Algal Mixotrophic Metabolism for The Production of Lipids

A Thesis presented to

The Faculty of Graduate Studies

of

Lakehead University

by

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In partial fulfillment of requirements

for the degree of

Master of Science in Biology

Thunder Bay, Ontario, Canada

Contents:

In the Name of Allah, the Entirely Merciful, the Especially Merciful

Acknowledgements

It's my sincere gratitude and warm regards that I dedicate this project to God, Prophet Mohammed (Peace be Upon Him) and my parents who have all been great pillars to me throughout my project work. You have granted me all the strength, knowledge and wisdom that I required. Mum, your prayers and words of encouragement always encouraged me even when I felt as if I was growing weaker. Thanks for your love. Dad, you've been my greatest inspirer. You always told me that I could make it and I surely made it. May Allah bless you abundantly. The completion of my project would not have been possible without the unwavering support that I received from my Husband and my family in law!

I sincerely thank my best friend Alaa, whose unending support, love and words of encouragement have been the reason as to why I persevered and thanks to my supervisor for his advice, guidance, keen interest and continuous reinforcement throughout the various stages of this project, till its completion. Thank you for being so caring, as well as correcting mistakes and being so prompt at responding to all questions!

I owe profound gratitude to Ministry of Higher Education and Saudi Bureau for supporting and funding which I would not have made it without them, Lakehead University for giving me the opportunity to do my research!

I feel highly indebted to the lab group for providing me with a conducive environment to carry out my practical's as well as providing me with any information that deemed necessary in my project!

Lastly, I am so fortunate to have supportive colleagues in the Biology department as well as friends whom I am heartily thankful for their prayers, moral support, and encouragement during tough moments that were encountered in the progress of this work!

Abstract:

The challenges associated with the use of conventional sources of crude oil increase the demand for alternative fuel sources. Biofuels offer the chance of replacing the depleting fossil fuels. However, the food versus fuel conflict that exists with the use of crops as source of biofuels means that algal biomass may be the only potentially viable biofuel source that ensures high production without trading off food production. Algal biomass may also ensure year-round production and uses minimal space. I confirm the known observation that the addition of glucose (0.2% w/v) to otherwise autotrophically grown algae (*Auxenochlorella pyrenoidosa* and isolate W7) increase lipid accumulation (based on chlorophyll content) 12-fold and 20-fold, respectively. More realistically, on per culture basis, lipid production increased 3-fold and 5-fold, respectively. Recently, cellulose was shown to be hydrolysed by algae and I provide preliminary evidence for the existence of extracellular cellulase produced by both algae used in my study. I show that on per culture basis, *A. pyrenoidosa* and isolate W7 supplemented with complex carbohydrates such as carboxymethyl cellulose, crystalline cellulose or paper mill sludge will increase their lipid content (up to triple for W7 supplemented with paper mill sludge). However, these carbohydrates as well as pure glucose depressed chlorophyll content of both algae, resulting in inflated apparent increase in lipid content based on chlorophyll concentration. This study adds important new information for the future potential commercialization of the mixotrophic production of algal lipid using cheap organic substrate in the form of pulp mill waste.

List of Figures:

List of Tables:

1. Introduction

1.1 Biofuels as a replacement for fossil fuels

My thesis investigates the use of mass produced microalgae as an alternative source of fuel oil. The world's population is growing exponentially and according to the population growth theory is expected to stabilize and enter stationary phase in the foreseeable future (Solow, 2000). Nevertheless, the increase in population is putting pressure on available energy sources such as fossil fuels (Jones & Mayfield, 2012). The continued exploitation of fossil fuels does not only lead to a decline in resources and attendant pollution, but also leads to global warming. This results in devastating effects such as flooding, spikes in global temperatures, and droughts (Panwar et al., 2011). Another great concern is the unsustainable cost of fossil fuels. As the challenges associated with the use of conventional sources of energy increase, the demand for alternative fuel sources also increases (Panwar et al., 2011). The concerns about the use of conventional fuels include the one raised by Malthus about the threat to food security as resources continue to diminish. The club of Rome also predicted imminent collapse in the economy and environmental use due to pollution and unmanaged use of resources (Rosegrant & Cline, 2003; Turner & Alexander, 2014). Major efforts and resources have been directed towards research into inexpensive, sustainable and ecologically friendly alternative energy sources.

Fuels derived from living matter biomass are being suggested as one alternative to fossil fuel. The production of biofuels does not only offer the chance to replace the environmentally unsustainable fossil fuels, but also helps to reduce environmental pollution by assimilating pollutants such as carbon dioxide from the environment, hence reversing the effects of global warming (Huang et al., 2010; Demirbas, 2011). The use of biofuels by Germans during World War II is one of the earliest examples of the important role played by alternative fuels in solving

shortage in conventional fuels (Cohen et al., 1995; Behera et al., 2015). Biofuel production is currently happening on a large scale in countries such as Brazil, Canada and the US. (Kumarappan et al., 2009; Sarkar et al., 2011; Pereira et al., 2012; Brown & Brown, 2013).

The production of biofuels can be achieved using crops as feedstock. Some of the crops that have been used in the production of biofuels include cereals such as maize and wheat. Others include sugar crops such as sorghum and sugarcane. Plants that are regarded as weeds (e.g. switchgrass) have also been suggested to be likely candidates for feedstock in the production of biofuels (Haas, 2005; Naik et al., 2010; Somerville et al., 2010). The production of biofuels using crops as feedstock is, however, faced with various challenges. Some of the challenges are the high costs associated with the intensive agricultural input requirement, and the fact that the venture needs the use of expansive tracts of land (Puri et al., 2012). The fact that there is a trade-off between the planting of crops for fuel production and for food production is a problem in a world that is faced with food shortages (Rajagopal et al., 2007; Murphy et al., 2011; Zilberman et al., 2012; Koizumi, 2013). Therefore, despite being environmentally friendly, the production of biofuels using crops as feedstock is not sustainable and only crop waste should be depended on to replace the use of fossil fuels.

The alternative production of biofuels through the use of lignocellulosic biomass (i.e. wood waste) may help to solve the problems associated with the use of crops as feedstock. The use of lignocellulosic biomass in fuel production is at an experimental stage. This approach is still faced with challenges associated with the need for agricultural waste input and freshwater availability (Limayem & Ricke, 2012; Roy et al., 2015). Due to the food versus fuel conflict that exists with the use of crops and to some extent lignocellulosic biomass, there is a need for an alternative biofuel source that ensures high production of biofuels without trading off food

production. The use of specifically produced biomass such as algal biomass potentially solves the conflict between fuel and food and offers the chance of enhanced production of biofuels (Chisti, 2007). My thesis focuses on the use of algae for the production of lipid under autotrophic conditions supplemented with heterotrophic carbon sources.

1.2 Literature review of factors affecting algal growth in relation to biofuel production

1.2.1 Influence and importance of algal biomass and biofuel production

1.2.1.1 Species used

Algal biomass provides an alternative fuel source that could, at least partially, satisfy the ever-increasing demand for fuel. Research on the use of fuels produced from algal biomass started as early as 1942 with the pioneering work by Harder and von Witsch (Cohen et al., 1995). Some of the algal species that have been shown to produce lipids include *Botryococcus braunii* (Lee et al., 1998; Ashokkumar & Rengasamy, 2012) *Dunaliella tertiolecta* (Chen et al., 2011) and *Gracilaria* (Shuping et al., 2010; Kumar et al., 2013), however this study will focus in the production of biofuels by the algae belonging to the genus *Chlorella* (Liu et al., 2010; Herrera-Valencia et al., 2011; Kaur et al., 2012; Gerken et al., 2013; Tate et al., 2013). These organisms are reported to produce lipids similar to vegetable lipids. One of the species known as *Chlorella vulgaris* produces a variety of lipids and can yield up to 40% of its dry weight as lipids in the natural environment. This production level can be increased tremendously under well-regulated conditions. The accumulation of lipid by autotrophic algae is dependent on the algal strain (Liang et al., 2006; Chisti, 2007; Chen et al., 2008) and growth conditions, particularly composition of the media (Illman et al., 2000; Solovchenko et al., 2008). Increased production of biofuels by algae can be realised by growing the algae in mixotrophic cultures to overcome the

challenge of light supply faced in autotrophic cultures (Chen et al., 2011; Das et al., 2011; Cheirsilp & Torpee, 2012).

Approximately, for the past two decades, there has been an upsurge of interest in the use of algae in the production of lipid. Some of the major steps towards the use of algal fuels include the use of algae-derived fuels in the aeronautical industry (Daggett et al., 2007; Rahmes et al., 2009; Hendricks et al., 2011). Although this is not yet happening on a large scale, it points to the increasing focus on algal fuel. Right up to the significant decrease in the cost of crude fossil lipid in 2014 (Baumeister & Kilian, 2016), large corporations were investing in algal biofuel technologies, enabling more research and development of technologies that could further boost the production and use of algal fuel (Um & Kim, 2009; Singh & Gu, 2010; Sims et al., 2010). Algal biomass has been used in the production of biodiesel with the highest lipid concentration being up to 80% of the biomass dry weight with concentrations of 20-50% being most common (Harun et al., 2011). Given the ability of algae to double their biomass within 24 hrs, algae have the potential to support an all year-round production of biofuels like biodiesel (Gallagher, 2011).

1.2.1.2 Types of biofuel

Some of the biofuels generated from algal biomass include ethanol, methane, hydrogen and biodiesel (Lee & Lavoie, 2013). These biofuels, if well managed and produced, offer the chance of replacing fuels such as diesel and petrol derived from fossils (Gallagher, 2011). A brief discussion of the various biofuels is presented below, however, my study will focus on lipid production under mixotrophic condition.

The production of biofuels from lipids by German scientists during World War II is one of the earliest examples of industrial production of biodiesel from algae (Cohen et al., 1995). The

biodiesel is made of esters composed of long chains of fatty acids (Behera et al., 2015). The lipids produced by algal biomass are used in the generation of biodiesel (Mata et al., 2010). Under normal environmental conditions production of lipids by algal organisms can reach up to 70% of the dry weight, however the production may be enhanced to 90% in the future, using advanced technologies (Rodolfi et al., 2009; Mutanda et al., 2011). The production of biodiesel from algal biomass holds great potential as an alternative fuel source as indicated by statistics showing that annual production of biodiesel by algal biomass can be as high as 15000 gal per acre (Spolaore et al., 2006).

In contrast to lipids, algal carbohydrates can be used in the production of ethanol through fermentation (Ho et al., 2013). The ability of the algae to accumulate large quantities of carbohydrates per dry weight makes them potentially useful for ethanol production (Behera et al., 2015). The use of algae in ethanol production is also preferred since these organisms have no lignin, hence the generation of simple sugars from starch and cellulosic cell wall material to be used in ethanol production is simpler than using wood waste containing inhibitors of fermentation derived from lignin (Singh et al., 2011; Nguyen, 2012; Chen et al., 2013). Various algae species such as *Chlorococcum* sp., *Laminaria* sp., and *Spirogyra* sp (Rajkumar et al., 2013) have been used in the production of ethanol, however, organisms of the genus *Chlorella* such as *C. vulgaris* are preferred, because of their high starch content per dry weight and efficient ethanol conversion rate (Lam & Lee, 2012).

The most direct route to bioenergy production from algae may be via hydrogen generation from algal cultures (Saleem et al., 2012). Some of the algae that have been used in the production of hydrogen include *Gelidium amansii* (Park et al., 2011) and *Chlamydomonas reinhardtii* (Saleem et al., 2012). The production of hydrogen from algae is achieved through

biophotolysis and photo fermentation (Shaishav et al., 2013). The obstacles with the generation of hydrogen from algal culture include the low production rate by organism such as *Tetraselmis* (D'Adamo et al., 2014). The little information on the structural complexity and dynamic response of algae in commercial autotrophic production impedes the optimisation of the light capture efficiency for enhanced hydrogen production (Oey et al., 2015). Additional challenges faced in the scale up of hydrogen production such as the need for an improved photobioreactor present obstacles to the use of algal culture for hydrogen generation (Oey et al., 2015).

One of the early fuels produced from algae was methane (Samson & Leduyt, 1986). The production of methane from algal biomass is achieved through anaerobic digestion of algal biomass by bacteria (Tan et al., 2015). The major biomass source organism that has been used in the production of methane is the seaweed (Murphy et al., 2015). However, other algae such as *Chlamydomonas reinhardtii* (Mussgnug et al., 2010), *Chlorella kessleri* (Mussgnug et al., 2010) and *Chlorella vulgaris* (Hernández & Córdoba, 1993) were also tested. *Emiliania huxleyi* (Lenhart et al*., 2016*) and *Trachydiscus minutus* (Alexandrov et al., 2013) are examples of the algae that are used as biomass to produce methane. However, the commercial production of methane from algal culture is also impeded by challenges associated with large-scale cultivation in open production system (Chisti, 2007). The high cost associated with the recovery and extraction of microalgal cultures is the other obstacle to commercial production of methane from algal cultures (Rösch et al., 2009).

Algal biomass for any of the biofuel processes described above can be produced under autotrophic, heterotrophic or mixotrophic conditions (John et al., 2011; Mitra et al., 2012; Wang et al., 2014). The production of biofuels through these various modes of nutrition will be discussed in the following sections.

1.2.2. Autotrophic biomass production

Autotrophic production of algae using simple salt media and solar energy employs the ability of these microorganisms to synthesize their own metabolites from inorganic substrates through the process of photosynthesis. The use of this approach appears to be the most effective, as it eliminates the costs of buying organic carbon sources (John et al., 2011). However, optimal production of algal biomass in autotrophic culture is affected by self-shading at high cell densities and various other environmental factors. These are discussed in the following sections.

1.2.2.1 Light

The autotrophic production of algae involves the growth in presence of radiant energy sources to allow the organisms to grow. The autotrophic production of metabolites by algae is dependent on the light intensity. Limited supply of light due to self-shading at increasing cell densities or due to the use of deep vessels leads to limiting photosynthesis, resulting in lower accumulation of biomass (Sorokin & Krauss, 1958; Tilzer & Dubinsky, 1987; Eilers & Peeters, 1988; Yeesang & Cheirsilp, 2011). The autotrophic production of algae in commercial set up is challenged by the inefficient penetration of solar or artificial light into the culture, leading to limited algal biomass production (Qiang & Richmond 1996; Singh et al., 2011). Liang et al. (2009) observed that the production of *C. vulgaris* under autotrophic conditions leads to limited algal biomass and low lipid production in comparison to the production under heterotrophic conditions. This will be discussed in greater detail in section 1.2.3.

1.2.2.2 Temperature

One of the environmental factors affecting algal biomass production is the prevailing temperature, which influences the growth rate. Temperature influences the activity of the

enzymes, which in turn affects the rate of metabolic reactions in the algal cells. Temperature ranges that facilitate optimum metabolic reaction rates specific to each organism lead to higher algal biomass growth (White et al., 1991; Hanagata et al., 1992; Renaud et al., 2002; Xin et al., 2011). The optimum temperatures allow for accumulation of biomass and subsequent switch to lipid production in the plateau phase (Juneja et al., 2013).

However, the increase in temperature beyond the optimum level results in the decline in algal growth (Roleda et al., 2013). Temperatures above the optimum level may affect the growth rate of algae by interfering with the algal protein accumulation ability. Protein metabolism in *Phaeodactylum tricornutum* and *Scenedesmus* sp. is reported to be higher at lower temperatures (Ras et al., 2013). The most likely mechanism of damage to metabolism at high temperatures are protein conformational changes and their ultimate denaturation at very high temperature (Hazel, 1984; Yamane et al., 1998). A study involving *Botryococcus braunii* showed that the intracellular lipid synthesis is lowered to about 5% dry weight when the temperature exceeds the organism's optimal growth temperature of 32 °C (Juneja et al., 2013). A similar reduction in the lipid content was observed in *C. vulgaris* biomass grown at supra-optimal temperatures (Sharma et al., 2012).

Reduction in temperature also causes decreased biomass (and lipid) accumulation, while the level of unsaturated fatty acids in the lipids tends to increase (Tonon et al., 2002; Guschina & Harwood, 2006; Rodolfi et al., 2009; Xin et al., 2011). It is suggested that the increase in the level of unsaturation is a response, which evolved to maintain the fluidity of the cell membrane. This metabolic adjustment is believed to be initiated by the decline in temperature (Holton et al., 1964; Renaud et al., 1995; Xin et al., 2011).

1.2.2.3 pH

The metabolic and physiological activities of living organisms are affected by the pH of the medium. The pH exerts its effects mainly by affecting the activity of the biological catalysts and by alteration of the ionic properties of biomolecules (Anderson & Domsch, 1993; Smolders et al., 1994; Lee et al., 2002). However, the tolerance of various organisms to pH varies, hence there is a need to understand the pH tolerance of the algal strain to be used in lipid production (Goldman et al., 1982; Visviki & Santikul, 2000; Solovchenko & Khozin-Goldberg, 2013). The pH of the growth medium affects the algal cells through the alteration of nutrient availability and interference with cell metabolism (Juneja et al., 2013). High biomass densities are normally obtained at a pH that is around neutral. It is, however, important to note that the optimum pH of the algal cells depends on the initial pH at which the organism was introduced into the media (Bhola et al., 2011). The effect of pH on the attainment of required algal biomass densities is associated with induction of metabolic inhibition. Nutrient uptake by heterotrophic algae is affected by acidic pH. A study carried out by Visviki and Santikul, (2000) on the growth of *Chlamydomonas applanata* under different pH conditions indicated that the algae do not grow at pH between 1.4 to 3.4 but maximum growth is observed at pH 7.4. However, pH preferences of different algal species vary, with some species including *Chlorella saccharophila* and *Euglena mutabilis* being able to tolerate acidic environments. The ability of the acid tolerant algal species to withstand low pH is associated with their ability to maintain constant intracellular pH. Reduction in the pH has also been observed to influence the composition of the algal biomass. A study on the effect of low pH on *Chlamydomonas* sp. showed that this organism accumulates more lipids at low pH (Sharma et al., 2012).

1.2.2.4 Salinity

Algae can thrive in environments with varying salinity level. The freshwater algae inhabit water with little or no salts. The establishment of algae in fresh water offers the advantage of increasing lipid production through the deprivation of nitrogen and phosphates (Khozin-Goldberg & Cohen, 2006; Widjaja et al., 2009; Xin et al., 2010; Yang et al., 2011). Some algae such as *Nannochloropsis oculata* are known to thrive in marine environments with varying concentration of salts. The culturing of algae in marine water minimizes the nutrients required in the production process (Pedersen & Borum, 1996).

The biochemical composition of the algal biomass is also affected by the concentration of salts resulting in the change in the growth rate of the biomass. It is indicated that the composition of lipids in the algal biomass is higher in conditions of high salinity. Various studies involving *Botryococcus braunii* and *Dunaliella tertiolecta* have shown that an increase in the level of NaCl in the media results in high accumulation of lipids (Sharma et al., 2012). However, other studies have indicated the opposite: that an increase in the salinity level results in reduced lipid accumulation and decline in the growth rate (Bartley et al., 2013). It is, therefore, important to establish the optimum salinity level for candidate algae to be used in the production of lipid for biodiesel.

1.2.2.5 Nutrients

The main macronutrients that are important for the autotrophic growth of the algal biomass are nitrogen and phosphate. Nitrogen is an essential nutrient that is required for the synthesis of algal macromolecules including the essential enzymes. However, limitation of nitrogen in algal culture leads to enhanced production of lipids (Stephenson et al., 2010) and low growth rate. Therefore, for commercially viable production of biodiesel, the level of nitrogen

should be regulated to ensure the initial attainment of high algal biomass, followed by high accumulation of lipids. Regulation of the amount of phosphorus applied should also be considered, since the element is necessary for algal growth. At low concentrations of phosphorus, the volume of lipids accumulated by the algal cells is high. Reduction in the initial phosphorus concentration from 2.0 to 0.1 mg/L has been shown to lead to an increase in the accumulation of lipids by *Scenedesmus* sp. from 23% to 53% (Xin et al., 2010).

The availability of trace metals such as iron, cobalt and nickel also affects the metabolic and behavior of algal biomass. The trace metals are required for growth of algal cells; however, growth inhibition occurs in the presence of high concentrations of the trace metals in the media. Metal toxicity can occur through the interference with the nutrient uptake efficiency (Monteiro et al., 2012).

It is evident that environmental and nutrient factors influence algal biomass in autotrophic environments. The development of high-density biomass for the production of biodiesel is dependent on how the different conditions are controlled to fit the algal species or genetic strain being used. In addition to manipulating autotrophic conditions for biomass and lipid production, it was found recently, that adding heterotrophic nutrients to the medium can

1.3 Algal heterotrophic and mixotrophic metabolism boost for increasing lipid production

Culturing of algae in heterotrophic conditions involves the provision of organic carbon nutrients, usually as soluble sugars, in the algal growth media (Mitra et al., 2012). In this section, an overview of the various substrates is presented, with specific focus on the substrates that may be used to boost lipid production. Discussion on the importance of cellulase enzymes in the utilization of lignocellulosic substrates by algae will also be presented.

The biomass yield of *Chlorella protothecoides* is increased by more than three times when grown under heterotrophic conditions through the provision of corn powder hydrolysate (Heredia-Arroyo et al., 2010). Growth of *C. vulgaris* is high under heterotrophic conditions in presence of acetate substrate, also leading to enhanced fatty acids production (Mitra et al., 2012). Despite the potential of heterotrophic conditions to enhance algal growth and subsequent biodiesel production, the costs associated with the inclusion of organic carbon can be prohibitive. There is, therefore, a need to evaluate other less costly alternatives. Liang et al. (2009) reported conflicting results that indicated that glycerol inhibits the heterotrophic growth of algae. The use of wastewater as a carbon source has been reported to reduce the costs associated with the heterotrophic production of algae (Li et al., 2011). The use of wastewater may be preferred because of its high nutrient content and the added benefit of reducing pollution caused by wastewater. The life forms present in wastewater have been indicated to have mutual dependency with algae leading to the enhanced algal growth (Li et al., 2011). The challenge with the use of wastewater for culturing of algae is associated with the high likelihood of contamination with undesirable organisms and the cost associated with its sterilization (Tercero et al., 2014). The possibility also exists that a temporary presence of a waste material toxic to algae may destroy the entire algae-dependent process.

Various approaches can be used to boost the production of lipid by autotrophically grown algae using heterotrophic substrate additives. In this section, the potential use of various carbon-based substrates such as glucose, carboxymethyl cellulose, solid cellulose and paper mill sludge as boosts for lipid production is discussed.

1.3.1 Glucose

The fact that glucose contains high energy chemical bonds makes it the preferred, albeit expensive, candidate for use in algal cultures. Incorporation of glucose into the algal growth media is associated with increased growth and respiration (Morales-Sánchez et al., 2013). Glucose is shown to enhance the physiological changes in algae such as *C. protothecoides* (Xiaoling et al., 2004) and *C. vulgaris* resulting in alteration of the metabolic pathways responsible for mediating the densities of energy stores such as the lipids. The glucose taken up is broken down and used to produce acetyl-CoA that does not enter the TCA cycle, but is fed into the lipid anabolic pathways. The added glucose enters the cells via hexose transport system into the pentose phosphate pathway that yields high quantities of NADPH. This also contributes to the overproduction of lipids (Wasylenko & Stephanopoulos, 2015). The pentose phosphate pathway likely occurs in the cytosol of the algal cells and depends on the concentration of glucose in the media (Klein, 1986). It is indicated that for *Chlorella sorokiniana* under heterotrophic conditions and when glucose is used as the sole source of carbon, pentose phosphate pathway accounts for close to 90% of the metabolism (Huang et al., 2016). However, Huang et al. (2016) did not study the localization of the pathway.

Liang et al. (2009) indicated that *C. vulgaris* grown in a culture amended with a high concentration of glucose produced high concentration of lipids, but do not offer a mechanism for this. Similarly, Miao and Wu, (2006) show that an addition of 10 g/L of glucose to the growth media results in an increase in the production of lipid by (Auxeno)*Chlorella pyrenoidosa* from 15% to 55%. These authors propose that the increase was due to increased cell densities. A study by Morales-Sánchez et al. (2013) reported enhanced biomass density of *Neochloris oleoabundans* grown in heterotrophic conditions with sole use of glucose as the carbon source.

The incorporation of glucose to the media affected the level of lipids produced by the *C. pyrenoidosa* in dose response manner (Bajwa et al. 2016). These researchers observed that the organism grown in absence of glucose produced nearly 5% total lipids, while the organism grown in 0.1% glucose produced twice as much lipids per dry weight. An increase of glucose concentration in the media to 1.0%, 1.5%, and 2.0% resulted in increased production of lipids to 11.7%, 12.1% and 14.1% of dry weight, respectively. These authors did not explain mechanism of the observed enhancement. As indicated by Bajwa et al. (2016), the timing of glucose addition and the concentration that is added to the culture media should be regulated, since high concentrations can result in end-product inhibition of the anabolic pathways and subsequent reduction in algal biomass and production of biodiesel. Therefore, to achieve a balance between biomass yield and lipid production, there is a need to tightly regulate the addition of glucose to the media to ensure proper timing and concentration.

1.3.2 Carboxymethyl cellulose

Carboxymethyl cellulose is an industrial polymer that has a highly crystalline, yet water soluble structure (Camacho et al., 2001). The polymer is a derivative of cellulose synthesized through a chemical combination of sodium hydroxide and chloroacetic acid. This form of cellulose was suggested to boost the production of lipids by heterotrophic algae by suppressing the damage of the algal cells by hydrodynamic stress (Camacho et al. 2001). These authors indicated that carboxymethyl cellulose has the potential to suppress cell damage to *P. tricornutum* through the addition of not more than 0.02% of carboxymethyl cellulose per weight. They also suggested, without convincing evidence, that carboxymethyl cellulose has no physiological effects on the algal biomass. Further discussion of the potential role of

carboxymethyl cellulose and cellulose in algal metabolism is offered below, and forms the basis of my thesis.

A potential way of utilizing carboxymethyl cellulose to boost lipid production by heterotrophic algae is by introducing a second organism capable of breaking down the polymer substrate to simple sugars (Shaobai et al., 2010). Breakdown of the polymer is achieved through the use of cellulase enzymes such as exoglucanases and beta-D-glucosidase. Microorganisms that have the capacity to breakdown the polymer include filamentous fungi such as *Aspergillus* and *Trichoderma* sp. The synergistic interaction (in co-culture system) between the algal cells and the other organism in the breakdown of carboxymethyl cellulose may enable the algal biomass to utilize the sugar produced to enhance lipid production (Shaobai et al., 2010).

1.3.3 Cellulose

The utilization of cellulose in the heterotrophic production of lipids is challenged by the crystalline and insoluble nature of the substrate. For cellulose to be utilized, there is a need for it to be depolymerized to monomeric sugars that can easily be used by the algal cells (Menon & Rao, 2012). Depolymerization of cellulose occurs with the help of several cellulase enzymes such as endo-1,4-β-glucanases, exo-1,4-β-glucanases (cellobiohydrolases) and β-glucosidases (Horn et al. 2012). Initially, with the action of endo-1,4-β-glucanases, cellulose polymer degrades into dimeric or trimeric form of glucose molecules. The dimeric form of cellulose, known as cellobiose will then be hydrolysed by exo-1,4-β-glucanases (cellobiohydrolases) and β-glucosidases resulting in simple sugars (glucose monomers) (Yoshida M et al., 2004).

The breakdown of cellulose to simple sugars also depend on the porosity of the substrate. The porosity of cellulose is determined by the surface area that is accessible to the enzymes and

is a function of its crystallinity (Blifernez-Klassen et al., 2012). These authors have provided the first evidence that a photosynthetic alga *Chlamydomonas reinhardtii* has the ability to utilize not only the soluble carboxymethyl cellulose, but also cellobiose and cellulose from filter paper as substrates for growth mediated by depolymerization of these substrates. This thesis attempts to expand on these findings, and investigates the potential to use industrial pulp mill waste as a substrate for algal growth.

1.3.4 Pulp mill sludge (Waste fiber from industry)

The sludge from the pulp mill industry is mainly composed of high amount of short cellulose fibers alongside other waste, such as microbial waste and inorganic residues of the pulping process (Tarlan et al., 2002). Tarlan and colleagues investigated the ability of algae to treat wood-based pulp. The high cellulosic fiber content and low heavy metals in the pulp mill sludge are the main differences compared to municipal sludge. The addition of pulp mill sludge to the heterotrophic algal biomass boosts the production of lipids by presumably releasing inexpensive carbon sources that are used by the algae to process the lipid precursors (Tarlan et al., 2002). Some of the simple sugars that are produced from the pulp mill sludge are glucose and xylose. The use of glucose by the various algal species is widely discussed. However, there is limited information on the utilization of xylose by algae to boost biomass density.

The use of paper pulp sludge in boosting the production of lipids by heterotrophic algae is limited by the need for additional pretreatment stages such as physical, biological processes such as enzymatic degradation (Tarlan et al., 2002). The byproducts of pulp mill sludge break down such as aliphatic and aromatic acids and ketones inhibit the growth of algal biomass (Herman et al., 1990), posing further challenges to the use of paper sludge. It is, therefore, important to evaluate the effect of the by-products of pulp mill sludge on the algae being used

before being added to the media. Future studies should also evaluate means of actively removing the byproducts from the media that cause algal growth inhibition.

The fact that most algae do not utilize xylose is a limitation in the use of cellulosic fiber found in the pulp mill sludge (Zheng et al., 2014). Research efforts should, therefore, focus on boosting the use of this substrate source by bio-prospecting for algal species that can autotrophically metabolize xylose (Hawkins, 1999; Liang, 2013). Future studies should also look into ways of altering the genetic makeup of existing strains to enhance their xylose-metabolizing ability under heterotrophic conditions. Synergistic interactions of heterotrophic algae with other organisms capable of breaking xylose down into sugars that can easily be used by the algal biomass in the production of sugars should also be assessed.

1.4 Mixotrophic utilization of cellulose: importance of cellulase enzyme:

The utilization of cellulose is largely dependent on the presence of a degradation system that is efficient and cost effective (Menon & Rao, 2012). However, little is known about cellulose degradation by algae. This section will therefore be based on the understanding of fungal and bacterial cellulases. Cellulase enzymes degrade cellulose through the cleavage of the β-1,4-glycosidic linkages at random sites in the cellulose chain or by the removal of oligosaccharides of particular length from one end of the cellulose chain (Goyal et al.,1991; Mandels & Reese, 1999). The eventual product of cellulose degradation is glucose that is produced from the conversion of cellodextrins by enzymes such as β-glucosidases and cellodextrinases (Gilbert & Hazlewood, 1993; Bayer et al., 1998). The efficient degradation of cellulose by microbial enzymes requires that the environment have optimum conditions of pH and temperature (Ghose, 1987; Reinikainen et al., 1995). Microbes with high cellulolytic action can form a synergistic relationship with an organism that has low levels of cellulolytic efficiency

resulting in complete breakdown of cellulose. Horn et al. (2012) indicate that the synergistic breakdown of cellulose results in higher specificity compared to the action of the cellulase from a single microorganism. Based on the discussion, it is possible that algae too posses cellulase enzymes capable of degrading cellulose biomass. This assumption is based on the fact that *Chlamydomonas reinhardtii* has been shown to heterotrophically grow in cellulose containing medium, suggesting that the organism has the ability to utilise the cellulose (Chen & Johns, 1994; Blifernez-Klassen et al., 2012).

2. Materials and Methods

2.1 Algal strains and storage

I used two algal species, isolate W7 collected from pieces of wood collected near Thunder Bay, Ontario, Canada and for comparison, the known lipid producer *Auxenochlorella pyrenoidosa* (abbreviated later as AP) (H. Chick) Molinari & Calvo-Pérez (Li et al., 2011; Kothari et al., 2012; Han et al., 2013; Calvo-Pérez Rodó & Molinari-Novoa, 2015). The standard algal medium (Sigma-Aldrich, St. Louis, MO, 17124) used in this study contains $NaNO_3$ (1 g/L), K_2HPO_4 (0.25 g/L), $MgSO_4$ (0.513 g/L), NH_4Cl (0.05 g/L), $CaCl_2$ (0.058 g/L), and Murashige and Skoog micronutrients (Murashige & Skoog, 1962). Carboxymethyl cellulose (1g/L) was used routinely to induce cellulase activity (Trivedi et al., 2013). Filter-sterilized carbendazim (5 mg/L) and Kasugamycin 100X stocks were added to the cooling medium to a final concentration (0.1 g/L) to prevent fungal and bacterial growth, respectively. Kasugamycin was prepared in DMSO, giving final medium concentration of 1%. The algal cultures were grown at 21 ±2 °C. Stock cultures were maintained on semi-solid agar (1.5%).

2.2 Reagent sources

Chemicals, including glucose were from Fisher Scientific (Mississauga, ON) or Sigma-Aldrich (St. Louis, Mo). Pure cellulose was a Bleached Softwood Kraft Pulp provided by AV Aditya Birla paper mill in Terrace Bay, ON, Canada and the primary paper sludge was from the same source.

2.3 Long-term cultures and Short-term cultures

Long-term cultures

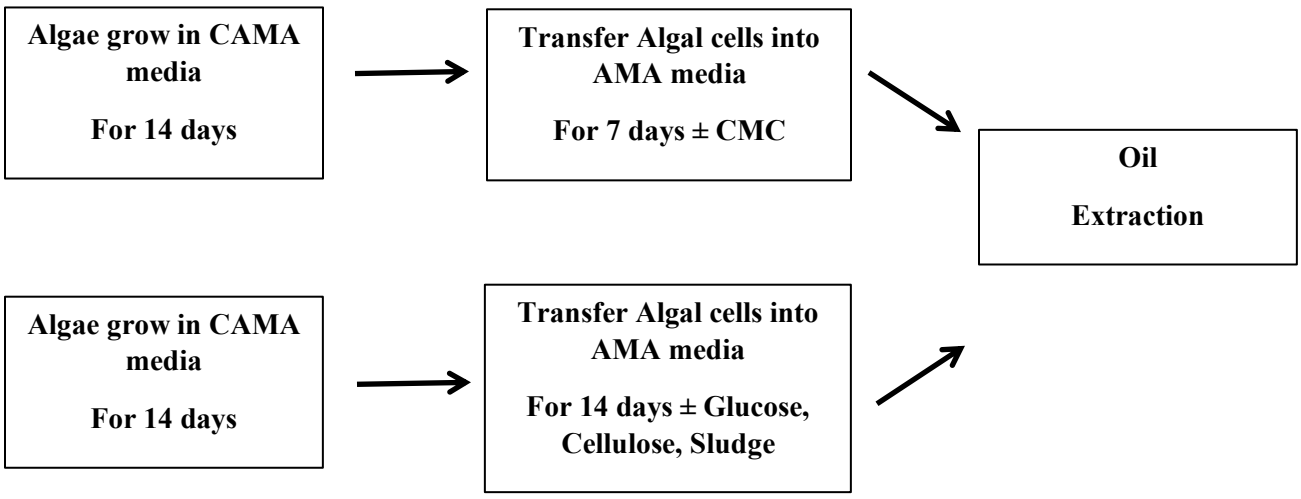
I prepared long-term (60 day) cultures to establish the ability of the algal isolates to degrade filter paper. A volume of 20 mL of algal medium was added to 9cm glass Petri dishes

with fibre glass (Whatman GF/A) or cellulose filter paper (Reeve Angel grade 202 filter paper, Whatman, Inc.) with circular inert plastic 4cm diameter support used to bring the filter paper surface into the air. A total of 1.2 x 10^6 cells was inoculated on the air-exposed surface and cultured for 60 days.

Short-term cultures

The AP and the W7 cells (1.2 million cells per mL) were inoculated into 250 mL Erlenmeyer flasks containing 20 mL of the sterile liquid medium from 2 weeks-old liquid stock cultures. During the three weeks of incubation, the light intensity under 4 fluorescent tubes was 30 µmoles/m^2/sec.

The substrates: A stock solution of 0.1g/mL glucose was prepared and autoclaved at 121 ^oC for 20 mins. From the stock solution 0.4mL of glucose was added 20mL of media (final concentration 0.2% w/v) after 2 weeks of incubation. In remaining experiments, un-autoclaved solid cellulose or sludge were added (0.1g/20mL, to a final concentration of 0.5%) to the media after 2 weeks of incubation.



2.4 Bradford protein assay protocol

This experiment was done based on the protocol of Bradford, (1976). I transferred 160 µL of each sample solution, which was prepared by ultrafiltration of the 40 mL of media on Millipore NMWL membrane, 5000 mol. wt. cut off to dryness and concentrate resuspended in 4 mL of 0.1mM pH 5.5 sodium acetate buffer and used immediately - 160 µL plus 40 µL of dye reagent concentrate (BioRad). The sample and reagent were mixed thoroughly by pipetting up and down. The plate was incubated at room temperature for 15 minutes prior to measuring the absorbance at 595 nm against appropriate BSA standard.

2.5 Cellulase assay and DNS reaction to measure glucose production

Enzyme reaction mixture consisted of 800 µL of active or boiled (5min) enzyme, 800 µL of 0.1mM pH 5.5 sodium acetate buffer with or without 1% CMC and incubated at 42℃. Triplicate aliquots of 60 µL were withdrawn hourly, from 0 to 3 hours, into a 96 well PCR plate. The reaction was stopped by freezing at -80°C.

The conversion of cellulose to glucose by the algal cultures was measured using the DNS reducing sugar assay (Markou et al., 2013; Kim et al., 2014). The assay works by evaluating the amount of the D-glucose based on the principle that the reducing sugars have the ability to reduce 3,5-dinitrosalicylic acid (DNS). The reduction of DNS by glucose results in the formation of 3 amino 5 nitro salicylic acid, accompanied by a color change (Saqib & Whitney, 2011). An aliquot of 120 µL of DNS reagent was added to each well with thawed enzyme sample or standard glucose and brought to 90°C for 5 minutes in PCR cycler (S1000™ Thermal Cycler,

Bio-Rad), cooled and 120 µL transferred to microplates for absorbance determination at 540nm (xMark™ Microplate Absorbance Spectrophotometer, Bio-Rad).

2.6 Lipid extraction and purification protocols

Lipid extraction and determination Method I

The algal cultures were centrifuged at 4000 RPM at 15°C for 30 min in pre-weighed centrifuge tubes. The centrifuge tubes containing the final algal pellet were placed in an oven at 90°C for 24 hours to weigh the dry mass of cells. The pellet was extracted with 10mL of methanol, followed by 5mL of chloroform. The samples were homogenized using a homogenizer (Ikka Werke - Finland) for 5 minutes. The homogenate was filtered through Whatman No. 1 filter paper on a Coors No. 3 Büchner funnel with light suction. Then, the filtrate was transferred into a 125mL separatory funnel after rinsed the previous beaker using 9 mL of water and 5 mL chloroform. Then the wash was added to the extract for a final solvent ratio of 2:2:1.8 (chloroform: methanol: water). The mixture in the funnels was thoroughly mixed by inverting the funnel multiple times and letting it settle for 2 hours. The bottom layer made up of chloroform is poured into a 20mL pre-weighed beaker. A small volume of the chloroform layer was left behind to prevent the collection of the top layer, which may lead to the possible contamination of the lipids. Fifty µg/mL of olive oil solution was used as the standard stock.

The samples and standards were kept in a water bath at 80 °C, followed by freezing for 3 minutes and added 100 µl of H_2SO_4 and mixed in a vortex mixer. I placed them again in a water bath at 90 °C and cooled for a few minutes. Then, transferred 50 µl of the reaction mixture to a 96-flat bottom well microplate. The background absorbance was read at 540 nm. Further, 50 µl of vanillin–phosphoric acid reagent (0.2 mg vanillin per ml 17% phosphoric acid) (1:1

phosphovanillin: rxn mixture) was added to each well and allowed 10 min for color developing. After developing, the absorbance was read at 540 nm once again.

Lipid extraction and determination Method II

A new method was developed to determine the concentration of lipid and chlorophylls in algae extracts simultaneously, using DMSO as extractant. I centrifuged cultures at 4000 rpm for 15 minutes, then removed the supernatant and added 5mL of DMSO to the pellet and homogenized the mixture on Turrax/Tissue Mizer (Ikka Werke, Finland) for 30 secs, centrifuged them at 4000 rpm for 15 minutes, then removed the supernatant. This was followed by an additional wash with 5mL of DMSO (as above) for a total of 10 mL of extract. Olive oil (1 mg/mL) in DMSO was used as a standard. I transferred 50 µl of each sample or standard into 10 x 75mm glass tubes and added 150 µL water and 5 µL Nile Red (5 µg/ml) to each tube. I then transferred 160 µL from each tube into microplate. The fluorescence at 570 nm following excitation at 530nm was measured using microplate fluorometer (BMG FLUOROstar Optima microplate reader). The amount of chlorophylls in the extract was determined immediately after extraction using Varian Cary 50 [California USA] spectrophotometer, using the following equation (Barnes et al., 1992):

$$\text{Total chlorophylls } (\mu g/mL) = 7.49 \times A_{665}\text{ nm} + 20.34 \times A_{648}\text{ nm}.$$

3. Results

3.1 Long-term cultures

Long-term cultures were prepared to evaluate the capability of the isolates to grow on cellulose filter paper compared to fiberglass (Fig. 1). Following an incubation period of 60 days, long-term cultures of isolate W7 was observed to grow on both, the fiberglass and cellulose filter. Similar results were also observed for *A. pyrenoidosa*: growth was evident on both, cellulose and fiberglass (Fig. 1). The cells did not colonize the substrate (i.e. did not attach themselves strongly), and were relatively easy to remove.

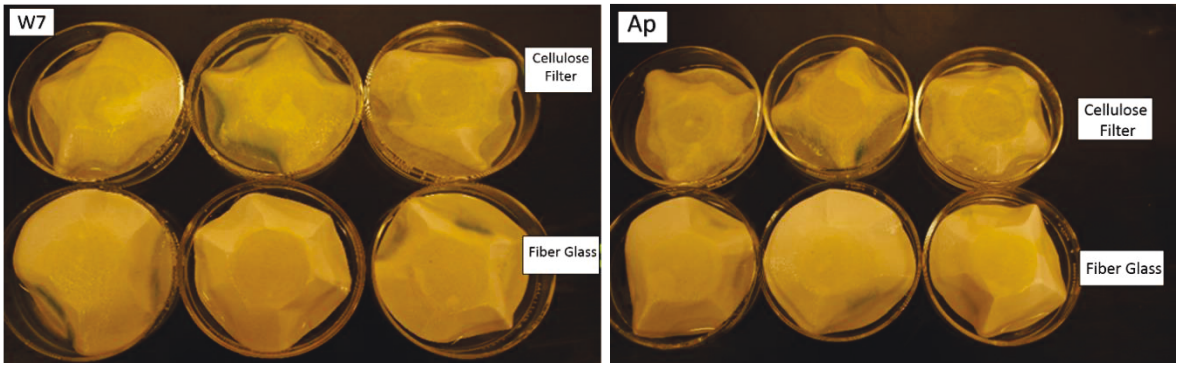


Figure 1: Long term cultures; The isolate W7 (left panel) and *A. pyrenoidosa* (Ap, right panel) inoculated on cellulose filter (top-row) and fiberglass (bottom-row), Both were grown for 60 days at 21±2 ℃.

3.2 Bradford protein assay

The Bradford assay (Bradford,1976) was carried out to determine the protein in concentrated media of isolates W7 and *A. pyrenoidosa* after 14 days in culture. Isolate W7 concentrate contained a higher protein concentration (0.031 mg/ml) as compared to the concentration in *A. pyrenoidosa* culture media concentrate, which was measured to be 0.024 mg/ml. The standard deviation was <5% of the mean value. The differences between the two

cultures may be in part due to differences in extracellular protein production by the two organisms, but are in part due to the different degree of concentration of the media.

3.3 Cellulase assay

There was no statistically significant difference between the four treatments (Fig 2). The concentration of sugars present in *A. pyrenoidosa* and W7 assays without additional CMC remained constant throughout the experimental period (0.38 µg/µl and 0.39 µg/µl respectively, Fig. 2, open symbols).

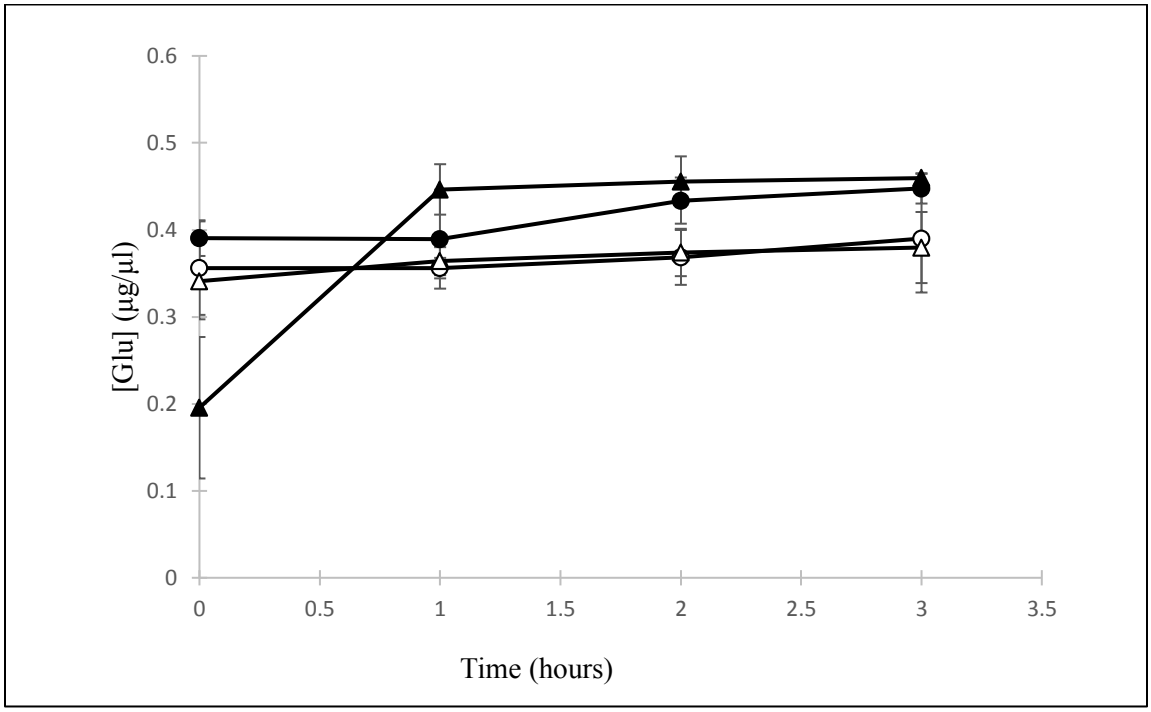


Figure 2: Activity of *A. pyrenoidosa* and isolate W7 algal extracellular cellulase enzyme concentrated by ultrafiltration tested over 3 hours. The amount of reducing sugar (glucose) produced by cellulase from two organisms (W7, circles), (AP, triangles) with 0.8% of CMC added after 2 weeks of culture (solid symbols) and not added (open symbols). Average of 3 experiments done in duplicate.

The addition of 0.8% CMC to the *A. pyrenoidosa* media apparently increased the amount of reducing sugar produced between 0 to 1 h of incubation. However, considering the large

standard deviation, i.e. variability in the data, there was no significant increase in reducing sugar yield over the 3 hour incubation (Fig. 2, solid symbols) for both organisms. A slightly perceptible, but statistically not significant, increase in glucose generation was detected when additional 0.8% CMC was present (Fig. 2, solid symbols). Further investigation of reducing sugar production from both organisms beyond 3 h might show statistically meaningful glucose generation from CMC. The specific activity of the enzyme concentrates was calculated to be about 0.012 µg glu/µg protein/3 hrs in both *A. pyrenoidosa* and isolate W7 supplemented with CMC.

3.4 Lipid extraction and quantification

3.4.1 Effect of the addition of CMC

As shown in Fig. 3, isolate W7 grown in CAMA media amended with 0.8% CMC produced higher concentration of lipids (3.5 mg/4mL) as compared to isolate W7 grown in CAMA alone, (3.1 mg/4mL). In a similar trend, *A. pyrenoidosa* grown in CAMA also produced lower concentration of lipids (1.6 mg/4mL) as compared to *A. pyrenoidosa* incubated in CAMA medium amended with 0.8% carboxymethyl cellulose (2.3 mg/4mL). The effect of adding more CMC later was not as pronounced as in CAMA medium experiments for isolate W7, but a slight, statistically not significant, improvement was observed in the case of *A. pyrenoidosa* algae regarding lipid production. Due to the co-extraction of chlorophyll in this method, an alternative was developed, which separately determined chlorophyll and lipid content.

The increase observed following growth in additional 0.8% CMC were statistically not different from the controls. However, as is apparent from the overlap in standard deviations (Fig. 3). Student's t-test (not shown) confirmed the lack of statistical differences between treatments. Isolate W7 as compared to *A. pyrenoidosa* appeared to contain more lipid (using Method I).

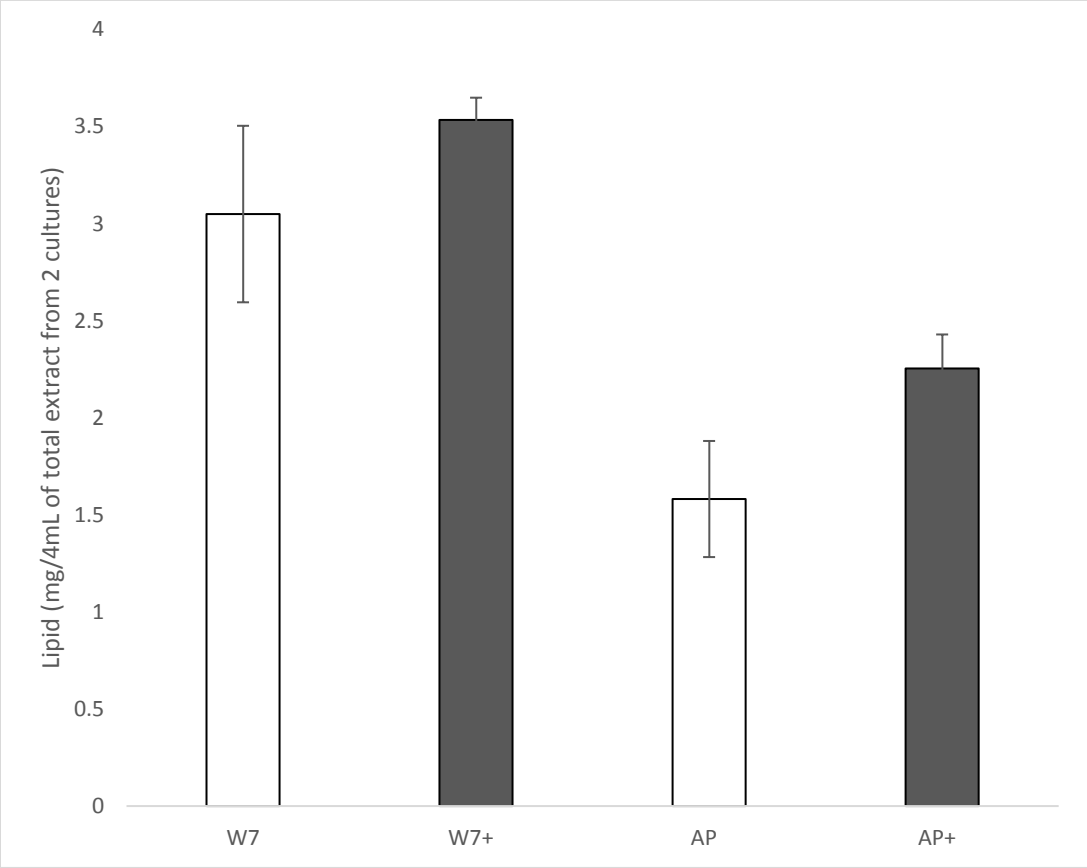


Figure 3: Lipid production by isolate W7 and *A. pyrenoidosa* cultures grown in CAMA media (open bars) and further addition of 0.8% of CMC on day 14 of growth (solid bars) followed by one week of growth. (Error bars represent standard deviation from average of 3 experiments run in duplicate).

3.4.2 Effect of the addition of glucose

The effect of added glucose on lipid content (using lipid assay II) of isolate W7 and *A. pyrenoidosa* is shown in (Fig 4). *A. pyrenoidosa* incubated in glucose-amended medium was observed to produce a higher concentration of lipids (14.8 µg lipid/µg chlorophyll) compared to control (1.2 µg lipid/µg chlorophyll), or about 12 fold increase. In contrast, isolate W7 responded by a 20-fold increase (0.7 µg lipid/µg chlorophyll to 14.3 µg lipid/µg chlorophyll, Figure 4).

A t-test analysis showed that amendment of media with glucose significantly improved the yield of lipid in both organisms.

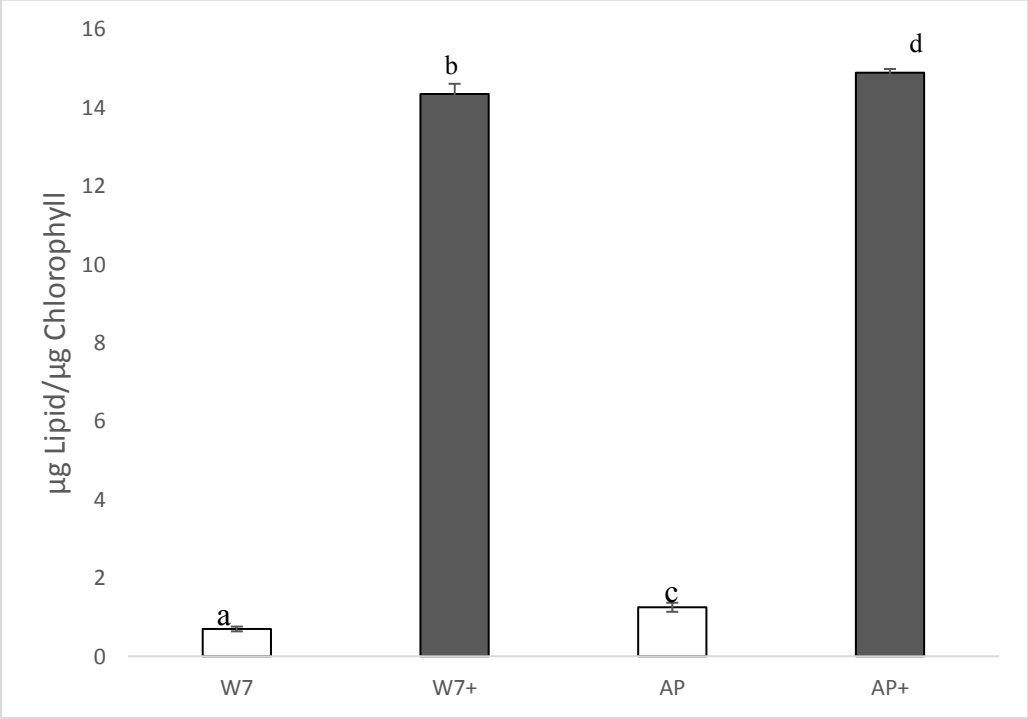


Figure 4: Lipid production by isolate W7 and *A. pyrenoidosa* (AP) cultures when grown in regular media (open bars) and when boosted with 0.2% of glucose on day 14 of growth (solid bars) followed by an additional week of growth. (Average of 3 experiments with duplicates in each, error bars represent standard deviation). Letters a,b,c,d indicate statistically significant differences between the means of lipid/chlorophyll ratio based on a t-test ($p < 0.05$). Columns with the same letter are not statistically significantly different from each other.

3.4.3 Effect of the addition of pure cellulose

A. pyrenoidosa produced the highest concentration of lipids (7.2 µg lipid/µg chlorophyll) compared to isolate W7 (1.86 µg lipid/µg chlorophyll) in response to added cellulose at 0.5% (Fig. 5). This represents nearly 7-fold increase for *A. pyrenoidosa* and about 3-fold increase for W7 (Fig. 5).

A t-test analysis was used for the pairwise mean comparison of lipid production by *A. pyrenoidosa* and isolate W7 by adding pure cellulose to the reaction media. The statistical analysis showed amendment with pure cellulose significantly improved the yield of lipid of both, *A. pyrenoidosa* and isolate W7. Production of lipid by W7 and AP was significantly different from each other without adding pure cellulose to the media (Fig. 5, open bars) and also when cellulose was added (Fig. 5, solid bars).

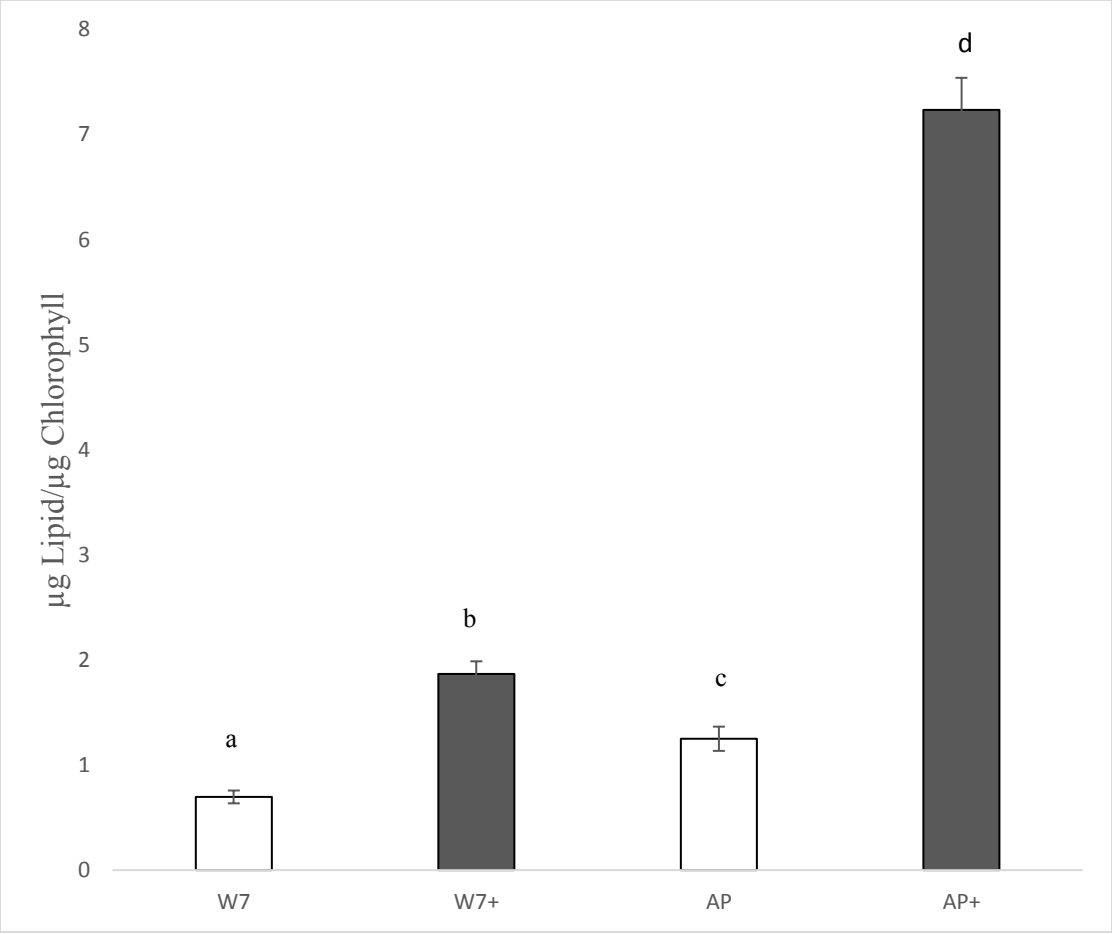


Fig 5: Lipid production by isolate W7 and *A. pyrenoidosa* (AP) cultures when grown in regular media (open bars) and when boosted with 0.5 % of pure cellulose on day 14 of growth (solid bars) followed by an additional week of growth. (Average of 3 experiments with duplicates in each). Letters a,b,c,d indicate statistically significant differences between the means of lipid/chlorophyll ratio based on a t-test ($p < 0.05$). Columns with the same letter are not statistically significantly different from each other.

3.4.4 Effect of the addition of paper sludge

A. pyrenoidosa incubated in paper sludge amended media was observed to produce the highest concentration of lipids (8.7 µg lipid/µg chlorophyll), about an 8-fold increase over the control (Fig. 6). Isolate W7 incubated in paper sludge amended media was also observed to produce a higher concentration of lipid (2.6 µg lipid/µg chlorophyll) - nearly four-fold compared to the control (Fig. 6).

A t-test analysis was used for the pairwise mean comparison of lipid production of *A. pyrenoidosa* and isolate W7 by adding paper sludge to the reaction media. Amendment of both *A. pyrenoidosa* and W7 cultures with paper sludge significantly improved the yield of lipid. Isolate W7 and *Auxenochlorella pyrenoidosa* treated with added sludge were significantly different from each other in terms of lipid production in response to 0.5% (w/v) paper sludge (Fig. 6)

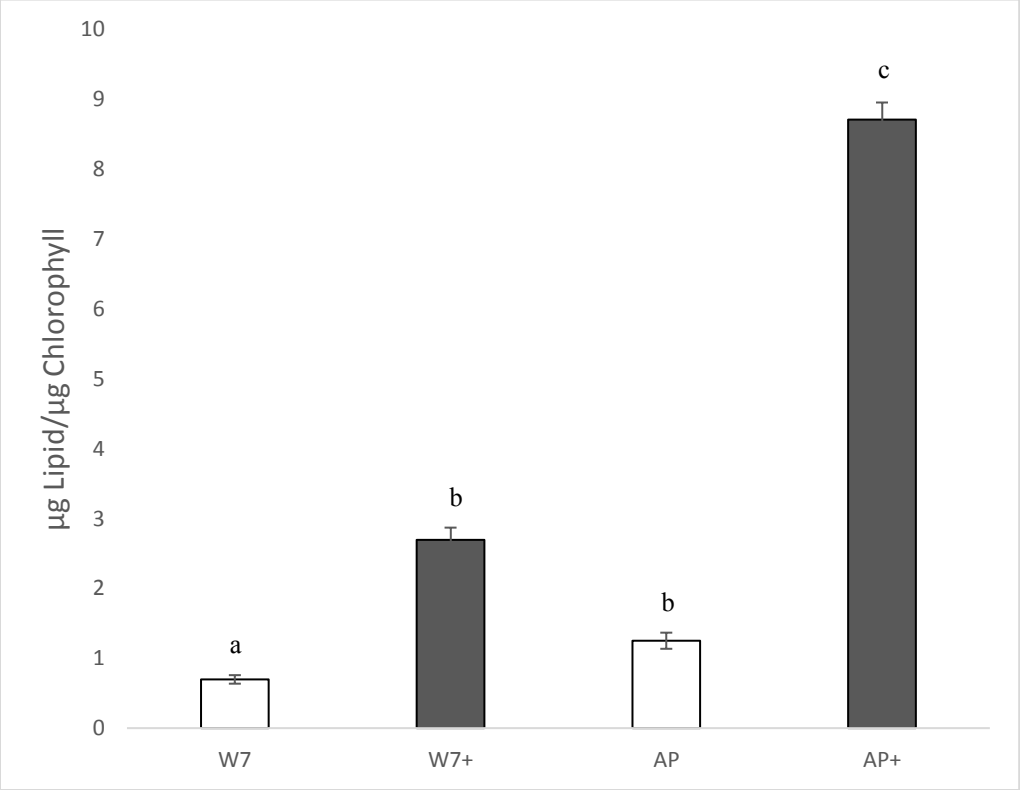


Fig 6: Lipid production by isolate W7 and *A. pyrenoidosa* (AP) cultures when grown in regular media (open bars) and when boosted with 0.5 % of paper sludge on day 14 of growth (solid bars) followed by an addition week of growth. (Average of 3 experiments with duplicates in each). Letters a,b,c indicate statistically significant differences between the means of lipid/chlorophyll ratio based on a t-test ($p < 0.05$). Columns with the same letter are not statistically significantly different from each other.

Table 1 summarizes the results of lipid production by isolate W7 and *A. pyrenoidosa* when glucose (0.2%w/v), pure cellulose (0.5%w/v), and paper sludge (0.5%w/v) were added to the culture medium. The amount of lipid produced relative to the chlorophyll content varied depending on the substrate used. Glucose addition yielded about triple the lipid content of *A. pyrenoidosa* per culture, but also depressed chlorophyll production (Table 1, Fig 4), resulting in about a 12-fold increase in lipid content per μg chlorophyll. A similar trend was observed in isolate W7 (Table 1, Fig. 4), but the lipid yield increase on a chlorophyll basis was even more pronounced resulting in about 20-fold increase in lipid content per μg of chlorophyll (Table 1). Again, this was partly due to the decrease in chlorophyll concentration (Table 1).

Addition of pure cellulose yielded about twice the lipid in *A. pyrenoidosa* cultures, but also depressed chlorophyll production (Table 1, Fig 5), resulting in about a 6-fold increase in lipid content per μg chlorophyll. Similar trend was observed in isolate W7 (Table 1, Fig. 5), but the lipid yield increase on a chlorophyll basis was about a 3-fold (Table 1).

Paper sludge addition yielded about twice the lipid in *A. pyrenoidosa* cultures, but also depressed chlorophyll production (Table 1, Fig 6), resulting in about a 7-fold increase in lipid content per μg chlorophyll. The same was observed in isolate W7 with paper sludge (Table 1, Fig. 6) so that lipid yield increase on a chlorophyll basis was about 4-fold based on μg of chlorophyll (Table 1). For isolate W7, addition of paper sludge yielded about 1.5 times the lipid as compared with the control.

Overall, *A. pyrenoidosa* was a much better producer of lipid than isolate W7, in all mixotrophic conditions used (Table 1) on a per culture basis. Chlorophyll concentration also

decreased in all mixotrophic situations in both organisms. The "boost" in lipid production was weaker in response to polysaccharides, compared to the monosaccharide glucose (Table 1).

Table 1: Lipid and chlorophyll production by *A. pyrenoidosa* (AP) and isolate W7 cultures under photoautotrophic (control) and mixotrophic (with glucose, pure cellulose and paper sludge) conditions. Errors represent standard deviation based on average of 3 experiments per culture run in duplicates.

		Control	**0.2 % w/v glucose**	**0.5 % w/v cellulose**	**0.5 % w/v sludge**
AP	lipid µg/10ml	584 ± 90.2	1782 ± 64.3	953.4 ± 2.6	973 ± 4.9
	chlorophyll µg/10ml	500 ± 70.7	120 ± 7.0	130 ± 7.0	110 ± 7.0
	lipid/chlorophyll **(µg/µg)**	1.2 ± 0.1	14.8 ± 0.1	7.2 ± 0.3	8.7 ± 0.3
W7	lipid µg/10ml	300 ± 70.8	1473.2 ± 18.2	354 ± 6.4	580.4 ± 29.5
	chlorophyll µg/10ml	420 ± 28.3	100 ± 14.1	190 ± 14.1	220 ± 14.1
	lipid/chlorophyll **(µg/µg)**	0.7 ± 0.1	14.3 ± 0.3	1.86 ± 0.1	2.6 ± 0.2

Values per 10mL represent lipid and chlorophyll content of the entire culture

4. Discussion

This study examines the ability of the newly isolated alga (isolate W7 growing on decaying wood) to produce lipids following the addition of various sugar sources. To compare the lipid production ability, *A. pyrenoidosa* was used because of its well-known ability to produce lipids. The ability of both algae to grow on cellulose filter paper and fiberglass was evaluated using long-term culture approach. After an incubation period of 60 days, *A. pyrenoidosa* and isolate W7 grew on both, the fiberglass and cellulose filter. Both organisms showed similar growth pattern on both, cellulose filter paper and fiberglass, suggesting that cellulose was not a preferred substrate compared to the inert glass (Fig. 1). The cells did not appear to attach strongly (i.e. form a coat or biofilm) to the cellulose filter paper, as would be expected in analogy to colony formation on decaying wood. However, the attachment may not be required for extracellular hydrolytic enzymes to act on potential woody substrates.

Both algae used in this study appeared to produce extracellular proteins with some capacity to degrade extracellular cellulose, in this case, CMC. There was no statistically significant difference in CMC-degrading activity (Fig. 3) between both the organisms. However, the protein was discharged into the media and could hydrolyze, albeit slowly, the substrate which confirms the cellulase activity. Subsequent experiments (longer time-course assays) demonstrate more convincingly the existence of CMC hydrolysis (Malek, pers. comm.). The specific enzyme activities of 0.012 µg glucose/µg protein/3hours in both isolate W7 and *A. pyrenoidosa*, respectively, are low compared to fungal cellulases produced by *Aspergillus flavus* BS1 (Sajith et al., 2016). Furthermore, the activity appears about 10x lower than that reported in Table 1 for *Chlamydomonas reinhardtii* by Bilfernez-Klassen et al., 2012. More precise specific activity value may be obtained over a longer assay period.

The degradation of complex carbohydrates into readily available monosaccharides is further indirectly confirmed in both, the isolate W7 and *A. pyrenoidosa* by the increased production of lipids. (Figs 5 and 6, Table 1). This response was not as strong as that to the monosaccharide glucose (Fig 4, Table 1) and as previously reported by Liang et al. (2009). The response to the various complex polysaccharides in terms of increased lipid production is discussed in subsequent paragraphs.

It appears that the addition of CMC leads to a higher production of lipids by isolate W7 compared to *A. pyrenoidosa* (Fig. 3). However, this analysis was based on an extraction and assay method, which combines lipid and chlorophyll, resulting in unreliable measure of lipid alone. The apparent higher lipid content of isolate W7 (Fig. 3) likely is due to high chlorophyll, which co-extracted with the lipid. Subsequent work shows that *A. pyrenoidosa* was a better lipid producer than isolate W7 in all conditions tested (Table 1). The inclusion of CMC in the growth media leads to the enhanced production of sugars by algae (Hong et al., 2012). In this study, the production of lipid by isolate W7 and *A. pyrenoidosa*, as a result of the addition of carboxymethyl cellulose, could be attributed to the ability of the algal isolate to produce carboxymethyl cellulase(s). CMC degrading enzymes have been indicated to aid in the release of sugars through the cleavage of the amorphous regions of cellulose and breakdown of cello-oligosaccharides to simple sugars (Klyosov, 1990; Béguin & Aubert, 1994; Zhou & Ingram, 2000). An enhanced lipid production due to more reducing sugar generated by the isolates from CMC could also be due to the protective effect of the substrate. Addition of CMC to the growth medium may prevent the algal cells from hydrodynamic stress that can damage the cells (Camacho et al., 2001). These authors observed that the amount of sugars produced by a given isolate depends on the number of viable cells present in the media. Low production of sugars by

the algal isolates grown in a medium that was not amended with carboxymethyl cellulose could be due to the reduction of the number of viable cells, which is attributed to cell damage. Hence, it is essential that the production of isolates W7 should be done in media in which CMC was added.

Highest lipid production by both algae investigated in this study was observed when glucose was added to the growth media (Table 1). The addition of glucose led to a higher production of lipids by the algal isolate W7 (14.8 µg lipid/µg chlorophyll) and *A. pyrenoidosa* (14.3 µg lipid/µg chlorophyll, Table 1) compared to the control concentrations at about 1 µg lipid/µg chlorophyll. Glucose addition has been reported to increase the metabolic activity of the algal cells, which resulted in enhanced growth and lipid production (Cheirsilp & Torpee, 2012; Xu et al. 2006). Similar studies demonstrating improved growth and lipid production were reported by several researchers (Krzemińska 2016; Liang et al. 2009). Glucose or external carbon effect might be attributed to the induction of hexose transport pathways, which play a key role in the production of lipids (Marshall et al., 1991). The detailed metabolic regulatory mechanism of the glucose effect needs to be elucidated by future work.

Chlorophyll content was observed to decrease in the presence of organic carbon. When the *Auxanochlorella pyrenoidosa* medium was supplemented with 0.2%w/v glucose the chlorophyll content decreased 4.2 fold (Table 1). Similarly, in the case of 0.5%w/v cellulose or sludge a 3.8 and 4.5 fold decrease in chlorophyll content was observed (Table 1). In the case of strain W7, the chlorophyll content decreased to 4.2, 2.2 and 1.9 fold in glucose, cellulose and sludge, respectively (Table 1). Similar, about 4.4 fold decrease in the chlorophyll content was reported in *C. vulgaris* when glucose was added to the media and this result clearly aligns with our findings (Kong et al., 2011; Smith et al., 2015). In a study on *N. oleoabundans* under strict

dark fermentation condition in the presence of glucose, the reduction in chlorophyll content was reported up to 2.3-3.5 fold (Morales-Sánchez et al.,2013), suggesting the sugar-induced chlorophyll loss may be more rapid in the presence of light. In contrast Cheirsilp and Torpee (2012) report a slight (15%) decrease in the chlorophyll content in mixotrophic condition, but nearly 90% decrease in dark, heterotrophic conditions. Under mixotrophic and photoperiodic conditions, the chlorophyll content of the cells may tightly depend on the duration of time that cells remain in light versus dark phase of the diurnal cycle (García, et al., 2005). The metabolic mechanism of chlorophyll degradation was studied in the heterotrophic culture of red alga *Galdieria sulfuraria*, where glucose was noticed to inhibit chlorophyll-a synthesis by partially blocking the transformation of a precursor molecule coproporphyrin III (Shugarman & Appleman, 1966; Stadnichuk et al., 1998; Feng et al., 2010). However, under mixotrophic condition inhibition on chlorophyll-a is reduced as light stimulates the production of coproporphyrin III.

Though the addition of glucose resulted in an enhanced lipid production (Table 1), investigation of the timing of glucose addition or glucose concentration was not my main objective. Rather, the addition of other substrates to the growth media was assessed. Cellulose addition to the cultures medium enhanced production of lipids by the two organisms (Fig. 5). This was presumably by hydrolysing cellulose (Fig. 2) making glucose available for the conversion into lipid. The results indicate (Figs. 5 and 6) that isolate W7 has a lower ability to use cellulose as compared to *A. pyrenoidosa*. It may be speculated that the genotype of both algae is responsible for the difference in lipid production. Each alga likely possesses a different and specialized enzyme subset to help in the breakdown of the substrate (Lynd et al., 2002). The ability of the algal strains to utilize cellulose is determined by their tolerance to lignocellulosic

hydrolysate (mainly lignin-derived phenolics), which has been shown to have an inhibitory effect on algal metabolic activities (Qi et al., 2017). The results obtained in this study were supported by various research findings that show the addition of cellulose hydrolysate to algal biomass leads to increased production of lipids (Liang et al., 2009; Joe et al., 2015). According to Joe et al. (2015), the addition of cellulose in the form of rice straw hydrolysate leads to increased production of lipids by *C. protothecoides*. *Chlorella vulgaris* is also reported to have the ability to convert cellulose-derived substrates to lipids, with evidence showing that this species has the ability to utilize biomass components such as xylose (Hawkins, 1999; Liang et al., 2009).

I opted for the use of un-autoclaved cellulose and sludge because such a step would add an unacceptably expensive (energy intensive) stage to the industrial use of my approach. I assumed that algal metabolic processes would outpace the possible contribution of contaminating microorganisms present in the polymeric substrate. The lack of significant statistical variability observed (Table 1) suggests that bacterial or fungal contaminants played a minor, if any, role in the apparent boost to lipid production. Further experiments are needed, and particularly a two-phase process initially using an active heterotrophic cellulose degrader, followed by glucose assimilation by the algal culture.

One of the approaches to improve cellulose availability to enzymes is by pre-treating the substrate (Selig et al., 2007; Alvira et al., 2010). Prior to being added to the medium, the complex structure of the cellulose can be disrupted by chemical or physical pre-treatment (Taherzadeh & Karimi, 2008). Another approach that can be used in improving the utilization of cellulose by isolate W7 is through the addition of accessory enzymes. Some of the accessory enzymes that can be added to enhance the utilization of cellulose in lipid production include β-glucosidases and hemicellulose (Waeonukul et al., 2012). Joe et al. (2015) reported that the

utilization of untreated cellulose source by *C. protothecoides* is enhanced by the addition of enzymes such as Cellic CTec2. However, it should be noted that the addition of accessory enzymes could make the lipid production expensive due to the high costs of enzymes (Li et al., 2013). Hence it is important to know the hydrolysing ability polymers such as cellulose and sludge by both isolates. Therefore, un-autoclaved (autoclaving the cellulose at 121ºC may induce hydrolysis; therefore, we used un-autoclaved substrate) cellulose and sludge (lignocellulose) was added to the medium after 2 weeks of incubation. As discussed earlier in the report, the extracellular protein production and the increase in glucose concentration in the media confirms the cellulase production. This opens a new possibility of simultaneous hydrolysis and biomass/lipid production by these isolates.

To recap, the current study indicates the external carbon (glucose, cellulose and CMC) addition can improve lipid production. However, use of glucose supplementation is associated with considerable cost (Bhatnagar et al., 2011) and may not be economically feasible on an industrial scale. Therefore, the addition of cheaper substrate was assessed - pulp and paper industry sludge. Addition of paper sludge yielded higher levels of lipids as compared to pure cellulose in the algal isolate W7 (2.6 µg lipid/µg chlorophyll) and *A. pyrenoidosa* (8.7 µg lipid/µg chlorophyll) (Table 1, Fig. 6). Paper sludge addition suppressed the amount of chlorophyll in isolate W7 and *A. pyrenoidosa* as was the case with other substrates. Paper sludge used in this study was probably clean and substantially devoid of lignin. Hence the enhanced production of lipids as was the case with pure cellulose. Therefore, it may be important to ensure cleanliness of the sludge used in algal lipid production. The observed increase in lipid production ability in isolate W7 and *A. pyrenoidosa* were due to the addition of paper sludge which suggested the possible application of the isolates in the commercial production of lipids from the

sludge derived from paper mills and any other waste material that contains a high content of waste paper. However, for algae to be used in the production of lipids from paper mill waste, it is highly recommended to investigate the effect of other potential effluent contaminants on algal growth. Factors such as the concentration of carbon dioxide and the presence of microbes have been indicated to affect the growth of algae (Roberts et al., 2013). Future studies should also examine how the growth of isolate W7 is affected by a high carbon dioxide concentration as suggested by Roberts et al., (2013). Culturing algal strains such as isolate W7 through substrate supplementation is key in boosting the production of lipid. The current study has assessed the influence of each substrate separately. To determine the substrate that offers greater lipid production efficiency, future studies should be based on methodologies that enable the comparison of the substrate effect on lipid production.

Conclusion and outlook for the future

In conclusion, the current study has demonstrated that isolate W7 has the potential to produce lipid, but is not better than the commonly used *Auxanochlorella protothecoides*. It provides tentative evidence that extracellular cellulolytic enzyme activity is produced by both algal species, potentially providing monosaccharides for lipid production. Complex sugars such as CMC, cellulose and paper sludge stimulate lipid production in the two organisms used in this study. Future studies should focus on identification of isolate W7 and on molecular level studies to evaluate the mechanism through which both the organisms produce cellulases, with the aim of identifying possible strategies of up-regulating the enzyme production. Furthermore, applied research is also necessary to develop efficient strategies for increasing biomass accumulation and lipid biosynthesis through the use of cost effective approaches.

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