Dark Stress to Improve Lipid Quantity and Quality in Acid-Tolerant Microalgae Exposed to Simulated (6% CO₂) Flue Gas

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Abstract

The use of CO₂ rich industrial flue gases to support cultivation of microalgae to produce lipids for biofuel and other applications is an increasingly researched option. However, this approach presents a challenge, as whilst flue gasses typically contain 6-10% CO₂, excessive medium acidification can be caused by the presence of NO_x and SO₂. The use of acidophilic or acid-tolerant species is a possible solution, but little is known about these microalgae. In this study we investigated the growth of a bioprospected acid-tolerant mixed photosynthetic green microalgae culture (91% dominated by a single *Coccomyxa* sp. taxon) at pH 2.5 and fed with a simulated flue gas containing 6% CO₂ and 94% N₂. At the end of the exponential growth phase, lipid accumulation and profiles, and the elemental composition of biomass were analysed over one week during which biomass was exposed to either continued light-dark cycle conditions or continual dark conditions. After three days of dark stress, the biomass consisted of approximately 28% of lipids, which was 42% higher than at the end of the exponential phase and 55% higher than the maximum lipid content achieved under light/dark conditions. Oleic acid (C18:1), pentadecanoic acid (C15:0), and palmitic acid (C16:0) were the dominant fatty acids at the end of the exponential phase, and light-dark and dark-treated biomass, respectively. Dark stress conditions favoured polyunsaturated fatty acid production and showed an increase in nitrogen content. This suggests that the use of dark stress to stimulate production of desirable lipids is a no-cost alternative to other commonly used stressors.

1. Introduction

Photosynthetic green microalgae are a diverse group of microorganisms often used to produce lipids for transesterification into biofuels [1] or human health products [2]. While microalgae biomass has the advantage of large yields per unit area [3] and the ability to grow on non-arable land, their high production costs can put them at a disadvantage when compared to other renewable and non-renewable resources, such as terrestrial plants or petrochemical oils. Various solutions to reducing the costs of cultivating microalgae at large scale include utilizing a biorefinery approach [4, 5], developing low-cost photobioreactors [6], and using industrial wastes, such as wastewater [7] and flue gas [8, 9] as sources of nutrients and CO₂. In particular, the use of industrial flue gases as sources of CO₂ has been shown to be effective, including that from coal-fired boilers from a beef processing plant [10], soda boilers at a pulp and paper mill [11], and from a combined heat and power plant [12].

The presence of NO_x and SO₂ in flue gases can, however, cause high acidification of the growth media, and therefore to avoid costs associated with a neutralizing agent, use of acidophilic or acid-tolerant microalgae species could be crucial to advance. In recent years, there has been increasing research towards understanding the biology and ecology of acidophilic and acid-tolerant species, with species diversity at low pH being higher than previously assumed [13]. While acidic conditions are not widely used in current (non-flue-gas-using) commercial large-scale microalgae cultivation, a controlled acidic medium could reduce contamination by non-target species, which is a common problem with less selective environments that favour a more diverse suite of neutrophilic taxa [14].

Acid stress experienced by acidophilic and acid tolerant microalgae typically leads to the accumulation of certain lipid and carbohydrate metabolites relative to growth under circumneutral pH conditions [15, 16]. Accumulation of highly-desirable bioactive compounds have consistently been shown to increase under nutrient starvation [17], increased irradiance [18], and increased salinity [19]. However, each of these stress-inducing environmental variables necessitate high cost at large-scale [20].

To induce the accumulation of desired biocompounds, a two-stage cultivation process is often used. Microalgae are grown to late exponential or early stationary phases under suitable growing conditions and then are put under environmental stress for a short duration. This encourages the cells to increase targeted secondary metabolites before harvesting. Whether adding a variable or taking one away, each stressor such as a modifying pH, represents an additional process cost, including an impact on subsequent downstream processing [21]. For example, flocculation harvesting methods are impacted by the pH of the liquid media [22, 23], which could lead to an increased use of pH modifying chemicals to achieve adequate harvest. Another common stress approach, use of salts, could negatively impact the ability to reduce costs through recycling media [24].

An approach to inducing the accumulation of target metabolites through a no-cost environmental stressor that also does not negatively affect downstream processes could be dark stress. There have been studies that have explored light stress via changing wavelengths [25], or by changing light intensities [26]. Increasing light penetration into a gas-lift bioreactor by modifying light exposure patterns (without increasing energy) increased lipid content by 16% [27], but total lipid content was found to double when microalgae were placed in constant dark [28]. Similarly, under combined dark and salinity stress, the marine microalgae *Dunaliella tertiolecta* was found to increase lipid content from 28% to 39% during 36 hours of dark [29]. Dark-induced senescence of the vascular plant *Arabidopsis thaliana* demonstrated an accumulation of triacylglycerides (TAGs) and lipoproteins via autophagy [30]. The process of autophagy (a mechanism induced by stress) in eukaryotic microalgae has been linked to increases in TAGs [31] and the degradation of ribosomal proteins [32] through nutrient starvation, but has yet to be linked to dark-induced stress.

In this study, we look at how applying dark stress influenced lipids of a mixed bioprospected microalgae culture enriched from a water body with a pH of 2.5. The culture was dominated (91%) by a photosynthetic, acid-tolerant green *Coccomyxa sp.* microalga. Growth curves in the laboratory (pH 2.5, 12-hour photoperiod, and a bubbled in synthetic flue-gas containing 6% CO₂) were obtained during the exponential growth phase. Post exponential phase, lipid accumulation and profiles were then studied over seven days in cells under either a light/dark cycle or dark only regime.

2. Material And Methods

2.1 Microalgae cultivation

A microalgae consortium was bioprospected from a water body (pH 2.8) on an active mine site and identified to be predominantly (91%) a single acid-tolerant *Coccomyxa* sp. taxon while grown at pH 2.5 based on high throughput SSU rRNA gene amplicon sequencing on the Illumina MiSeq platform using the 18S primer set and methods described in Bradley et al. [33]. The remaining 9% was made up of other green microalgae, protozoa and fungi. The microalgae were inoculated in 2 L flasks (triplicated) containing 1.2 L of acidified (pH 2.5) Bold's basal media (BBM; [34]) with a starting density of approximately 100 mg L⁻¹ dry weight biomass. The pH of the flasks was lowered from neutral pH using 1 M sulfuric acid and measured daily using a calibrated Oakton™ pHTestr™ 30 (Fisher Scientific; CAT#13200263). The flasks were placed on a gyratory shaker (Model G2, New Brunswick Scientific Co.) at 125 rpm and maintained under red and blue LEDs (approximately 22 mmol s⁻¹ m⁻²) with a 12-hour light/dark cycle. A simulated flue gas mixture of 6% CO₂ and 94% N₂ was continuously bubbled through the flasks for eight hours, starting at the beginning of the 12-hour light photoperiod. This mixture, along with the pH modification was used to simulate acidic flue gas produced by a nickel smelter.

Daily biomass productivity was measured based on the daily increase of dry weight biomass throughout the growth period and expressed as mg_{dw} L⁻¹ day⁻¹. Average biomass productivity (mg_{dw} L⁻¹ day⁻¹) was measured based on the increase of dry weight biomass from the beginning of the growth period to the end of exponential phase, and specific growth rate (day⁻¹) calculated using Eq. (1):

$$\mu = \frac{\ln X_2 / X_1}{t_2 - t_1} \quad (1)$$

where X_1 and X_2 are the biomass densities (mg_{dw} L⁻¹) at the beginning and end of the exponential phase, respectively, t_1 and t_2 are the time in days at which exponential phase begins and ends, respectively.

Individual samples for lipid analysis (duplicated) and elemental analysis (duplicated) were removed from the flasks once the highest density was achieved (end of exponential phase), to be used as controls. The CO₂ biofixation rate was calculated for the control sample using Eq. (2):

$$CO_2 \text{ biofixation rate (mg}_{dw} \text{ L}^{-1} \text{ day}^{-1}) = P C_{carbon} \left(\frac{M_{CO_2}}{M_C} \right) \quad (2)$$

where P is the average biomass productivity, C_{carbon} the carbon content (fractional), which was determined by elemental analysis (described in section 2.4), M_{CO_2} the molecular weight of CO₂, and M_C the molecular weight of carbon.

2.2 Microalgae under light-dark and dark conditions

After exponential phase had been reached under the same growing conditions as previously mentioned (1.2 L of BBM, 12-hour light/dark cycle, pH 2.5, 6% CO₂), the biomass experienced either light-dark or dark treatments. During light-dark treatment, the flasks remained on the gyratory shaker and the biomass after

the exponential phase was kept under 12-hour light/dark cycle with 6% CO₂ for 7 days. Individual samples for lipid analysis (duplicated) and elemental analysis (duplicated) were removed on days 1, 2, 3, 4, and 7. These samples represent ordinary conditions for cells during stationary and/or decline phase, and were used as comparison against the dark-treated cells. All samples were stored at -80°C until the biochemical and elemental analyses were conducted.

During dark treatment, biomass obtained after the exponential phase was placed on a gyratory shaker at 125 rpm under constant darkness for 7 days. Unlike the flasks exposed to the normal 12-hour light/dark cycle, these flasks did not receive any CO₂ since photosynthesis does not occur in the dark for photosynthetic green microalgae. After the flasks were placed in the dark, individual samples for lipid analysis (duplicated) and elemental analysis (duplicated) were removed from the flasks on days 1-4, and 7 and stored as noted above.

2.3 Lipid and elemental analysis

For lipid analysis, frozen, wet samples were sent to Lipid Analytical Laboratories Inc. at the University of Guelph, Ontario, Canada. Lipids were extracted based on the Bligh and Dyer method [35] using tridecanoin and methyl tricosanoate (NuChek Prep, Elysian, MN, USA). The total lipid extract was quantified via transmethylation [36] to produce fatty acid methyl esters (FAMES). Analysis of FAMES was completed using an Agilent 7890B gas-liquid chromatograph (GC-FID) with a 60 m DB-23 capillary column (internal diameter of 0.32mm). Determination of the fatty acids was carried out by a 90-110% match of the peak area of the known internal standards from Sigma-Aldrich (Oakville, ON, Canada) and Nu Chek Prep.

For elemental analysis, samples were lyophilized and ground down using a mortar and pestle before being sent to the Perdue Central Analytical Facility at Laurentian University, Ontario, Canada, for analysis. Vanadium pentoxide was added to samples weighing 3 – 3.5 mg to help facilitate sulfur combustion, and a Thermo ScientificTM FlashSmartTM elemental analyzer with a 2m PTFE column was used to determine the relative abundance of C, H, N, and S in the biomass. Calibration curves were created using BBOT (2,5-Bis(5-tert-butyl-benzoxazol-2yl)thiophene), and cysteine was used as a certified reference material to evaluate recovery.

Data presented are the averages ± standard errors within their respective technical replicates. Statistical analysis of the data was conducted in R [37], and all graphical representations of the data were created using the R package ggplot2 [38]. The statistical significance threshold was placed at 0.05.

3. Results

3.1 Microalgae growth

The *Coccomyxa* sp. consortium showed atypical exponential growth (Fig. 1), where the lag phase included two reductions in density and where the exponential phase included varying slopes until it

reached the highest biomass density ($550 \pm 36.1 \text{ mg L}^{-1}$), which was observed on day 19. The largest increase in biomass was observed between day 17 and 18, with a daily productivity of $130 \text{ mg L}^{-1} \text{ day}^{-1}$. The average biomass productivity was $25.71 \text{ mg L}^{-1} \text{ day}^{-1}$ and the average CO_2 biofixation rate was $44.64 \text{ mg L}^{-1} \text{ day}^{-1}$. The exponential phase was determined to be between days 9 and 19, with a specific growth rate of $0.136 \pm 0.004 \text{ day}^{-1}$.

3.2 Lipid accumulation after exponential phase

After the exponential phase, the highest total lipid content accumulated by light-dark treated cells occurred after 7 days of exposure ($17.81 \pm 1.04\%$) whereas continual dark-treated cells accumulated more total lipids after only 3 days of exposure ($27.67 \pm 9.92\%$) (Fig. 2). The main fatty acids found in all treatments are outlined in Table 1.

Table 1. Select fatty acid profiles of the biomass with post-exponential treatment (light-dark or dark) over 7 days.

	Saturated (%)			Monounsaturated (%)		Polyunsaturated (%)		
	C15:0	C16:0	C18:0	C16:1	C18:1	C18:2n6	C18:3n3	C18:3n6
Light-dark								
Day 1	29.53	2.16	18.79	2.62	20.96	0.00	0.31	19.87
Day 2	27.48	1.55	18.92	2.90	21.78	0.00	0.35	22.13
Day 3	28.84	2.27	16.48	2.70	19.88	0.00	0.55	21.89
Day 4	35.54	3.93	16.04	5.04	16.55	0.31	0.86	8.59
Day 7	30.69	1.15	37.55	6.44	8.72	0.03	0.18	8.31
Dark								
Day 1	13.17	14.10	8.19	1.55	19.53	11.03	13.28	13.48
Day 2	0.99	26.53	1.15	2.10	15.94	23.08	24.67	0.00
Day 3	1.05	31.64	1.03	2.34	18.91	20.04	18.46	0.01
Day 4	1.07	36.77	1.26	2.56	21.28	15.68	14.07	0.04
Day 7	1.10	42.90	1.31	2.77	23.90	14.10	7.22	0.10

Dark treatment resulted in a larger ratio of omega-3 and omega-6 polyunsaturated fatty acids (PUFAs; 19.47% and 21.04%, respectively) compared to 7-days of light-dark treatment, which had a lower ratio (1.94% and 9.53%, respectively). While there were no significant differences between the treatments and the types of fatty acids (two-way ANOVA, $p = 0.98$), as can be seen from Fig. 3 there were significant

differences between saturated fatty acids (SFAs) and the monounsaturated (MUFAs) and polyunsaturated fatty acids within the samples (two-way ANOVA, $p < 0.05$).

3.3 Elemental analysis after exponential phase

Light-dark treated cells achieved their highest lipid content 7 days after the exponential growth phase, whereas dark-treated cells achieved it at 3 days after the exponential growth phase. The C:N ratio was highest ($11.9 \text{ mol mol}^{-1}$) for biomass at the end of exponential growth phase and on day 7 of light-dark stress (Table 2). Whereas, it remained generally unchanged during dark stress ($6.2 - 6.5 \text{ mol mol}^{-1}$). The highest sulfur content during dark stress was on day 7 at 0.67%, while highest sulfur content during light-dark stress was 0.34% on day 4. However, the elemental analysis of the biomass showed no significant differences between the stress treatments (Kruskal-Wallis rank sum, $p > 0.05$).

Table 2. Elemental composition of the algal biomass at the end of exponential phase (control) and on the day of the highest lipid content obtained for each post-exponential treatment

Treatment	Highest lipid content (%)	Carbon:nitrogen (mol mol^{-1})	Sulphur (%)
Control	19.48 ± 1.1	11.9	0.11
Light-dark	17.81 ± 1.04	11.9	0.11
Dark	27.67 ± 9.92	6.4	0.28

4. Discussion

Under standard growing conditions, the maximum daily productivity of $130 \text{ mg L}^{-1} \text{ day}^{-1}$ of the acid-tolerant bioprospected *Coccomyxa* sp. dominated consortium is similar to those for bioprospected acidophilic *Coccomyxa onubensis* [39, 40]. However, higher productivities presented by Vaquero et al. [41] and Casal et al. [42] for *C. onubensis* ($410 \text{ mg L}^{-1} \text{ day}^{-1}$) and *Coccomyxa acidophila* ($130\text{-}250 \text{ mg L}^{-1} \text{ day}^{-1}$), suggest that further improvements can be made to enhance biomass productivity at low pH. Increases in biomass productivity could be obtained by utilizing different growing media [43]. The BBM utilized in this experiment had an original pH of 6.6 whereas modified acid media (MAM), with an original pH of 4 [44], has been successfully used as a source of nutrients for acidophilic microalgae such as *Chlamydomonas acidophila* [45]. Furthermore, while the extent of bacterial and protozoan populations was not explored in this consortium, differing bacterial strains and protozoan species negatively affecting growth could be also likely [46, 47].

The average CO_2 biofixation rate during the cultivation period ($44.64 \text{ mg L}^{-1} \text{ day}^{-1}$) was approximately half of the reported biofixation rate of the acidophilic species *Chlamydomonas acidophila* [48]. It is, however, worth noting that their reported specific growth rate was considerably larger (0.92 day^{-1}) than our results (0.136 day^{-1}). According to [49], the neutrophilic *Chlorella fusca*, with a growth rate of

approximately 0.14 day^{-1} , also had an increased biofixation rate of $171.7 \text{ mg L}^{-1} \text{ day}^{-1}$, suggesting the *Coccomyxa sp.* consortia would not be an ideal candidate in systems with high CO_2 levels.

When cells undergo three days of dark stress after experiencing exponential phase growth, total lipid content in the cells increases. Interestingly, the most abundant fatty acids are different for each treatment. Lipid from biomass sampled at the end of the exponential phase favoured oleic acid (C18:1), whereas dark-light-treated cells favoured pentadecanoic acid (C15:0), and dark treated cells, palmitic acid (C16:0). Typically, biomass with high saturated fatty acid (SFA) and monounsaturated fatty acid (MUFA) contents are favoured for biodiesel production, as these fatty acids are easily transesterified [50]. However, C15:0 has been found to have positive outcomes on human health by having anti-inflammatory, antifibrotic and red blood cell-stabilizing properties [51].

Overall, dark-treated cells had a higher variance of PUFAs (50% on day 2) compared to the light-dark treated cells (25% on day 3) and the control (38%), whereas the light-dark treated cells favoured SFA production (73% on day 7) and would be more suitable for biodiesel production. A possible explanation for the increase in PUFAs in dark-treated cells is that the absence of photosynthesis has been found to correlate with fatty acid composition, in particular a significant increase in PUFAs as photosynthesis rates decline [52]. Furthermore, nitrogen depleted environments can increase PUFA content within *Coccomyxa* microalgae [53]. However, since an increase in PUFAs is not as apparent in the light-treated cells, it can be assumed that the lack of photosynthesis attributed more to the increase in PUFAs than a lack of available nitrogen. Therefore, the higher PUFA content in dark-treated cells suggest they could be an ideal dietary supplement as these fatty acids are known for their health beneficial properties [54, 55]. In particular, dark-treated cells had a high abundance of omega-6 fatty acids (15-26%) including linoleic acid (C18:2n6), which has the potential to reduce the risk of cardiovascular disease and type-2 diabetes [56, 57].

Elemental analysis showed the C:N ratio for all treatments to be slightly higher than the mean C:N of extremophilic polar microalgae (5.8 mol mol^{-1}) whereas the mean C:N of dark stress (6.4 mol mol^{-1}) and light-dark stress (6.8 mol mol^{-1}) biomass was lower than the mean C:N of temperate, non-extremophilic microalgae (7.5 mol mol^{-1}) [58]. While the changes of C:N ratios between treatments were found to be statistically insignificant, the decreased C:N ratio of the dark-stressed microalgae could be an indication of an increase in amino acid synthesis [59], which would have a positive outcome on total protein content in the biomass and, therefore, support the use of this algae as a food nutrient enhancer. Increased nitrogen could also make lipid extracted biomass (LEB) a possible land ameliorant [60, 61]. The microalgae or LEB could be used as either a liquid fertilizer [62] or dry fertilizer in the form of biochar [63], to increase crop yield and quality [64].

The highest sulfur content in the *Coccomyxa sp.* consortia was found to be 0.67% on day 7 of dark-treatment. The sulfur content in the biomass can be present in bio-oil when the lipids are extracted, which is not beneficial for biofuel as it can lead to storage instability [65]. Therefore, use of a catalyst, such as a mesoporous silica nanoparticle described in Huang et al. [66], as a means to reduce sulfur content may be required [67]. Furthermore, the presence of sulphur could lead to SO_2 emissions if the biomass was

used for energy recovery. For example, pyrolysis using a fluidized bed reactor could be employed as a method to recover energy and nutrients from lipid-extracted biomass, and can recover more than 90% of the energy content in the biomass [68]. However, microalgae residue with a sulfur content of 0.44% was found to produce SO₂ emissions when combusted using a fluidized bed reactor between 200-800°C, but sharply decreased above 800°C [69].

Conclusion

The acid-tolerant microalgae consortia were found to grow on par with other *Coccomyxa* strains at low pH (pH 2.5), with a specific growth rate of $0.136 \pm 0.004 \text{ day}^{-1}$ and a maximum density of $550 \pm 36.1 \text{ mg L}^{-1}$. The dominant fatty acids were found to be oleic acid (end of the exponential phase), and post exponential phase, pentadecanoic acid (light-dark treated biomass), and palmitic acid (dark-treated biomass). From the results, the mixed microalgae culture accumulated the maximum level of lipids, which was after three to four days of dark treatment after the exponential growth phase. Dark stress predominantly favoured the production of polyunsaturated fatty acids (PUFAs) and resulted in a lower C:N ratio. This makes imposing dark conditions a potential stressor for photosynthetic microalgae designated for dietary supplements. By using dark stress conditions to increase total lipids and the production of PUFAs, addition of additional chemicals or energy (e.g., temperature change, increased salinity, or increased illumination) is avoided. Therefore, dark stress incurs no extra costs and has no impacts on downstream processes, allowing for easier recycling of media after microalgae harvesting. Future work includes optimizing cell densities and increasing growth rates to compete with microalgae species growing in more neutral environments.

Declarations

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Availability of data and material:

Not applicable

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Not applicable

Author contributions:

All authors contributed to the study conception and methodology. Investigation, data collection, visualization, and original draft preparation were performed by Sabrina M Desjardins. Resources were supplied by Sari Muinonen and John A Scott. Formal analysis was done by Sabrina M Desjardins and Corey A Laamanen. Review and editing was completed by Sabrina M Desjardins, Corey A Laamanen, Nathan Basiliko, and John A Scott. Supervision was provided by Nathan Basiliko and John A Scott. Funding acquisition was done by Sari Muinonen and John A Scott. All authors read and approved of the final manuscript

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Figures

Figure 1

Growth curve (mean $\pm$ standard error) of the *Coccomyxa* sp. consortium exposed to a 12-hour light/dark cycle and 6% CO₂

Figure 2

Total lipid content (% , mean $\pm$ standard error) of the dried biomass after the exponential phase

Figure 3

Lipid profile of *Coccomyxa* sp. consortium during A) light-dark growing conditions (12-hour light/dark cycle, 6% CO₂) and B) under continual dark conditions. The control is the lipid profile at the end of the exponential phase