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Title: Effect of pH on neutral lipid and biomass accumulation in microalgal strains native to the Canadian prairies and the Athabasca oil sands

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Running Title: EFFECT OF PH ON ALGAL LIPID AND BIOMASS ACCUMULATION

Abstract

Algal biodiesel has been a subject of growing importance in the realm of renewable energy due to carbon capture properties and its potential for photosynthetic efficiency with high lipid output. This study identified five isolates of freshwater green algae, belonging to the *Chlorellaceae*s, and measured the lipid classes and fatty acid profiles of these species to determine if they were suitable for biodiesel production. To induce the greater accumulation of lipids, especially in the form of triacylglycerols (TAGs) desired for biodiesel, we examined the lipid accumulation in cells stressed by nitrogen deficiency, sulfur deficiency or pH stress. Increases in biomass were monitored in order to determine if adjusting pH incrementally over the course of the experiment had any effect on the growth and lipid accumulation of several isolates. TAG accumulation was visually evident by Nile Red fluorescence and further assessed by gas chromatography. Lipid amounts were observed to be comparably equal or better for pH-stress treatments than for standard nutrient-deprivation treatments. Incremental pH treatments were found to have lipid accumulation comparable to constant pH treatments, yet biomass growth was equivalent to unstressed growth. One of the isolates obtained from the Athabasca oil-sands region of Alberta is a good candidate for biodiesel production, having accumulated over 45% of its dry weight as lipid, with over 80% of the lipid as triacylglycerols, and contains an abundance of 18:1 fatty acids. This class of fatty acids improves the cold flow and oxidative stability of biodiesel, and is ideal for biofuel used in a Canadian climate.

Key Words: Biodiesel · Lipid · Oil Sands · pH Stress · Nile Red · Biomass · TAG

1 Introduction

2 As the supply of non-renewable energy resources declines and becomes more costly to
3 extract, the search for economical, renewable biofuels has become more important. Biofuel from
4 algae is often cited as being one of the more promising alternatives to fossil fuels, despite some
5 current limitations (Chisti 2007, Dismukes et al. 2008, Hannon et al 2010, Larkum 2010). Algal
6 biomass production has a smaller impact on food supplies and competition for prime agricultural
7 land compared to other biofuel stocks such as corn and soybean (Day et al. 1999, Chisti 2007,
8 Chisti 2008). The use of algae would also bypass the need for secondary fermentation, as is the
9 case for cellulosic fermentation.

10 A key factor in the production of algal biofuels is the identification of strains which
11 combine high yields of desirable bioproducts with ease of growth and extraction. Researchers are
12 looking for algae that can produce large quantities of oil (Abou-Shanab et al. 2011, Kaur et al.
13 2011, Larkum et al. 2012), survive harsh environments (Zhou et al 2011) or organisms which
14 can be used in remediating and ameliorating the effects of municipal and industrial waste water
15 (Craggs et al. 2011, Park et al. 2012). The green algae have often been a focus of bioproduct
16 research, partly due to their ease of growth and the degree to which they have been previously
17 characterized at the physiological and molecular level. In particular a number of studies have
18 identified species and isolates of the genus *Chlorella* as being of interest for biofuel production
19 due to their rapid growth rates and accumulation of TAGs (Iwamoto 2007, Gouveia and Oliveira
20 2009).

21 In addition to identifying the appropriate microalga to grow in culture for biofuel
22 production, optimal growth parameters are also necessary to accumulate sufficient biomass with
23 high lipid content. Microalgae have been shown to produce substantial portions of their dry

weight (50-80%) as lipids when grown in physiologically stressful conditions such as nutrient deprivation, temperature change, and photooxidative stress (Spoehr and Milner 1949, Renaud et al. 2002, Hu et al. 2008, Abou-Shanab et al. 2011). Until recently, most of the research directed towards increasing oil yield of algae has focused on nitrogen or sulfur deprivation. As the cells become depleted of nitrogen, carbon fixed by photosynthesis is partitioned into lipids, as growth is halted by a lack of protein and nucleic acid building blocks (Shifrin and Chisholm 1981). This partitioning of carbon into lipids triggers triacylglycerol (TAG) accumulation (Leman 1997, Illman et al. 2000, Siaut et al. 2011). Difficulties and costs associated with the monitoring and adjustment of nitrogen levels in large cultures such as open ponds or large photobioreactors are significant. Thus, there is a fundamental downside with using nutrient deprivation to induce TAG accumulation in algal cells. Guckert and Cooksey (1990) demonstrated that cellular TAG accumulation can be induced in a single species of *Chlorella* by growing it at a pH higher than normal. This process was explored further and has shown this to be true for other microalgal genera such as *Scenedesmus* and *Coelastrrella* (Gardner et al. 2010). Utilizing pH as a stressor for microalgae eliminates the difficulty of measuring nitrogen concentration, because all that is needed is a simple pH probe or indicator dye. The costs of this approach are also decreased as using NaOH or HCl to adjust pH is a cost efficient and simple method to achieve pH stress.

This work had two main goals. The first was to obtain a number of freshwater green algal isolates belonging to the genus *Chlorella*, or closely related genera, and to investigate their potential use as biofuel stocks. The second goal was to characterize the lipid production of these new isolates with respect to simple physiological stresses, to further explore their biofuel potential. The algae chosen are from different environments such as rivers, saline ponds and lakes located in Saskatchewan and Alberta, and are meant to represent the variety of ecosystems

from which potential candidates for biofuel could be drawn, and as a function of maximizing variability in species selection. Algae from the oil sands region are of interest since there has been sparse research on organisms found in tailings ponds; their proximity to industrial oil effluent and bi-products could indicate a tolerance that allows them to effectively handle extremely stressful conditions. Two isolates in particular were obtained from tailings ponds in the Athabasca oil sands region of Alberta in order to examine their potential for biodiesel production. These two isolates were chosen for a comparison to algae not adapted to the harsh conditions of tailings ponds, and these two isolates responded well to decontamination and culturing efforts. All of these algal isolates are exposed to dramatically fluctuating light and temperatures, and they are also adapted to relatively saline water at alkaline pH. However, the isolates from the oil sands region are also adapted to exposure to heavy metals, organic pollutants such as naphthenic acids, and highly alkaline pH (Allen 2008, Headley et al. 2009). Because tailings ponds are typically very alkaline ($\text{pH} \approx 10$), we explored the possibility of using a lower pH to stress algae from this environment.

Materials/Methods

Algal culturing

Five strains of algae were collected from soil and water samples. Three isolates from rivers, ponds, and lakes of Saskatchewan Canada (FGP5, Rb1a, RBD8) as well as two strains isolated from soil samples taken from tailings ponds in the Athabasca oil sands of Alberta Canada (OS1-3, OS4-2). The Saskatchewan strains were grown on plates with TAP media (Gorman and Levine 1965, Harris 1989) while the oil sands strains were grown on modified WC medium (Andersen 2005). The Saskatchewan algal stock cultures were grown in 250mL flasks

1 containing TAP medium, pH 7.2, and the Athabasca oil sands algae, due to the alkaline state of
2 tailings ponds were grown in modified WC medium at pH 10. Experimental treatments were
3 conducted over 7 days using 200 mL culture tubes at 21°C under constant illumination (110
4 $\mu\text{mol}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$) with continuous mixing by bubbling filtered ambient air (Wilson and Huner 2000).
5 A hemocytometer was used to determine cell numbers in cultures as required.

6 *PCR and DNA sequencing of rDNA*

7 The identity of the algal isolates was examined based on DNA sequence analysis of the
8 18S, ITS1 and ITS2 regions of rDNA. DNA extraction and PCR were carried out in a single step
9 using Phire® Plant Direct PCR Kit (Finnzymes) according to manufacturer's specifications
10 using the oligonucleotides listed in Table 1. Following PCR amplification, samples were
11 separated on 1% agarose TAE gels stained with ethidium bromide. The resulting fluorescent
12 bands were excised from the gel, purified using the EZ-10 Spin Column DNA gel extraction kit
13 (Bio Basic Canada Inc., Markham, ON, Canada), ligated into the pJET 1.2 vector using the
14 CloneJet kit (Fermentas Canada Inc, Burlington, ON, Canada), and transformed into DH10 β
15 *E. coli* by electroporation. Plasmids were extracted from transformed *E. coli* and DNA
16 sequencing was performed by the NRC Plant Biotechnology Institute DNA sequencing
17 laboratory using the pJet1.2 Forward and Reverse primers supplied with the cloning kit.

18 *Nutrient deprivation experiments*

19 To compare lipid accumulation under pH stress to other stress conditions, all cultures
20 were grown under four separate treatments: standard growth conditions, nitrogen limited, sulfur
21 depleted, and constant high/low pH stress for Saskatchewan and Alberta algae, respectively.
22 Nitrogen limitation was achieved by using 20% of normal nitrogen levels and allowing the
23 culture to deplete it naturally as this has been shown to produce higher lipid yields (Stephenson

et al., 2010). MgCl_2 was substituted for MgSO_4 for sulfur deprivation treatments to remove sulfur from the growth media. Sulfur limitation was considered, however the effects of sulfur limitation have not been adequately researched and a comparison is beyond the purview of this study.

pH adjustment experiments

FGP5, OS1-3 and OS4-2 were chosen for incremental pH adjustment experiments. Each experiment used three culture tubes containing either unstressed (pH 7.2 for FGP5 or pH 10 for OS1-3 and OS4-2), constant pH stressed (pH 9.5 for FGP5 or pH 7.6 for OS1-3 and OS4-2), or incrementally pH stressed algae, with FGP5 cells started at a pH of 7.2 and increased by 0.5-1.0 per day to pH 9.5 by the addition of 1M NaOH. The OS1-3 and OS4-2 cell cultures were started at pH 10 and gradually the pH was lowered to pH 7.6 by the addition of 20% HCl. Initial cell counts were taken to compare to later measurements for overall biomass increase in cells. Each experiment was repeated four times, with new cells. Due to the daily changing of growth conditions, specific growth rates could not be accurately assessed.

Nile Red staining and confocal microscopy

To visually assess neutral lipid accumulation across treatments, aliquots of cells were taken after 7 days. Cells were stained according to Chen et al. (2009) with a 2.4 $\mu\text{g/mL}$ concentration of Nile Red (9-diethylamino-5H-benzo[α]phenoxazine-5-one) obtained from Molecular Probes Inc. (Junction City, Oregon) dissolved in acetone (w/v), supplemented with 10% DMSO (v/v). Cells were incubated with the dye solution at 37°C and agitated at 220 rpm on an orbital shaker for 20 minutes immediately followed by confocal microscopy using a Zeiss LSM510/ConfoCor 2. Excitation and emission wavelengths were 514nm and 560-615nm respectively.

1 *Analytical analysis of lipids*

2 Cells were harvested by centrifugation at 4500 rpm in 50 mL falcon tubes, and
3 transferred to 2.0 mL screw cap microcentrifuge tubes, centrifuged at 12000 rpm and the pellet
4 was flash frozen in liquid nitrogen. Samples were lyophilized and the dry weight determined
5 prior to lipid extraction.

6 The fatty acid/oil content determination was performed using the solvent system
7 proposed by Lee et al. (1998). A 1 mL solution of 2:1 chloroform:methanol containing 500
8 µg/mL 15:0 fatty acid as internal standard was added to the 2.0 mL centrifuge tubes along with
9 100-200 µL 0.5 mm glass beads and agitated in a bead-beater for 3 x 60 s at 4°C. The contents of
10 the tube were then transferred to glass tubes with Teflon-lined screw-caps and the bead-beater
11 tubes were rinsed with 3 x 1 mL of 2:1 chloroform:methanol which was then added to the glass
12 tubes. After centrifugation, a 300 µL aliquot was taken and evaporated under a gentle stream of
13 N₂. After evaporation, 1 mL of 1% sulfuric acid in methanol (v/v) was added and the samples
14 were incubated at 80°C for two hours. Samples were then cooled to 4°C and 1 mL of 0.9% NaCl
15 (w/v) was added to remove water-soluble impurities. Samples were extracted twice with 1 mL
16 hexane, then evaporated under N₂ and 100 µL of 0.1100 mg/mL 17:0 and 23:0 in hexane were
17 added as external standards. For gas chromatography with FID, 30 µL of the resulting solution
18 was used and run in an Agilent 6890N network GC system with Agilent Chemstation software at
19 an initial temperature of 160°C for 1 min., followed by an increase in temperature of 4°C/min. up
20 to 240°C, and held at this temperature for 10 min.

21 To quantify neutral lipid classes and amounts, thin-layer chromatography was performed
22 (Miquel and Browse 1992, Lightner et al. 1994, Christie 2003). Lipids were extracted similarly
23 to the bead-beater method described above, and were spotted on to Silica Gel 60 F₂₅₄ TLC plates

for separation alongside known lipid standards. The mobile phase used was 70:30:1 hexane: anhydrous ethyl ether: glacial acetic acid. To visualize the neutral lipids, the plates were developed using 0.05% primulin in acetone (w/v). TLC scrapings of individual spots were transferred to glass tubes containing 100 µg 15:0 fatty acid as internal standard, and 2 mL of 3 N methanolic HCl was added, followed by incubation at 80°C for two hours. After incubation, 2 mL 0.9% NaCl was added and extracted twice with 2 mL hexane. After evaporation, 15 µL of 0.1100 mg/mL 17:0 and 23:0 in hexane were added as external standards and the full 15 µL was used for GC analysis, as described above.

Results

Isolation and identification of algal strains

The five algal strains characterized in this study were isolated from soil and water samples. Two of the three Saskatchewan strains: FGP5 and Rb1a originated in Saskatoon, Canada, on the campus of the University of Saskatchewan and South Saskatchewan River, while the third, RBD8 was collected near Redberry lake, Saskatchewan. The two additional strains originated in tailings ponds from the Athabasca oil sands region of Alberta, Canada. Based on microscopic imaging, all five strains had characteristic morphological markers of the genus *Chlorella*. Using rDNA and ITS sequence alignments, it was possible to further categorize the five isolates (White, 1990). As presented in Figure 1, a phylogenetic tree suggests that FGP5, Rb1a and RBD8 are most similar to the *Micractinium*. FGP5 and RBD8 share more than 99% sequence similarity over the 2430 bases, and segregate with CCAP 248/2, an uncharacterized *Micractinium* species. Rb1a also falls within the *Micractinium*, but on a relatively deep branch, with no closely related characterized species.

1 The OS1-3 and OS4-2 strains appear to fall within the genus *Chlorella*. They appear to
2 belong to the same branch of the phylogenetic tree as *C. vulgaris*, *C. lobophora* and an
3 uncharacterized *Chlorella* species UTEX 938, which was recently recharacterized (Luo et al
4 2010). It was formerly annotated as *Dictyosphaerium planctonicum*. When comparing the
5 sequences obtained in this study, there were no identical sequences present in genbank, thus we
6 are unable to give a more specific phylogenetic characterization at this time.

7 *Lipid accumulation and growth measurements under managed culture conditions*

8 Measurements were taken of the total amount of lipids present in the samples for each
9 culture condition to determine suitability for biofuel production, and which treatments produce
10 the greatest change in total lipid amount. Algae are extremely variable in their reaction to
11 changing stimuli, and we hoped to mitigate this variability by using similar genera of algae for
12 our experiments.

13 Figure 2 shows a comparison of the three Saskatchewan isolates studied with regards to
14 their total lipids measured as a percent of dry weight. The three Saskatchewan isolates vary in
15 terms of total lipid amounts for the unstressed (pH 7.2) condition, and in their responses to the
16 other experimental treatments as the effect most of the other treatments have on each organism.
17 As shown in Figure 2, the FGP5 strain exhibited an approximately 50% increase in total oil
18 content as a percent dry weight when exposed to either nitrogen or sulfur limitation. However,
19 high pH media elicited a 100% increase, regardless of whether the high pH was immediately or
20 incrementally introduced. The Rb1a strain accumulated lower levels of oil following nitrogen
21 limitation, and only a 25% increase in oil content as a percent dry weight following sulfur
22 deprivation or high pH exposure (Fig. 2). The RBD8 strain was the most responsive to the
23 experimental treatments, with nitrogen limitation eliciting a 300% increase in oil accumulation

1 and a 500% increase following sulfur deprivation or high pH exposure (Fig. 2). Thus, the RBD8
2 strain appeared to be the most responsive to the experimental treatments, and all of the strains
3 showed increased oil accumulation when stressed by sulfur limitation or high pH.

4 Figure 3 shows total lipid accumulation for oil sands isolates OS1-3 and OS4-2. In
5 contrast to the Saskatchewan isolates, OS1-3 and OS4-2 displayed enhanced oil accumulation
6 under neutral pH. They accumulated the greatest oil as a percent of dry weight when the pH was
7 incrementally decreased from 10 to 7.6 (Fig. 3). The incremental decrease produced similar
8 levels of oil as the constant pH stress condition (pH 7.6) with OS4-2 showing an approximately
9 300% increase in overall lipids when the pH was incrementally adjusted over the course of the
10 experiment. Under nitrogen limitation and sulfur deprivation, the OS1-3 strain accumulated only
11 50% and 25% of the total oil found in the non-stressed culture. However, based on the Nile Red
12 confocal imaging of the OS1-3 cells, this appeared to be due to decreased cell survival.
13 Following growth at pH 7.6, pH 10 or following the incremental change from pH 10 to 7.6, there
14 was less change in the total oil accumulated by the OS1-3 cells.

15 Conversely, all of the experimental treatments increased oil content of the OS4-2 cells.
16 Under nitrogen and sulfur deprivation, the oil yield for OS4-2 increased by 300% and 200%
17 respectively, with the incremental pH condition faring slightly better than nitrogen limitation,
18 and constant pH stress performing marginally better than sulfur deprivation.

19 To ascertain the effect of pH stress on biomass accumulation, growth measurements for
20 FGP5 and OS1-3 were taken during incremental pH experiments for unstressed, constant pH
21 stressed, and incrementally adjusted pH stressed conditions. These two isolates responded most
22 favourably to pH treatments in terms of lipid accumulation, and thus were chosen for
23 incremental pH experiments and subsequent biomass measurements. Due to extreme cell

1 clumping, precise counting of cells for OS4-2 was not possible and biomass increase was
2 omitted for this isolate. Table 2 shows relative biomass increase after the seven day incubation.
3 Both isolates' growth was diminished in the constant pH stress condition compared to the
4 unstressed condition. However, the growth of cultures undergoing incremental pH stress was
5 slightly increased relative to unstressed algae (Table 2). In the case of FGP5 the unstressed and
6 incremental pH treatment biomass were significantly greater than the constant pH stress
7 treatment (ANOVA; $p < 0.01$, Tukey's HSD test; $p < 0.01$). OS1-3 varied slightly, with
8 incremental pH stress only being significantly greater than constant pH stress (ANOVA; $p < 0.05$,
9 Tukey's HSD test; $p < 0.05$). There was no significant difference between unstressed and
10 incrementally pH stressed biomass accumulation for any isolate.

11 *Phospholipid and TAG fatty acid composition*

12 In order to determine how much of the total lipid amount measured was comprised of
13 TAGs and the changes in the fatty acid profile for specific lipid classes, thin-layer
14 chromatography was performed. Because our goal was to examine the isolates' potential for
15 biodiesel production, it was essential to know which isolates produce the highest amount of
16 TAGs under specific growth conditions. Table 3 compares the effects of the experimental
17 treatments on the amount of TAGs as a percent of total lipids present in strains FGP5 and OS4-2.
18 In the FGP5 cultures, the treatment that produced the most TAGs as a percent of the total lipids
19 was nitrogen limitation. While high pH stress and incremental pH elicited a doubling in total oil
20 content (Fig. 2), they only resulted in a 60% increase in TAGs. OS4-2 accumulated 89% of its
21 total lipids as TAGs, even under the unstressed condition (Table 3). As a result, there was very
22 little difference in TAG accumulation for OS4-2 following exposure to any of the stresses tested
23 in this study (Table 3).

1 The fatty acid profiles of specific lipid classes were analyzed. Figure 4a shows changes
2 in the phospholipid component of the fatty acid profile for FGP5. Overall there was little
3 variation for most of the fatty acids, however there is a trend of most monounsaturated fatty
4 acids (MUFAs) decreasing, especially with the incremental pH stress condition. This decrease in
5 MUFAs is contrasted by an increase in most polyunsaturated fatty acids (PUFAs), with the only
6 exception being 16:2-n6,n9 (hexadecadienoic acid). Although nitrogen limitation produced
7 higher quantities of 16:0 (palmitic acid), incremental pH stress appears to have the most effect
8 overall, with decreases in 16:1-n7 (palmitoleic acid), hexadecadienoic acid, and 18:1-n9 (oleic
9 acid). Incremental pH stress also produced the highest quantity of 18:2-n6,n9 (linoleic acid). As
10 shown in Figure 4b, the TAGs measured from the different treatment regimes for FGP5 were
11 composed of fatty acids dominated by palmitic acid, oleic acid, and linoleic acid, although in the
12 unstressed condition there was a significantly higher amount of palmitoleic acid (ANOVA;
13 $p < 0.01$, Tukey's HSD test; $p < 0.01$). There was an observed increase in the amount of PUFAs in
14 TAGs when FGP5 was stressed, specifically hexadecadienoic acid, 16:3-n3,n6,n9
15 (hexadecatrienoic acid), linoleic acid and 18:3-n3,n6,n9 (α -linolenic acid). Constant pH stress
16 had less effect than incremental pH stress on the PUFAs typically, and all stress treatments
17 produced increased amounts of the saturated long-chain fatty acid 18:0 (stearic acid).

18 Fatty acid composition of phospholipids for OS4-2 is shown in Figure 5a. There was
19 little variation in the fatty acid profile of the phospholipids in general. Specifically, nitrogen
20 limitation had the greatest effect on hexadecatrienoic and linoleic acids with increases and
21 decreases respectively, as well as producing minor increases in saturated fatty acids 14:0
22 (tetradecanoic acid) and stearic acid. Specific fatty acid composition of TAGs in OS4-2 is seen in
23 Figure 5b, with palmitic, oleic, and linoleic acids being most prominent. There is very little

change in the overall fatty acid profile of OS4-2 TAGs for many fatty acids. The most notable change is that of oleic acid, which is significantly increased (ANOVA; $p < 0.01$) for nitrogen limitation (Tukey's HSD test; $p < 0.05$) and both pH stress conditions (Tukey's HSD test; $p < 0.01$). OS4-2 appears to have a different reaction to pH stress conditions than FGP5 for TAG composition, with an increase in oleic acid and a decrease in linoleic acid, which was increased in FGP5.

Nile Red Fluorescence

In order to visually assess neutral lipid accumulation in terms of amount and spatial conformation, aliquots taken directly before cell harvesting were stained with Nile Red and analyzed by confocal microscopy. For the FGP5 strain, some fluorescence was observed after the 7-day incubation for the unstressed condition as shown in Fig. 6a, and distinct lipid bodies can be seen in the cells, indicating some accumulation of storage TAGs. A change in lipid body size and abundance occurred in the FGP5 cells when exposed to either constant high pH stress (Fig. 6b) or following incremental pH stress (Fig. 6c).

The OS1-3 and OS4-2 algal strains obtained from the Athabasca oil sands were less variable in terms of the Nile Red fluorescence (Fig. 7). In both OS1-3 and OS4-2 cultures, there were observable, discrete lipid bodies in the unstressed (pH 10) condition (Fig. 7a and 7d), with lipid bodies predominantly forming near the cell periphery. There was also an observable clustering of these oil bodies, making it difficult to distinguish discrete droplets. The effect of pH stress seemed to promote the production of more lipid bodies for OS4-2, as shown in Fig. 7e and 7f corresponding to constant low pH stress (pH 7.6) and incremental pH stress (pH 10-7.6), respectively. These lipid bodies were larger, taking up a greater portion of the cell volume. Oil bodies for OS1-3 in the incremental pH treatment condition (Fig. 7c) were more localized than

1 when grown under constant pH stress (Fig. 7b), which had neutral lipid fluorescence spread out
2 around the entire cell. For all three strains (FGP5, OS1-3 and OS4-2) the differences in neutral
3 lipid fluorescence reflected the more detailed and precise measurements made using TLC and
4 gas chromatography.

5 6 7 Discussion

8 We have identified five new *Chlorophyceae* strains that based on morphological and
9 molecular sequencing data belong to the *Chlorella* clade as defined by Luo et al (2010). Further
10 breakdown places the two strains isolated from the Athabasca oil sands region of Alberta as
11 being part of the genus *Chlorella* (Fig. 1). The two strains segregate together within the genus,
12 but significant sequence differences exist and the strains behave differently in terms of their oil
13 production and response to stress conditions. The two strains may reflect novel *Chlorella*
14 species, but further sequencing and taxonomic analyses are required to confirm this suggestion.
15 The three isolates from the Saskatchewan appear to belong to the genus *Micractinium* (Fig. 1).
16 The FGP5 and RBD8 strains are very highly similar at the sequence level and have similar lipid
17 accumulation responses to stress. Based on molecular sequence comparisons, they are most
18 similar to an uncharacterized *Micractinium* species (CCAP 248/2), but fall within a branch
19 shared by *M. pusillum*. In contrast, the Rb1a strain appears to be significantly different based on
20 both sequence and lipid accumulation data. Similar to the two oil-sands isolates, these three
21 strains may represent two new species of the genus *Micractinium*, but further characterization is
22 needed.

23 The type of organism used for biodiesel fuel is important for the different properties of
24 the end product (Singh & Singh 2010). The main goal of this work was to determine if any

1 green-algal isolates from the Canadian prairies could be useful for biofuel production. We found
2 that the five isolates characterized could accumulate 20 to 45% of their dry weight as lipid.
3 These results suggest that based on total lipid accumulation, strains OS1-3 and OS4-2, fall within
4 the range of productivity that may make them suitable for biodiesel production (Abou-Shanab et
5 al. 2011, Zhou et al. 2011). In addition, OS4-2 accumulates 80-90% of its total lipids as TAGs.
6 However, FGP5, the lowest lipid accumulator grows 50% faster than OS1-3. Therefore, the cells
7 need to be grown under photobioreactor conditions to carefully assess the lipid production per
8 day that each of these strains produces. One of the persistent problems associated with biodiesel
9 is cold flow performance and oxidative stability, due to the mix of lipids found in vegetable and
10 algal oil. One of the aims of this study was to determine if Canadian algal isolates could be
11 identified that provide biodiesel fuel with properties such as cold flow and oxidative stability
12 optimized for use in the Canadian climate. Thus, the fatty acid profiles of the algae and how
13 stress affects them were of interest. The key factors regulating biodiesel quality are fatty acid
14 chain length, and the degree of saturation of those fatty acids (Knothe 2005, Scragg, 2009). As
15 shown in Figure 8, unsaturated fatty acids give better lubricity and cold flow than saturated fatty
16 acids and as such are more desirable when considering the cold climate of Canada. However,
17 oxidative stability concomitantly decreases as the level of fatty acid unsaturation increases,
18 which means less degradation in storage. An optimal fatty acid profile for Canadian biodiesel,
19 then, would incorporate more long-chain fatty acids, with a moderate degree of unsaturation.
20 However, for use as a biodiesel, the level of polyunsaturated lipids must be low (Greenwell et al
21 2010). The OS4-2 strain met this requirement, especially when the fatty acid profiles of the
22 TAGs were examined. The greatest proportion of TAG fatty acids in OS4-2 was 16:0 and 18:1.
23 Importantly, the high levels, as a molar percent, of 18:1 fatty acids observed in the TAG fraction

1 of OS4-2 cells is highly desirable in biodiesel production (Knothe 2005). The fatty acid profiles
2 of the different lipid classes for FGP5 and OS4-2 changed in response to physiological stress
3 (Figs 3 and 4, respectively). Changes in the fatty acid profile of the TAG fraction of FGP5 show
4 a decrease in MUFAs and an increase in PUFAs, while at the same time there is a decrease in the
5 amount of palmitic acid, the most abundant saturated fatty acid for this isolate, under most stress
6 conditions. OS4-2 has a very stable fatty acid profile for the TAG fraction, with the only changes
7 of note being an increase in oleic acid and a decrease in linoleic acid. The pH stress condition
8 actually further enhanced oleic acid accumulation in the TAG fraction. This is opposite to the
9 changes observed in FGP5, and emphasizes the importance in algal strain selection for biofuel
10 production. Fluorescence of Nile-Red-stained neutral lipids in the algal cells, combined with our
11 estimations of total lipid and TAG changes, clearly demonstrated TAG accumulation under pH
12 stress in all of our algal isolates (Figs 6 & 7). The intensity of fluorescence for OS1-3 and OS4-2
13 cells following pH stress in particular showed that the lipid bodies formed are much larger and
14 more tightly clustered than those in the unstressed condition. Chlorophyll fluorescence was
15 accounted for as the negative control displayed no fluorescence, suggesting that all fluorescence
16 observed was TAG accretion, which is in agreement with the literature (Cooksey et al. 1987,
17 Priscu et al. 1990) and our TLC data.

18 Decreases of total lipid amount for Rb1a and OS1-3 in response to nitrogen limitation can
19 be explained by the presence of increased dead cell biomass, which was observed for both
20 isolates undergoing this treatment regime (data not shown). Indeed, for OS1-3 which also
21 showed a similar response to sulfur deprivation, it is noteworthy that both pH stress experiments
22 had the opposite effect on lipid accumulation as a percent dry weight in this isolate. This both
23 highlights the detrimental effects that stress conditions can have on cell survival and hence oil

1 production and demonstrates the robustness and effectiveness of the pH stress treatments. In the
2 case of these two isolates, it appeared that nutrient stress was harmful to the cells, while pH
3 stress was more easily tolerated. Thus for optimal oil production, algal cell cultures will need to
4 be examined individually to determine the most productive set of growth conditions.

5 The need to assess growth conditions was also highlighted with the oil-sands strains, by
6 their adaptation to high pH conditions. Thus, exposure to pH 7.6 initiated a lipid stress response.
7 As for biofuel production, the effect of incremental pH stress on growth was important because
8 the biomass accumulation measured for FGP5 and OS1-3 was greater than that observed in the
9 unstressed condition. We surmise that this was due to the algae growing to a greater density
10 while under a more permissive pH, before being induced to increase oil production as the cell
11 cycle became affected by the pH of the growth media. Because there is normally an inverse
12 relationship between growth and oil production, incrementally inducing pH stress was the most
13 encouraging alternative. There was no apparent loss in growth with the added benefit of
14 increased oil production. We suggest this process would be easier to consistently scale up to the
15 photobioreactor or mesocosm level, due to the ease and cost of measuring and maintaining pH
16 compared to nutrient content.

17 In conclusion, we isolated and identified five green algal strains from the prairie region of
18 Canada. At least one of these strains, OS4-2, an apparently new species of *Chlorella* has
19 significant potential as a source of biodiesel. It accumulates approximately 15% of its dry weight
20 as lipid under optimal growth conditions, and can be induced to accumulate over 45% of its dry
21 weight as lipid when exposed to incremental pH increases. Two other characteristics give it even
22 greater potential: it accumulates over 80% of its lipids as TAGS and over 60% of its fatty acids
23 as oleic acid. Further research into algal metabolism in response to pH stress could also provide

valuable information. In yeast, decreases in intracellular pH influences the binding of PA to a transcription factor, Opi1, leading to a coordinated repression of phospholipid metabolic genes (Young et al., 2010). Interestingly, among the algal strains of this study opposing modes of response in storage lipid accumulation were found, likely reflecting the versatile metabolic adaptation strategies of the algal strains to their natural habitat. Finally, one of the greatest concerns of algal biofuel production is the economic viability of the enterprise. Another advantage that the OS4-2 strain has is its isolation from a heavily polluted and alkaline oil-sands tailing pond. If OS4-2 can be further optimized for lipid accumulation and used in a bioremediation program, it may be possible to get an added side benefit that would greatly decrease the cost of biodiesel production (Mehta & Gaur, 2005). It is apparent that there is large potential in the oil sands for high producing, efficient biodiesel feedstock; more research into algae inhabiting the oil sands is necessary to investigate this possibility.

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