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# Mixotrophic and heterotrophic production of lipids and carbohydrates by a locally isolated microalga using wastewater as a growth medium

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ABSTRACT

The biomass production and changes in biochemical composition of a locally isolated microalga (*Chlorella* sp.) were investigated in autotrophic, mixotrophic and heterotrophic conditions, using glucose or glycerol as carbon sources and municipal wastewater as the growth medium. Both standard methods and Multivariate Curve Resolution-Alternating Least Squares (MCR-ALS) analysis of data acquired by Fourier-transform IR (FTIR) spectrometry showed that autotrophic and mixotrophic conditions promoted carbohydrate accumulation, while heterotrophic conditions with glycerol resulted in the highest lipid content and lowest carbohydrate content. Heterotrophic conditions with glycerol as a carbon source also resulted in high oleic acid (18:1) contents and low linolenic acid (18:3) contents, and thus increasing biodiesel quality. The results also show the utility of MCR-ALS for analyzing changes in microalgal biochemical composition.

## 1. Introduction

Microalgae have recently received significant attention due to their potential as feedstocks for biofuel production and usefulness in bioremediation (Liu & Benning, 2013). Microalgal biomass comprises three major components: lipids, carbohydrates and proteins. The lipids can be transesterified to generate biodiesel (Abinandan & Shanthakumar, 2015), while the carbohydrates can be fermented to produce bioethanol (Jones & Mayfieldt, 2012). Production of biodiesel from microalgae rather than crops is advantageous due to the lower land requirement and high level of lipids in microalgal biomass (Jones & Mayfieldt, 2012).

Treatment of wastewater is required to remove nutrients such as nitrogen and phosphorus, which if left unchecked could otherwise promote excessive growth of plants and/or algae in aquatic systems known as eutrophication (Cai et al., 2013). Within this context, microalgae is known to thrive in wastewater and efficiently remove the above-mentioned nutrients (Brennan & Owende, 2010) while the resulting biomass can be used to produce biofuels, thereby offsetting costs (Daroch et al., 2013). Thus, integrating wastewater treatment and biofuel production using microalgae is an attractive option for producing environmentally and commercially viable biofuels.

Light availability is one of the major factors limiting algal growth in boreal regions, due to the long winters with low solar fluxes (Brennan & Owende, 2010). However, some microalgal strains are heterotrophic, i.e. they can grow using organic compounds without light (Venkata Mohan et al., 2015). Moreover, some microalgae can grow mixotrophically, i.e. utilize light and organic compounds simultaneously. Glucose is the most frequently used organic compound to cultivate microalgae in heterotrophic conditions, and it promotes higher growth than other tested compounds (Venkata Mohan et al., 2015). However, it is too expensive to support viable large-scale microalgal cultivation (Li et al., 2007).

Conversely, glycerol is an organic compound that is produced as a byproduct during biodiesel production and thus can be obtained very cheaply (Vivek et al., 2017). However, fewer strains can utilize glycerol than glucose in mixotrophic and heterotrophic conditions (Perez-Garcia et al., 2011), because glycerol inhibits growth of some strains (Heredia-Arroyo et al., 2011). Nevertheless, some species reportedly produce more biomass and lipids in mixotrophic conditions with glycerol than in corresponding autotrophic conditions, and there is substantial variation among strains as well as species (Paranjape et al., 2016). Thus, effects of glycerol on the biomass and biochemical composition of isolated strains that could potentially be used for biofuel production clearly warrant attention.

Since relative amounts of biochemical components vary among strains and can be strongly affected by growth conditions (Paranjape et al., 2016), various methods have been used to examine the biochemical composition of microalgae. Fourier-transform infrared (FTIR) spectroscopy, can provide estimates of lipid, carbohydrate and protein content in a single scan (Pistorius et al., 2009), because each element has a specific infrared absorption signature. FTIR has recently been

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used to analyze biochemical composition of microalgae. During FTIR data analysis, two characteristic absorption bands of amides in proteins ("amide bands I and II") are often used as calibrating signals to estimate contents of lipids and carbohydrates, assuming that the protein content remains roughly constant. However, protein content may be more variable than is sometimes thought, so compositional inferences resting on this assumption may be erroneous (Dean et al., 2010; Meng et al., 2014). A potential solution to this problem, which has recently been applied in analyses of plant and animal samples, is to subject FTIR data to Multivariate Curve Resolution-Alternating Least Squares (MCR-ALS) analysis, which can identify biochemical differences between samples and relate them to differences in conditions (Felten et al., 2015). Hence, the goal of this study was to use FTIR coupled with MCR-ALS to analyze biochemical composition changes of algal in autotrophic, mixotrophic and heterotrophic conditions and compare results to standard methods. Municipal wastewater was employed as a growth medium and glycerol or glucose as a carbon source.

## 2. Materials and methods

### 2.1. Algal isolation and municipal wastewater

To isolate a suitable microalgal strain, municipal wastewater was collected from the community wastewater treatment plant at Umeå, Sweden, then 35 ml of wastewater in 50 ml falcon tube were incubated at 25 °C under 16 h light (120 μmol/$m^2$ s) /8h dark cycles, and bubbling with 5% $CO_2$ (0.1 L $min^{-1}$). After five days, the wastewater (then green) was streaked on BG11 plates. Colonies obtained from BG11 plates were repeatedly subcultured until pure colonies were obtained. The isolated strain was cultured in the dark on BG11 plates containing 3 g/L glucose twice and no contamination was detected.

To identify the microalgal strain, genomic DNA was isolated from it using a Qiaprep Spin Miniprep kit, following the manufacturer's instructions (Qiagen). 16S rRNA gene was amplified using CYA359 forward primer (GGGGAATYTTCCGCAATGGG), and an equimolar ratio of CYA781Ra (GACTACTGGGGTATCTAATCCCAT T) and CYA 781Rb (GACTACAGGGGT ATCTAATCCCTTT) reverse primers, as described by Nubel et al. (1997). The amplified region was then purified and sent to Eurofinns for sequencing. Finally, the obtained sequence was compared to other microalgal sequences in the NCBI database using Basic Local Alignment Search Tool (BLAST) software.

### 2.2. Algal cultivation and experimental design

To examine effects of the growth conditions on the alga's biomass, triplicate cultures of the isolated microalgal strain were grown, in autotrophic, mixotrophic and heterotrophic conditions for eight days. In all cases, the basal growth medium was municipal wastewater collected and immediately stored at 4 °C for at most 10 days. Before experiments, large particles were removed by filtration through filter paper (pore size of 10 μm, Munktell AB, Sweden) and wastewater portions were autoclaved. Inoculum was grown in 100 ml portions of BG11 in 500 ml flasks autotrophically in 16 h light (120 μmol/$m^2$s)/ 8 h dark cycles, with bubbling by 5% $CO_2$, at 25 °C. 200 ml of wastewater in each one liter flask was inoculated with inoculum to an optical density ($OD_{630}$) of 0.06 (0.005 g). In autotrophic and mixotrophic conditions, samples were bubbled with 5% $CO_2$, and the cultures were supplemented with either glycerol or glucose (VWR International) to a final concentration of 37.5 mM. Growth was monitored by collecting 10 ml of each culture every two days, and after washing twice with distilled water, freeze-drying the samples for 3 days and weighing them. At the end of the 8-day cultivation period, biomass was recovered by centrifugation at 3700*g* for 6 min, washed twice with distilled water. Each wash was followed by centrifugation at 3700*g* for 6 min and the supernatant was discarded, and samples were freeze dried for 3 days. Freeze dried biomass was used for FTIR, lipid and carbohydrates extraction in this study. Samples of the wastewater were collected before and after each experiment to measure nitrogen (kit LCK 138) and phosphorus (kit LCK349) using a DR 3900 spectrophotometer (Hach Lange, Germany) following the manufacturer's instructions.

### 2.3. FTIR analysis

To identify changes of alga's biochemical composition in different growth conditions, freeze-dried samples were mixed with KBr at a 1:10 ratio, and ground to a fine powder using a mortar and pestle. The powder was loaded into sample containers of a carousel plate, with portions of KBr powder alone for background measurements. FTIR spectra were acquired (400–5200 $cm^{-1}$ from 128 scans per sample, spectral resolution 4 $cm^{-1}$) using an IFS 66 FTIR spectrometer equipped with OPUS 6.5 software (Bruker Optik GmbH, Ettlingen, Germany). FTIR spectra were analyzed using MCR-ALS as described by Felten et al. (2015). Briefly, FTIR spectra were first narrowed to 800–1850 $cm^{-1}$. After baseline collection to eliminate broad baseline features and maintain small intensity bands (AsLS $\lambda = 100{,}000$, AsLS $P = 0.001$), numbers of components were then identified by singular value decomposition (SVD) and relative proportions of the components in each sample were estimated.

### 2.4. Lipid quantification

Lipids were extracted from the algal samples following (Bligh & Dyer, 1959) with slight modification. Briefly, a 20 mg portion of freeze-dried sample was ground in 5 ml of a 4:1 methanol:$H_2O$ mixture then 4 ml of chloroform and 1.2 ml of 0.73% NaCl solution were added to the ground mixture. The mixture was shaken and centrifuged for 2 min at 1250 rpm (Wifug, Doctor, Sweden), and the lower phase was collected in a pre-weighed screw-cap tube. Part of the lower phase (1/10) was saved and later used for transmethylation. The rest was completely dried by sparging with nitrogen gas, and the screw cap tube was re-weighed to measure the lipid content.

### 2.5. Transmethylation

Lipids extracted and saved as described above were completely dried, by sparging with nitrogen gas, then transmethylated by adding 200 μl of internal standard (20 mg of methyl-15:0/100 ml dry methanol) and 1 ml of 2% $H_2SO_4$ in dry methanol. Samples were then sparged for a further two minutes, then the tubes were immediately closed to avoid oxygen entering and heated for 1 h at 90 °C. Transmethylated fatty acids were extracted by adding 1 ml of $H_2O$ and 2 ml of petrol ether, then mixing and centrifuging the mixture for 2 min at 1250 rpm (Wifug, Doctor, Sweden). The top phase was collected in a new screw-cap tube and the process was repeated using only 2 ml of petrol ether. The petrol ether was removed by sparging with nitrogen gas and samples were dissolved in 100 μl of heptane in preparation for gas chromatography (GC) analysis.

### 2.6. Fatty acids analysis

Fatty acids methyl ester (FAME) composition was assessed using GC (Thermo scientific Trace 1310). The GC contained FAME WAX column (30 m × 0.32 mm × 0.25 μm) and flame ionization detector, and data were analyzed using chromeleon 7.2 software. 1 μl of each sample was injected into GC and FAME Standards that are: 14:0 (myristic acid), 16:0 (palmitic acid), 16:1 (palmitoleic acid), 17:0 (margaric acid), 18:0 (stearic acid), 18:1 (oleic acid), 18:2 (linoleic acid), 18:3 (linolenic acid), 20:0 (arachidic acid) and 22:0 (behenic acid), purchased from Sigma Aldrich were employed to identify FAME in samples. Internal standard C15:0 (pentadecylic acid) was employed to quantify FAME in samples using peak area of C15:0 relative to peak area of sample.