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Mixotrophic cultivation of green microalgae *Scenedesmus obliquus* on cheese whey permeate for biodiesel production

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ABSTRACT

Microalgae mass cultivation for biodiesel production might very well become the next marketable biofuel. The main challenge to overcome however is the development of high efficiency strategies for the large-scale production of oleaginous microalgae at low costs. In the present work, the use of cheese whey permeate (WP) in mixotrophic microalgae cultures is proposed. Pure lactose, the main constituent of WP (>80% w/w of the total dissolved solids), can support *Scenedesmus* growth under heterotrophic culture conditions (absence of light). Substituting 40% (v/v) of the culture medium with WP significantly stimulates *Scenedesmus obliquus* growth under mixotrophic ($\mu_{max} = 1.083 \pm 0.030$ day^{-1}) and heterotrophic ($\mu_{max} = 0.702 \pm 0.025$ day^{-1}) conditions, compared to photoautotrophic control cultures ($\mu_{max} = 0.267 \pm 0.083$ day^{-1}). As growth occurs in the presence of lactose, a significant reduction of its concentration is observed, while the galactose and glucose concentrations actually increase in the culture medium. Culture medium analyses showed complete exhaustion of extracellular nitrogen (nitrate and ammonium), while intracellular lipid analyses showed neutral lipid (NL) accumulation, particularly under conditions of high pH (>9.5). Photoautotrophic control cultures accumulated more lipids (per dry weight) than WP-supplemented cultures, an aspect which is discussed in the context of lipid enrichment strategies. A fast and simple method for NL cellular content estimation is also described.

1. Introduction

Microalgae are largely under investigation for their ability to produce oils for a variety of markets [1]. Marine species usually contain large amounts of polyunsaturated fatty acids (PUFAs) and are often good candidates for the production of the following ω-3 fatty acids: arachidonic acid (ARA), eicosapentenoic acid (EPA) and docosahexenoic acid (DHA) for the aquaculture and human food supplement industries [2]. Freshwater species, on their behalf, mainly accumulate saturated fatty acids (SFAs) and monounsaturated fatty acids (MUFAs). These can often be transformed into high quality biodiesel or even jet fuel [3]. In particular, triglycerides (TAGs) are a class of neutral lipids (NLs) that can be converted into fatty acid methyl esters (FAMEs) through transesterification, a catalytic reaction that involve alcohols [4,5]. FAME is a source of biodiesel compatible with most conventional diesel engines, thus could readily replace fossil diesel sources. However, the high costs of microalgae production still hinder the advent of this product [6].

A significant advantage of microalgae-produced biodiesel is that it limits the net amount of carbon dioxide (CO_2) emitted into the atmosphere [7]. This advantage has yet to be supported by more efficient industrial processes allowing the production of large amounts of microalgal biomass at lower costs. Mixotrophic growth, for some microalgae species, can significantly improve biomass productivity (acting on both the maximum cell density and growth rate), thus lowering the production costs [8,9]. In this nutrition mode, the microalgae may assimilate different dissolved organic carbon (DOC) sources in addition to the inorganic carbon (CO_2) fixed through photosynthesis [10]. For example, a 10-fold increase in EPA productivity has been observed under mixotrophic conditions (glycerol as the DOC source) with the marine diatom *Phaeodactylum tricornutum* [11]. Glucose is also often used as a carbon source for mixotrophic cultivation of various microalgae as it is easy to assimilate: numerous microalgae species including *Scenedesmus obliquus* and *Chlorella protothecoides* can grow on this substrate [12,13]. Although its use is pertinent for research, this DOC source would however be too expensive to depend on for an actual (economically viable) biodiesel production process.

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The present work therefore investigates the possibility of integrating microalgae biomass production into an existing industrial production cycle: the dairy product industry. While microalgae can help eliminate phosphorus, nitrogen and sugars from different industrial effluents, the equipment and expertise already available on site (in most dairy product factories) could readily be used for microalgae production. As of fundamental concern, mixo/heterotrophic culture conditions require good control of bacterial populations [14], thus it is necessary to pre-process the DOC source. In a cheese factory for example, large amounts of whey are produced and passed through ultrafiltration devices to separate the proteins from other constituents, leaving mainly lactose. The costs of this ultrafiltration step are well compensated by the high-value of the whey proteins on the human food supplement market (~$30 kg^{-1} dry weight). The remaining lactose concentrate (or whey permeate, WP), is likewise free of contamination and could readily be used as a source of carbon (plus other nutrients) for microorganism cultivation. Presently, WP is often used directly for livestock feed without significant profit, or on occasion dried by a rather energy consuming process to produce lactose powder with low market value [15]. As the number of people subject to lactose intolerance increases, the demand for such a product seems an unlikely growth market. Therefore, the hypothesis whether the lactose-rich WP could serve as a DOC source for feeding microalgae in a biodiesel production process merits further investigation, complementarily to the work presented in [16,17].

To that end, as WP is mainly composed of lactose, the hypothesis whether pure lactose can support and/or stimulate growth of the *Scenedesmus* and *Chlorella* microalgae strains is first evaluated. These freshwater species are selected since they are known to exhibit endogenous β-galactosidase activity [18]. They could also easily be produced as mass cultures and have good lipid profiles for biodiesel [19,20]. The specific objectives of this the study are thus: 1) to select a microalgae strain capable to use lactose for growth, 2) to quantify the growth and lactose consumption rates by microalgae in the presence of WP for both mixotrophic and heterotrophic conditions and 3) to analyze NL content and the fatty acid profile under culture conditions that emulate a realistic industrial (scaled-up) implementation.

2. Materials and methods

2.1. Culture maintenance and medium

S. obliquus and *Scenedesmus acutus* strains were obtained from the Canadian Phycological Culture Center in Waterloo, Canada (CPCC 5 and CPCC 10). The *Chlorella vulgaris* and *C. protothecoides* strains were obtained from the University of Texas at Austin algae collection (UTEX 2714 and UTEX 255). All cultures were kept in heat sterilized (121 °C, 15 min) Bold's basal medium (BBM), with an adjusted pH of 6.8 [21]. For *C. protothecoides*, 1 g L^{-1} of soy protein peptone (Sigma-Aldrich) was added to the medium (BBMP). All manipulations were done under sterile conditions in a laminar flow biological hood and axenic culture conditions were confirmed periodically, at least at the beginning and end of each culture as follows: samples were fixed with glutaraldehyde (0.1% v/v) and analyzed using an Epic Altra flow cytometer (Beckman Coulter Inc., Fullerton, BC, CA) fitted with a 488 nm laser operated at 15 mW under a flow rate of 60 μL per minute. Data were analyzed with the Expo32 v.1.2b software (Beckman Coulter Inc., Fullerton, CA). Heterotrophic bacteria were quantified in diluted samples stained with SYBR Green I nucleic acid bounder (Molecular Probes Inc., OR, USA), and were separated according to their nucleic acid content (LNA and HNA for low and high nucleic acids, respectively) [22].

A concentrate of pure D-lactose monohydrate (Sigma-Aldrich) was prepared in BBM medium at 100 g L^{-1}, filtered on 0.2 μm syringe filters, and then diluted to the desired concentration before inoculation. Cheese WP was received from a dairy transformation plant in Quebec, Canada (shipped on ice). Upon reception, the pH of the WP was 6.2 ± 0.1 (Supplementary material). Neutralization tests were performed with 1 M NaOH and 1 M Na_2CO_3 solutions, which led to the formation of a white precipitate possibly due to calcium phosphate precipitation. To avoid potential problems or influencing the results, no pH adjustments were further made to the WP solution, which was used directly in the cultures while ensuring rigorous pH monitoring during experimentation. Natural lake water (LW) from the Lac-St.-Jean (latitude 48.4666, longitude −71.8180; QC, Canada) was obtained, sampled on July 1st 2011, 30 m from the lakeside. All these solutions were filtered (0.2 μm filters) and stored at 4 °C until further use. Detailed chemical analyses of each solution are provided as Supplementary material.

2.2. Experimental design

Erlenmeyer flasks were incubated on an orbital shaker at 120 rpm (C1 platform shaker, New Brunswick Scientific, Edison, NJ, USA) under constant temperature (22.5 °C) and a light intensity of 100 μmol m^{-2} s^{-1} (when applicable), measured on the external surface of the flasks using a Q201 quantum radiometer (Macam Photometrics Ltd., Livingston, Scotland). The initial cellular concentration for each experiment was set to 1×10^6 cells mL^{-1} (day 0), using manual cell counts on a hemocytometer. All experiments were performed in three replicates (n = 3).

2.2.1. Microalgae strain selection for lactose conversion

S. obliquus, *S. acutus*, *C. vulgaris* and *C. protothecoides* were grown in triplicate under heterotrophic conditions (continuous darkness) in 20 mL of sterile BBM (or BBMP for *C. protothecoides*) containing 5 g L^{-1} D-lactose monohydrate in 50 mL Erlenmeyer flasks. Photoautotrophic control cultures were grown in identical conditions (also in triplicate), except for the absence of lactose in their culture medium. Samples were taken at days 6 and 13 post-inoculation to determine the cellular concentration through manual cell count using a hemocytometer.

2.2.2. WP use and growth optimization

Partial substitution of the BBM medium with WP in *S. obliquus* cultures was tested in various proportions (5, 10, 20, 40, 60 and 80% WP (v/v)) under mixotrophic conditions. Results showed an increased growth of up to 40% substitution (data not shown). Higher initial WP concentrations prompted a significantly longer lag phase, possibly due to substrate inhibition at low cell concentrations [23]. Thus for the mixotrophic and heterotrophic treatments, 80 mL of sterile mixture (60% BBM and 40% WP v/v) was used in a 250 mL Erlenmeyer flask for the experiments (13 day duration). Photoautotrophic treatment used 80 mL of sterile BBM only (no DOC source).

The photoautotrophic and mixotrophic cultures were kept under continuous illumination, while the heterotrophic cultures were kept in the dark. All experiments were performed in triplicate. Every day the cultures were sampled to monitor growth. Biomass concentration was estimated by measuring absorbance at 750 nm with a fixed wavelength DU640 UV–visible spectrophotometer (Beckman Coulter Inc., Fullerton, BC, CA) in 1 cm test tubes. Cultures were diluted (1/10) with distilled water to keep readings in the interval 0 to 1.2. Biomass yield was analyzed using dry weight (DW) measurements. Cells were washed 3 times at 4 °C with an equivalent volume of distilled water to get rid of excess sugars (centrifuged for 5 min at 1500 g between each washing). Culture samples were then filtered on a 24 mm glassfiber filter (GF/C, Whatman Ltd., Maidstone, UK) and dried for 24 h at 70 °C. A good linear regression fit was obtained (R^2 value of 0.9953) between the dry weight and absorbance measurements at 750 nm, which was observed for dry weights ≤2.0 g L^{-1} and $OD_{750\ nm}$ ≤0.5767. Specific growth rate (μday^{-1}) was calculated from the increase in cell density according to the equation $\mu = \ln(X_2 / X_1) / (t_2 - t_1)$, where X is the biomass yield (g L^{-1}) and t is the time in units of days. Cultures were sampled at days 0, 3, 7, 10 and 13 for sugar and ion analyses (see Sections 2.3.1 to 2.3.2).

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2.2.3. Lipid enrichment

Lipid accumulation while growth occurs on WP was tested with different initial nitrogen levels (Supplementary material). *S. obliquus* was grown in triplicate on 150 mL of sterile BBM, BBM + WP (60% and 40% v/v, respectively) and LW + WP (60% and 40% v/v, respectively) media in 250 mL Erlenmeyer flasks. All cultures were kept in 16 h:8 h light–dark regimes with air bubbling at an aeration rate of 0.3 L min^{-1}. Samples were taken at days 7 and 13 for growth and lipid analyses (see Section 2.3.3).

2.3. Analytical methods

2.3.1. Sugar analysis

Culture samples were centrifuged (1500 g, 5 min) and the supernatants were transferred in other tubes before freeze-drying. Lyophilized material was analyzed as described in [24] by GC–MS.

2.3.2. Ion analysis

Culture samples (1 mL) were centrifuged (10,000 g, 15 min) and the supernatant fraction was stored at −20 °C until analysis. The analytical procedure was performed with an ion chromatography system (ICS-1000, Dionex Corporation, Sunnyvale, CA) equipped with a high-pressure isocratic pump and conductivity detector (DS6) set to 35 °C. Volumes of 25 μL were injected. Cation analyses were performed with a security guard cartridge (IonPac CG12A 4 × 50 mm) coupled to the analytical column (IonPac CS12A, 4 × 250 mm). The eluent was 20 mM of methylsulfonic acid and a flow rate of 1.0 mL min^{-1} was used throughout the analysis (15 min per run). Suppressor (CSRS Ultra II, 4 mm) current was set to 59 mA. Anion analyses were performed with a pre-column (IonPac AG14A, 4 × 50 mm) coupled to the analytical column (IonPac AS14A, 4 × 250 mm). An aqueous solution of 8 mM Na_2CO_3 and 1 mM $NaHCO_3$ was used as eluent at a flow rate of 0.8 mL min^{-1}. The total run time of each sample was 18 min. Suppressor (ASRS Ultra II, 4 mm) current was set to 45 mA. Calibration curves were used to quantify the major ions: sodium (Na^+), potassium (K^+), ammonium (NH_4^+), magnesium (Mg^{2+}), calcium (Ca^{2+}), chloride (Cl^-), nitrite (NO_2^-), nitrate (NO_3^-), phosphate (PO_4^{3-}) and sulfate (SO_4^{2-}), and all samples were diluted so their concentration would fall within the boundaries of these curves. The chromatograms were monitored and integrated by the Chromeleon® software version 6.8.

2.3.3. Intracellular lipid analysis

Culture samples were filtered on 24 mm glass-fiber filters (GF/C, Whatman Ltd., Maidstone, UK) and the lipids were extracted using a dichloromethane:methanol (2:1 v/v) solution, following the Folch procedure [25]. Extracts were subdivided into two subsamples: one for fatty acid profile characterization on total lipids and the other for fatty acid characterization of NL (reserve lipids, mainly TAG) after separation from polar lipids (structural lipids, mainly phospholipids) by column chromatography on silica gel micro-columns (30 × 5 mm I.D. Kieselgel 70–230 mesh Merck) using chloroform:methanol (98:2, v/v) to elute NL [26]. Fatty acid profiles were determined on FAME obtained by esterification using sulfuric acid:methanol (2:98, v/v) in toluene. FAMEs were concentrated in hexane before analysis by GC–MS (Thermo Fisher Scientific Inc., GC model Trace GC Ultra and MS model ITQ900) equipped with a Supelco Omegawax 250 capillary column (30 m × 250 μm × 0.25 μm film thickness). Initial oven temperature was 100 °C for 2 min, then 140 °C for 1 min and was increased at a rate of 10 °C min^{-1} until it reached 270 °C. Injector temperature was 90 °C and a constant helium flow of 1.0 mL min^{-1} was used. Volumes of 1 μL were injected. Fatty acids (FAs) were identified by comparing the retention times and mass spectrum with known standards (Supelco 37 Component FAME Mix; Supelco Inc., Belfonte, PA, USA) with the use of Xcalibur v.2.1 software (Thermo Fisher Scientific Inc., Mississauga, ON, CA).

2.4. Data analysis

For evaluating the ability of the microalgae strains to use lactose (Fig. 1), the cellular concentrations obtained at day 13 were analyzed using Student's *t*-test. The effects of WP supplementation on *S. obliquus* growth (Fig. 2) were evaluated through mixed-model analyses of variance (ANOVA) performed on culture absorbance at 750 nm for all three trophic culture conditions (photoautotrophy, mixotrophy and heterotrophy). The results at two different time conditions (days 6 and 13) were used as repeated measures. Maximum specific growth rates ($\mu_{max}day^{-1}$) were evaluated by one way ANOVA with the trophic condition as treatment. Dissolved sugar contents of the culture media were analyzed by mixed-model ANOVA and the results obtained at two different time conditions (days 3 and 13) were used as repeated measures. Mixed-model ANOVA was also performed on the lipid enrichment results to evaluate the temporal variation (between days 7 and 13) in cellular total lipid content (mg g^{-1} dry weight), % saturated fatty acid (SFA), % monounsaturated fatty acid (MUFA), % polyunsaturated fatty acid (PUFA) and neutral lipid (NL) ratio to total lipids (TL) for the microalgae grown in three different medium formulations (BBM, BBM + WP, LW + WP). When variations were noted, a posteriori Student–Newman–Keuls (SNK) multiple comparison tests were conducted to determine whether the difference in the results was statistically significant. Normality was verified by a Shapiro–Wilk test. When necessary, data were log + 1 or arcsine square-root (for % data) transformed to achieve homogeneity of variances. Analyses were carried out using SAS v.9.2 software (SAS Institute Inc., Cary, NC, USA).

3. Results and discussion

3.1. Microalgae strain selection

Four microalgae strains (*S. obliquus*, *S. acutus*, *C. vulgaris* and *C. protothecoides*) were identified as potential candidates for WP conversion (mainly lactose), based on their endogenous β-galactosidase activity [18], their ability to accumulate high lipid contents and their well-adapted fatty acid profiles for biodiesel production [3,27]. They were cultivated in the presence of pure D-lactose monohydrate under strict heterotrophic conditions for comparison. Results in Fig. 1 clearly show that only the *Scenedesmus* strains were able to grow in the 5 g L^{-1} pure lactose-supplemented medium: both cultures showed significant differences relative to their respective control cultures at day 13 of the experiment ($T_{(4)} = -8.66$, $p < 0.001$ and $T_{(4)} = -6.24$, $p = 0.003$, respectively). *S. obliquus* cultures reached the highest cellular concentrations ($25 \pm 4 \times 10^6$ cells mL^{-1}). To our knowledge, it is only the second time that lactose is shown to support *Scenedesmus*'s growth [28]. A similar utilization of lactose was observed in certain plant cell suspensions [29–31]. Since *C. protothecoides* cannot assimilate nitrate [32], soy protein peptone was added to the BBM medium (BBMP) so it would support its growth in photoautotrophic conditions. In heterotrophic conditions, results showed that lactose did not support the growth of either of the *Chlorella* spp.: no significant difference has been observed between the controls and the lactose-supplemented cultures.

3.2. WP use and growth optimization

Substituting 40% (v/v) of BBM with WP led to important growth stimulation in *S. obliquus* cultures under both mixotrophic and heterotrophic conditions (Fig. 2). Culture growth was estimated using absorbance (at 750 nm) and dry weight measurements. Under mixo- and heterotrophic conditions, pH remained stable over the whole experiment at an average value of 6.4 ± 0.1. Mixotrophic conditions induced rapid growth stimulation until day 6, after which it considerably slowed down to a somewhat stationary growth phase. Heterotrophic conditions induced moderate though sustained growth stimulation until

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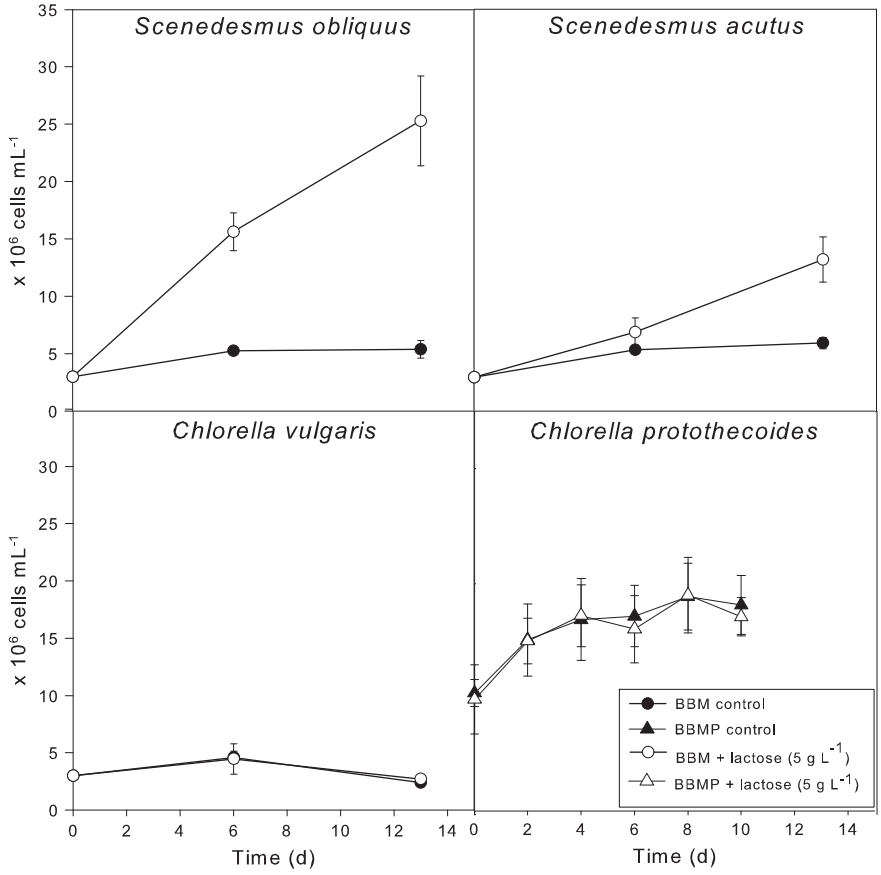

Fig. 1. *S. obliquus*, *S. acutus* and *C. vulgaris* cultured in Bold's basal medium (BBM), and *C. protothecoides* cultured in BBM + 1 g L^{-1} soy protein peptone (BBMP), were supplemented with 5 g L^{-1} pure lactose under strict heterotrophic conditions (absence of light). Results are presented in cell concentration ($\times 10^6$) per mL and expressed as the mean ± SD (n = 3).

day 13. At that time, absorbance measurements were 636% and 575% (respectively for the mixotrophic and heterotrophic conditions) to that of photoautotrophic cultures. A significant interaction between time and treatments was observed ($F_{(2, 12)} = 387.30$, $p < 0.0001$).

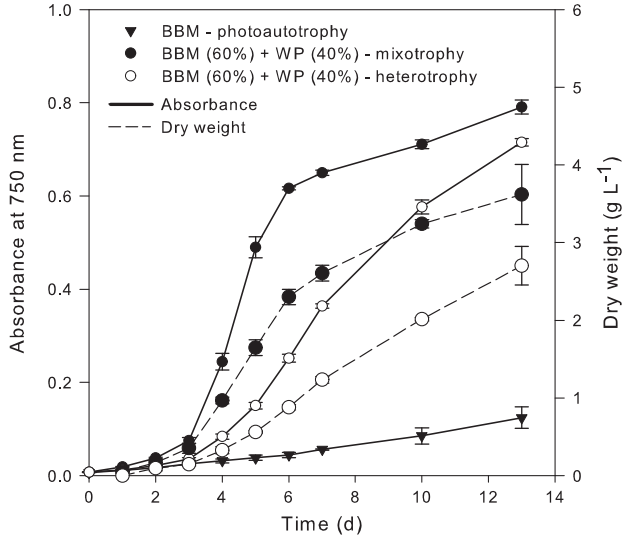

Fig. 2. Absorbance at 750 nm and dry weight (DW) of *S. obliquus* cultures grown in photoautotrophy, mixotrophy or heterotrophy. For mixotrophic and heterotrophic conditions, 40% (v/v) of the Bold's basal medium (BBM) was substituted by cheese whey permeate (WP). Results are expressed as the mean ± SD (n = 3).

Dry weight (DW) measurements were performed for the mixotrophic and heterotrophic WP supplemented cultures throughout the experiment. The highest biomass yield was obtained in mixotrophic conditions, 3.6 ± 0.4 g L^{-1} DW after 13 days, versus 2.7 ± 0.2 g L^{-1} DW (also after 13 days) for heterotrophic cultures. A linear regression was performed between DW and absorbance data (Supplementary material). Different linear relationships were obtained for biomass concentrations above and below 2 g L^{-1}. For this reason, only the values under 2 g L^{-1} were used for the linear regression equation. The correlation shift coincides with the decrease in growth observed from day 6 in the mixotrophic group. A similar phenomenon was also observed in *Haematococcus pluvialis* cultures in [33], which was attributed to a change in the cellular state or cell contents due to variations in nutrient availability. These results further enforce the recommendation in [34] that correlating culture absorbance with dry weight (for standard curve generation) should only be done during the exponential and linear growth phases.

In photoautotrophic BBM control cultures, the maximum specific growth rate (μ_{max} day^{-1}) was reached at day 3 (0.267 ± 0.083), while it occurred at day 4 for both the mixotrophic (1.083 ± 0.030) and the heterotrophic (0.702 ± 0.025) groups. The mixotrophic conditions resulted in a significant increase of the growth rate ($F_{(2, 6)} = 179.94$, $p < 0.0001$), which is consistent with other results found on *S. obliquus* in mixotrophic conditions (and various other DOC sources) [19,35–37]. Another interesting observation in this case is that the maximum growth rate (μ_{max}) obtained in mixotrophic mode corresponds approximately to the sum of the maximum growth rates obtained in the photoautotrophic and heterotrophic modes ($\mu_{mixo} = \mu_{photo} + \mu_{hetero}$). Similar observations can be found in the literature with other microalgae species also: *Chlorella regularis*, *C. vulgaris*, *Euglena gracilis*, *H. pluvialis* and *Spirulina platensis* [38].

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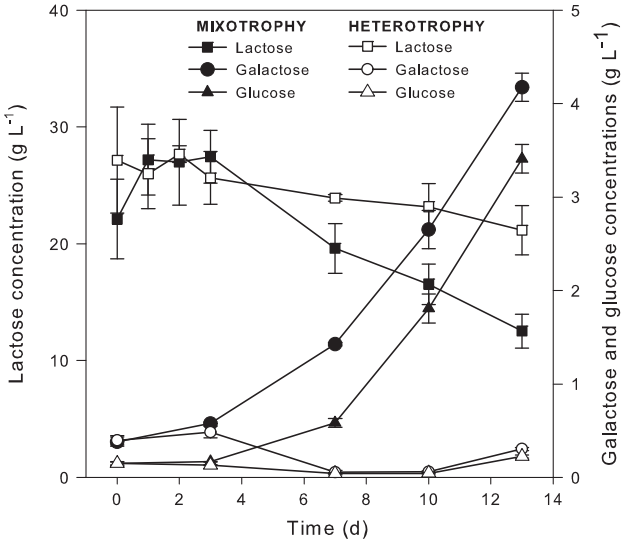

Fig. 3. Lactose, galactose and glucose concentrations in *S. obliquus* cultures grown under mixotrophic or heterotrophic conditions with 40% (v/v) cheese whey permeate (WP) substitution of the culture medium (BBM). Results are expressed as the mean ± SD (n = 3).

3.2.1. Lactose hydrolysis from WP by S. obliquus

Significant differences in the measurement of initial lactose concentration (at day 0) were observed between the two treatments (Fig. 3) despite an identical medium formulation. This could be due to 1) an incomplete solubilization in the culture medium at the start of the experiment, 2) adsorption by freshly inoculated cells or 3) another phenomenon. Due to this unexplained difference, statistical significance of results in sugar concentrations was analyzed between days 3 and 13 of the experiment.

Significant differences attributable to the trophic conditions and times were observed for each individual sugar (galactose: $F_{(3,\ 4)} = 1133.4$, $p < 0.0001$; glucose: $F_{(3,\ 4)} = 872.6$, $p < 0.0001$; lactose: $F_{(3,4)} = 31.19$, $p = 0.0031$). Heterotrophic conditions however showed no significant differences in lactose concentrations between days 3 and 13 ($p = 0.0603$). Also in this case, the glucose and galactose concentrations fell below the detection limit of the instrumentation between days 7 and 10 (Fig. 3), suggesting that the monosaccharides were consumed by the microalgae.

Mixotrophic conditions showed a significant decrease in lactose concentration (−54.4%) between days 3 and 13 ($p = 0.001$). It was accompanied by an accumulation of galactose and glucose in the culture medium, suggesting extracellular hydrolysis of lactose. Such a phenomenon would make more glucose and galactose available to the cells (for growth), though in this case these metabolites were mainly accumulated in the medium (up to 4.2 ± 0.1 g L^{-1} for galactose and 3.5 ± 0.2 g L^{-1} for glucose) while the onset of the stationary growth phase occurred (Fig. 2). Exhaustion of nitrogen (or other nutrient limitations) in the culture media would explain this occurrence (see Section 3.2.2).

To assimilate lactose, living organisms have to synthesize a β-galactosidase enzyme to hydrolyze lactose into glucose and galactose and internalize the molecules through the adequate transmembrane proteins. In *Escherichia coli*, the *lac* operon is responsible for the expression and regulation of these enzymes as a function of lactose and glucose availability in its environment [39]. Certain plant and bacterial β-galactosidases can even be secreted outside the cell [40,41], resulting in a necessary internalization of the glucose and galactose molecules instead. In the microalgae *Chlorella kessleri*, transmembrane transporters HUP1 and HUP2 allow for this internalization to occur, but with different affinities for glucose and galactose [42]. Interestingly, some point mutations in the HUP1 gene (Q298N and N436Q) can modulate this affinity [43]. The ability of *S. obliquus* to grow on glucose suggests that a similar sugar transport mechanism is present in its genus [19]. Although endogenous β-galactosidase activity has already been demonstrated in *S. obliquus* cultures [18], whether this enzyme can be secreted by these cells remains to be investigated.

Differences in lactose consumption rates between the mixo- and heterotrophic conditions may be explained by the different growth rates obtained. In both occasions, glucose and galactose began to accumulate as the biomass reached 2 g L^{-1} DW (day 7 for mixotrophy and day 13 for heterotrophy) which corresponds to the onset of a stationary phase for the mixotrophic group (Fig. 2). Also, different patterns of gene expression and metabolic regulation under mixo- and heterotrophic culture conditions could be responsible for this result. For instance, light regulates various cellular metabolisms, such as photosynthesis and glycolytic pathways [44]. For instance, it was shown that for *Chlorella sorokiniana* grown in the dark, the metabolic flux of glucose was mainly being routed (90%) through the pentose phosphate pathway (PPP), while only 10% was routed through the Embden–Meyerhof pathway (EMP). In the presence of light, the opposite was actually observed (EMP was the main glycolytic pathway) [45].

3.2.2. Nitrogen depletion

The media was sampled at days 0 to 4, 7, 10 and 13 for major ion (Na^+, K^+, NH_4^+, Mg^{2+}, Ca^{2+}, Cl^-, NO_2^-, NO_3^-, PO_4^{3-}, SO_4^{2-}) analyses by ion chromatography (Supplementary material). Nitrate (NO_3^-) and ammonium (NH_4^+) concentrations fell below the detection limit of the method (<1 ppm) from day 7 of the experiment for both the mixotrophic and heterotrophic cultures. Under mixotrophic conditions, NH_4^+ was consumed more rapidly, probably due to a faster growth rate. Since nitrogen depletion coincided with the end of the exponential growth phase, such evidence points toward this nutrient to be the limiting factor. In most microalgae cultures, total consumption of nitrogen from the culture media is necessary before significant lipid accumulation (mainly NL) occurs: thus, the design of an industrial application should carefully consider the biomass yields before introducing nitrogen deficiency conditions [46–48].

3.3. Lipid enrichment

Several aspects of a realistic industrial implementation were considered in the design of this experiment. First, the culture volume in the shake-flasks was twice the usual volume, and an air bubbling system was added. The idea was to emulate the strategy used in larger tanks (with smaller surface-to-volume ratio) to avoid excessive CO_2–O_2 accumulation [49]. A light–dark cycle (16 h:8 h) was also imposed to reduce the operation costs versus continuous illumination. Finally, the BBM medium formulation was replaced by natural lake water (LW) composition, sampled from a lake that could serve as water supply for large scale microalgae culturing.

Cultures were sampled during linear (day 7) and early stationary growth phases (day 13) for dry weight and lipid analyses. Table 1 presents the global NL and TL productivities (mg L^{-1} day^{-1}) at day 13 and biomass yields at days 7 and 13 for all three experimental conditions. Despite biomass yields 1.8 to 2.6 times higher, mixotrophic

Table 1
Biomass yields at day 7 and day 13, and lipid productivity at day 13 of *S. obliquus* cultured in Bold's basal medium (BBM), BBM + 40% (v/v) whey permeate (WP) or lake water (LW) + 40% (v/v) whey permeate.

	Biomass yield (g L^{-1})		Lipid productivity (mg L^{-1} day^{-1})	
	Day 7	Day 13	Total lipids	Neutral lipids
BBM	0.7 ± 0.1	1.9 ± 0.1	36.9 ± 1.0	31.2 ± 2.1
BBM + WP	2.6 ± 0.1	4.9 ± 0.2	37.8 ± 4.3	19.2 ± 1.8
LW + WP	1.9 ± 0.1	3.5 ± 0.1	28.3 ± 6.0	17.6 ± 4.5

Results are expressed as the mean ± SD (n = 3).

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Table 2
Total and neutral lipid fatty acid composition at day 7 and day 13 of *S. obliquus* cultures in BBM, BBM + 40% (v/v) whey permeate and lake water + 40% (v/v) whey permeate[A].

Day	7			13			
	BBM	BBM + WP	LW + WP	BBM	BBM + WP	LW + WP	Statistical analysis
Total lipids (% molar)							
16:0	21.9 ± 0.4	22.1 ± 0.3	22.8 ± 1.1	23.6 ± 0.2	21.7 ± 0.2	23.0 ± 0.2	
18:0	1.0 ± 0.3	1.4 ± 0.1	1.0 ± 0.1	2.1 ± 0.1	2.4 ± 0.1	2.2 ± 0.2	
16:1	TR	5.0 ± 0.3	5.5 ± 0.7	2.0 ± 0.2	4.5 ± 0.4	5.8 ± 0.4	
17:1	2.5 ± 0.2	4.8 ± 0.4	7.1 ± 0.3	1.4 ± 0.1	3.7 ± 0.1	4.6 ± 0.3	
18:1 n-9	27.1 ± 5.2	25.2 ± 2.0	22.4 ± 1.6	43.9 ± 1.0	29.3 ± 0.5	28.7 ± 0.4	
18:2 n-6	8.8 ± 1.0	15.1 ± 1.5	18.7 ± 0.8	8.4 ± 0.8	17.4 ± 0.7	21.3 ± 0.4	
18:3 n-3	2.2 ± 0.2	1.7 ± 0.1	1.3 ± 0.1	1.3 ± 0.1	1.3 ± 0.1	0.5 ± 0.1	
18:4 n-3	34.4 ± 4.4	22.8 ± 0.2	19.4 ± 0.7	15.7 ± 0.2	17.7 ± 0.7	12.3 ± 0.5	
Sum SFAs	24.0 ± 0.5^{c}	24.5 ± 0.4^{bc}	24.9 ± 1.1^{bc}	26.4 ± 0.2^{a}	25.1 ± 0.3^{b}	26.1 ± 0.3^{a}	t∗trt ($F_{(2, 12)} = 4.17$, $p = 0.0421$)
Sum MUFAs	30.2 ± 4.9^{d}	35.4 ± 1.4^{c}	35.3 ± 1.9^{c}	48.0 ± 0.9^{a}	38.0 ± 0.8^{bc}	39.5 ± 0.3^{b}	t∗trt ($F_{(2, 12)} = 20.64$, $p < 0.0001$)
Sum PUFAs	45.8 ± 5.3^{a}	40.1 ± 1.6^{b}	39.7 ± 1.0^{b}	25.6 ± 1.1^{d}	36.9 ± 1.0^{bc}	34.4 ± 0.3^{c}	t∗trt ($F_{(2, 12)} = 23.26$, $p < 0.0001$)
Neutral lipids (% molar)							
16:0	20.6 ± 1.6	19.8 ± 0.1	21.6 ± 1.2	19.2 ± 0.5	18.1 ± 0.7	19.1 ± 0.3	
18:0	2.1 ± 0.2	3.1 ± 0.1	2.8 ± 0.2	2.2 ± 0.1	3.5 ± 0.1	3.1 ± 0.1	
16:1	4.5 ± 2.9	2.4 ± 0.4	2.5 ± 1.4	1.5 ± 0.1	2.8 ± 0.7	4.1 ± 0.2	
17:1	0.9 ± 0.1	1.9 ± 0.3	3.6 ± 0.2	0.8 ± 0.1	2.0 ± 0.1	3.3 ± 0.1	
18:1 n-9	51.2 ± 2.5	48.1 ± 2.4	34.9 ± 2.2	58.0 ± 1.0	46.5 ± 0.8	36.8 ± 0.1	
18:2 n-6	4.8 ± 0.3	11.9 ± 1.8	20.1 ± 2.2	6.8 ± 0.6	16.1 ± 0.6	23.7 ± 0.3	
18:3 n-3	1.0 ± 0.2	1.1 ± 0.1	0.8 ± 0.1	0.7 ± 0.1	0.9 ± 0.1	0.4 ± 0.1	
18:4 n-3	11.6 ± 0.4	8.9 ± 0.7	10.1 ± 0.2	9.3 ± 0.1	7.9 ± 0.3	7.5 ± 0.3	
Sum SFAs	24.1 ± 1.3	24.2 ± 0.6	26.2 ± 1.6	21.9 ± 0.5	22.6 ± 0.8	23.3 ± 0.4	t ($F_{(1, 12)} = 26.19$, $p = 0.0003$); trt ($F_{(2, 12)} = 6.06$, $p = 0.0152$)
Sum MUFAs	58.4 ± 1.2^{b}	53.6 ± 0.5^{c}	42.3 ± 1.5^{e}	61.1 ± 1.0^{a}	52.2 ± 0.4^{c}	44.7 ± 0.2^{d}	t∗trt ($F_{(2, 12)} = 4.63$, $p = 0.0323$)
Sum PUFAs	17.6 ± 0.4	22.2 ± 2.4	31.4 ± 2.3	16.9 ± 0.6	25.3 ± 0.4	31.9 ± 0.5	trt ($F_{(2, 12)} = 159.49$, $p < 0.0001$)
Lipids (mg g^{-1} dry biomass)							
Total lipids (TLs)	109 ± 3^{b}	91 ± 7^{bc}	78 ± 1^{c}	249 ± 10^{a}	99 ± 7^{b}	105 ± 25^{b}	t∗trt ($F_{(2, 12)} = 54.39$, $p < 0.0001$)
Neutral lipids (NLs)	46 ± 15^{bc}	36 ± 6^{c}	25 ± 4^{c}	211 ± 27^{a}	50 ± 4^{bc}	65 ± 18^{b}	t∗trt ($F_{(2, 12)} = 44.52$, $p < 0.0001$)
NL/TL	0.43 ± 0.14^{cd}	0.40 ± 0.04^{cd}	0.32 ± 0.05^{d}	0.85 ± 0.08^{a}	0.51 ± 0.04^{bc}	0.62 ± 0.06^{b}	t∗trt ($F_{(2, 12)} = 6.25$, $p = 0.0138$)

Results are expressed as the mean ± SD (n = 3). BBM: Bold's basal medium; LW: lake water; WP: whey permeate; SFAs: saturated fatty acids; MUFAs: monounsaturated fatty acids; PUFAs: polyunsaturated fatty acids; T: time; trt: treatment; and d.f.: degree of freedom.

[A] After mixed model repeated two-ways ANOVA, Student–Newman–Keuls (SNK) multiple comparison test results are arranged in decreasing order from left to right: a > b > c > d > e (effect of time (t) and treatment (trt), SNK test, $P < 0.05$).

cultures resulted in lower NL productivities even when compared to the photoautotrophic control cultures. This is attributable to lower NL cellular contents at the time of the sampling (Table 2).

Fatty acid profiles (TL and NL fractions) are presented in Table 2 for each treatment. At day 7, the presence of PUFA was dominant for all treatment conditions, with stearidonic acid (18:4 n-3) as the main one. The relative proportion of each fatty acid class changed with time and the presence of MUFA, with oleic acid (18:1 n-9) as the main component, became dominant at day 13, again in all three situations. This increase in oleic acid is the result of NL accumulation between days 7 and 13: oleic acid was the prominent fatty acid of the NL fraction and an increase in the NL/TL ratio was observed for all treatments over this time interval. This ratio even reached 0.85 ± 0.08 in the photoautotrophic BBM control cultures at day 13. These cultures also showed the highest TL accumulation (249 ± 10 mg g^{-1} dry weight).

Differences in DOC and overall nutrient availability, growth stages, osmolality and pH could explain the more important NL accumulation in the photoautotrophic BBM control cultures than in the WP supplemented cultures. As in Section 3.2, the pH of these cultures remained stable over the whole experiment (6.1 ± 0.3) while in photoautotrophic cultures it raised rapidly to reach 9.7 ± 0.2 (in average) between days 7 and 13 of the experiment. High pH conditions combined with nitrogen deficiency have been known to induce TAG accumulation in *Scenedesmus* sp. [48]. Finally, higher NL accumulation was observed in LW + WP cultures (2.6 fold) compared to BBM + WP cultures (1.4 fold) between days 7 and 13 of the experiment. Lower initial nitrogen availability in LW could explain this difference.

Fatty acid profile analyses revealed good suitability of *S. obliquus*'s oil for biodiesel production. The enrichment in SFA and MUFA fractions observed between days 7 and 13 has been favorable to this end, as biodiesel produced from these classes of fatty acids has better physicochemical properties (particularly cetane number and oxidative stability) than when produced from PUFA [5]. About the global availability and potential of whey as a DOC source for microalgal biodiesel production, there is currently over 150 M tons of whey produced each year globally, of which 4.9% (w/w) is lactose [50]. According to the results obtained here, these sugars could yield between 3.5 and 7 M tons of microalgal biomass and between 50 and 500 M gallons of microalgal biodiesel annually.

3.4. NL cellular content estimation

Various methods are available to estimate NL cellular content (often expressed in mg g^{-1} DW). Nile Red fluorescence is a widely used method which was proven successful with a good variety of microalgae species [51–53]. However, this method implies significant costs, time and manipulation, thus is not well adapted for industrial implementation. The NL cellular contents (Table 2) were therefore correlated with the culture absorbances at 540, 680 and 750 nm (Supplementary material). The hypothesis is that for a given cell concentration, the difference in composition (lipids vs. proteins vs. carbohydrates) would result in different light absorption patterns, providing information that could be translated into an indication of the NL cellular content. The best correlation results ($R^2 = 0.82$) were obtained with log transformed data at a 680 nm wavelength. Differences in cell size, shape and organization may also greatly influence the cell's absorption/light scattering patterns, especially at 750 nm where light scattering is predominant. A particularity of *S. obliquus* cells (which individual size can vary from 4 to 12 μm) is their common organization in groups of four cells (or in pairs) depending on the growth stage. Despite these important changes in physiological patterns, the NL content (in mg g^{-1} DW) was estimated with relatively good accuracy simply by measuring the absorbance of

S. obliquus cultures at 680 nm and 750 nm ($R^2 = 0.78$). This suggests that the NL content is inversely correlated with the chlorophyll content, since chlorophyll *a* is known to absorb light at 680 nm [54]. Whether this relation would be useful to estimate NL cellular content in other microalgae species is yet to be demonstrated.

4. Conclusion

In conclusion, cheese whey permeate has been investigated as a potential DOC source for microalgae cultivation for biodiesel production. Evaluation of microalgae strains with an ability to use lactose for growth showed *S. obliquus* to be the most promising candidate. Substituting 40% (v/v) of the default culture medium (BBM) with WP resulted in higher specific growth rates and biomass yields under mixotrophic conditions than heterotrophic and photoautotrophic cultures. Mixotrophic growth allowed a 54.4% reduction in lactose concentration while during the stationary growth phase, an accumulation of glucose and galactose was observed in the culture medium, suggesting extracellular lactose hydrolysis by the microalgae. Future work is however necessary to optimize the biomass yields and lactose consumption, and determine the culture conditions that will allow increased NL productivity in *S. obliquus* WP supplemented cultures.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.algal.2014.03.002.

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