DARK GROWN *CHLORELLA KESSLERI* FED CORN, SORGHUM AND LIGNOCELLULOSIC HYDROSYLATES FOR ALGAL BIODIESEL PRODUCTION

A Thesis by

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Bachelor of Technology, Jawaharlal Nehru Technological University, 2010

Submitted to the Department of Biological Sciences
and the Faculty of the Graduate School of
Wichita State University
in partial fulfillment of
the requirements for the degree of
Master of Science

July 2013

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The following faculty members have examined the final copy of this thesis for form and content, and recommend that it be accepted in partial fulfillment of the requirement for the degree of Master of Science with a major in Biological Sciences.

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Dedication

To my parents, sister and all my dear friends

ACKNOWLEDGEMENTS

I warmly realize that this thesis project would never be the same if it were not for the many people around me. First and foremost I wish to express my sincere and deep felt gratitude to my advisor Dr. Mark A. Schneegurt, Ph.D., Professor, Department of Biological Sciences, for guiding me at every step with his valuable suggestions and advice, his incessant encouragement and allowing me to analyze my ideas with a degree of independence

I would like to extend my gratitude to my committee members Dr. Chris Rogers, Dr. George Dehner for their helpful comments and suggestions at different stages of the project

I express my heartfelt thanks to Dr. Donghai Wang (Kansas State University) for providing the hydrosylates for the project.

I owe my thanks to Dr. Belinda Sturm (University of Kansas) for GC-MS analysis of algal samples for biodiesel production

I am thankful for Kansas NSF EPSCoR for funding this research. I am thankful to the office staff, Department of Biological Sciences, Wichita State University especially Maria Martino and Debbie Werth for being so helpful always.

Last but not the least; I am deeply indebted and thankful to my family who played an important role in my endeavors. The almighty has always blessed us - Thank you god for everything

ABSTRACT

Biodiesel production by microalgae is typically driven by photosynthetic light harvesting. Production in open ponds is hampered by high water requirements and contamination. Photobioreactor are highly engineered systems with high capital costs. Both approaches are limited by a requirement for high surface area-to-volume ratios that promote light penetration. The current study avoids these issues by growing algae heterotrophically. If algae are grown in the dark, existing bioethanol facilities may be used to co-produce, or readily be changed to produce, biodiesel. Here we fed *Chlorella kessleri* grown in the dark with the same corn and sorghum hydrosylates used for bioethanol production. *Chlorella* cultures also were grown heterotrophically in the dark on pure sugars (fructose, glucose, sucrose and a mixture of three sugars) in shake-flasks or continuously sparged and stirred bioreactors. The rate of growth in heterotrophic cultures was comparable to light-grown autotrophic cultures and mixotrophic cultures supplemented with exogenously added sugars in the light. Strong heterotrophic growth was observed for *Chlorella* maintained heterotrophically on corn mash, sweet sorghum juice, and sorghum mash prepared by hydrolysis of bulk grains. When these bioethanol feedstocks were added to a final concentration of 1% sugar, growth rates were comparable to pure sugar substrates. Biodiesel production was low in these experiments since no attempt was made to promote nitrogen starvation. The current study suggests that algal biodiesel production may be possible during heterotrophic growth in the dark on sugar feedstocks already in use by the biofuel industry. This provides new opportunities for flexible production of renewable liquid fuels using existing infrastructure.

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LIST OF ABBREVIATIONS

°C	Degrees Celsius
µL	micro liter
CO_2	Carbon dioxide
EPSCoR	Experimental Program to Stimulate competitive Research
FAME	Fatty acid methyl esters
$FeCl_3$	Ferric trichloride
h	hours
kg	kilograms
L	Liter
mgL^{-1}	milligrams per liter
mL	milliliter
mM	millimolar
mV	milliVolts
$Na_3C_6H_5O_7$	Sodium citrate
NaOH	Sodium hydroxide
ORP	Oxidation Reduction Potential
Rpm	rotations per minute

CHAPTER 1

INTRODUCTION

"We, the human species, are confronting a planetary emergency — a threat to the survival of our civilization (climate change) that is gathering ominous and destructive potential even as we gather here. But there is hopeful news as well: we have the ability to solve this crisis and avoid the worst — though not all — of its consequences, if we act boldly, decisively and quickly" (Al Gore, acceptance speech for the Nobel Peace Prize 2007).

Finding sufficient supplies of clean energy for the future is one of society's most daunting challenges and is intimately linked with global stability, economic prosperity, and quality of life. Fuels represent around 70% of the total global energy requirements, particularly in transportation, manufacturing and domestic heating. Electricity only accounts at present for 30% of global energy consumption (Gouveia & Oliveira, 2009). Petroleum-based fuels are reserves which are usually concentrated in certain regions of the world. These reserves are on the verge of reaching their peak production, these resources are depleting day by day. This scarcity of petroleum-based fossil fuels will make renewable sources of fuel an attractive choice (A. Demirbas, 2009). Rapid growth of population and a concomitant agricultural and industrial expansion has lead to a rapid increase in atmospheric carbon dioxide and approximately 0.6°C rise in global temperatures which has led to accelerated sea level increase and other climate changes and is mainly attributed to the increasing anthropogenic CO_2. This dominance of fossil fuels and sources reflect their convenience and relative use of production in comparison to other sources of fuel especially oil and gas. The International Energy Agency (IEA) estimated that in 2002 79.6% of energy requirements were established through fossil fuels in comparison to a

mere 0.5% for non-carbon sources. Despite the concerns of about climate change which led to the Kyoto protocol, which seeks to decrease global CO_2 emissions by 5% from 1990 levels to 2008-2012 levels on the contrary it appears that fossil fuel use will continue to grow for decades to come. It is anticipated that at the current levels of fossil fuel use will result in an increase in global temperatures which will lead to inundations of low-lying areas and changes in agricultural productivity and disease patterns (Lincoln, 2005).

1. 1. What are Biofuel and its prospects?

Biofuel is a fuel whose energy is derived from biological carbon fixation which is being promoted as being the most promising pathways in reducing the world's dependence on fossil fuels, lowering greenhouse gas emissions and supporting growing economies and local agriculture (Smith et al., 2010). Continued use of petroleum based fuels is now widely recognized as unsustainable with depleting supplies and relatively high contribution of these fuels to carbon dioxide in the environment. Thus there is imminent need to lower dependence on petroleum and the most practical and least disruptive approach to achieve this is to use alternative renewable fuels (Abou-shanab et al., 2006). First generation biofuels have been traditionally extracted from rapeseed oil, sugarcane, sugar beet and maize as well as vegetable oils and animal fats using conventional technology. First generation biofuels have now reached economic levels of production, though they have generated a lot of controversy due to their impact on global food markets and security. Thus first generation fuels have raised doubts over their potential to replace fossil fuels or act as sustainable sources of renewable energy (Moore, 2008). Currently, around 1% of the world's cultivable land is used to fuel approximately 1 % of the world's energy requirements; increasing that to 100% is impractical due to concerns with food security (Brennan & Owende, 2010). Second generation biofuels utilize whole plant matter

of dedicated crops or agricultural residues, forest harvesting residues or wood processing wastes (Moore, 2008). However, the technology for conversion has not reached the scales of commercially economical viability. This has further inhibited any significant growth and exploitation of this feedstock for fuel production. A viable alternative energy source, a biofuel should provide a net gain, have environmental benefits, be economically competitive and be producible in large quantities without reducing food supplies (Hill et al. 2006) Production of biofuels from microalgae is the third generation of biofuel production which is an alternative to minimize the noxious environmental and agricultural effects associated with current land-based biofuel feedstock (Chisti, 2007).

1. 2. Why Biodiesel only through algae?

Photosynthesis is one of the basic biochemical processes of photosynthetic micro-organisms which convert solar energy into chemical energy. Man has used this natural process of harvesting the sun in the development of algal cultivation systems for secondary waste water treatment (Noüe et al,. 1992) for the production of food for humans and animal feeds.

1. 3. *Chlorella kessleri* as an organism for biodiesel production

Chlorella is a single celled spherical non motile green alga, 2.0 to 10.0μm diameter. When dried it is about 45% protein, 20% fat, 20% carbohydrate, 10% various minerals and vitamins (Kay & Barton, 1991). *Parachlorella kessleri* also known a green alga commonly is also *Chlorella kessleri*. Although most microalgae are strictly photosynthetic, i.e. they need light and carbon dioxide as energy and carbon sources, some algal species are capable of growing in the dark while utilizing organic carbon sources. We obtained our organism from The Culture Collection of Algae at the University of Texas at Austin. We choose *Chlorella kessleri* as the organism to study as it has been found to have high lipid content and is capable of heterotrophic

growth under specialized conditions. Decreases in nitrogen concentration lead to increase in lipid productivity of approximately up to 45% of biomass in *Chlorella* species (Piorrecket al., 1984). Green algae require light, carbon dioxide, water and inorganic salts. Temperature must remain generally between 20 to 30°C. The growth media should provide inorganic elements that constitute the algal cell. Essential elements include nitrogen, phosphorus and iron (Chisti, 2007). Micro algae are good candidates for biodiesel production because of their higher lipid content, shorter time growth cycle and need for less land compared to other energy crops (Milne et al., 1990). Higher biomass and lipid production under heterotrophic conditions have been achieved with *Chlorella protothecoides* by using different carbon sources (Miao & Wu, 2006)(H. Xu, Miao, & Wu, 2006). With previous studies (Xu et al., 2006) proving *Chlorella* species being successfully used to grow alga heterotrophically which have also demonstrated to produce higher biomass and higher lipid content than in photoautotrophic or autotrophic conditions. Nitrogen limitation increased the lipid content of heterotrophically chlorella species four times higher than in photoautotrophic conditions (Miao & Wu, 2006; Xu et al., 2006). The growing interest in microalgae, either non-recombinant or with appropriate genetic modification (Potvin & Zhang, 2010; Weinstein & Beale, 1985) suggests that heterotrophic micro algal processes offer significant commercial opportunities (Spechtet al., 2010). Cell densities of more than 100 gL^{-1} cell dry weight, achieved with *Chlorella*, *Crypthecodinium* and *Galdieria* species, highlight the potential of heterotrophic micro algal processes (Rosenberg et al., 2008;De Swaaf et al,. 2003; Graverholt & Eriksen, 2007). Moreover, systematic screening for new compounds is only feasible provided that sufficient quantities of concentrated biomass from axenic cultures are attainable (Olaizola, 2003; Wu & Shi, 2007). *Chlorella* species are robust organisms that can grow in many conditions around the world; they can serve as an example for heterotrophic and

mixotrophic growths supplied with glucose, glycerol, acetate or other carbon compounds from waste resources with zero or negative costs as carbon source to accumulate lipids for biodiesel production (Heredia-Arroyo et al., 2010).

1. 4. Can algae grow in heterotrophic media?

Products harvested from micro algal cultures are low and is restricted by low productivities found in light – dependent cultures of phototrophic microorganisms. The productivity of heterotrophic micro algal cultures can be significantly be much higher in comparison to photoautotrophic cultures Heterotrophic algal cultivation has been reported to provide high algal biomass productivity; it has also been reported to produce equally higher cellular oil content as well (Miao & Wu, 2004) (H. Xu et al., 2006) (X. Li et al.,, 2007). In *Chlorella protothecoides*, heterotrophic growth on corn powder hydrolysate resulted in 3.4 times higher biomass yield than those from autotrophic while the lipid content increased by 4.2 times (Liang et al., 2009). Even though the biomass and lipid productivities are significantly higher than autotrophic growth, the cost of organic carbon nutrients like glucose is higher compared with that for autotrophic growth (Liang et al., 2009). To overcome these high carbon input costs, we intend to utilize ethanol plant-based feedstock to act as nutrients for the micro alga. The prospect of autotrophic growth of microalgae for biodiesel production is interesting, tempting and technically feasible; this culture mode though presents hurdles as it is difficult to reach high densities of microalgae biomass since light penetration is inversely proportional to the cell concentration (F. Chen & Johns., 1995). Furthermore, low biomass concentration also increases the biomass harvesting costs (John R., 1994) (Wen & Chen, 2003). To date, only a small number of micro algal species have been cultured in conventional bioreactors (F. Chen, 1996)(Garcia et al., 2011). The commercialized processes in which microalgae are grown under

heterotrophic conditions are on the manufacture of polyunsaturated fatty acids in 100- m^3 scale (Andersen, 2005). Several other heterotrophic processes that utilize microalgae have been established at a laboratory scale to deliberately enrich the biomass with compounds such as pigments and antioxidants (Pulz & Gross, 2004)(Raja et al., 2008) (Spolaore et al., 2006). L-Ascorbic (Running et al., 2002) acid and polysaccharides (Ramus, 1972) are examples of commercially available extracellular products obtained from micro-algae. The growing interest in microalgae either non- recombinant or with genetic modification (Potvin & Zhang, 2010; Specht et al., 2010), suggests that heterotrophic microalgal processes offer significant commercial opportunities (Rosenberg et al., 2008). Cell densities of more than 100 gL^{-1} cell dry weight has been achieved with *Chlorella, Crypthecodinium* and *Galdieria* species demonstrating the potential of heterotrophic microalgal processes (De Swaaf et al., 2003;Doucha & Lívanský 2011; Graverholt & Eriksen, 2007). Moreover systematic screening for new compounds is only feasible provided that sufficient quantities of concentrated biomass from axenic cultures are attainable (Olaizola, 2003;Wijffels, 2008;Miao & Wu, 2006) studies *Chlorella protothecoides* state that the lipid content in heterotrophic cells could be as high as 55%, which was found to be 4 times higher than in autotrophic cells at 15% under similar conditions. Micro algal lipids have been found to be good candidates for biodiesel production because of their higher lipid content, shorter growth cycle and minimal land requirement when compared to terrestrial energy crops like soybean, palm and other starch crops (Milne et al., 1990). Hence it is evident that heterotrophic cultivation of *Chlorella* cells will result in higher production of biomass with accumulation of high lipid content in the cells.

1. 5. Is it feasible to grow algae in open ponds or closed reactors for heterotrophic media?

In comparison to open – air systems, there are several distinct advantages to use closed systems, but technical hurdles still remain. In view of potential applications, development of a more controllable, economical, efficient closed culturing system is needed (Xu et al., 2009). Unlike open-air systems, closed bioreactors are aimed at maintaining axenic single-species culture of microalgae. Bioreactors have been successfully used for producing large quantities of microalgal biomass (Chisti, 2007). Open-culture systems have almost always been located outdoors and rely on natural light for illumination. Although they are relatively much more inexpensive in comparison to closed bioreactors, they suffer from many problems like cultures that are not axenic so contaminants may out-compete the desired algal species; predators like rotifers can decimate the algal cultures and vagaries of weather can make proper control of nutrients, light intensity and CO_2 challenging (Carvalho et al., 2006). Many algae can also grow heterotrophically and although this is a boon in terms of remediation of liquid waste streams, the presence of any reduced carbon can also wreak havoc in non axenic algal systems because microbial contaminants with their faster growth rates can easily outgrow algae. Consequently the supply of carbon to microalgal mass culture systems represents a principal difficulty and limitation in obtaining cost effective production of algal biomass (Sevilla & Grima, 1998; Benemann et al., 1987Olaizola et al., 1991;García Sánchez et al., 2003). Chisti (2007) evaluated the technical feasibility of microalgae for biodiesel production. In reviewing production practices the study finds that the current technology in micro algal production is feasible. Open ponds and photo bioreactor (PBR) production systems are described in detail, with discussion about prospects for improvements in each. There is a limited section on the economics for biodiesel production. For PBR and open pond production of 100,000kg of biomass annually Chisti (2007) estimated the cost per gallon of production to be $2.95 and $3.80 for PBRs and open ponds,

respectively (2006 dollars). A major limitation to this research is that there are no details for how Chisti arrived at these cost estimates. It must be assumed that capital costs are not taken into account and the lower price of producing biomass in PBRs is solely based on the fact that higher yields can be achieved in PBRs (Richardson et al., 2012)

1. 6. Growth of algae in heterotrophic media: Is it feasible in sterile fermenters or in open ponds or in photo bioreactors?

The ability of certain algae to grow heterotrophically in the dark at the expense of various substrates has been known for a relatively long period of time. According to Barker., (1935), this phenomenon was first reported by Beijerinck in 1890. Since then, many investigations have been undertaken to ascertain some of the mechanisms involved. Certain investigations like (Roach, 1926;Skinner & Gardner, 1930) revealed a variety of organic compounds found to be assimilated by algae growing in the dark including sugars, proteins, peptones, amino-acids, organic acids and alcohols. Recently there have been reports of algae growing in water mains located several feet beneath the surface of the soil and in pools of water used to cool atomic reactors. These pools of water are shielded from sunlight by one or more inches of steel plus concentrate of considerable thickness Seilheimer & Jackson, (1963). (Parker, Bold, & Deason, 1961) reports several species of algae isolated from soil to have grown as facultative heterotrophs and suggests that organic substrates for this type of growth may be supplied by certain heterotrophic bacteria. Rodhe, (1955) work reported algal growth in subarctic lakes during winter beneath a cover of ice and snow of almost 2.5 meters thick, providing substantial evidence that the phenomenon of algal growth in the dark was more widespread than previously expected. Richardson et al., (2012) studies on the dividends, production and interest costs for open pond and photo bioreactor algal farms that produce 10MG/year of algal oil noted that 57% of total production costs in open

pond and 43% of total production costs in photo bioreactor. The literature for microalgae production is quite extensive; however, there are still few studies on the feasibility of commercial scale algae farm. Studies by Davis et al., (2011) also study the feasibility of open ponds and PBR's but it never includes the cost of production in fermenters. (Andersen, 2005) provides an economic comparison of photo bioreactors and fermenters on various scales. Studies by (Andersen, 2005) demonstrate the calculated producing dry algal biomass from a bioreactor and from a open pond, for simplicity only the cost of energy is considered i.e. light for photo bioreactor and glucose for the fermenters. The actual costs for producing 1kg of algal biomass are given in (Table 1 page 62), for phototrophs the actual costs are much greater than $11.22 because, the assumptions are aggressive. For heterotrophs, the cost of energy per kilogram of dry algal biomass is based on experimental data. The tables clearly show that the costs of phototrophic growth greatly exceed the cost of heterotrophic growth, largely because of the inefficiency of converting electricity into ATP and NADPH via photosynthetic electron transport (Table 1 page 62). These inefficiencies are not present for heterotrophs. When the (Table 3 page 64) cost data is coupled with productivity data (Table 1 and 2 pages 62-63) it is clear that the economics of biomass production greatly favor fermenters over photo bioreactors. Therefore, photo bioreactors are only feasible when the value of the products are high enough to justify the costs of production (Andersen, 2005).

1. 7. How does algal growth in dark differ from in light?

Among all the environmental factors, light supply is one of the most important factors that not only affects algae photosynthesis and productivity but also cell composition, metabolic pathways and algal cultivation process (Cuaresma et al., 2009). Many strains of photosynthetic algae are capable of heterotrophic growth, using organic carbon, under dark conditions and their

heterotrophic growth capabilities can be used for efficient production of biomass. However, for some species light is absolutely essential for efficient production of biomass and other metabolites, since the growth rate of mixotrophic culture is approximately the sum of specific growth rate of cells grown under heterotrophic and photoautotrophic conditions (Richmond, 2008). Algae have been found to utilize the organic compounds for growth in the dark. Initial investigations revealed that the organic compounds found to be assimilated by algae growing in the dark were sugars, proteins, peptones, amino-acids, organic acids and alcohols (Skinner & Gardner, 1930). Studies by Li et al., (2012a) comparing the electricity conversion efficiency between *Chlorella kessleri* and *Chlorella protothecoides* illustrated a higher efficiency of *Chlorella kessleri* in dark than in light. *Chlorella kessleri* also had better electricity conversion efficiency than *Chlorella protothecoides* with 0.7677 $\mu molm^{-2}s^{-1}$ and 0.6000 $\mu molm^{-2}s^{-1}$ respectively. Despite attaining a higher electricity conversion factor using low light intensities, this often resulted in lower specific growth rate and having lower biomass productivity than those under higher intensities. Therefore one needs to compromise between higher production and low energy input when working with actual algae cultivation systems (Y. Li et al., 2012a). For algae strain Chlorella kessleri it was observed that the growth of biomass increased with the increase in light intensity from 0 to $120\mu molm^{-2}S^{-1}$, while further increase of light intensity to $200\mu molm^{-2}S^{-1}$ repressed the accumulation of biomass probably due to light inhibition (Ogbonna & Tanaka, 2000). Though biomass production was low in the dark, organic carbon can expedite the growth of algae very rapidly and allows for growth of the desired organism without any contamination by other organisms.

1. 8. Feedstock's for biodiesel production

Petroleum and diesel come in a category of non-renewable fuels and will last only for a limited time. Need for renewable fuels is the utmost priority of today. Typical raw materials of biodiesel are rapeseed oil, canola oil, soybean, sunflower and palm oil. There are various other biodiesel sources like almond, andiroba (*Carapa guianensis*), babassu (*Orbignia* sp.), barley,amelina (*Camelina sativa*), coconut, copra, cumaru (*Dipteryx odorata*), Cynara cardunculus, fish oil, groundnut, *Jatropha curcas*, karanja (*Pongamia glabra*), laurel, *Lesquerella fendleri, Madhuca indica*, microalgae (*Chlorella vulgaris*), oat, piqui (*Caryocar* sp.), poppy seed, rice, rubber seed, sesame, sorghum, tobacco seed, and wheat (Pinto et al., 2005).

Zea mays (corn) is the most widely grown grain crop in the Americas with 332 million metric tons grown in United States alone. Approximately 40% of the crop i.e. 130 million tons is used for corn ethanol. We decided to utilized mashed corn as a substrate which is found in abundance and is a cost effective substrate. Lignocellulosic biomass is a plentiful and renewable resource for fuels and chemicals. The efficient conversion of lignocellulosic materials into biofuel is expected to be needed as a breakthrough in producing affordable and renewable fuel (Petersson et al., 2007). Biofuel can be produced by fermentation from sugars, starch and various lignocellulosic materials such as corn cob, wood and waste (Wackett, 2008). Microbes annually hydrolyze 10^{11} tons of plant biomass, principally plant cell wall material that contains the energetic equivalent of 640 billion barrels of crude oil (Ragauskas et al., 2006). The eventual shift from petroleum hydrocarbons to highly oxygen-functionalized, bio-based feedstocks will create remarkable opportunities for the chemical processing industry wherein by using carbohydrates as chemical raw materials it will eliminate the need for several capital-intensive,

oxidative processes used in the petroleum industry. Biomass carbohydrates will provide a viable route to products such as alcohols, carboxylic acids and esters.

These natural products are also stereo chemically and regio chemically pure, thereby reducing dependence on expensive chiral catalysis and complex syntheses that are currently required to selectively install chemical functionality in petrochemicals. Bio-based feedstocks are already having an impact on some practical applications including solvents, plastics, lubricants and fragrances. Further reductions in cost are expected with improvements in the fermentation process and the use of waste agricultural materials as feedstocks. The major drawback of biomass use is the development of methods to separate, refine and transform it into chemicals and fuels. Of these step separations currently accounts for 60 to 80% costs of the process costs. As we progress from refineries to bio-refinery, the costs and hurdles associated might simplify but also might never diminish. Distillation which is critical and dominate the refinery separation will be replaced by solvent extraction for biofuel (Ragauskas et al., 2006). Today's bioethanol plant processes rely largely on the fermentation of starch from corn in the United States and sugar cane in Brazil (Lovins, 2004; Parikka, 2004). In view of changing world energy needs, we can utilize the high production crop waste of the respective countries (e.g. corn in United States) as a feedstock and as algae can grow in fresh to brackish water. We can utilize this ability of algae to cultivate and extract algal oils for our future energy needs.

1. 9. Lignocellulosic feedstocks for Biodiesel production

Lignocellulosic biomass in the form of plant materials like grasses, crop and wood residues offers the possibility of a renewable, geographically distributed and relatively greenhouse gas-favorable source of sugars that can be converted to ethanol and other liquid fuels

(Hill et al., 2006). Lignocellulosic biofuel production involves collection of biomass, deconstruction of cell wall polymers into component sugars by pretreatment and saccharifaction and conversion of the sugars to biofuel by fermentation (Rubin, 2008). Plants can be viewed as solar energy collectors and thermochemical energy storage systems. It is the storage of energy in a form that can be accessed later via thermochemical or enzymatic conversion that distinguishes between biomass and other energy sources. Cellulosic biomass also referred to as lignocellulosic biomass, is an abundant renewable resource that can be used for the production of biofuel (Himmel et al., 2007). The three main components of lignocellulose are cellulose, hemicellulose and lignin with the relative proportion of the three components dependent on the material source (Reddy & Yang, 2005). Cellulose, the main structural component of plant cell walls, is a long chain of glucose molecules, linked to one another primarily by glycosidic bonds. Hemicellulose, the second most abundant constituent of lignocellulosic biomass, is not a chemically well defined compound but rather a family of polysaccharides, composed of different 5- and 6- carbon monosaccharide units, that links cellulose fibers into microfibrils and cross-links with lignin creating a complex network of bonds that provide structural strength (van Wyk, 2001). Finally lignin, a three dimensional polymer of phenylpropanoid units, can be considered as the cellular glue providing the plant tissue and the individual fibers with comprehensive strength and the cell wall with stiffness (Del Río et al., 2007). The breakdown of biomass involves the release of long-chain polysaccharides, specifically cellulose and hemicellulose with the subsequent hydrolysis of these polysaccharides into their component 5- and 6- carbon chain sugars (van Wyk, 2001;LaForge & Hudson, 1918). Most plants assimilate their CO_2 first into a C_3 compound, whereas smaller subsets use a C_4 compound. Plant using C_4 photosynthesis tend to be among the most productive, having higher maximum efficiencies of light, nitrogen and water use

in assimilating carbon. The C_4 group of potential energy crops includes various perennial grasses such as switchgrass and *Miscanthus.* These grasses have the advantages of not requiring replanting after a yearly harvest, rapid growth, high biomass density per unit area and low nutrient and water needs enabling growth on marginal agricultural lands (Rubin, 2008). These favorable aspects have prompted us to investigate the use of switchgrass as a cellulosic feedstock which is native to Kansas and abundantly found.

1. 10. Strategies to increase lipid production

Lipid accumulation can be influenced by temperature, light intensity, pH, salinity, mineral salts and nitrogen source. Among them nitrogen limitation is the most reliable and commonly used strategy to increase lipid content of the microalgae (Hsieh & Wu, 2009; Illman, Scragg, & Shales, 2000; Solovchenko, 2008). However, employing of nitrogen starvation strategies usually decreases the growth rate, thereby lowering overall lipid productivity. Therefore, there is still an immediate need to develop an efficient lipid production strategy (Chen et al., 2011; Posten, 2009). Lipid content and liquid productivity are the two commonly used indicators assessing the performance of lipid production by lipid-rich microalgae. Lipid contents, biomass and lipid productivities, although key characteristics for biodiesel production are not the only characteristics to be considered to ensure a cost-effective and feasible biodiesel production process. Resistance to contamination, tolerance of operating conditions such as light, temperature, ionic strength and flue gas toxins, nutrient requirements, as well as ease of harvesting and downstream processing also impact the success of large-scale culture. However, insufficient published information currently exists to enable comparison across these aspects of a variety of species (Griffiths & Harrison, 2009). A study on nitrogen limitation found that in cells

of the green alga *Scenedesmus obliquus,* the average lipid content rose from 12.7% dry cell weight when grown under normal conditions to 43% under nitrate-deficient conditions. It is understood that the lack of nitrogen promotes lipid accumulation because when limited, all available nitrogen is utilized for synthesis of essential enzymes and cellular structures. Since the amount of CO_2 being taken up by the cells remains the same, most of the carbon is used to make carbohydrates or lipids instead of proteins (Mandal & Mallick, 2009). In addition to limitation of nitrogen, phosphate limitation in the heterotrophic media used to grow algae also led to substantial enhancement in lipid accumulation of (Khozin-Goldberg & Cohen, 2006) *Monodus subterraneus* with decreasing phosphate availability from 175 to 52.5, 17.5 and 0µm (K_2HPO_4), the total lipid content of starved cells increased, mainly due to the drastic increase in TAG levels. In the absence of phosphate, the proportion of phospholipids was reduced from 8.3% to 1.4% of total lipids. Not only phosphate, but iron deficiency has also also been reported to stimulate lipid accumulation in microalgae *Chlorella vulgaris,* which accumulated up to 56.6% of biomass by dry weight under the optimal condition ($1.2X10^{-5}$ M $FeCl_3$) (Liu et al., 2008). A major disadvantage of the biochemical engineering approach of nutrient-starvation for increased lipid production is drastic reduction in cell division and subsequent biomass production. The rapidly growing interest is the use of transgenic microalgae for industrial applications is powered by the developments in microalgal biotechnology. Nuclear transformation of various microalgal species is now a routine, chloroplast transformation of various microalgal species and further success in organelle transformation is likely as the number of sequenced plastid, mitochondrial and nucleomorph genomes continue to grow (Walker et al., 2005). Even though there is no success with respect to lipid overproduction of microalgae using the genetic engineering approach to date, a solid understanding towards the global triacylglycerols biosyntheses which is generally

accepted to be identical throughout all species except the differences in the location of reactions and the structure of key enzymes has been established. Extensive studies have also been carried out regarding the enhancement of lipid production using the genetic engineering approach in different species. These results provide a valuable background for future studies for microalgae (Courchesne et al., 2009). The use of regulatory factors such as transcription factors to control the abundance or activity of multiple enzymes relevant to the production of desired products has provoked widespread interests (Capell & Christou, 2004). This approach is referred to as transcription factor engineering and can be more precisely described as a novel technology employing the over expression of transcription factors that up- or down regulate the pathway being involved in the formation of target metabolites for their excess production (Courchesne et al., 2009) Though genetic engineering of organisms sounds promising, the lack of established research in the literature only adds to the disadvantages whereas transcription factor engineering is still in embryonic stage and producing metabolically engineered microalgal strains is not cost effective for a low value product like biodiesel. There is decrease in biomass accumulation by depreciated nitrogen and phosphorus which applies physiological stress to channel metabolic influx to lipid syntheses. This method is still the most mature and widely employed strategy among the three.

1. 11. Lipid extraction protocols

After successful cultivation and harvesting microalgae, the most critical step is the ability to successfully and efficiently extract oil or more specifically valuable fatty acids. Additionally, there is a concern to extract the lipids in the safest and most environmentally sustainable manner while subsequently extracting the maximum possible lipids from the biomass.

Organic solvents such as benzene, hexane, cyclohexane, acetone and chloroform have shown to be effective when used on microalgal paste; they degrade microalgal cell walls and extract the oil because oil has a high solubility in organic solvents (Harun et al., 2010). It has been feasible to extract fatty acid methyl esters without damaging cell walls; this is possible if the solvents are not toxic to cells. A stable solvent is one which is insoluble in water; preferably solubilize the compound of interest, has a low boiling point to facilitate its removal after extraction and has a considerable different density than water. To be economically feasible it should be inexpensive and reusable (Banerjee et al., 2002). Due to these qualities, hexane is typically the solvent of choice for large scale extractions (Mercer & Armenta, 2011). An efficient extraction requires the solvent to fully penetrate the biomass as well as match the polarity of the targeted compounds. This in conjugation with the ability to make physical contact with the lipid material and solvate the lipid, makes for a successful extraction solvent, this can be facilitated by mechanical disruption of cells prior to exposure to solvent (Cooney et al., 2009). Shen, Pei, Yuan, & Mao., (2009) found that for *Scenedesmus dimorphus* (an autotrophic alga) wet milling followed hexane extraction gave the best recovery, with 25.3% compared to 6.3% using soxhlet extraction. They also demonstrated that for *Chorella protothecoides* (a heterotrophic alga) using a bead beater followed by hexane extraction, was most effective at recovering oil; 18.8% compared to 5.6% for soxhlet extraction. The organism in my research is *Chlorella kessleri* which is a freshwater heterotrophic micro alga, it is only sensible to mechanically disrupt the cells by bead before exposing it to solvents. Traditionally, lipids have been extracted from biological matrices using a combination of methanol, chloroform and water (Bligh & Dyer, 1959). Bligh and Dyer method, originally designed to extract lipids from fish tissue, has been a benchmark in extraction of lipids from biological materials. Similar to other extraction

procedures, moisture can prove troublesome with solvents as well, restricting solvent access to cells by acting as a barrier between cells and solvents (Mercer & Armenta, 2011). A similar experiment (Cer n et al., 2008) was performed using mechanical cell disruption by bead mill before saponification with potassium hydroxide and hexane extraction, this experiment showed that mechanical cell disruption was indeed necessary to achieve acceptable yields of oil, as well as being a scalable process with the potential to be applied to industrial level.

To date, extraction of microbial oils is extracted mainly by solvents such as hexane, coupled with mechanical disruption techniques. The other non- solvent extraction technologies include the use of pulse electric field, enzymes, microwaves, ultrasonic energy and mechanical disruption. Few of these have shown significant yields at laboratory scales but haven't been tested at large scale (Mercer & Armenta, 2011). This demonstrates that the present technology of solvent extraction is the most feasible for commercial production due to low costs of the solvents involved though this also represents an opportunity to research efficient extraction technologies involving biological systems without residue solvents in the desired end product.

1. 12. Advantages of Biodiesel over Conventional diesel fuel

An alternative fuel to petroleum derived diesel must be technically feasible, economically competitive, environmentally acceptable and easily available. The current alternative diesel fuel is Biodiesel (A. H. Demirbas & Demirbas, 2007). Biodiesel is the first and the only alternative fuel to commercial diesel to have a complete evaluation of emission results. A renewable fuel such as biodiesel, along with lesser exhaust emissions is the need of the present scenario worldwide (A. Demirbas, 2008). Approximately, half of the dry weight of the microalgal biomass is carbon (Mirón et al., 2003), which is typically derived from carbon dioxide.

Therefore, producing 100 tons of algal biomass fixes roughly 183 tons of carbon dioxide. This carbon dioxide must be fed continually during daylight hours. Microalgal biomass production can potentially make use of some of the carbon dioxide that is released in power plants by burning fossil fuels; this carbon dioxide is often available at little or no cost. (Sawayama, Inoue, Dote, & Yokoyama, 1995) (Yun, Lee, Park, Lee, & Yang, 1997). The oxygen content of biodiesel improves the combustion process and decreases its oxidation potential. The combustion efficiency is improved by the increase in the homogeneity of oxygen with the fuel during combustion due to which the combustion efficiency of biodiesel is higher than petroleum diesel. Biodiesel contains 11% oxygen by weight and no sulphur which gives better lubrication properties than petroleum diesel (A. Demirbas, 2008). The advantages also include lower emissions in terms of carbon monoxide, hydrocarbons, particulate matter, polycyclic aromatic hydrocarbon compounds and nitrated polycyclic aromatic hydrocarbon compounds (A. Demirbas, 2008). The most important advantage observed in the majority of literature is its domestic origin, its potential to minimize an economy's dependence on imported oil, biodegradability, higher flash point and inherent lubricity. The high flash point of biodiesel makes it an easy storage and transportation fuel. Blends up to 20% with petroleum diesel can be used in almost all the diesel equipment. It doesn't contribute to net carbon or sulphur and emits less gaseous pollutants.

The major disadvantages of biodiesel as a fuel are higher viscosity, lower energy content, higher cloud point and pour point, higher nitrogen oxide emissions, lower engine speed and power, injector coking, engine compatibility and high prices of production (A. Demirbas, 2009). The major portion of the production costs involves the downstream process involving harvesting and extraction of lipids. The other major problem involved is crystallization of fuel at colder

temperatures giving a cloudy appearance. The wax solidifies while thicken the oil and eventually clogs fuel filters and injectors in a diesel engine.

1. 13. Hypothesis

1. Growth of Chlorella kessleri in the dark will be lower than in light

    Algae are mainly autotrophic organisms which require light to grow and carry out the metabolic processes. Though many algae can grow heterotrophically with supply of carbon sources the growth rates in light will be higher than in dark.

2. Higher growth rate of algae will be observed in feeds with grain based biomass than in cellulosic biomass suggesting lower quality cellulosic biomass than grain biomass.

    Any organism will grow rapidly when carbon sources are readily available thus algal growth will be higher in feeds like mashed sorghum and corn than in cellulosic feeds such as big blue stem, switch grass etc.

3. Growth rates will be higher in mixotrophic growth conditions than in heterotrophic conditions

Algae are photosynthetic organisms which utilize light as a source of grow and reproduce in number. Mixotrophic conditions offer light source and a carbon source to feed on which allows for rapid growth whereas heterotrophic conditions offer carbon source only in absence of light

CHAPTER 2

MATERIALS AND METHODS

2.1. Algal Culture: Laboratory Scale

2.1.1 Pure Culture

Algal Samples were obtained from UTEX, the Culture Collection of Algae at The University of Texas at Austin. UTEX number 263 a strain of *Chlorella kessleri*, class *Chlorophyceae* was selected. Erlenmeyer shake-flasks with 100 mL of proteose medium (Table 4 page 65) was autoclaved on wet cycle for 30 minutes at 121°C before being inoculated with 1 mL of pure culture sample received from UTEX. A New Brunswick Scientific rotary platform shaker at a speed of 150 rpm was used to continuously stir the culture under artificial light (cool white fluorescent of approximately 50 µE). The cultures were grown in Erlenmeyer shake flasks with 100 mL of heterotrophic growth media (Table 6 page 67) and inoculated with 5 mL of algal stock culture before growth in the dark. The cultures were maintained by transferring 5 mL of dense algal culture into a new flask containing 100 mL of heterotrophic media every 15 d.

2.1.2 Estimation of Growth on different pure sugars

Growth curves were calculated on different pure sugars viz. glucose, sucrose, fructose and the combination of all three sugars in equal proportions. Heterotrophic growth media (Table 6 page 67) were prepared with each sugar replacing carbon substrate. Sugar was at 1% loading rate. One hundred mL of heterotrophic growth media (Table 6 page 67) was added to 250-mL Erlenmeyer shake flasks and autoclaved for 30 min at 121°C and 1 mL of ampicillin at a concentration of 0.25kg/m^3 was added to prevent bacterial contamination along with 1 mL of

carbendazim (dissolved in ethanol) at a concentration of 10% was added to avoid fungal contamination issues. Five mL of previous dense axenic cultures of *Chlorella kessleri* was added and maintained on a New Brunswick Scientific rotary platform shaker set at a speed of 150 rpm. The shaker was set under artificial light (cool white fluorescent of approximately 50 μE) for mixotrophic and autotrophic growth. The shaker was moved under a cabinet with a thick black cloth to prevent any light exposure for heterotrophic growth conditions. Sampling was performed every 24 h in a laminar flow hood with aseptic technique to prevent contamination issues. Three mL aliquots of sample were transferred to a clean test tube using sterile pipettes to analyze various growth factors.

2.2. Estimation of Growth using different hydrosylates

All the hydrosylates were filtered using a whatman filter paper by vacuum filtration prior to inoculation to prevent binding of algal cells to hydrosylates. Filtration also removes the particulate matter which can interfere with the cell count. Each hydrosylate was diluted to 1% glucose concentration and pH adjusted to 7.0 prior to autoclaving. Autoclaved media was inoculated with 1 mL of dense axenic cultures. One mL of carbendazim (concentration 1μgmL^{-1}) (Mahan, Odorn, & Herrin, 2005) and 1 mL of 25 mgL^{-1} ampicillin (Kvíderová & Henley, 2005) also was added to avoid contamination issues with bacteria and fungi. Streptomycin is an aminoglycoside antibiotic and could inhibit eukaryotes because it blocks protein synthesis at the 30S mitochondrial and chloroplast ribosome (Cammarata, 1973). Only aliquots of 1 mL were transferred to a clean test tube under aseptic conditions and 10 μl of sample was pipetted onto a hemocytometer to observe the number of algal cell under a light (Olympus BH-2) microscope.

2.2.1 Algal Cell Count

A Hausser Scientific Hemocytometer was used for cell counts. Cells that fall in five squares of the grid were counted along with the centre square. If the cell numbers were high, then the culture sample was diluted to usable counts of algal cells. The Hemocytometer was washed with ethanol before and after each use and 10 µL of sample was pipetted onto the hemocytometer. Hemocytometric cell count gives the number of cells per cubic millimeter.

2.2.2 Chlorophyll Measurement

Chlorophyll measurement was based on a modified Arnon's whole cell extraction protocol. The culture was used without disrupting the cells. A Thermo Scientific Genesys 10S UV-VIS spectrophotometer was used to measure the absorbance. Blank was the respective autoclaved media used without any live algal cultures. Absorbance was measured and concentrations based on Arnon (1949) calculations.

2.3. Cellulosic feedstock pretreatment and hydrolysis

Big blue stem was collected during the month of July from the WSU Biological Field Station located on the Ninnescah Reserve along a mile of the Ninnescah River. The collected biomass was ground using a coffee bean grinder. Four g of the biomass was added to 40 mL of 1% NaOH at 50°C±1°C for 12 h to pretreat the biomass. After the initial pretreatment, 0.1M, pH 4.8 $Na_3C_6H_5O_7$ (sodium citrate) buffer was added. One hundred µL of 20kg/^{m3} sodium azide solution was added. To this 5 mL of water was added. The contents were bought to 50°C±1°C in a water bath. The temperature of contents was held at 50°C±1°C to allow enzyme activity after which 100 µL of Accelerase® 1500 was added. This mixture was maintained in an incubator

shaker at 150 rpm maintained at 50°c±1°C for 48 h to allow the release of glucose from the cellulosic biomass.

2.3.1 Glucose Estimation from cellulosic feedstock

Glucose was estimated by anthrone analysis(Yemm & Willis, 1954) . Anthrone reagent was prepared by adding 2g of anthrone to a liter of sulphuric acid and allowed to incubate for 2 h. A comparison standard curve was prepared with a known standard concentration.

2.4. Lipid Analysis

One hundred mL of 1% feedstocks were prepared by diluting the feedstock appropriately and pH was adjusted to pH 7.0. The medium was inoculated with 1 mL of dense algal culture using aseptic techniques under a Biosafety cabinet in 250 mL Erlenmeyer shake flasks. These flasks were maintained on a New Brunswick Scientific rotary platform shaker placed under a cabinet covered by a thick black cloth to mimic dark room conditions. Lipid analysis was performed according to a modified Bligh & Dyer, (1959) method. Algal cells were harvested after reaching stationary phase of growth by centrifugation at 12,000 rpm for 10 min. The pelleted cells were re-suspended in 10 mL of water and 20 mL a of chloroform and methanol mixture in the ratio of 1:2. The mixture was centrifuged again at 12,000 rpm for 10 min. This created 3 layers with methanol and water on top followed by lipid and larger particles in the last phase. The lipid phase was extracted carefully using a 5 mL pipette and transferred to a clean test tube that was washed with the chloroform and methanol mixture to remove any lipids. The lipids were concentrated and the solvents evaporated under a stream of dry nitrogen gas. The lipids were suspended in 5 mL of ice-cold acetone to remove any non-polar lipids and concentrated

again under nitrogen gas. The concentrated lipids were suspended in 1 mL of chloroform prior to GC-MS analysis. Fatty acid methyl esters analysis was performed by gas chromatography and mass spectrometry (Smith et al., 2010).

CHAPTER 3

RESULTS

3.1 Preliminary Laboratory Algae Culture

The algae of interest, *Chlorella kessleri* obtained from the culture collection of algae at the University of Texas at Austin, was successfully cultured in laboratory using proteose medium (Table 4 page 65) as specified by UTEX. *Chlorella kessleri* showed very good rates of growth in proteose medium, clear autoclaved media normally shows signs of green cell mass in a period of 5-7 days under ambient light conditions. The culture was sub cultured every 10 d to maintain fresh algal cultures

3.2 Growth curves on Pure sugars

3.3 Autotrophic growth mode

Growth was measured by chlorophyll content and cell count. Modified heterotrophic media (Table 7 page 67) were adjusted to pH 7.0 prior to algal culture inoculation. Chlorophyll measurements were analyzed over a duration of 264 h from the time of inoculation with samples taken every 24 h. The chlorophyll measurements (Fig. 5 page 53) showed very slow growth rates with a long lag phase. Algal growth in autotrophic growth mode peaked at 28.2 mgL^{-1} at 240 h. Cell counts were performed by hemocytometery (Fig. 4 page 52). Cell counts were observed to be 9.43 $X10e^{5}$ $cellsmL^{-1}$ at 240 h after the time of inoculation.

3.4 Heterotrophic growth mode

Chlorella kessleri was grown in heterotrophic growth media (Weinstein & Beale, 1985). The carbon substrate (originally glucose) was replaced by sucrose, fructose and an equal mixture of glucose, sucrose and fructose. Measurements of growth were performed both by cell count and chlorophyll content. Sucrose appeared to be the best source of carbon for heterotrophic growth at 15.49 mgL^{-1} chlorophyll at 120 h after inoculation. The combination of glucose, sucrose and fructose was found to be the next best carbon source in the heterotrophic growth mode at 11.49 mgL^{-1} chlorophyll at 168 h after inoculation. Fructose proved to be the third best carbon source at 10.96 mgL^{-1} chlorophyll at 144 h after inoculation. Glucose-fed cultures had the lowest chlorophyll content at 10.21 mgL^{-1} at 120 h (Fig. 7 page 55) after inoculation.

Cell counts were performed in addition to chlorophyll measurements on cultures in heterotrophic growth mode (Fig. 8 page 56). Sucrose-fed cultures sugar had the highest cell numbers at $3.3X10e^{5}$ $cellsmL^{-1}$ at 168 h after inoculation. Glucose supported the next highest cell counts at $12.4X10e^{5}$ $cellsmL^{-1}$ at 144 h from inoculation. Fructose had a cell count of $7.38X10e^{5}$ $cellsmL^{-1}$ at 168 h from inoculation. The mixture of glucose, sucrose and fructose had the lowest cell number 4.32 $X10e^{5}$ $cellsmL^{-1}$ at 144 h from inoculation.

3.5 Mixotrophic growth mode

Mixotrophic growth conditions included placing the algal cultures under ambient light conditions as well as providing a carbon source such as glucose, sucrose, fructose and a mixture of glucose, sucrose and fructose in equal proportions. Growth was measured by cell count and chlorophyll calculation. Heterotrophic growth media (Weinstein & Beale, 1985) was used with a

carbon source that supplemented the natural light to create mixotrophic conditions. Glucose-fed cultures registered the highest chlorophyll content at 21.84 mgL^{-1} at 144 h after inoculation followed by sucrose at 13.17 mgL^{-1} chlorophyll content at 144 h from inoculation. The culture fed mixture of glucose, sucrose and fructose had a chlorophyll content of 12.97 mgL^{-1} at 144 h from inoculation. Fructose fed cultures had a chlorophyll content of 4.58 mgL^{-1} at 144 h after inoculation (Fig. 9 page 57) which was the least preferred sugar for maximum growth under mixotrophic growth conditions.

Cell counts also were performed with the pure sugars under mixotrophic conditions. Glucose-fed cultures demonstrated the highest cell number at $8.92X10e^5$ cells mL^{-1} followed by sucrose at $8.74X10e^5$ cellsmL^{-1} at 72 h after inoculation. The cultures fed mixture of glucose, sucrose and fructose gave the third highest cell number at 5.38 cells mL^{-1} at 72 h after inoculation. Fructose-fed cultures had the lowest cell counts at $3.76X10e^5$ cells mL^{-1} at 96 h (Fig. 10 page 58) from the time of inoculation.

3.6 Growth patterns in corn, sorghum and big blue stem hydrosylates

Growth patterns were analyzed in corn, sorghum and lignocellulosic hydrosylates by cell count in mixotrophic and heterotrophic growth modes. Each hydrosylate was diluted to 1% sugar content from its initial higher concentration and the media was adjusted to pH 7.0 prior to inoculation by algal cultures.

3.7 Mixotrophic growth mode

Growth in mixotrophic mode was determined by cell count method for 144 h with a 24 h interval between each sample measurement (Fig. 11 page 59). Corn mash as a carbon substrate

gave the highest cell numbers at 5.05X10e^{5} cellsmL^{-1} at 96 h from inoculation in mixotrophic growth mode. Sorghum Mash followed closely at 4.74X10e^{5} cellsmL^{-1} at 120 h from inoculation. The lignocellulosic feedstock comprised of big blue stem had a maximum cell count of 4.00X10e^{5} cellsmL^{-1} at 120 h. Sweet sorghum juice gave the lowest cell counts of 1.66X10e^{5} cellsmL^{-1} at 120 h after inoculation.

3.8 Heterotrophic growth Mode

In heterotrophic growth mode, big blue stem, the lignocellulosic hydrolysate gave the highest cell numbers at 1.25X10e^{5} cellsmL^{-1} at 96 h after inoculation. Corn mash-fed cultures gave a cell count of 6.99X10e^{5} cellsmL^{-1} at 120 h after inoculation. Sorghum Mash followed closely at 6.48X10e^{5} cellsmL^{-1} at 120 h from inoculation. Sweet sorghum juice gave the least cell number at 4.38X10e^{5} cellsmL^{-1} at 120 h after inoculation. It can be observed the cell number in lignocellulosic hydrolysate was nearly twice as high as the next best performing corn hydrolysate (Fig.12 page 60).

3.9 Fatty acid methyl esters analysis by Gas Chromatography-Mass spectrometry

FAME analysis was performed on the lipids extracted from the biomass grown on the different hydrosylates. Biodiesel is composed mainly of 16-carbon and 18-carbon methyl esters. Total lipids extracted from biomass grown on sweet sorghum juice were 1.08X10e^{2} mgkg^{-1} (Table 11 page 71). Total lipids of 6.21*10e^{2} mgkg^{-1} was extracted from biomass grown on sorghum mash hydrosylate (Table 12 page 72). The highest lipid content was found in algal biomass grown on corn mash hydrosylate at 6.82X10e^{2} mgkg^{-1} (Table 13 page 73). The lowest

lipid yield was extracted from biomass grown on big blue stem hydrosylate at $0.543X10e^{2}mgkg^{-1}$ (Table 14 page 74) of biomass extracted.

CHAPTER 4

DISCUSSION

Light intensity is the most critical component which determining the growth characteristics of *Chlorella kessleri*. Light is the inducer and regulator of the production of algal products such as pigments and fatty acids (Sukenik, Carmeli, & Berner, 1989). Light intensity greatly affects the composition and content of biodiesel derived from algal cells cultivated under different light conditions (Table 5 page 66). Our aim to grow high algal biomass and high lipid content in heterotrophic conditions with *Chlorella kessleri* is focused on the use of different terrestrial crop and lignocellulosic feedstock under heterotrophic conditions to produce biodiesel. Use of grain and lignocellulosic feedstocks without nitrogen limitation likely caused a major hindrance to lipid production. In comparison to previous studies such as (Bumbak, Cook, Zachleder, Hauser, & Kovar, 2011; Wu & Shi, 2007) which reported lipid yields of 15%-25% with no nitrogen limitation, the lipid yields in this study were less than 1% in all the feedstocks grown under heterotrophic conditions. It should be noted that the earlier study did not grow algae on the laboratory scale fermenters as we did; all previous studies emphasize the importance of fed-batch culture mode to achieve high biomass concentrations. The present study's experiments were conducted on a much smaller scale in a 100mL (Fig. 13 page 61) Erlenmeyer flasks coupled with lower algal biomass concentrations, the presence of nitrogen in feedstock and the lack of nitrogen limitation conditions which we speculate might have caused such low lipid yields.

Use of algal species to produce biodiesel has been practiced for decades. Heterotrophic growth of *Chlorella* species like *C. protothecoides* supplied with acetate, glucose or other carbon

sources resulted in high biomass and high contents of lipids in the cells (Endo et al., 1977) (Qingyu et al., 1994). It has been observed that nitrogen limitation plays an important role in cellular lipid level which can be increased from 20-30% to 60-70% with nitrogen limitation. Nitrogen limitation was not initiated in the current project as the feedstocks usually have a predefined nutritive value. Techniques such as using high amounts of inoculums to create nutrient starvation environments that mimic nitrogen starvation were attempted but the lipid contents remained unaltered from the normal inoculations.

As reported, some strains of *C. vulgaris* can only grow in the dark (Haass & Tanner, 1974) while others can only grow in the light (Karlander & Krauss, 1966) . The strain tested in the current study can grow both in light and dark conditions. Chlorophyll content was higher in pure sugars in heterotrophic growth mode for sucrose in comparison to mixotrophic growth. All sugars including glucose, sucrose, fructose and a mixture of glucose, sucrose and fructose as well as corn, sorghum and lignocellulosic feedstocks demonstrated that they can be used to support the growth of *C. kessleri* in heterotrophic and mixotrophic growth modes. In the comparison of algal growth by cell number in mixotrophic, autotrophic and heterotrophic conditions most favored. Heterotrophic conditions favor rapid algal growth. The maximum cell number in autotrophic (Fig. 6 page 54) conditions was found to be $9.43X10e^5$ cellsmL^{-1} at 240 h after inoculation whereas in heterotrophic conditions (Fig. 8 page 56) the cell number reached close to the autotrophic cell count by 96 h in pure sucrose and glucose sugars. The cell numbers and the biomass of the *C. kessleri* in heterotrophic conditions was nearly thrice as much as in autotrophic conditions. It has been observed (Y. Li et al., 2012) that *C. kessleri* growth was suppressed by increases beyond 120 $\mu molm^{-2}s^{-1}$ of light. The dependence of algal growth and cell composition on light intensity relies heavily on the genetic makeup of the organisms. In a comparison of

mixotrophic and autotrophic growth modes in pure sugars the cell count in mixotrophic cultures in different sugars peaked at 72 h (Fig. 10 page 58) after inoculation whereas the same cell count in autotrophic cultures (Fig. 6 page 56) was reached at 216 h after inoculation. Martínez & Orús, (1991) observed that increasing light increased specific growth rates while the same was not observed in photoautotrophic cultures due to the stimulatory effects of light on metabolism of sugar by mixotrophic cells. The stimulatory effects of light on metabolism of sugars may have played a role in the sudden decrease in the cell count we observed chlorophyll contents kept increasing.

The corn, sorghum and lignocellulosic hydrosylates are the most critical part of this study as there growth patterns are used to analyze the lipid content and profile extracted from the biomass grown on these hydrosylates. It was observed that big blue stem had the highest cell number in heterotrophic culture mode (Fig. 12 page 60). Studies by Mutlu, Işık, Uslu, Koç, & Durmaz, (2011) showed that though biomass was high, it need not necessarily translate to higher lipid content; the highest lipid content was produced in media deprived of nitrogen up to 100%. Lack of nitrogen leads to lack of chlorophyll *a* pigment which leads to low protein content and accumulation of lipids. Studies by Sukenik et al., (1989) and Shifrin & Chisholm, (1981) state that decreases in nitrogen caused decrease in cell number and chlorophyll *a* which increased the lipid contents. In addition, the carotene content increased and turned the cultures yellow. This allows us to make the assumption that there were significant nitrogen source in big blue stem which kept the cell biomass at such high values but couldn't produce the higher lipid contents observed with corn and sorghum hydrosylates. Miao & Wu, (2006) cultured *Chlorella protothecoides* autotrophically and found 52.64% protein and 14.57% lipid. In the same study it was reported that protein levels decreased to 10.28% and lipid level increased to 55.20% during

heterotrophic growth. This study supports the conclusion that heterotrophic growth is the best mode of culture to achieve increased lipid yields. Thus only heterotrophic growth was carried out in the current study on *Chlorella kessleri* with hydrosylates to achieve highest possible lipid yields.

CHAPTER 5

CONCLUSION

Chlorella kessleri is a freshwater autotrophic alga. The accumulation of algal biomass as well as the composition of algal cells is highly dependent on the light supply, though a number of abiotic stresses also play a vital role in defining the growth characteristics. The first hypothesis was based on the natural growth characteristics of *Chlorella kessleri* under autotrophic conditions. In the current study it was observed that growth rates measured by chlorophyll content and cell number were higher under heterotrophic conditions than under autotrophic conditions. There was a lag phase in autotrophic cultures and growth continued until 240 h after inoculation, whereas in the heterotrophic cultures the growth had reached stationary phase by 120 h after inoculation. Autotrophic growth mode is not viable for biodiesel production when the cultures biomass productivity is low and more than twice the time is required to attain high cell numbers in autotrophic media.

Many previous studies have shown that nitrogen limitation greatly enhances the production of lipids in algae, but growth rates are drastically reduced in comparison to a nutrient rich media. In the current study, the second hypothesis was shown to be false as higher growth rates were observed in heterotrophic conditions in lignocellulosic feedstock than in corn and sorghum based feedstocks (Fig. 12 page 60). The lipid contents of the biomass harvested from heterotrophic cultures were analyzed for FAME by GC-MS which showed that much higher lipid contents were found with corn and sorghum-based feedstocks than in lignocellulosic-based feedstocks. We can speculate from this that the lignocellulosic feedstock was high in nitrogen content which did not hinder the growth of the algal cells. However in case of corn and sorghum

based feedstocks the cell number was lower and higher amounts of lipids were recovered (Tables 12, 13, 14 pages 73-75). Though a number of factors affect the growth conditions, higher nitrogen content in lignocellulosic feedstock may have led to better growth and lower lipid yields (Mutlu et al., 2011).

Light is the most important parameter that affects the growth and lipid accumulation characteristics of algae. The third hypothesis was based on the assumption that with availability of light and a carbon source, growth rates would be much higher than in heterotrophic conditions. Studies by Yeh, Chang, & chen (2010) reported that increase in light intensity increased the growth rates. The study also reported that light saturation occurred when cultures were subjected to continuous light. In this study, in mixotrophic cultures of pure sugars and feedstocks, the growth rates were higher with high cell numbers observed in feedstock-based cultures than in pure sugars. However in heterotrophic conditions, the growth rates were higher in pure sugars than in feedstock-based media. Sorokin & Krauss, (1958) also report that the dependence of algal growth on light intensity and the utilization efficiency of incident light differ widely with the different strains of green algae. They also reported that the stimulatory effect of light on metabolism of sugar by mixotrophic cells is altered by light intensity and the carbon substrate. This third hypothesis can neither be rejected nor accepted as the growth responses rely on a variety of conditions with such complex carbon substrates.

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APPENDICES

APPENDIX A

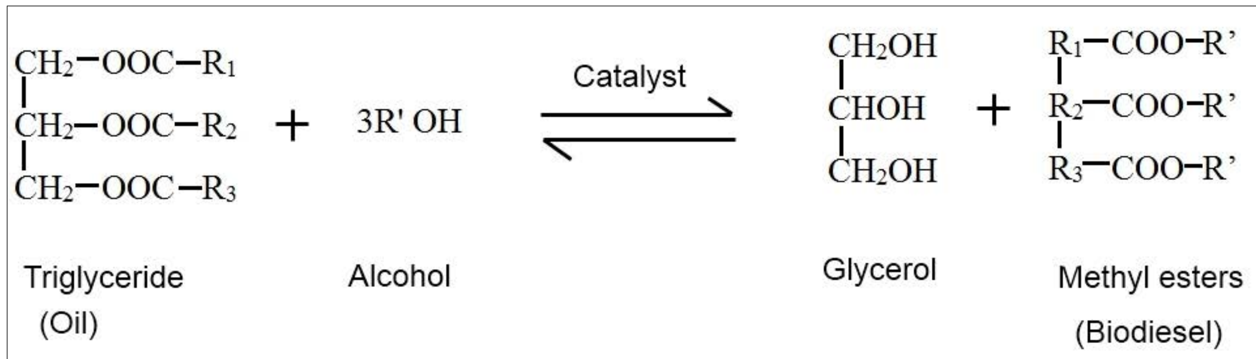

Figure 1. Biochemical reaction of Transesterification

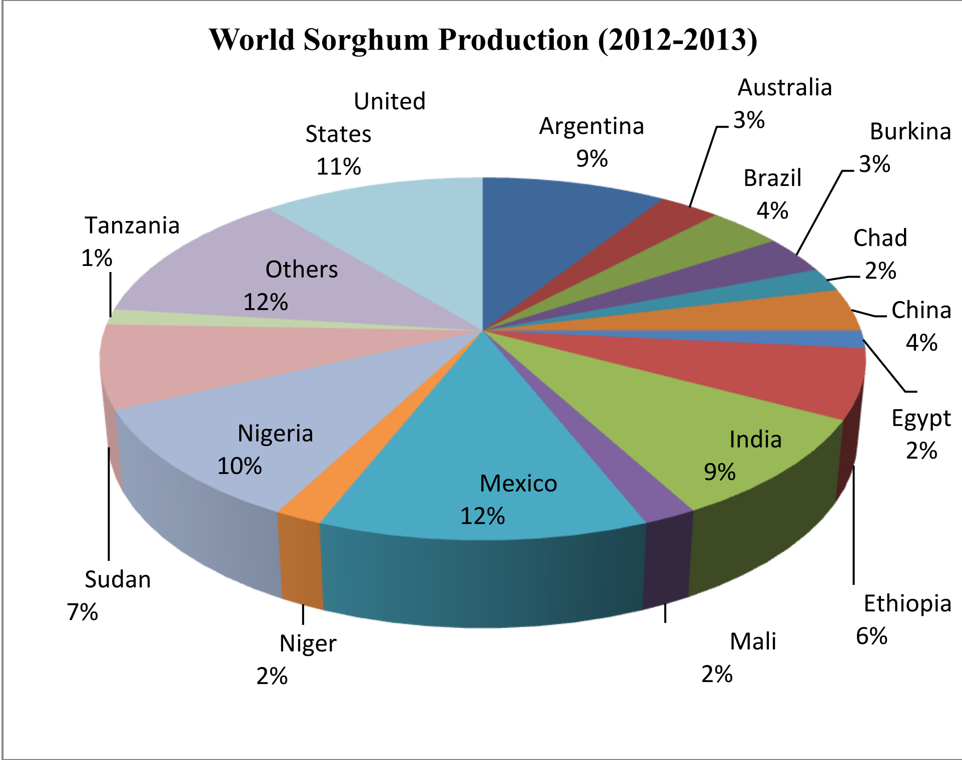

Figure 2. World Sorghum Production

Data Sourced: http://fas.usda.gov/psdonline/psdHome.aspx

United States Department of agriculture

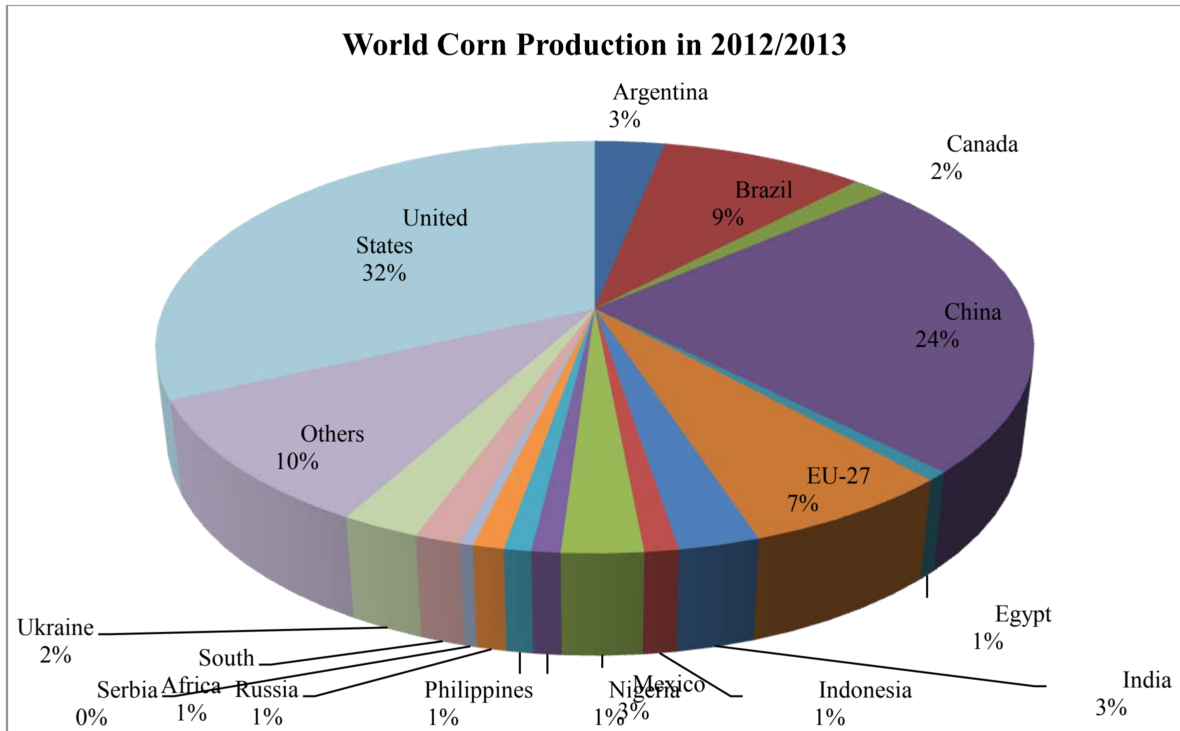

Figure 3. World Corn production (2012/2013)

Data Sourced: http://fas.usda.gov/psdonline/psdHome.aspx

United States Department of agriculture

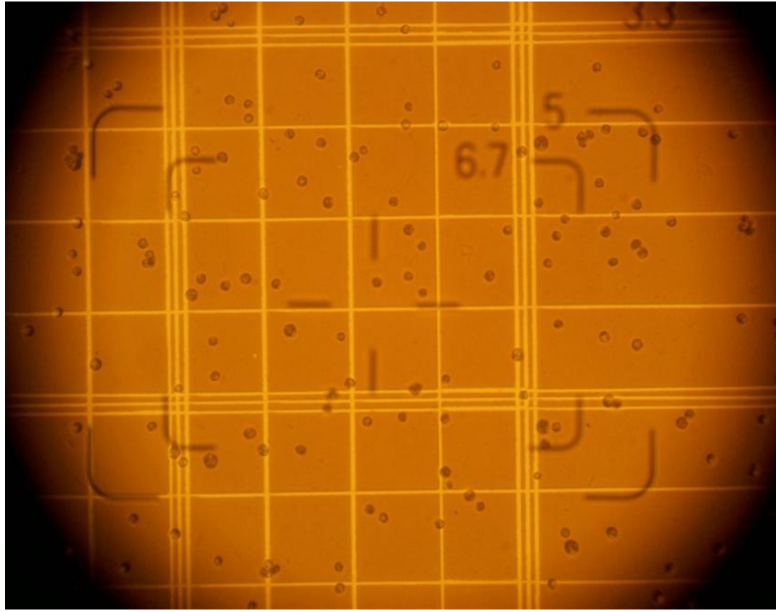

Figure 4. 40X magnification of Chlorella kessleri cells observed under a light microscope

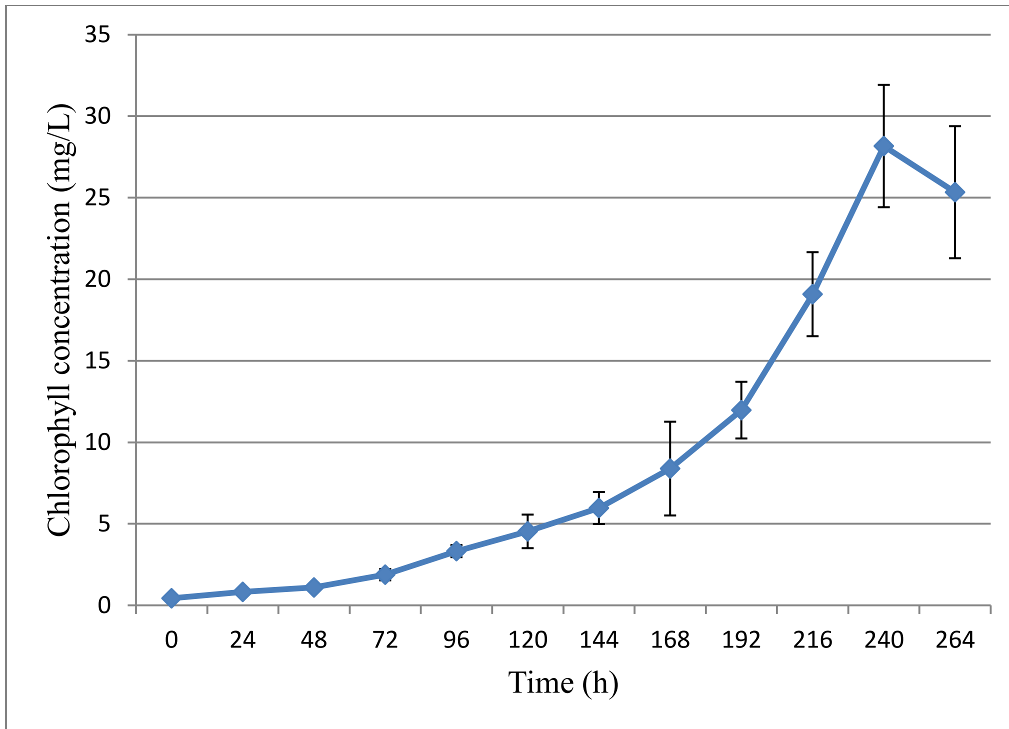

Figure 5. Algal growth in autotrophic media

Algal growth curve was measured in autotrophic culture using chlorophyll concentration from 0 to 264 hours from inoculation.

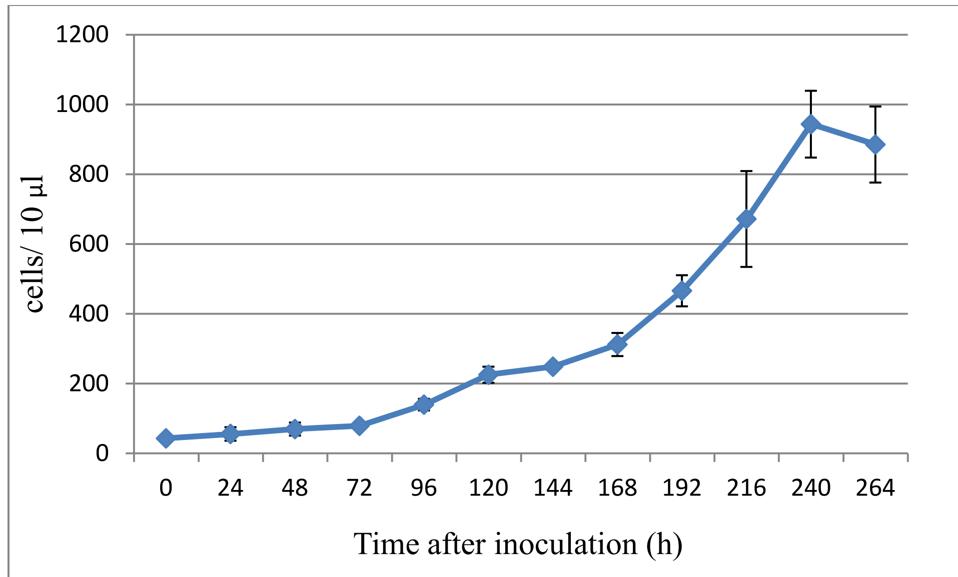

Figure 6. Algal growth by cell number measured by Hemocytometer in a autotrophic medium from 0 to 264 hours from inoculation

Algal growth by cell number measured by Hemocytometer in an autotrophic medium from 0 to 264 hours from inoculation

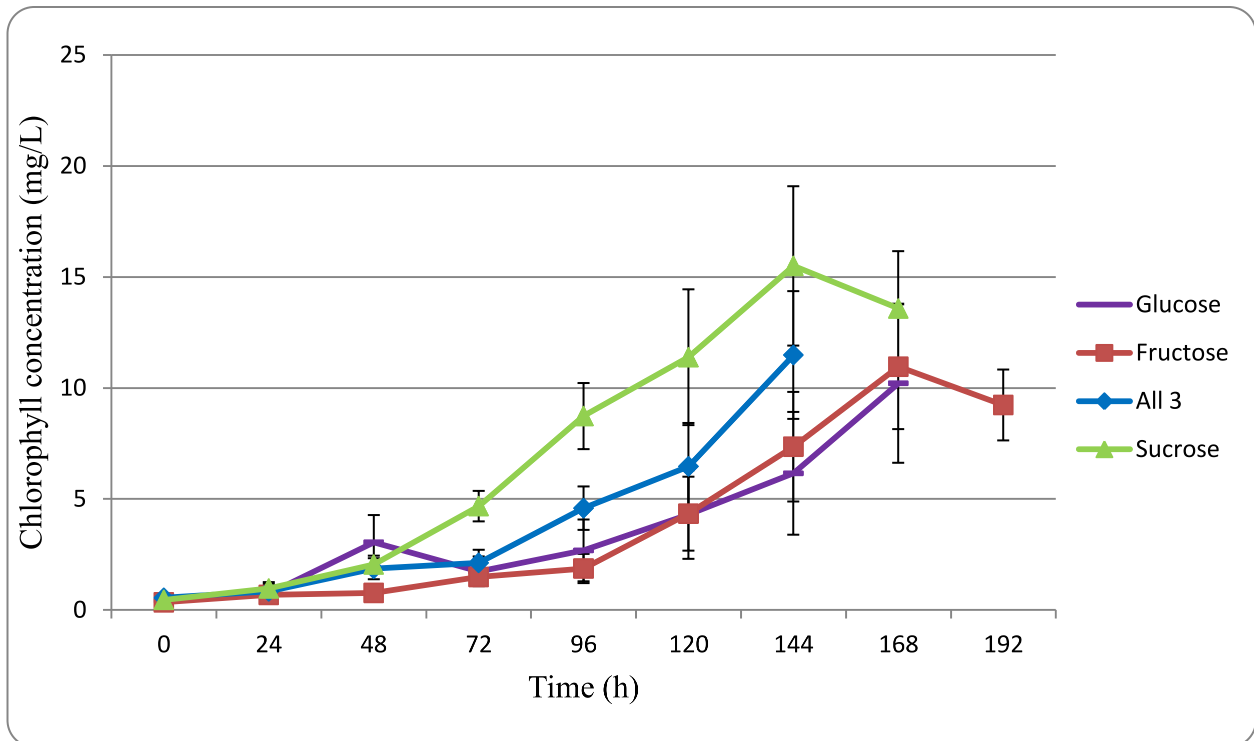

Figure 7. Algal growth by chlorophyll measurement in heterotrophic media (Weinstein & Beale, 1985) in different sugars and a combination of all three sugars

Sucrose as a carbon substrate performed better than all other sugars in the measure of chlorophyll concentration

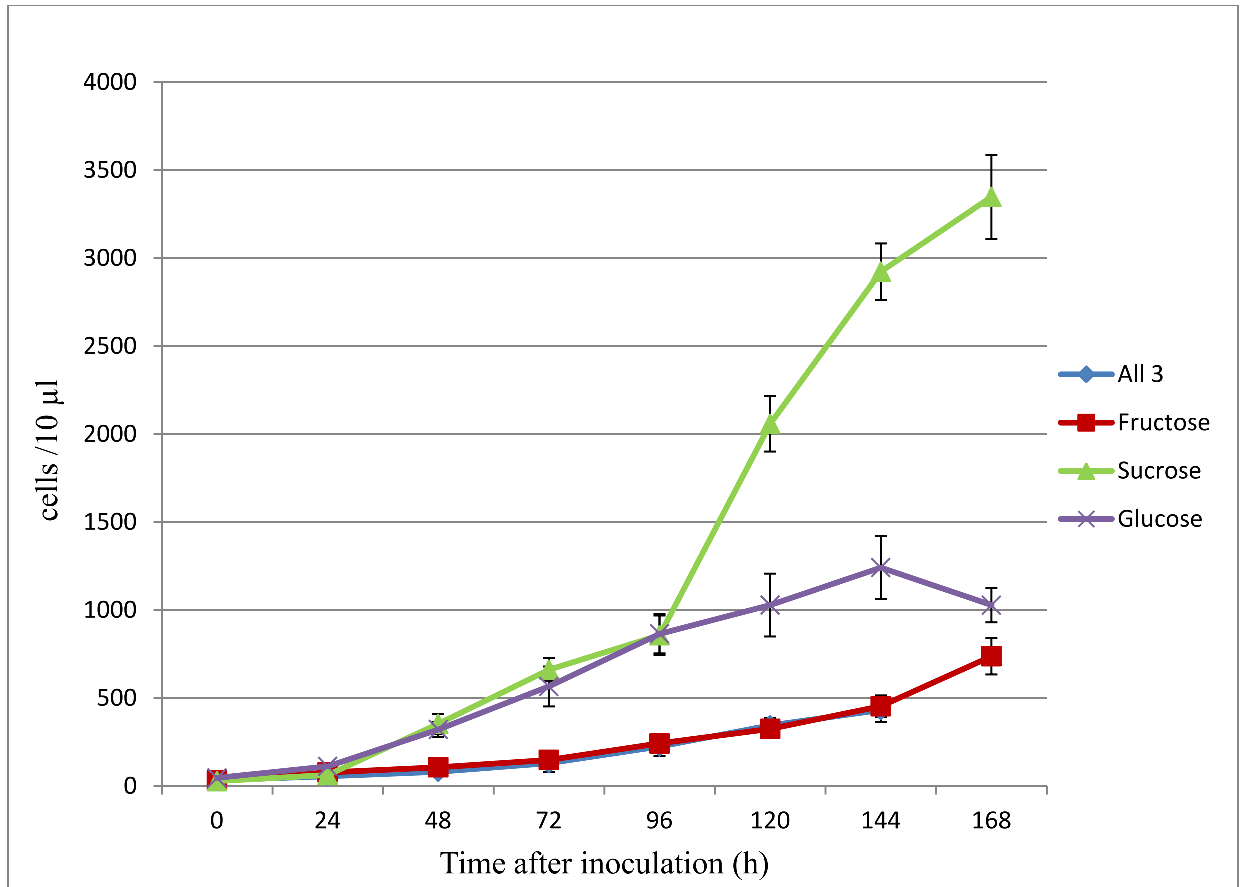

Figure 8. Cell number in Heterotrophic growth media (Weinstein & Beale, 1985) observed in heterotrophic growth mode

Cell number was observed highest in sucrose in Heterotrophic mode of growth

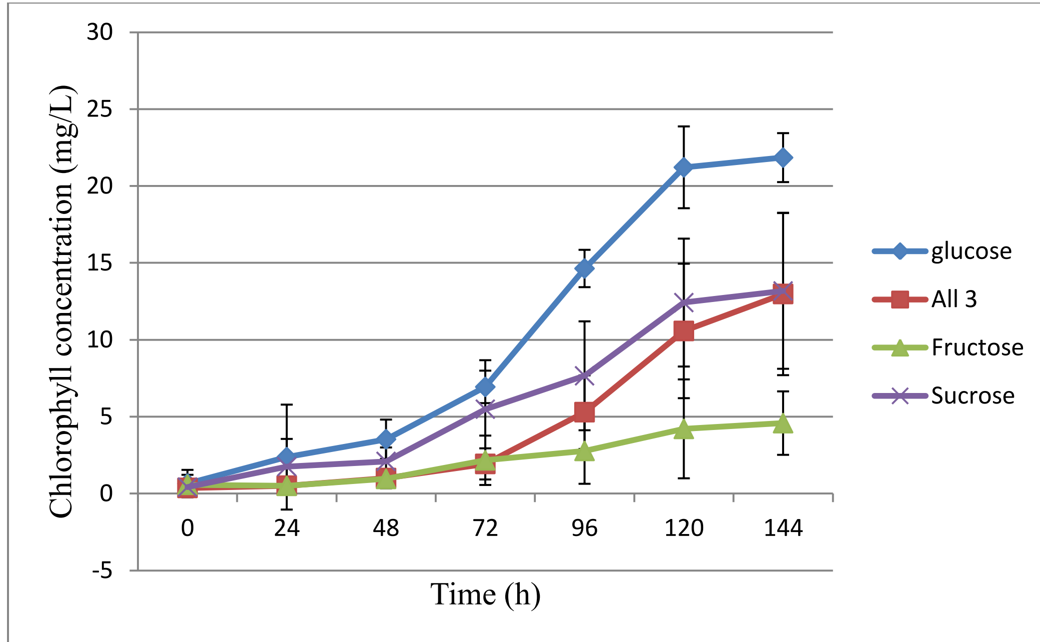

Figure 9. Chlorophyll concentration observed in algal growth in mixotrophic growth mode in pure sugars

Sucrose as a carbon substrate was observed to have the highest chlorophyll concentration among all three sugars in mixotrophic growth mode

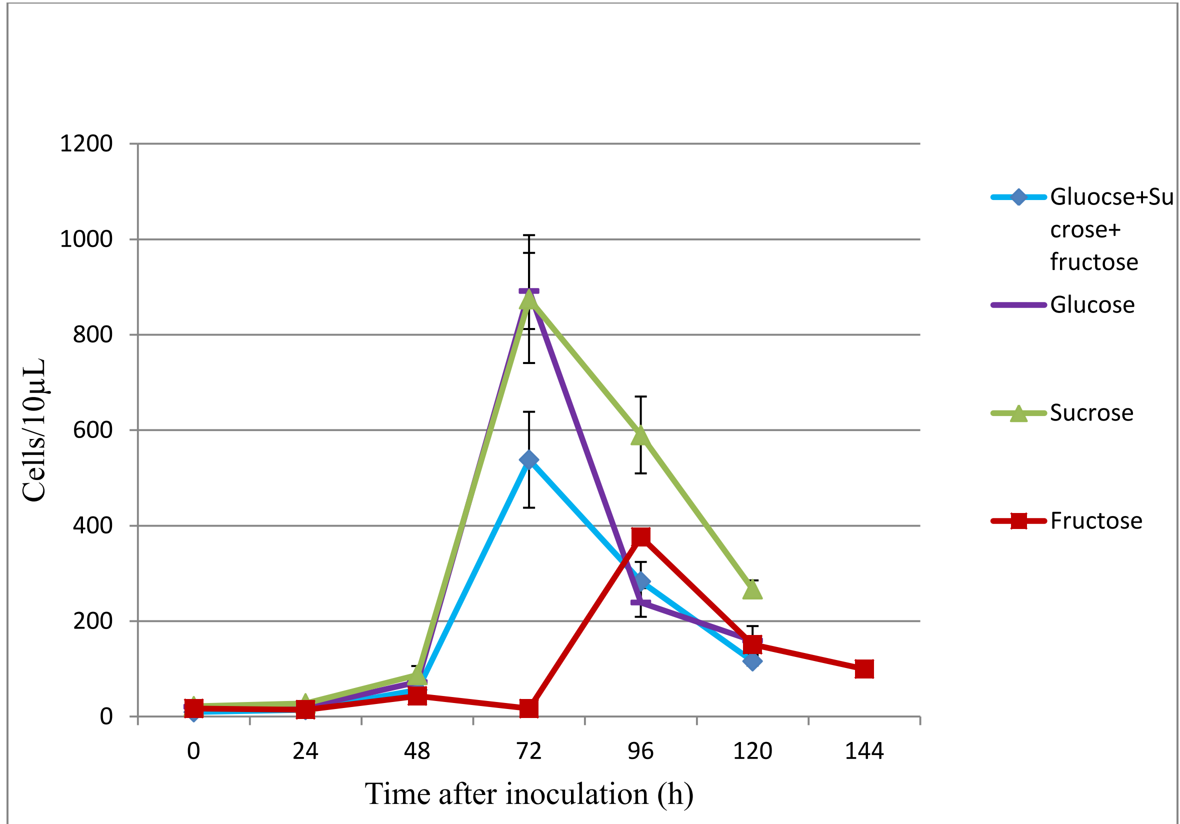

Figure 10 Growth in algal cultures observed by cell number in mixotrophic growth mode in pure sugars

Sucrose and Glucose were predominantly dominating carbon substrates observed in mixotrophic growth mode

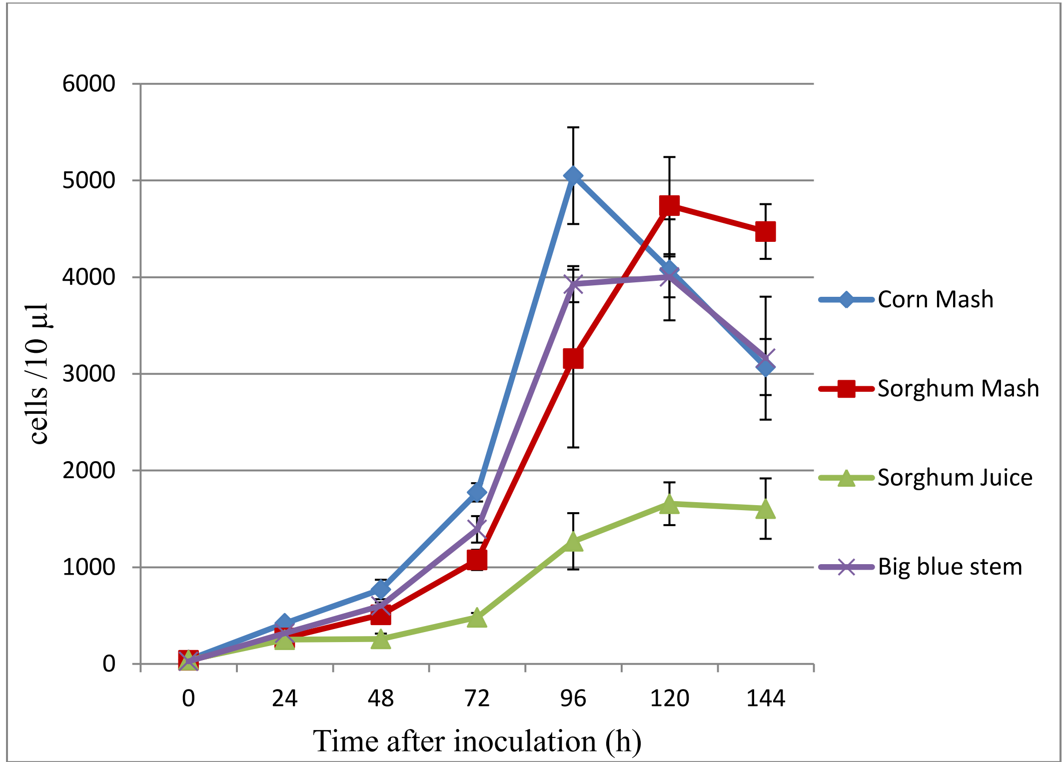

Figure 11 Growth in algal cultures observed in corn, sorghum and lignocellulosic hydrosylates in mixotrophic growth mode

Growth observed by hemocytometer in a 10µl culture demonstrated maximum cell number in

Corn mash feedstock in mixotrophic growth media

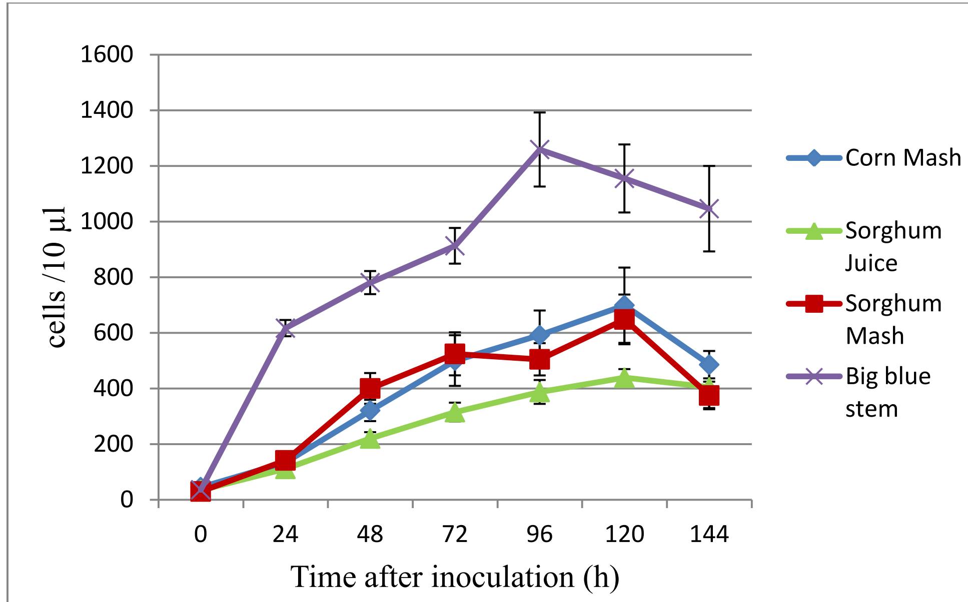

Figure 12 Growth observed in heterotrophic growth mode by cell number on a hemocytometer in corn, sorghum and lignocellulosic feedstocks

Algal growth observed by cell number in a 10µl media on a hemocytometer in heterotrophic growth mode in corn, sorghum and lignocellulosic hydrosylates

Figure 13 Algal biomass grown on corn and sorghum hydrosylates to evaluate their lipid profile

APPENDIX B

Table 1

Comparison of open and closed systems for microalgae (Grobbelaar, 2000; O. Pulz, 2001)

Qualitative	Open systems	Closed systems
Contamination Risk	High	Low
CO_2 Loses	High	Low
Evaporation Issues	High	Low
Light use efficiency	Poor	Excellent
Area to volume ratio	Low	High
Area Required	High	Low
Process control	Difficult	Easy
Biomass Productivities	Low	High
Investment Costs	Low	High
Operation Costs	Low	High
Harvesting Costs	High	Relatively lower
Scale- up	Easy	Difficult

Table 2

Energy costs to produce a kilogram of biomass (Andersen, 2005)

Cost Factor	Photobioreactor	Fermenter
Construction Method	Individually constructed	Mass produced by craftsmen
Scale	Relatively Small scale	Up to 500,000 liters
Algal Concentration	Dilute	High
Energy Source	Light	Organic carbon

Table 3

Major cost factors for photo bioreactors and fermenters (Andersen, 2005)

	Phototroph	**Heterotrophs**
Energy Source	Light	Glucose
Energy Cost	$0.07/kW-hr	$0.67/kg
Estimated cost/kg of dry weight	$11.22	$0.81
Actual cost/kg of dry weight	Less than $11.22	$2.01
Productivity	$0.4 g.L^{-1}.day^{-1}$	$5.8 g.L^{-1}.day^{-1}$

Table 4

Protease Media

Component	Amount	Stock Solution concentration	Final Concentration
Sodium Nitrate	10mL/L	10 g/ 400mL dH_2O	2.94mM
Calcium Chloride	10mL/L	1g/400mL dH_2O	0.17mM
Magnesium sulfate	10mL/L	3g/400mL dH_2O	0.3mM
Potassium phosphate dibasic	10mL/L	3g/400mL dH_2O	0.43mM
Mono-potassium phosphate	10mL/L	7g/400mL dH_2O	1.29mM
Sodium Chloride	10mL/L	1g/400mL dH_2O	0.43mM

Add 5 grams protease peptone to one liter media.

University of Austin (TX) UTEX the culture collection of algae (n.d). Retrieved from http://web.biosci.utexas.edu/utex/mediaDetail.aspx?mediaID=65)

Table 5

Biodiesel productivity and composition under different light intensities for Chlorella kessleri (Y. Li et al., 2012)

	Light Intensity µmol$m^{-2}S^{-1}$					
	0	15	30	60	120	200
Content of 16-C FAME (% of total FAME)	22.93	34.77	53.98	38.65	25.08	26.38
Content of 18-C FAME(% of total FAME)	77.06	58.37	24.40	58.45	74.55	73.26
% of FAME (% of total TVSS)	14.42	15.73	14.39	17.88	22.76	24.19

FAME: Fatty acid methyl esters; TVSS: Total volatile suspended solids

Table 6

Composition of the medium for the Heterotrophic growth

Ingredient	Final Concentration (mM)
$C_6H_{12}O_6$ (carbon source)	50.0
KNO_2	30.0
$MgSO_4$	5.0
KH_2PO_4	5.0
$CaCl_2$	0.1
The following ingredients are added from a 1000- fold concentrate (µm)	
Na_4EDTA	20.0
Na_2EDTA	20.0
$FeCl_2$	20.0
$MnCl_2$	10.0
$ZnSO_4$	10.0
H_3BO_3	1.0
$CuSO_4$	1.0
Na_3VO_4	1.0
$(NH_4)_6Mo_7O_{24}$	1.0 (as Mo)
$CoCl_2$	0.1

All of the ingredients are mixed and autoclaved together. The final pH is approximately 4.4

Table 7

Autotrophic growth media

Ingredient	Final Concentration (mM)
KNO_2	30.0
$MgSO_4$	5.0
KH_2PO_4	5.0
$CaCl_2$	0.1
The following ingredients are added from a 1000- fold concentrate (µm)	
Na_4EDTA	20.0
Na_2EDTA	20.0
$FeCl_2$	20.0
$MnCl_2$	10.0
$ZnSO_4$	10.0
H_3BO_3	1.0
$CuSO_4$	1.0
Na_3VO_4	1.0
$(NH_4)_6Mo_7O_{24}$	1.0 (as Mo)
$CoCl_2$	0.1

Modified Heterotrophic growth media (Weinstein & Beale, 1985)

All of the ingredients are mixed and autoclaved together. The final pH is approximately 4.4.

pH was adjusted to 7.0 before inoculation

Table 8

Hydrosylates parameters

Hydrosylates	pH	ORP/mV	Glucose content
Corn Mash	4.94	129.9	20%
Sweet Sorghum Juice	4.28	168.6	15%
Sorghum Mash	5.33	110.4	20%
Big Blue Stem	10.45	-180.6	6.54%

Table 9

Natural occurrence of the main pigments of the Chlorophyll group

Pigment	Occurrence
Chlorophyll a	All photosynthetic algae (except prochlorophytes) and higher plants
Chlorophyll b	Higher plants, green algae and symbiotic prochlorophytes
Chlorophyll c	Chromophyte algae, and brown seaweeds

Table 10

Technical properties of Biodiesel

Properties of Biodiesel	
Common Name	Biodiesel
Chemical Name	Fatty Acid (m)ethyl ester
Chemical formula range	C_{14}-C_{24} methyl esters or $C_{15-25}H_{28-42}O_2$
Kinematic viscosity (mm^2/s, at 313K)	3.3 – 5.2
Density range (kg/m^3, at 288K)	860-894
Boiling point range (K)	>475
Flash point range (K)	420-450
Distillation Range (K)	470-600
Vapor pressure (mm Hg, at 295K)	<5
Solubility in water	Insoluble
Physical appearance	Light to dark yellow, clear liquid
Odor	Light musty/ Soapy odor
Biodegradability	More biodegradable than petroleum diesel
Reactivity	Stable, but avoid strong oxidizing agents

Table 11

Lipid profile of Fatty acid methyl esters extracted from Sweet sorghum juice hydrosylate

Lipid	Lipid	µg/mL extract
Decanoic acid, methyl ester	C10:0	0
Dodecanoic acid, methyl ester	C12:0	0
Methyl tetradecanoate	C14:0	4.22801679
Hexadecanoic acid, methyl ester	C16:0	142.46321
9-Hexadecenoic acid, methyl ester, (Z)-	C16:1	9.187461127
9,12-hexadecadienoic acid, methyl ester	C16:2	7.896104778
7,10,13-hexadecatrienoic acid, methyl ester	C16:3	6.473307567
Octadecanoic acid, methyl ester	C18:0	51.53019208
9-Octadecenoic acid (Z)-, methyl ester	C18:1	190.3323032
9,12-Octadecadienoic acid, methyl ester	C18:2	495.5540751
9,12,15-Octadecatrienoic acid, methyl ester	C18:3	164.0778551
Eicosanoic acid, methyl ester	C20:0	6.602337751
Docosanoic acid, methyl ester	C22:0	2.761081695
13-Docosanoic acid, methyl ester	C22:1	0
Tetracosanoic acid, methyl ester	C24:0	4.884136316
other saturated		
other unsaturated		
pentadecanoic acid	C15:0	
heptadecanoic acid	C17:0	
Sum Saturated Lipids (ug/g)		212.4689746
Sum Unsaturated Lipids (ug/g)		873.5211069
Sum Saturated: Unsaturated Lipids		0.243232788
Sum Unsaturated: Saturated Lipids		4.111287817
Sum Lipids (ug/g)		**1085.990082**

Table 12

Lipid profile of Fatty acid methyl esters extracted from Sorghum Mash hydrosylate

Lipid	Lipid	µg/mL extract
Decanoic acid, methyl ester	C10:0	0
Dodecanoic acid, methyl ester	C12:0	0
Methyl tetradecanoate	C14:0	30.31726
Hexadecanoic acid, methyl ester	C16:0	843.4147
9-Hexadecenoic acid, methyl ester, (Z)-	C16:1	59.95491
9,12-hexadecadienoic acid, methyl ester	C16:2	22.23652
7,10,13-hexadecatrienoic acid, methyl ester	C16:3	10.75627
Octadecanoic acid, methyl ester	C18:0	538.9397
9-Octadecenoic acid (Z)-, methyl ester	C18:1	1141.482
9,12-Octadecadienoic acid, methyl ester	C18:2	2873.565
9,12,15-Octadecatrienoic acid, methyl ester	C18:3	527.8259
Eicosanoic acid, methyl ester	C20:0	67.31801
Docosanoic acid, methyl ester	C22:0	22.02945
13-Docosanoic acid, methyl ester	C22:1	23.39105
Tetracosanoic acid, methyl ester	C24:0	30.06311
other saturated		5.831143
other unsaturated		12.37587
pentadecanoic acid	C15:0	
heptadecanoic acid	C17:0	
Sum Saturated Lipids (ug/g)		1537.913
Sum Unsaturated Lipids (ug/g)		4671.587
Sum Saturated: Unsaturated Lipids		0.329206
Sum Unsaturated: Saturated Lipids		3.037614
Sum Lipids (ug/g)		**6209.5**

Table 13

Lipid profile of Fatty acid methyl esters extracted from Corn Mash hydrosylate

Lipid	Lipid	μg/mL extract
Decanoic acid, methyl ester	C10:0	0
Dodecanoic acid, methyl ester	C12:0	0
Methyl tetradecanoate	C14:0	12.74223
Hexadecanoic acid, methyl ester	C16:0	810.9357
9-Hexadecenoic acid, methyl ester, (Z)-	C16:1	34.06045
9,12-hexadecadienoic acid, methyl ester	C16:2	11.01684
7,10,13-hexadecatrienoic acid, methyl ester	C16:3	0
Octadecanoic acid, methyl ester	C18:0	288.9634
9-Octadecenoic acid (Z)-, methyl ester	C18:1	1600.384
9,12-Octadecadienoic acid, methyl ester	C18:2	3560.751
9,12,15-Octadecatrienoic acid, methyl ester	C18:3	357.2884
Eicosanoic acid, methyl ester	C20:0	50.22362
Docosanoic acid, methyl ester	C22:0	26.70852
13-Docosanoic acid, methyl ester	C22:1	2.47396
Tetracosanoic acid, methyl ester	C24:0	24.95468
other saturated		3.969164
other unsaturated		21.88615
pentadecanoic acid	C15:0	
heptadecanoic acid	C17:0	11.84833
Sum Saturated Lipids (ug/g)		1230.346
Sum Unsaturated Lipids (ug/g)		5587.861
Sum Saturated: Unsaturated Lipids		0.220182
Sum Unsaturated: Saturated Lipids		4.5417
Sum Lipids (ug/g)		6818.207

Table 14

Lipid profile of Fatty acid methyl esters extracted from big blue stem hydrosylate

Lipid	Lipid	µg/mL extract
Decanoic acid, methyl ester	C10:0	0
Dodecanoic acid, methyl ester	C12:0	0
Methyl tetradecanoate	C14:0	0
Hexadecanoic acid, methyl ester	C16:0	5.465583
9-Hexadecenoic acid, methyl ester, (Z)-	C16:1	0
9,12-hexadecadienoic acid, methyl ester	C16:2	0
7,10,13-hexadecatrienoic acid, methyl ester	C16:3	0
Octadecanoic acid, methyl ester	C18:0	2.114495
9-Octadecenoic acid (Z)-, methyl ester	C18:1	21.88462
9,12-Octadecadienoic acid, methyl ester	C18:2	15.46617
9,12,15-Octadecatrienoic acid, methyl ester	C18:3	9.404057
Eicosanoic acid, methyl ester	C20:0	0
Docosanoic acid, methyl ester	C22:0	0
13-Docosanoic acid, methyl ester	C22:1	0
Tetracosanoic acid, methyl ester	C24:0	0
other saturated	0	
other unsaturated		
pentadecanoic acid	C15:0	
heptadecanoic acid	C17:0	
Sum Saturated Lipids (ug/g)	7.580078	
Sum Unsaturated Lipids (ug/g)	46.75485	
Sum Saturated: Unsaturated Lipids	0.162124	
Sum Unsaturated: Saturated Lipids	6.168123	
Sum Lipids (ug/g)	**54.33493**	

Table 15

Compositions of 18C fatty acid methyl esters in different hydrosylates

Hydrosylate	18C Methyl ester of fatty acids composition mg/kg biomass
Corn Mash	6.76
Sweet Sorghum Juice	0.84
Sorghum Mash	6.16
Big Blue Stem	0.05