, their biomass for bioethanol production can reduce greenhouse gas levels. In addition, ethanol is less toxic, is readily biodegradable and its use produces fewer air-borne pollutants than petroleum fuel. Under the Kyoto Protocol, the Government of Canada has committed to reduce the greenhouse gas emissions by 6% from 1990 levels between 2008 and 2012 (Champagne, 2007).

Research on improving biofuel production has been accelerating for both ecological and economic reasons, primarily for its use as an alternative to petroleum based fuels (Prasad *et al*., 2007a) Microbial fuel cells (MFCs) are also getting attention but they need huge improvements in technologies and also not suitable for transport (Pant *et al*., 2010).

Biofuels could play an essential part in reaching targets to replace petroleum based transportation fuels with a viable alternative, and in reducing long term CO_2 emission, if environmental and economic sustainability are considered carefully (Yuan *et al*., 2008) they can be direct and immediate replacements for the liquid fuels used for transport and can be easily integrated to the logistic systems that are operating today (Escobar *et al*., 2009).

2.1 Classification of Biofuel

Concerns about shortage of fossil fuels, increasing crude oil price, energy security and accelerated global warming have led to growing worldwide interests in renewable energy sources such as biofuels. An increasing number of developed and rapidly developing nations see biofuels as a key to reducing reliance on foreign oil, lowering emissions of greenhouse gases (GHG), mainly carbon dioxide (CO_2) and methane (CH_4), and meeting rural development goals (Koh *et al* ., 2008).

Biofuels are referred to solid, liquid or gaseous fuels derived from organic matter but as earlier discussed in this chapter; the research is strictly based on liquid bio-fuel. They are generally divided into primary and secondary biofuels (Fig. 2.1). While primary bio-fuels such as fuel wood are used in an unprocessed form primarily for heating, cooking or electricity production, secondary bio-fuels such as bio-ethanol and biodiesel are produced by processing biomass and are able to be used in vehicles and various industrial processes. The secondary biofuels can be categorized into three generations: first, second and third generation bio-fuels on the basis of different parameters, such as the type of processing technology, type of feedstock or their level of development (Nigam *et al*., 2010).

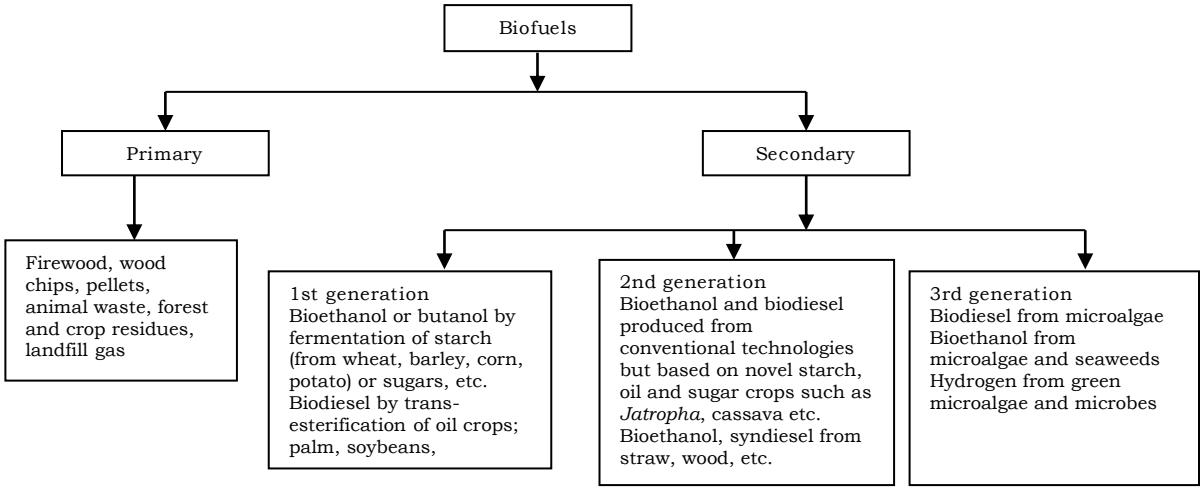

Figure 1 Classification of biofuels *(Giuliano et al., 2010)*

2.2 First and Second- Generation Biofuels

First generation biofuels which have attained economic levels of commercial production, have been mainly extracted from food and oil crops (viz. rapeseed oil, palm oil, sugarcane, sugar beet, wheat, barley, maize. etc.) as well as animal fats using conventional technology (Nigam *et al*., 2010). The liquid biofuels production and consumption growth is increasing day by day, but their impact towards meeting the overall energy demands in the transport sector will remain limited due to competition with food and fiber production for the use of arable land, high water and

fertilizer requirements, lake of well managed agricultural practices in emerging economies, biodiversity conservation and regionally constrained market structures.

Global biofuel production has been increasing rapidly over the last decade, but the expanding biofuel industry has recently raised important concerns. In particular, the sustainability of many first generation biofuels (primarily from food crops such as grains, sugar cane and vegetable oils) has been increasingly questioned over concern such as reported displacement of food crops, effects on the environment and climate change. The limitation of first generation biofuels produced from food crops have caused greater emphases to be placed by second generation biofuels produced from lignocellulosic feed stocks, although significant progress continue to be made to overcome the technical and economic challenges, second generation biofuels production will continue to face major constraints to execute commercial deployment (Sims *et al*., 2010).

2.3 The Third Generation Biofuels.

Algae are gaining wide attention as an alternative renewable source of biomass for the production of bioethanol, which is grouped under the "third generation biofuels" (Nigam *et al*., 2010). The major drawbacks of first and second generation biofuels are overcome to a greater extent by third generation biofuels. The concept of using algae as energy feedstock dates back to the late 1950s (Chen *et al*., 2009) but a concerted effort began with the oil crisis in 1970s. Over the last three decades there has been extensive research on algal biofuels production and the use of algae for CO_2 bioremediation (Borowitzka, 2008). The US Department of Energy (DOE) devoted $25 million to algal fuels research in its aquatic species program at the National Renewable Energy Lab (NREL) in Golden, Colorado from 1978 to 1996. The program gave way to mile stone advances that set the stage for algal biofuel research today (Waltz, 2009). Algae represent a vast variety of photosynthetic species dwindling in diverse environments (Mata *et al*., 2010; Nigam *et al*., 2010). They may be autotrophic or heterotrophic.

The cultivation of microalgae does not compete with other crops for space in agricultural areas, which immediately excludes them from the 'biofuels versus food' controversy. Similar to other oil crops, microalgae exhibit a high oil productivity potential, which can reach up to 100,000 L/hr^{-1}. This productivity is excellent compared to more productive crops, such as palm, which yield 5,959 L/hr^{-1} and thus contribute to the alleviation of the environmental and economic problems associated with this industry (Demirbas *et al*., 2011). Although the productivity of microalgae for biofuel production is lower than traditional methods, there is increasing interest and initiatives regarding the potential production of microalgae in conjunction with wastewater treatment, and a number of experts favour this option for microalgae production as the most plausible for commercial application in the short term (Harmelen *et al*., 2012).

The use of microalgal biomass for the production of energy involves the same procedures used for terrestrial biomass. Among the factors that influence the choice of the conversion process are the type and amount of raw material biomass, the type of energy desired, and the desired economic return from the product (Brennan *et al*., 2010). Microalgae have been investigated for the production of numerous biofuels including biodiesel, which is obtained by the extraction and transformation of the lipid material, bioethanol, which is produced from the sugars, starch, and carbohydrate residues in general, biogas, and bio-hydrogen, among others (Demirbas, 2011).

2.4.1 Comparison of potential oil yields of algae and other oil seeds.

The potential oil yields of algae compared with other oil seeds are shown in Tables 1 and 2 respectively.

Table 1: Oil content of microalgae.

Microalga	Oil content (% dry weight)
Botryococcus braunii	25-75
Chlorella sp.	28-32
Crypthecodinium cohnii	20
Cylindrotheca sp.	16-37
Nitzschia sp.	45-47
Phaeodactylum tricornutum	20-30
Schizochytrium sp.	50-77
Tetraselmis suecia	15-23

Source: Chisti *et al*., (2007)

Table 2: Oil yields based on crop type

Crop	Oil yield (gallons/acre)
Corn	18
Soybeans	48
Canola	127
Jatropha	202
Coconut	287
Oil Palm	636
Microalgae	6283-14641
10g/m²/day at 15% Triglycerides of algae	1,200
50g/m²/day at 50% Triglycerides of algae	10,000

Source: Chisti *et al*., (2007)

Algae is considered to be one of the most efficient organisms on earth, because of their rapid growth rate (some species can double their biomass in a day). Algae grow best in seawater, which comes in virtually unlimited supply. The growing needs of Algae are very different from biofuel crops which have been blamed for land grabbing in 3rd world countries and rising food prices. There are two main ways in which Algae is produced in a large scale.

2.4.2 Characteristics of microalgae

Microalgae, recognized as one of the oldest living organisms, are thallophytes (plants lacking roots, stems, and leaves) that have chlorophyll *a* as their primary photosynthetic pigment and lack a sterile covering of cells around the reproductive cells (Brennan *et al*., 2010). While the mechanism of photosynthesis in these microorganisms is similar to that of higher plants, they are generally more efficient converters of solar energy because of their simple cellular structure.

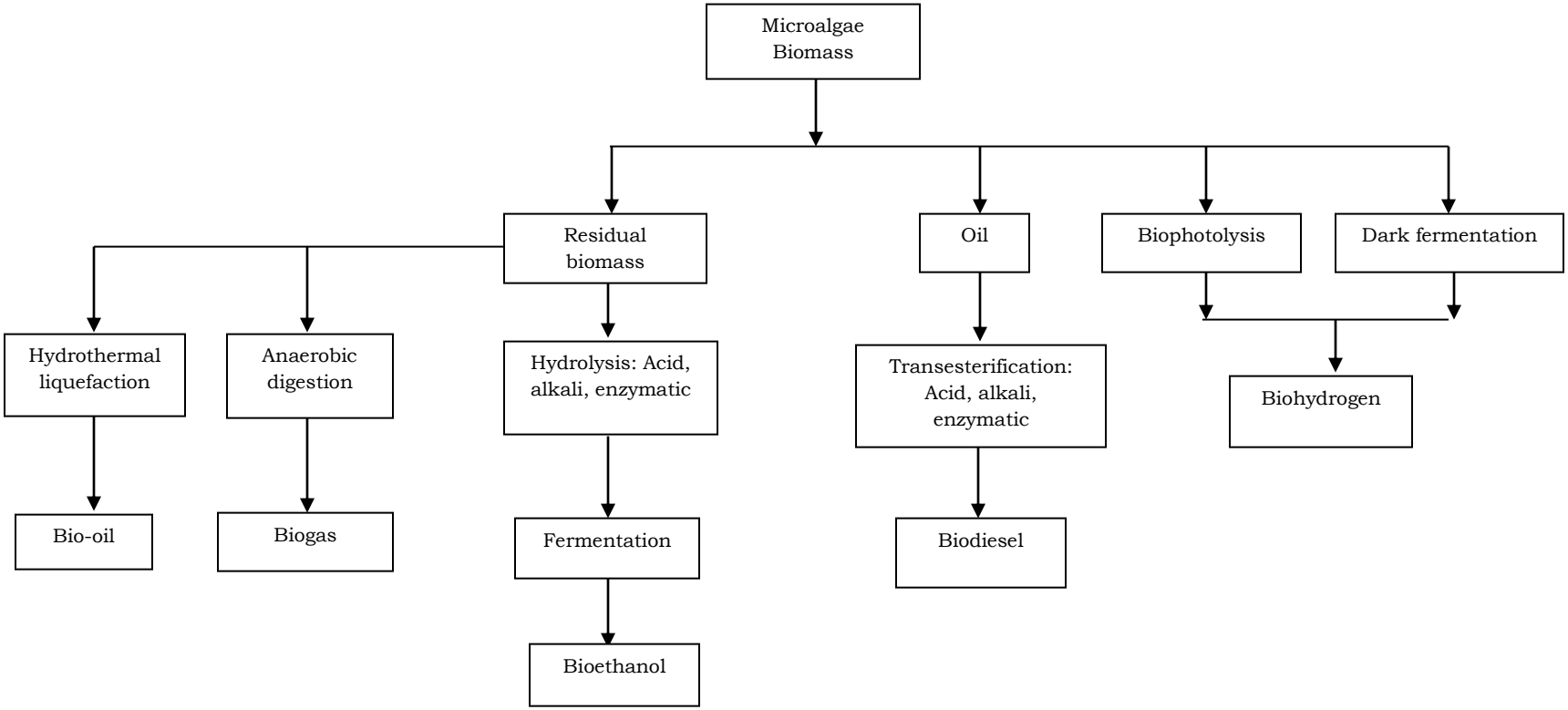

Figure 2 *Diagram of the principal microalgae biomass transformation processes for biofuel production (*Rosana *et al*., 2013)

In addition, because the cells grow in aqueous suspension, they have more efficient access to water, CO_2, and other nutrients (Chisti, 2007).

2.5 Bioethanol

The principle fuel used as a petroleum substitute is bioethanol. Bioethanol is mainly produced by the sugar fermentation process, although it can also be produced by the chemical process of reacting ethylene with steam. The main source of sugar required to produce ethanol comes from fuel or energy crops and plant. These fuel crops are normally grown specifically for energy use and include maize, corn and wheat crops, waste straw, willow, sawdust, reed canary grass, cord grasses, Jerusalem artichoke, myscanthus, sorghum plants and algae which are basically spirogyra biomass as feedstock used in this research. Bioethanol produced from pretreatment and microbial fermentation of biomass has great potential to become a sustainable transportation fuel in the near future (Thomsen *et al*., 2003). Bioethanol is a renewable domestically produced liquid fuel that can help reduced the country independence on foreign oil imports. Recent environment and economic concerns have prompted resurgence in the use of bioethanol throughout the world.

2.5.1 Benefits of using Bioethanol

The physical characteristics of Bioethanol fuel alternative, off-several environmental and performance advantages over petroleum based fuel which requires excessive processing. Bioethanol is produced using familiar methods, such as fermentation, and it can be distributed using the same petrol forecourts and transportation systems as before.

The studies at the European Union (EU) and the United States have determined that the use of bioethanol for transportation purposes produces less environmental hazards like pollution as compared to that produced by fossil fuel. Bioethanol is used as a source of generating power and as earlier discussed, its increasing use as transport fuel is in this three different ways namely:

- Bioethanol has an octane rating of 113-115 and therefore is used as an octane enhancer which is replacing MTBE, a hydroscopic octane enhancer that has caused significant ground water ground water contamination and has major legal liabilities associated with its use.
- Bioethanol is used to meet the minimum oxygen content requirements for gasoline. Some oxygen is required in gasoline to minimize carbon monoxide pollution from vehicles and pollutants that produce ozone.
- Bioethanol is a fuel when mixed with the gasoline and when used alone.

Also, other benefits of bioethanol include:

- Bioethanol can be produced from sugar crops such as sugar beet and sugarcane or starch crops such as barely, wheat, maize, and cassava or rice husk hence renewable.
- Bioethanol is bio degradable
- It provides a 90% reduction in cancer tasks since there is reduced amount gaseous pollutants.
- Bioethanol is conserving natural resources.

2.5.2 Advantage of using algae for Bioethanol production

- It can be naturally occurring in the sea or grown (it does not need soil or other producer substances to grow) and there would be no imbalance that would happen when it is harvested.

- The food price hike must be avoided if the new technology must be utilize as land to farm crops will no longer be taking away.
- Algae / seaweed grow unbelievably faster. Around 10 times as fast as sugar cane. It is actually the fastest growing crop known to man.
- It produces up to 300 times more oil per acre than conventional food crops such as palms, rapeseed, soya beans, atrophy, etc.
- It has a life cycle of approximately 10days, and thus, it allows several harvests in a very short time frame.

2.5.3 Emission of Bioethanol

One of the major advantages of bioethanol is that, it is environmental friendly with reduced emission of gaseous pollutants when compared to fossil fuel as seen in the following:

- Reduction of net carbon monoxide (CO_2) emission by 10-40%
- Reduction of net carbon dioxide (CO_2) emissions by 100%
- Reduction of sulphur dioxide (SO_2) emissions by 100%
- Reduction of hydrocarbon (HC) emissions by 10-50%
- Reduction of all poly cyclic aromatic hydrocarbons (PAHS)

2.5.4 Bioethanol as a fuel

The transport sector is today almost entirely dependent upon the oil-based fuels. In EU the transport sector accounts for more than 30% of the total energy consumption and it is 98% dependent on fossil fuels where the crude oil feedstock is largely being imported. This leads to changes: the transport sector is extremely vulnerable to any market disturbance and it contributes with about 28% to the CO_2 emissions. It is expected that 90% of the increase of CO_2 emission between 1990 and 2012 will be attributed to transport. It is therefore important that we develop renewable fuels and here biofuel such as bioethanol provide the best option to replace a significance share of fossil fuels.

2.5.5 Advantages of bioethanol over other fuel types

Bioethanol as a transport fuel tables numerous advantages over traditional fuel such as:

- Premature ignition and prevention of cylinder knocking due to the higher octane number and higher heat of vaporization compared to traditional fuel (Balat *et al*., 2008)
- Reduction in hydrocarbon and carbon monoxide exhaust emission based on the higher oxygen content of bioethanol (Demirbas, 2008).
- In an internal combustion engine, the lower energy content of bioethanol fuel blend as the compression ratio is higher and burn time is shorter (Lucia, 2010).
- The blending or mixing of bioethanol with traditional or other kinds of fuel is compatible with current engine designs. (Jegennathan *et al*., 2009)
- Bio-ethanol is chemically miscible in petrol (Sanchez and Cardona, 2008)

However, there are disadvantages associated with bio-ethanol which are as follows:

- Combustion of bio-ethanol when blended with petrol, releases formaldehyde and acetaldehyde, which are toxic to human (Demirbas, 2008).
- The use of agricultural products such as cereal grains will limit food and feed reserves in developing countries, leading to food crisis. (Chakauya *et al.*, 2009).

The important properties of bioethanol as compared to the properties of fossil gasoline (PMS) are shown in Table 3 below.

Table 3 Parameters of bioethanol in comparison with petrol

Fuel	Density (kg)	Viscosity (mm^2/s)	Flash point (^0C)	Caloric value MJ/kg)	Caloric value (MJ/1)	Octane number (RON)	Fuel Equivalent
PMS	0.76	0.6	<21	42.7	32.45	92	1.0
Bioethanol	0.79	1.5	<21	26.8	21.17	>100	0.65

Source: *Adapted from (Bugaje et al., 2008)*

2.6 Basic Chemistry of Ethanol

During ethanol fermentation, glucose is decomposed into ethanol and carbon dioxide.

$$C_6H_{12}0_6 \rightarrow 2CO_2 + 3H_20 \quad (1)$$

During combustion ethanol reacts with oxygen to produce carbon dioxide, water, and heat: (other air pollutants are also produced when ethanol is burned in the atmosphere rather than in pure oxygen).

$$C_2H_6+30_2 \rightarrow 2CO_2+3H_20 \quad (2)$$

Together, they add up to:

$$C_6H_{12}+60_2 \rightarrow 6CO_2+6H_20 + heat \quad (3)$$

This is reverse of the photosynthesis reaction, which shows that the three reactions completely cancel each other out, only converting light into heat without leaving any byproducts:

$$6CO_2+6H20+ light \rightarrow C_6H_{12}O_6+6O_2 \quad (4)$$

2.7 Bioethanol Production from Microalgae

Bioethanol production from microalgae has received remarkable attention because of the high photosynthetic rates, the large biodiversity and variability of their biochemical composition, and the rapid biomass production exhibited by these microorganisms (Derner *et al*., 2006). Furthermore, bioethanol derived from microalgae biomass is an option that demonstrates the greatest potential. (John *et al*., 2011) assessed microalgae biomass as a raw material for bioethanol production and argued that it is a sustainable alternative for the production of renewable biofuels. Examples of the genera of microalgae that fit the parameters for bioethanol production include the following: *Chlorella*, *Dunaliella*, *Chlamydomonas*, *Scenedesmus*, *Arthrospira*, and *Spirulina.* These microorganisms are suitable because they contain large amounts of starch and glycogen, which are essential factors for the production of bioethanol. The carbohydrate composition of these genera can be 70% of the biomass (Harun *et al*., 2011b).

In bioethanol production, the processes vary depending on the type of biomass and involve the pretreatment of the biomass, saccharification, fermentation, and recovery of the product. The pre-treatment of the biomass is a critical process because it is essential for the formation of the sugars used in the fermentation process (Table 2.5). Before the traditional fermentation process, acid hydrolysis is widely used for the conversion of carbohydrates from the cell wall into simple sugars. The acid pretreatment is efficient and involves low energy consumption (Harun *et al*., 2011a). Other techniques, such as enzymatic digestion (Chen *et al*., 2012) or gamma radiation (Yoon *et al*., 2012), are interesting alternatives for increasing the chemical hydrolysis to render it more sustainable. Through analysis of the process in terms of energy, mass, and residue generation, it is possible to determine the best route. With enzymatic hydrolysis, the process can be renewable. Another technique for pretreatment of the biomass

is hydrolysis mediated by fungi. (Bjerk, 2012) investigated the *Aspergillus* genera for this purpose, and the bioethanol produced was monitored by gas chromatography using a headspace autosampler. The study demonstrated that seven strains (four isolates from *A. niger*, one from *A. terreus*, one from *A. fumigatus*, and one from *Aspergillus* sp.) were more efficient at hydrolyzing the residual biomass.

3 Materials and Methodology

An adopted modification to the materials and methods used by (Fuad *et al.*, 2010, 2011), was used in this work as stated below.

3.1 Materials

Table 4 depict the materials and equipment used in this research for the production of bioethanol with specification and their respective manufacturer listed below.

Table 4: List of materials used with specification and their manufacturer

Materials/Equipment	Specifications	Manufacturer
Basket	Plastic	-
Bucket	Plastic	-
Laboratory trail	Plastic	-
Mortar and pistle	Ceramic	-
Sieve	1.00mm Laboratory test sieve. PAT No. 667924	ENDECOTTS LTD, London, England
Algal biomass	*Spirogyra* species	Faculty of general Agriculture, University of Maiduguri, Borno State, Nigeria
Potato dextrose agar (PDA) medium	-	-
Yeast extract, peptone and dextrose (YPD) agar media	-	-
Distilled water	-	-
Conical flask	-	-
Beaker	-	-
Glass rod	-	-
Erlenmeyer flasks	500ml	-
Autoclave	Model: YN-280, Rated working pressure: 0.14MPa, Serial No. 3247	Seradon
Thermometer	-	-
Stop watch	-	-
Petri dish	-	-
Pipette	-	-
Test tube	-	-
Spatula	-	-
Electric precision balance	Model: TL-5000, Capacity:	

	5000g, diameter = 0.1g	METRA
Inoculating niddle	-	-
Apergillus niger	-	-
Capillary tube	-	-
Saccharomyces cerevisiae	-	-
U-tube	-	-
Balloon	-	-
Measuring cylinder	10ml graduated-plastic	-
Lighter	-	-
Cotton wool	-	-
Foil paper	-	
Spirit lamp	-	-

3.2 The Composition of Synthetic Media

Components of synthetic media used in this research study and their respective percentages are shown in Table 5 below:

Table 5 Compositions of Chemically Defined Media

S/No	Component	Percentage (%)	Specification	Manufacturer
1	Monosodium glutamate	0.03	99+ % pure	Ajino-Moto Co, inc. India, Japan
2	NH_4NO_3	0.14	-	-
3	KH_2PO_4	0.2	Assay 99.2% min (on dried material), PH of 2% solution 4.3 to 4.6 maximum limit of impurity.	HOPKIN & WILLIAMS; General purpose reagent (G.P.R).CHADWELL HEALTH ESSEX, ENGLAND.
4	$CACL_2$	0.03	Product number: 27584	BDH Chemicals Ltd poole, England.
5	$MgSO_4.7H_2O$	0.03	Assay not less than 99.5% and not more than the equivalent of 103.0% $MgSO_4.7H_2O$	AnalaR (analytical reagent), BDH Chemicals Ltd poole, England
6	Bacto-Peptone	0.75	Control: 643765, store at 15-30^{0}C. DIFCO certified.	Difco Laboratories; Detroit Michigan, USA
7	$FESO_4.7H_2O$	0.5	A green or bluish-green crystals or crystalline powder. Assay, minimum of 99.0%. A.R grade	General Laboratory supplies: High street, Tatten hall, Nr.Chester.
8	$MnSO_4.4H_2O$	0.16	Assay. Not less than 98.0% and not more than the equivalent of 101.0% $MnSO_4.4H_2O$. PH of 5.0	AnalaR (analytical reagent), BDH Chemicals Ltd poole, England
9			Assay: Not less than	

	$ZnSO_4.7H_2O$	0.14	99.5%	-
10	Tween 80	2	-	-
11	Lactose	0.5	Product number: 10139	AnalaR (analytical reagent), BDH Chemicals Ltd poole, England. Holland

Source: *Modified from (Fuad et.al 2010, 2011).*

Figure 3 Collection of algae spirogyra samples

3.3 Methodology

3.3.1 Sample preparation

The algal sample was identified upon microscopic examination as *Spirogyra* species. The alga *Spirogyra* was collected from pond situated at the orchard in the Faculty of Agriculture, University of Maiduguri, Borno state, Nigeria as shown below. Two fungal cultures *Aspergillus niger* and *Saccharomyces cerevisiae* were procured from LAB 113 of Biological science department, university of Maiduguri. The fungi *Aspergillus niger* was cultured and maintained on potato dextrose agar (PDA) medium at 30^0C. The yeast *Saccharomyces cerevisiae* was cultured and maintained on Yeast extract, peptone and dextrose (YPD) agar media at 30^0C as depicted in Figures 3.2 and 3.3 respectively.

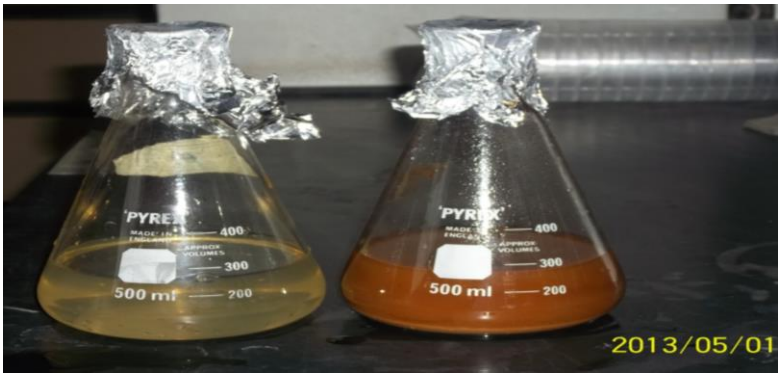

Figure 4 Preparation of the media

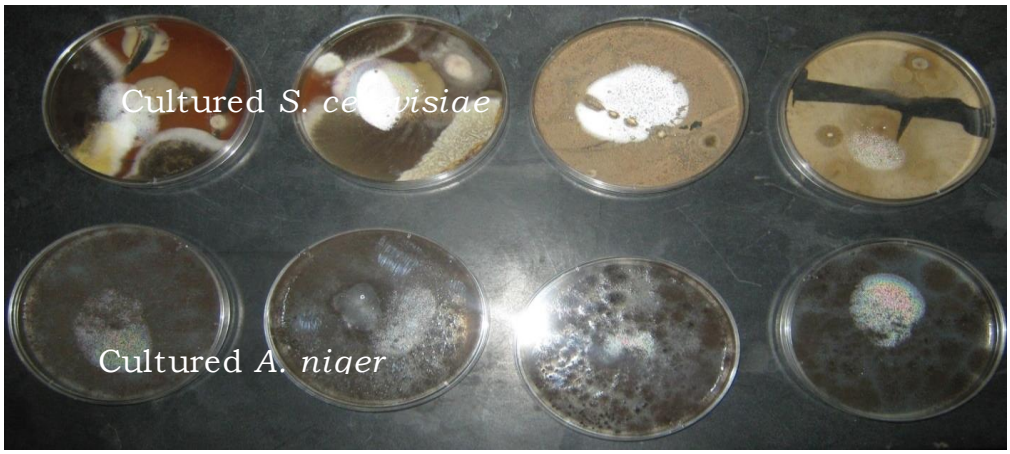

Figure 5 *Cultured Aspergillus niger on P D A medium and Saccharomyces cerevisiae on Y P D agar.*

3.3.2 Processing of biomass

The *Spirogyra* biomass was subjected to sun dryness which removes the moisture content (Figure 6).

Figure 6 Subjecting the Spirogyra biomass to sun dryness

The dried *Spirogyra* biomass was grinded and filtered through 1mm sieve as presented in their respective Figures 7 and 8. Fine powder of *Spirogyra* biomass thus obtained (Figure 9) was used for all fermentation experiments by taking two variations: half of the biomass was chemically pre-treated and remaining biomass was left untreated.

3.3.3 Chemical pre-treatment of *Spirogyra* biomass:

The *Spirogyra* biomass was chemically pretreated with 1% NaOH for a period of 2 hours.

Figure 7 dried Spirogyra biomass

Figure 8 Filtering of the biomass

Figure 9 Fine powdered Spirogyra biomass

3.3.4 Saccharification and Fermentation

Spirogyra biomass was saccharified by enzymes produced from *Aspergillus niger* (amylase and cellulase). Biomass was divided into two equal halves (50% + 50%); one part of the biomass was directly used for saccharification and fermentation, remaining part of powder was treated chemically and then used for saccharification and fermentation. In the first step the biomass was subjected to saccharification by *Aspergillus niger* and in the second step *Saccharomyces cerevisiae* was added for fermentative process to produce bioethanol. Three experiments were designed and designated as experiment-1, 2 and 3 in the following manner:

Biomass + Aspergillus niger + Saccharomyces cerevisiae (5)
Biomass + Lactose + Aspergillus niger + Saccharomyces cerevisiae (6)
Biomass + Nutrients + Aspergillus niger + Saccharomyces cerevisiae (7)

Fermentation studies were conducted in 500ml Erlenmeyer flasks. Experiments were carried out in the following manner shown in Table 6.

Table 6 Design of Fermentation experiments

Experiment No's	Spirogyra Biomass	Chemically Treated Spirogyra Biomass
1	5g of the biomass + 100ml of distilled water + *A. niger* + *S. cerevisiae*	5g of the biomass + 100ml of distilled water + *A. niger* + *S. cerevisiae*
2	5g of the biomass + 100ml distilled water + 0.5% of Lactose + *A. niger* + *S. cerevisiae*	5g of the biomass + 100ml distilled water + 0.5% of Lactose + *A. niger* + *S.cerevise*
3	5g of the biomass +100ml of synthetic media + *A. niger* + *S. cerevisiae*	5g of the biomass + 100ml of synthetic media + *A. niger* + *S. cerevisiae*

Source: *Adapted from Fuad et.al (2010, 2011).*

In the fermentation process, for comparative studies; *Spirogyra* biomass was used for fermentative production of bioethanol in two variations- chemically pre-treated form and untreated form. Fermentation studies performed in the 500ml Erlenmeyer flasks has the following three different variations:

- 5g of the biomass in 100ml of distilled water,

- 5g of the biomass in 100ml distilled water containing 0.5% of lactose and
- 5g of the biomass in 100ml of synthetic media containing the components as shown in Table 6.

The flasks were autoclaved at 15lbs for 15 minutes and inoculated with mycelial mat of *Aspergillus niger* presented in Figure 5. The same process was followed for both the chemically pretreated biomass and the untreated biomass (Figure 10).

Figure 10: *Autoclaved flasks inoculated with mycelial mat of Aspergillus niger.*

3.3.4.1 Saccharifiation of *Spirogyra* biomass by *Aspergillus niger*

For the saccharification of algal biomass developed mycelial mat of *Aspergillus niger* was used. *Aspergillus niger* is cellulolytic and amylolytic in nature as it produces cellulases and amylases. These enzymes hydrolyze the cellulose and starch present in *Spirogyra* and releases free sugars. The saccharification was carried out for a period of six days and the process was monitored every 24 hours for sugars released using empty balloons by measuring the weight as it is inflamated by the released gas.

3.3.4.2 Fermentation by *Saccharomyces cerevisiae*

After six days of saccharification mycelial mat of *Aspergillus niger* was removed under aseptic conditions and 10% of *Saccharomyces cerevisiae* was added to the flasks for fermentative production of bioethanol. The process was carried out for a period of another six days at about 78^{o}C (since ethanol has a boiling point of 78.4^{o}C) during which every 24 hours each of the flasks were heated to about the same temperature and ethanol was collected as displayed in Figure 11.

Figure 11: *Heating of the Erlenmeyer flask for bioethanol production*

Amount of the bioethanol produced was released through a collecting tube connected to the experimental flasks and finally drop into a U-tube as indicated in Figure 12 and 13.

Figure 12 Experimental setup

Figure 13 Collection of the bioethanol

Thereafter, the samples were measured in a plastic measuring cylinder and stored in plastic sample bottles tightly covered to avoid evaporation (Figure 14).

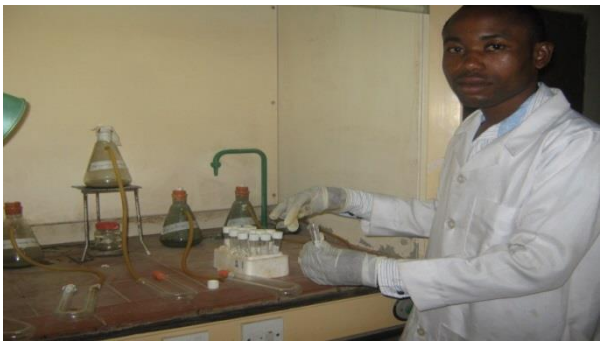

Figure 14 Displayed *measured samples*

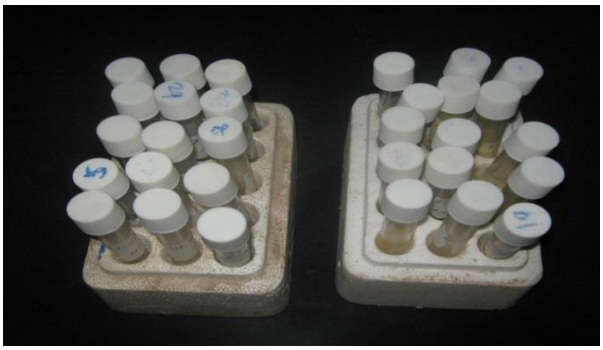

Figure 15 Stored bioethanol in sample bottles

The sample bottles containing the bioethanol produced were then labeled accordingly and kept under aseptic condition to avoid contamination.

4 Experimental Results and Discussions

4.1 Saccharification and Ethanol Production from Spirogyra Biomass

4.1.1 Saccharification of *Spirogyra* biomass by *Aspergillus niger* *(un-treated)*

Figure 16 depict the saccharification of the un-treated *Spirogyra* biomass was carried out for a period of six (6) days at 30^0C for sugar released after 24 hours.

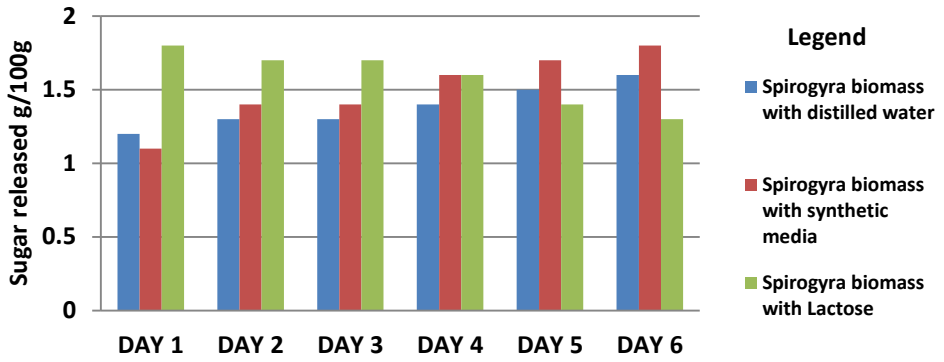

Figure 16(a) Histogram of bioethanol production in submerge fermentation from un-treated substrate (sugar released g/100g)

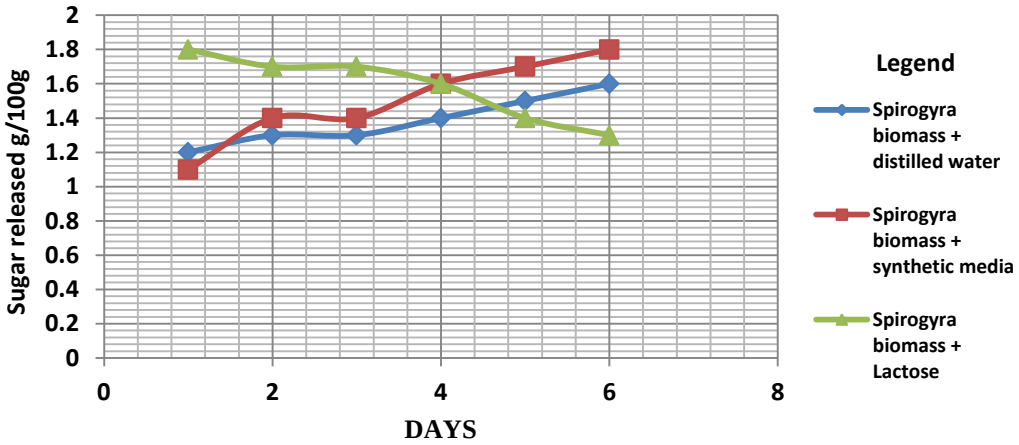

Figure 16(b) Profiles of bioethanol production in submerge fermentation from un-treated substrate (sugar released g/100g)

4.1.2 Saccharification of *Spirogyra* biomass by *Aspergillus niger* *(pre-treated)*

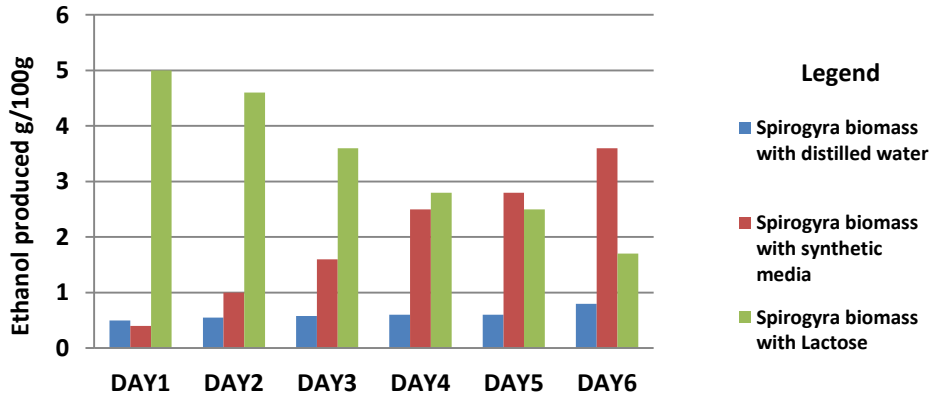

Figure 17(a) Histogram of bioethanol production in submerge fermentation from pre-treated substrate (sugar released g/100g)

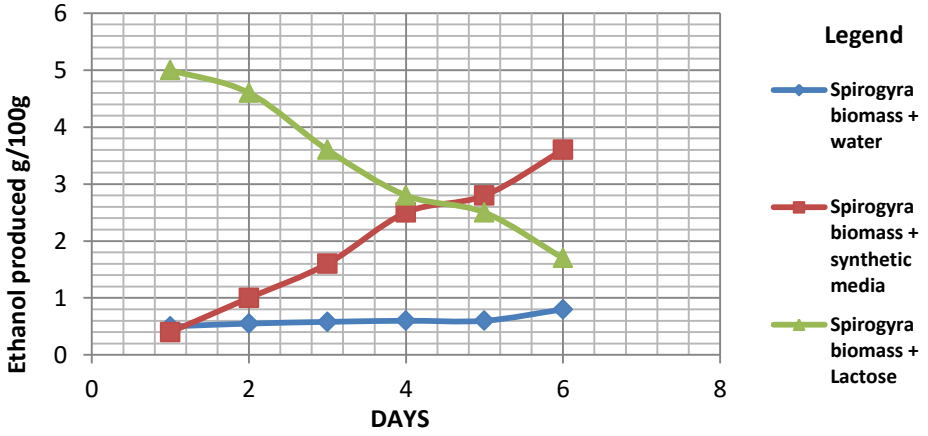

Figure 17(b) Profiles of bioethanol production in submerge fermentation from pre-treated substrate (sugar released g/100g)

4.2 Bioethanol production from pre-treated substrate (Sugar released g/100g)

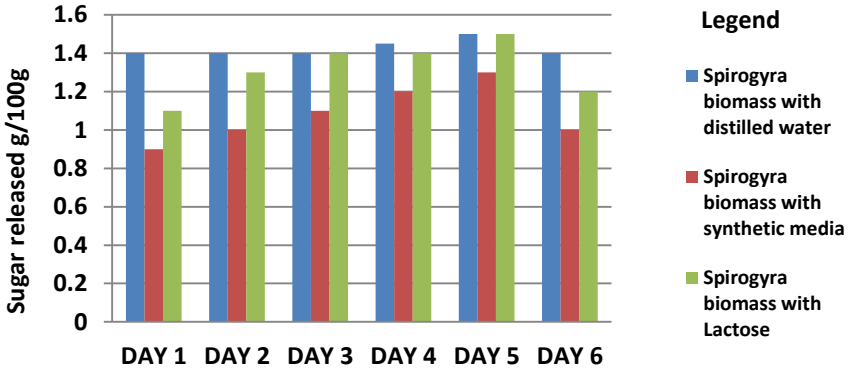

Figure 18(a) Profiles of bioethanol production from pre-treated substrate (sugar released g/100g)

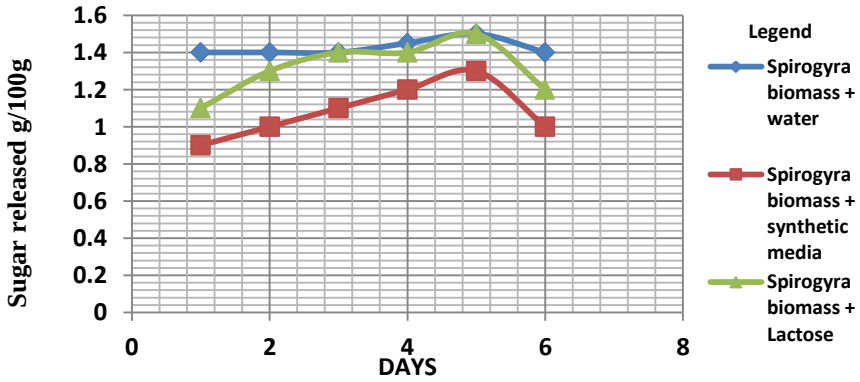

Figure 18(b) Profiles of bioethanol production from pre-treated substrate (sugar released g/100g)

4.3 Bioethanol production from pre-treated *Spirogyra* biomass with Lactose (ethanol produced g/100g)

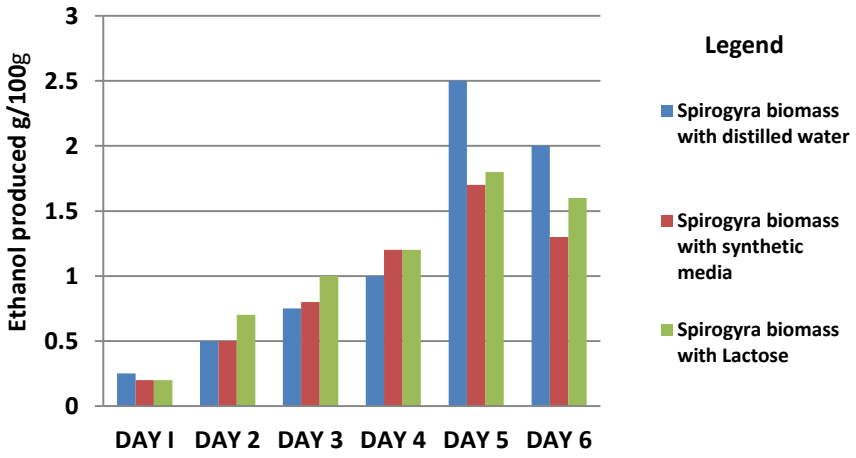

Figure 19(a) Profiles of bioethanol production in sub-merge fermentation from pre-treated substrate (sugar released g/100g)

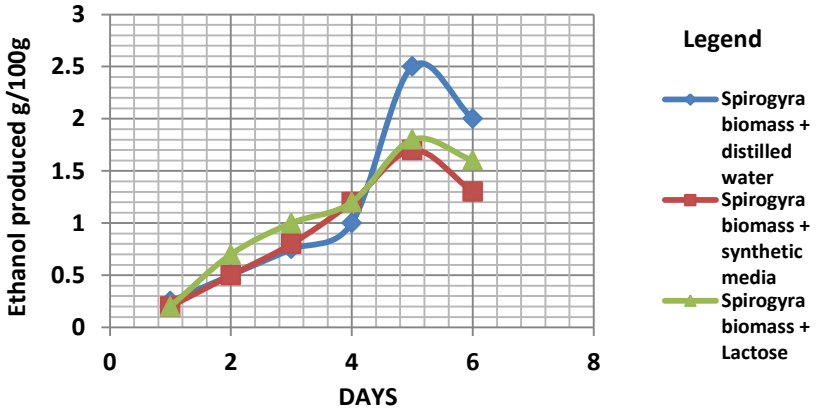

Figure 19(b) Profiles of bioethanol production in sub-merge fermentation from pre-treated substrate (sugar released g/100g)

5.0 Discussion of Results

In this study we use algal-*Spirogyra* biomass as a substrate for bioethanol (ethanol) production which is rich in polysaccharides- starch and cellulose. A weak alkali treatment was used instead of acid for the pre-treatment of the processed *Spirogyra* biomass; as acid pre-treatment results in production of toxic substances which decreases the fermentation efficiency of *Saccharomyces cerevisiae.* As a source of cellulase enzyme, *Aspergillus niger* was used for saccharification of *Spirogyra* biomass into simple sugars. Saccharomyces cerevisiae is then procured to ferment the saccharified algal biomass. Since the industrially used *Saccharomyces cerevisiae* is non-cellulolytic and non amylolytic in nature, the fungal culture *Aspergillus niger* was employed to hydrolyse and produce simple sugars which can be

directly utilized by *Saccharomyces cerevisiae* for ethanol production (Fuad *et al.,* 2010). Aspergillus niger was used as it is an enzyme source of cellulase and amylase for saccharification of *Spirogyra* biomass into simple sugars, since pure commercially available enzymes are very expensive. This explained the fact that enzyme hydrolysis is a natural and ideal method for conversion of cellulose materials to sugars which could be used as a source of food, fuel or chemicals (Martin *et al*., 1982).

5.1 Saccharification and Bioethanol Production from Un-Treated Spirogyra Biomass

The fungi *Aspergillus niger*, as a source for starch and cellulose hydrolysis was used and saccharification process was performed under optimal conditions. The degree of saccharification was evaluated by monitoring the amount of sugar released by the liquefaction of starch and cellulose using empty balloons by measuring the weight as it is inflamated by the released gas. The effective usefulness of the un-treated *Spirogyra* biomass as a medium for yeast growth was further estimated by checking the ethanol production through a capillary tube as it is been released into a U-tube when subjected to heating as discussed and shown in the materials and methods chapter of this research. The results of sugar released for saccharification of the un-treated *Spirogyra* biomass are shown in Figures 16(a) and (b) and various grades of ethanol production were shown in Figures 17 to 18. The highest sugar was released on sixth (6^{th}) day of saccharification in all the flasks with distilled water, and synthetic media but on the first (1^{st}) day of saccharification in the flask with lactose. Accordingly, the amount of bioethanol produced was also more on the 6^{th} day in the flasks containing distilled water, and synthetic media but less in the flask with lactose. The same trend was observed in the flask which had nutrient media along with the biomass where the highest sugar released and bioethanol produced was on the 6^{th} day in an increasing order as shown in Figure 16(a), for the saccharification process by *Aspergillus niger* depicted in Figure 17 for the fermentation process by *Saccharomyces cerevisiae*. In this flask, comparatively more sugar was released with respective amount of bioethanol produced on this last day than those flasks which did not have nutrient media. In Figure 17, the fermentation process but there is a great difference in the flask with lactose when it was added; as the rate of sugar released and bioethanol produced is highest on the 1^{st} day but decreases subsequently till the 6^{th} day of saccharification which has the lowest sugar released and fermentation with lowest amount of ethanol produced as depicted in Figure 17(b). This changed the trend of sugar released and bioethanol produced completely because lactose is an enzyme inducer which speed-up the conversion activity of cellulose into sugars.

The result indicates that the trend of sugar released and bioethanol production from un-treated *spirogyra* biomass gradually increased from 1^{st} day to 6^{th} day with slight fluctuations except with lactose where the highest quantity of sugar was released on the 1^{st} day and gradually decreased up to 6^{th} day leading to highest bioethanol production on the 1^{st} day and lowest on the 6^{th} day as shown in Figures 18(a) and (b), indicating that lactose is acting as inducer for cellulase activity, hence more sugar was released on the 1^{st} day leading to highest production of bioethanol. This trend gradually decreased as the quantity of lactose decreased from 1^{st} day to 6^{th} day. It seems profound effect of an inducer on *A. niger* stimulates the organism to release more enzyme, hence efficiency of saccharification enhanced from 1st day itself. Thus In both cases, the trend indicates that production of bioethanol was directly proportional to availability of sugar for fermentation (Fuad *et al.*, 2010).

5.2 Saccharification and Bioethanol Production from Chemically Pre-treated *Spirogyra Biomass*

The two organisms (*Aspergillus niger* and *Saccharomyces cerevisiae*) were proved very promising in this research study as in these sets of experiments, the *Aspergillus niger* was used for saccharification of *Spirogyra* biomass and *Saccharomyces cerevisiae* was used for fermentative production of the bioethanol. The total sugar released, increased gradually with increase in time of incubation in all the flasks containing chemically treated biomass where more quantity of sugar was released on 5th day but drops on the 6th day leading to more production of bioethanol on the 5th day but less amount was produced on the 6th day as shown in Figures 18 for the sugar released and Figures 19 for the bioethanol produced respectively. Thus, in the fermentation with chemically treated *Spirogyra* biomass the same trend was observed in each of the flask which had distilled water, lactose and synthetic media. In these flasks, comparatively there was a constant sugar released on the 1st three days consecutively in the flask containing *Spirogyra* biomass with distilled water which gradually increased on the 5th day where the highest released of sugar was recorded but drops on the 6th day of saccharification as shown in Figure 19 (a) and (b). The bioethanol produced in this flask with distilled water increased from the 1st day to the 6th day with the highest production rate on the 5th day but decreases on the last day of fermentation.

Therefore, it was noticed that as compared to this flask containing distilled water, there was also an increased level of sugar released in the flaks which had lactose and synthetic media with the highest amount observed on the 5th day of saccharification. The increased in sugar released in this two flasks (lactose and synthetic media) alongside with the *Spirogyra* biomass led to a substantial increase in the amount of bioethanol produced accordingly with the highest production obtained on the 5th day which also drops on the 6th day.

The result also shows that; in ethanol production from chemically pre-treated *spirogyra* biomass, highest sugar released was observed on the 5th day of saccharification in all flasks containing distilled water, lactose and synthetic media. Similarly, the ethanol was also produced more on the 5th day of fermentation in the various flasks of substrates 19 (a) and (b) which confirmed the fact that increased in the flasks of cellulosic materials due to conversion into sugars, caused an increased in the conversion activity of enzyme leading to gradual increase in total sugars for fermentation and vice-visa.

5.3 Comparison of Bioethanol Production from Un-treated and Pre-treated *Spirogyra Biomass*

The result obtained show that the cellulose is more suitable for saccharification than the chemically treated biomass where the biomass may be damaged due to chemical treatment and partially lost its suitability for the saccharification process. In the un-treated *Spirogyra* biomass with all the substrates in their respective elermenyer flasks, sugar released was monitored to be a gradual increased from the first day to the sixth day in both the flasks with distilled water and synthetic media but a periodic decreased in the flask of lactose with highest sugar released on the first day of saccharification. The amount of bioethanol produced with the un-treated *Spirogyra* biomass in the fermentative process was carefully taken into cognizant and proved a corresponding increased to the sugar released except with lactose which also has a corresponding decreased from the first to the last day and highest production on the first day, as shown in Figures 18(a) and (b). This shows that the microbial production of ethanol from cellulosic material is mainly dependent on saccharification enzymes produced by *Aspergillus niger,* as the trends indicate that production of bioethanol was directly proportional to availability of sugar for fermentation. These enzymes converted the biomass into sugars then the sugars released were fermented by *Saccharomyces cerevisiae* (Fuad *et al.,* 2011).

Bioethanol production from pre-treated *Spirogyra* biomass comparatively has the same trend observed in the amount of sugar released to the rate of ethanol produced. As compared to the un-treated *Spirogyra* biomass, the result from the experiments showed that; pre-treated *Spirogyra* biomass also depends on saccharification enzymes produced by *A. niger* for bioethanol production. The variation mostly arises in the trend of sugar released during saccharification process and fermentative production of bioethanol as highest sugar released was observed on the 5th day of saccharification in all flasks containing distilled water, lactose and synthetic media with chemically pre-treated *Spirogyra* biomass leading to the production of more ethanol on the 5th day of fermentation unlike the un-treated biomass. Also, the trend encompasses a gradually increased for the pre-treated substrate in all the flasks with no exception from 1st day to the 5th day where the highest released was noticed and drops on the 6th day; proved quite different from the un-treated substrate which when lactose was added, the trend of sugar released was completely changed.

Generally, in all the experiments conducted and the results obtained for both the un-treated and pre-treated *Spirogyra* biomass, it was observed and proved that conversion efficiency of cellulosic materials into sugars was dependent on the suitability of cellulose for saccharification and the activity of enzymes and enhancement in total sugars especially when lactose was added for the un-treated *Spirogyra* biomass as vividly discussed in this chapter. The production of ethanol is dependent on the availability of sugars and the activity of enzymes (cellulase and amylase) produced by the *A. niger*. Therefore, it is noticed that saccharification and fermentation are moving hand in glove with each other. Thus, increased production of ethanol was observed when sugars are more in the suspension and the activity of enzymes decreased gradually when sugars were decreased gradually (Fuad *et al.*, 2010) as experimentally justified and presented in the results of this study.

6.0 Conclusions

In this research study, we have seen that algal biomass is more beneficial as raw material than agro-based raw materials for the production of bioethanol, because it is renewable and available abundantly in fresh water as well as marine ecosystem. The work also shown generally that when the un-treated biomass were compared with the chemical pre-treatments samples, is not required for the algal material (biomass) particularly for *Spirogyra*. These chemical treatments were usually employed in practice to remove or denature unwanted materials which are present along with cellulose and starch in agriculturally based raw materials used in bioethanol production. The spirogyra cell wall does not even demand any form of pre-treatment as it is made up of simple starch and pure cellulose which are employed in the saccharification and fermentation processes for the production of bioethanol. The un-treated *Spirogyra* biomass produces more yield of ethanol as the cellulose can easily be damaged through pre-treatment.

Furthermore, the comparative studies revealed that, for ethanol production from *Spirogyra* using *Aspergillus niger*, chemical pre-treatment is not necessary for high yield of products and effective production rate. An enzyme inducer such as the Lactose was used in this experiment played a vital role in the enhancement of bioethanol production.

Finally, the production of biofuel (bioethanol) from algae-specifically *Spirogyra* biomass as feedstock is possible and more beneficial when compared to the fuel produced from other agro-based raw materials (biomass) and fossil fuels which justified the aim and objective of this research studies. Also, the comparative study confirmed the production of bioethanol by using chemically pre-treated and un-treated *Spirogyra* biomass through saccharification

process by Aspergillus *niger* and fermentation process by *Saccharomyces cerevisiae*. The un-treated *Spirogyra* biomass is more economical and risk free than the chemical pre-treatment as it gives room for less expensive method by cutting off the pre-treatment cost.

Recommendation

The following should dully be taken into consideration:

1. The use of alga material (biomass) as a substrate for bioethanol production is advisable than agro-based materials as it's more beneficial and renewable.
2. Pre-treatment with chemicals are not required for the algal material particularly for *Spirogyra*. Thus, un-treated *Spirogyra* biomass is strongly recommended for any bioethanol production from *Spirogyra* feedstock as it is more economical.
3. The production of bioethanol is dependent on the availability of sugars and the activity of enzymes produced by the *Aspergillus niger*, therefore addition of Lactose is highly recommended for the saccharification process for more amount of ethanol to be produced; as saccharification and fermentation are moving hand in glove with each other.
4. The department of Chemical engineering should help in the awareness and provision of chemicals such as L-Glutamic acid, Tween 80, etc. for any further research on bioethanol production from *Spirogyra* biomass.
5. The Federal Government of Nigeria should take an active part in renewable energy and embark on this project on a large scale.

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