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DESENVOLVIMENTO E APLICAÇÃO DE MÉTODOS ANALÍTICOS PARA CARACTERIZAÇÃO DE BIOMASSAS MICROALGAIS

Santa Maria, RS
2020

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Tese apresentada ao Curso de Pós-Graduação em Ciência e Tecnologia dos Alimentos, da Universidade Federal de Santa Maria (UFSM, RS), como requisito parcial para obtenção do título de **Doutora em Ciência e Tecnologia dos Alimentos**

Orientador: Prof. Dr. Roger Wagner

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RESUMO

DESENVOLVIMENTO E APLICAÇÃO DE MÉTODOS ANALÍTICOS PARA CARACTERIZAÇÃO DE BIOMASSAS MICROALGAIS

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ORIENTADOR: Roger Wagner

A biomassa microalgal apresenta compostos de importância comercial, lipídios, proteínas, carboidratos, pigmentos e toxinas são os mais pesquisados e aplicados. Entretanto, intermediários da síntese também podem ser estudados, para auxiliar no entendimento das vias metabólicas desempenhadas por esses microrganismos. O objetivo desse trabalho foi avaliar metabólitos microalgais e caracterizar biomassas obtidas a partir de cultivos com diferentes condições externas. As análises foram realizadas com métodos integrados de preparo de amostra e para pigmentos, especificamente, foi desenvolvido um novo método de extração. Com exceção dos pigmentos que são termo instáveis e determinados por espectrofotometria ou cromatografia líquida (LC), a cromatografia em fase gasosa (GC) se apresenta como uma ferramenta analítica adequada para caracterização de metabólitos. Essa técnica tem como vantagens separação e detecção de gases, compostos orgânicos voláteis, metabólitos polares (ácidos orgânicos, aminoácidos, açúcares e açúcares álcoois) e não polares (ácidos graxos e esteróis), além de elevada resolução e seletividade. A GC foi emprega no estudo da metabolômica de *Scenedesmus obliquus*, permitindo a identificação e quantificação de ácidos orgânicos, aminoácidos livres e ácidos graxos. Além dos precursores, foram determinados proteínas, lipídios e clorofilas totais. A síntese dos metabólitos precursores foi afetada pelas condições externas dos cultivos, diferentes fotoperíodos, 24:0 e 12:12 (hora:hora; claro:escuro), e também pelas fases do crescimento celular, exponencial, estacionária e de declínio. Ao final dos cultivos as maiores concentrações de biomassa (4020 mg L^{-1}) e proteína (47,30%) foram obtidas no cultivo com iluminação constante. Já no cultivo com fotoperíodo 12:12 (claro:escuro) a *S. obliquus* desencadeou maior produção de lipídios (23,00%) e clorofilas (26,4 mg g^{-1}). Os pigmentos majoritários presentes em microalgas são as clorofilas e os carotenoides e ainda existem limitações nos métodos de extração aplicados nessas biomassas, como elevado consumo de solventes e tempo. Para o desenvolvimento do método de extração simultânea de clorofilas e carotenoides das microalgas *Spirulina* sp. e *S. obliquus*, foram avaliados diferentes tempos de homogeneização mecânica e solventes extratores. Valores de recuperação satisfatórios, de 101,11-124,18% para maioria dos pigmentos, em relação método de maceração (MM) utilizado como referência, foram obtidos com 30 min de homogeneização e extração com clorofórmio seguida por etanol (PM30E). Em geral, os perfis dos pigmentos do PM30E apresentaram menos isômeros e possíveis compostos de degradação, além da redução de cerca de cinco vezes no volume de solventes orgânicos e ser quatro vezes mais rápido que o MM. Posteriormente, foi realizada a caracterização de biomassas de *S. obliquus* cultivadas com 3, 5, 10, 15, 20 e 25% de CO_2. Cultivos com 3, 5 e 10% de CO_2 apresentaram maior síntese de aminoácidos e proteínas (superior a 60% da biomassa seca). As maiores concentrações de clorofilas foram encontradas nos cultivos com 15, 20 e 25% CO_2, 24,23, 23,06 e 30,79 mg g^{-1}. Os carotenoides totais foram três vezes maiores cultivos com 3, 5 e 10% de CO_2 e all-*trans*-β-caroteno foi o composto principal. A síntese de lipídeos foi intensificada com o aumento das porcentagens de CO_2, assim como o acúmulo de ácidos graxos poli-insaturados.

Palavras-Chave: *Scenedesmus obliquus*. *Spirulina* sp. Cromatografia em Fase Gasosa. Ácidos Orgânicos. Aminoácidos. Ácidos Graxos. Proteínas. Lipídios. Pigmentos.

ABSTRACT

DEVELOPMENT AND APPLICATION OF ANALYTICAL METHODS FOR MICROALGAL BIOMASSES CHARACTERIZATION

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Microalgal biomass has compounds of commercial importance, lipids, proteins, carbohydrates, pigments and toxins are the most researched and applied. However, synthesis intermediates can also be studied to help understand the metabolic pathways performed by these microorganisms. The objective of this work was to evaluate microalgal metabolites and to characterize biomass obtained from cultivations with different external conditions. Analyzes were carried out with integrated sample preparation methods, and for pigments, specifically, a new extraction method was developed. Except for pigments that are thermally unstable and determined by spectrophotometry or liquid chromatography (LC), gas chromatography (GC) presents itself as a suitable analytical tool for characterizing metabolites. This technique has the advantage of separating and detecting gases, volatile organic compounds, polar (organic acids, amino acids, sugars and sugar alcohols) and non-polar (fatty acids and sterols) metabolites, in addition to high resolution and selectivity. GC was used in the study of the *Scenedesmus obliquus* metabolomics, allowing the identification and quantification of organic acids, free amino acids and fatty acids. In addition to the precursors, proteins, lipids and total chlorophylls were determined. The synthesis of precursor metabolites was affected by the external cultivation conditions, different photoperiods, 24:0 and 12:12 (hour:hour; light:dark), and also by the phases of cell growth, exponential, stationary and declining. At the end of the cultivations, the highest concentrations of biomass (4020 mg L^{-1}) and protein (47.30%) were obtained in the cultivation with constant lighting. In the 12:12 photoperiod (light:dark) culture, *S. obliquus* triggered a higher production of lipids (23.00%) and chlorophylls (26.40 mg g^{-1}). The major pigments present in microalgae are chlorophylls and carotenoids, and there are still limitations in the extraction methods applied in these biomasses, such as high solvent consumption and time. For the method development of simultaneous extraction of chlorophylls and carotenoids from the microalgae *Spirulina* sp. and *S. obliquus*, different mechanical homogenization times and extracting solvents were evaluated. Satisfactory recovery values, from 101.11-124.18% for most pigments, in relation to the maceration method (MM) used as a reference, were obtained with 30 min of homogenization and extraction with chloroform followed by ethanol (PM30E). In general, the PM30E pigment profiles showed fewer isomers and possible degradation compounds, in addition to a reduction of about five times in the volume of organic solvents and is four times faster than MM. Subsequently, the characterization of *S. obliquus* biomasses cultivated with 3, 5, 10, 15, 20 and 25% of CO_2 was carried out. Cultures with 3, 5, and 10% CO_2 showed greater synthesis of amino acids and proteins (greater than 60% of dry biomass). The highest concentrations of chlorophylls were found in cultures with 15, 20 and 25% CO2, 24.23, 23.06 and 30.79 mg g^{-1}. The total carotenoids were three times bigger cultures with 3, 5 and 10% of CO_2 and all-*trans*-β-carotene was the main compound. The synthesis of lipids was intensified with the increase of CO_2 percentages, as well as the accumulation of polyunsaturated fatty acids.

Keywords: *Scenedesmus obliquus*. *Spirulina* sp. Gas Chromatography. Organic Acids. Amino acids. Fatty acids. Proteins. Lipids. Pigments.

SUMÁRIO

APRESENTAÇÃO

A tese de doutorado segue as normas estabelecidas na Estrutura e Apresentação de Monografias, Dissertações e Teses – MDT da UFSM (UNIVERSIDADE FEDERAL DE SANTA MARIA, 2015). Os resultados obtidos no decorrer dos anos de doutoramento estão apresentados na forma de quatro artigos científicos, um deles de revisão bibliográfica e os demais com dados experimentais da pesquisa que foi desenvolvida.

1 INTRODUÇÃO

Metabólitos são compostos químicos transformados durante metabolismo e seu estudo, também conhecido como “ômicas”, está assumindo elevada importância no cenário científico biológico, pois possibilita o conhecimento e compressão das rotas metabólicas existentes em determinada matriz biológica (PATTI, YANES & SIUZDAK, 2012). Avanços rápidos nas tecnologias "ômicas" tem auxiliado na elucidação do potencial das microalgas como matéria-prima multiuso, seja em biotecnologia industrial, biocombustíveis, ciência de alimentos e aplicações biomédicas (GUARNIERI & PIENKOS, 2015).

Microalgas são organismos procariontes ou eucariontes, que apresentam versatilidade no que se refere a manutenção de suas estruturas, usufruindo de diferentes metabolismos energéticos, fotossintético, heterotrófico e fixação de nitrogênio (MORALES-SÁNCHEZ et al., 2014; ZHU, 2015). Dependendo da espécie, as microalgas são capazes de produzir quantidades variáveis de lipídios, pigmentos, esteróis, proteínas, enzimas, toxinas e compostos antioxidantes, os quais apresentam aplicações em distintos setores industriais (biocombustíveis, cosméticos, farmacêuticos, nutrição humana e animal) (MORENO-GARCIA et al., 2017; CHEW et al., 2017).

A maioria das análises metabólicas realizadas em microalgas tem foco na identificação e quantificação de metabólitos secundários com valor econômico. Metabólitos microalgais com interesse comercial são amplamente estudados para diferentes espécies. Já estudos metabólicos sobre a resposta de um microrganismo frente a estímulos ou estresse devido diferentes condições ambientais são realizados em menor quantidade (JAMERS, BLUST & COEN, 2009). Neste sentido, pesquisas recentes se concentram principalmente na elucidação dos mecanismos responsáveis pela carência de nutrientes e indução do acúmulo de lipídios microalgais na perspectiva de produção de biodiesel (RAI et al., 2016).

Em um estudo de metabólitos, o método de preparo de amostra e a ferramenta analítica a serem utilizados para determinação devem ser considerados. Normalmente, a extração dos metabólitos de uma amostra é realizada utilizando mais de um solvente ou método para cobrir uma maior gama de compostos, como por exemplo hidrofóbicos e hidrofílicos (RAI et al., 2016). Em contrapartida, atualmente são requeridos métodos com redução no número de etapas, uso de solventes menos tóxicos, economia de energia, integração de processos, redução do volume ou eliminação dos solventes utilizados, assim como dos resíduos gerados (TOBISZEWSKI et al., 2015).

Em relação as ferramentas analíticas, a Ressonância Magnética Nuclear (NMR) e a Espectrometria de Massas (MS) estão entre as tecnologias disponíveis mais populares para identificação de perfis metabólicos de médio a alto nível. Já as técnicas mais comuns para separação dos compostos antes da identificação, são a Cromatografia Líquida (LC) e a Cromatografia Gasosa (GC) (GOULITQUER, POTIN & TONON, 2012). A GC apresenta como vantagens a separação e detecção de gases, compostos orgânicos voláteis, compostos polares (ácidos orgânicos, aminoácidos, açúcares e açúcares álcoois) e não polares (ácidos graxos e esteróis) com elevado poder de resolução e sensibilidade, além de permitir a análise simultânea de diferentes classes químicas (LEE et al., 2013; VENDRUSCOLO et al., 2018; VENDRUSCOLO et al., 2019).

Nesse sentido, o desenvolvimento de novos métodos de preparo de amostra e aplicação de técnicas analíticas que permitam monitorar cultivos microalgais, bem como caracterizar as biomassas obtidas ao final desses cultivos são relevantes no cenário científico-tecnológico atual.

2 OBJETIVOS

2.1 OBJETIVO GERAL

Monitorar os metabólitos microalgais de cultivos sob diferentes condições ambientais e caracterizar suas biomassas, empregando e desenvolvendo métodos analíticos integrados, mais rápidos, com menor uso de solventes orgânicos e, consequente, menor geração de resíduos.

2.2 OBJETIVOS ESPECÍFICOS

- Apresentar a GC como uma ferramenta analítica viável para o monitoramento e otimização de processos biotecnológicos microalgais, bem como destacar as vantagens e as implicações práticas de seu emprego.

- Monitorar os ácidos orgânicos, aminoácidos e ácidos graxos produzidos em cultivos fotossintéticos de *S. obliquus* com diferentes fotoperíodos nas diferentes fases do crescimento celular através de extração com volume reduzido da mistura metanol-clorofórmio e análise por GC. Além da determinação do conteúdo de proteínas, lipídios e clorofilas totais.

- Desenvolver um método para extração simultânea de clorofilas e carotenoides de *Spirulina* sp. e *S. obliquus* sem o emprego de tecnologias de alto custo e com menor consumo de solventes orgânicos e tempo.

- Avaliar o efeito das concentrações de CO_2 (3, 5, 10, 15, 20 e 25%) em compostos polares livres, proteína total e perfil de aminoácidos proteicos, lipídios totais e perfil de ácidos graxos, clorofilas e carotenoides totais, bem como seus perfis, em cultivos fotossintéticos de *S. obliquus*.

3 REVISÃO BIBLIOGRÁFICA

3.1 ESTUDO DOS METABÓLITOS

A metabolômica faz parte dos estudos ômicos e tem por objetivo identificar e quantificar os metabólitos presentes em sistemas biológicos. Os metabólitos são produtos finais de interações complexas que ocorrem no interior das células e refletem a expressão gênica, o metabolismo intrínseco e também a relação dos organismos com o meio em que se encontram, o que permite que sejam utilizados como marcadores (GOLDANSAZ et al., 2017). A metabolômica pode apresentar uma abordagem direcionada ou não, quando direcionada, é estabelecida uma hipótese que poderá ser confirmada através dos metabólitos. Em abordagens não direcionadas, o objetivo é determinar o maior número de compostos possíveis (DUDZIK et al., 2018).

Os avanços, principalmente, em química analítica e análise de dados fizeram da metabolômica uma ciência interdisciplinar. Atualmente, é utilizada rotineiramente em pesquisas biomédica, análise de alimentos, nutrição, monitoramento ambiental e culturas de microrganismos, como as microalgas (BEALE et al., 2018). As microalgas são candidatas promissoras como fonte renovável de compostos para diversas aplicações industriais (lipídios, proteínas, carboidratos e pigmentos) e a metabolômica têm por objetivo auxiliar no entendimento do metabolismo desses biocomponentes nas diferentes espécies, assim como frente a diversas condições ambientais (SALAMA et al., 2019).

As principais ferramentas analíticas empregadas na identificação de compostos em pesquisas metabolômicas são a ressonância magnética nuclear (NMR) e espectrometria de massas (MS). Devido à complexidade do metaboloma, técnicas de separação como a cromatografia gasosa (GC) e a cromatografia líquida (LC) são utilizadas acopladas a MS (YANG et al., 2019). A GC aliada a diferentes detectores é uma técnica eficaz e versátil para pesquisas ômicas, que apresenta,

robustez, grande faixa linear dinâmica e permite analisar vários metabólitos simultaneamente. Com exceção de compostos termo instáveis, é possível realizar análise direta de metabólitos com baixo peso molecular, assim como não-voláteis após métodos de preparo de amostra adequados (KANGINEJAD & MANI-VARNOSFADERANI, 2018).

3.2 MICROALGAS

Microalgas são microrganismos microscópicos procariontes ou eucariontes, sendo as organelas as principais responsáveis por esta diferenciação. Procariontes não possuem cloroplastos, mitocôndrias e núcleo. As microalgas procariontes possuem representantes nos grupos *Cyanophyta* e *Prochlorophyta*, já as microalgas eucariontes nos grupos *Glaucophyta, Rhodophyta, Ochrophyta, Haptophyta, Cryptophyta, Dinophyta, Euglenophyta, Chlorarachniophyta* e *Chlorophyta* (MUTANDA et al., 2010). No entanto, destacam-se sob o aspecto de exploração biotecnológica os grupos: cianobactérias (*Cyanophyta*), clorofíceas (*Chlorophyta*) e diatomáceas (*Ochrophyta*) (JACOB-LOPES et al., 2019).

As microalgas são a base da cadeia alimentar e estão presentes em todos os ecossistemas existentes, devido a capacidade de se desenvolverem em ampla gama de condições ambientais, como temperaturas severas, salinidades e diversas intensidades de luz (MISHRA et al., 2019). Esses microrganismos podem ser classificados também com base no metabolismo desempenhado. No metabolismo autotrófico, as microalgas utilizam carbono inorgânico, como CO_2, e realizam fotossíntese utilizando a luz como fonte de energia para obtenção de energia e manutenção de suas estruturas. Já o metabolismo heterotrófico requer nutrientes orgânicos como matéria prima, muitas espécies acabam metabolizando por essa via por se encontrarem em ambientes extremos onde há privação de radiação solar. Por fim, microalgas mixotróficas possuem capacidade de utilizar as duas estratégias metabólicas simultaneamente (VUPPALADADIYAM et al., 2018).

Ao desempenhar seu metabolismo as microalgas acumulam quantidades substanciais de lipídios, carboidratos, proteínas, pigmentos e vitaminas, tornando-as uma fonte renovável e sustentável desses bioprodutos, que apresentam aplicabilidade industrial na área energética, farmacêutica, de alimentos e suplementos. Durante seu desenvolvimento também podem ser utilizadas em sistemas de tratamento de águas residuárias e de mitigação de CO_2 (KHAN et al., 2018). Ademais, apresentam elevadas taxas de crescimento celular, não necessitam de grandes

extensões de terras aráveis e não contribuem para o aumento da poluição ambiental como as culturas tradicionais (RÖSCH, ROBMANN & WEICKERT, 2018).

3.3 METABÓLITOS MICROALGAIS

3.3.1 Ácidos Orgânicos

Os ácidos orgânicos são caracterizados por apresentarem átomos de carbono em sua estrutura, os ácidos carboxílicos são os mais comuns, cuja acidez está associada a presença de um ou mais grupamentos carboxil (-COOH) (SHAIKH & QURESHI, 2013). A presença do grupo funcional -COOH os caracteriza como ácidos fracos em meio aquoso e que apresentam elevados pontos de ebulição devido a facilidade de formarem interações intermoleculares do tipo ligações de hidrogênio (HARRIS, 1999).

Nos sistemas biológicos, incluindo microalgas, o metabolismo dos ácidos orgânicos é de fundamental importância a nível celular para várias vias bioquímicas, incluindo produção de energia, formação de precursores para a biossíntese de aminoácidos, além de atuarem na regulação osmótica dos vacúolos das células (OIKAWA et al., 2011). Nesses microrganismos, os ácidos orgânicos também estão relacionados a adaptação frente a condições ambientais desfavoráveis, como deficiência de nutrientes, temperaturas elevadas e tolerância a metais (YE et al., 2016; SU et al., 2017; KOVÁČIK et al., 2018).

As microalgas são organismos promissores para produção de compostos orgânicos de cadeia curta, como álcoois e ácidos orgânicos (WIJFFELS, KRUSE, HELLINGWERF, 2013). Os ácidos orgânicos foram os primeiros compostos a serem explorados pela biotecnologia. Atualmente, devido à crescente demanda por compostos de origem biossintética houve um aumento na diversidade de ácidos orgânicos com aplicação industrial (BECKER et al., 2015), os quais estão sendo utilizados como blocos de construção química e polímeros biodegradáveis em substituição a produtos químicos sintéticos à base de petróleo (YANG, LÜBECK, LÜBECK, 2014).

3.3.2 Aminoácidos e Proteínas

Os aminoácidos são as unidades estruturais básicas das proteínas, macromoléculas biológicas mais abundantes. Os 20 aminoácidos primários que compõe as proteínas são α-aminoácidos, possuem um grupo carboxila e um grupo amino ligados ao mesmo átomo de carbono

(carbono α). Eles diferem entre si apenas por sua cadeia lateral, a qual é responsável pela sua função e propriedades bioquímicas (WU, 2009).

Além de serem os blocos construtores das proteínas e o ácido glutâmico da clorofila em microalgas (KANG et al., 2011), os aminoácidos desempenham um papel fundamental no metabolismo do carbono e nitrogênio. As concentrações totais e as proporções entre alguns aminoácidos são moduladas pelo processo fotossintético e pela assimilação de nitrogênio. Assim, os aminoácidos livres podem ser utilizados como marcadores para caracterizar diferentes condições fisiológicas das microalgas (VENDRUSCOLO, et al., 2019).

As proteínas são um dos compostos orgânicos majoritários que constituem as microalgas e sua composição em aminoácidos essenciais é o principal fator da qualidade proteica da biomassa microalgal. Algumas espécies de microalgas apresentam elevado potencial para produção de aminoácidos essenciais, superando proteínas alimentares de referência como a soja (VANTHOOR-KOOPMANS et al., 2013), aumentando assim o interesse no isolamento e caracterização dos aminoácidos de microalgas para aplicações farmacológicas e nutracêuticas (DO NASCIMENTO et al., 2019).

O teor de proteínas em células microalgais pode atingir até 70% em base seca, *Arthrospira maxima*, *Spirulina platensis*, *Chlorella vulgaris* e *Scenedesmus obliquus* são espécies que apresentam maiores concentrações de proteínas (EJIKE et al., 2017). As aplicações das proteínas microalgais incluem enzimas, anticorpos, hormônios, proteínas com atividades antibacteriana e antioxidante, proteínas quelantes, alimentação humana e animal. Em 2015, cerca de 54% da receita total do mercado de microalgas foi atribuída a venda de proteínas e a previsão é que este mercado tenha aumento anual de 6,5% de 2016 a 2024 (MORENO-GARCIA et al., 2017).

3.3.3 Ácidos Graxos e Lipídios

Os lipídios são compostos orgânicos de origem biológica, solúveis em solventes orgânicos não polares e geralmente insolúveis em água (AFANAS'EVA et al., 2011). Os triacilgliceróis, ácidos graxos esterificados com glicerol, são os constituintes majoritários dos lipídios, enquanto os minoritários são monoglicerídeos e diglicerídeos, ácidos graxos livres, fosfatídeos, esteróis e vitaminas lipossolúveis (ASIF, 2011). Os ácidos graxos que compõem os lipídios de microalgas apresentam, geralmente, uma faixa de 12 a 22 carbonos (MATOS, 2017).

Nos sistemas biológicos, os lipídios desempenham funções únicas, como formação de membranas celulares, estabilização das proteínas ligadas a membrana, reserva energética, principalmente na forma de triacilgliceróis, além de serem, juntamente com seus derivados, moléculas biologicamente ativas (hormônios, vitaminas) (AHMED et al., 2015).

Várias espécies de microalgas apresentam a capacidade de acumular lipídios na forma de triacilgliceróis (KUMAR e SHARMA, 2014). Através da manipulação das condições de cultivo, como elevada relação carbono/nitrogênio e também condições ambientais de estresse (mudanças no pH e temperatura elevada, por exemplo) é possível induzir a produção de quantidades substanciais destes compostos (20 a 50% em peso seco) com diferentes composições em ácidos graxos (ZHAO et al., 2019).

As microalgas são, teoricamente, capazes de produzir mais lipídios do que qualquer fonte convencional, assim se apresentam como potenciais fontes para produção de biodiesel (SUN et al., 2019). Entretanto, os lipídios obtidos de algumas espécies de microalgas apresentam elevadas concentrações de ácidos graxos ômega-3, como o ácido linolênico (C18:3n3), além dos ácidos graxos de cadeia longa, como EPA e DHA, podendo ser aplicados com fins farmacêuticos, nutracêuticos e terapêuticos, pois apresentam maior valor agregado que biocombustíveis (SANTOS-SÁNCHEZ et al., 2016).

3.3.4 Pigmentos

Os pigmentos são considerados os compostos com maior potencial de mercado, devido seu elevado valor agregado. Nas microalgas são responsáveis pela captura da luz, fixação do CO_2, proteção frente a iluminação excessiva e coloração das células (KOLLER, MUHR & BRAUNEGG, 2014). Três grupos de pigmentos são encontrados nesses microrganismos, os principais são as clorofilas (coloração verde) e os carotenoides (coloração do amarelo ao laranja), mas em cianobactérias também ocorrem as ficobilinas (com coloração vermelha ou azul) (CHEW et al., 2017).

As clorofilas são pigmentos verdes lipossolúveis que são encontrados em todas células fotossintéticas (cianobactérias, micro e macroalgas e plantas superiores). Estruturalmente, as clorofilas apresentam uma calda de fitol e um macrociclo de porfirina que compreende quatro anéis de pirrol e um átomo central de magnésio (MATOS, 2017). As ligações duplas conjugadas da

fração porfirina são as responsáveis pela captura da energia luminosa e o átomo de magnésio pelo transporte de elétrons necessários as reações da fotossíntese (CARVALHO et al., 2011).

Os principais tipos de clorofila são a clorofila *a* e a clorofila *b*, mas microalgas ainda podem apresentar clorofila c_1, clorofila c_2 e clorofila *d*, além de vários produtos de degradação formados por exposição a ácidos fracos, luz e oxigênio (DA SILVA & LOMBARDI, 2020). Em relação a fotossíntese, a molécula mais importante é a clorofila *a* que está presente no complexo do centro da reação, enquanto as clorofilas *c* e *d*, assim como os carotenoides são pigmentos auxiliares no processo fotossintético. Esses pigmentos absorvem diferentes comprimentos de onda do espectro eletromagnético e transferem a energia para a clorofila *a*, também é atribuído aos carotenoides efeito fotoprotetor da clorofila (CARVALHO et al., 2011).

A clorofila é aprovada e tem registro para o uso como corante em alimentos. A maioria das clorofilas disponíveis no mercado está na forma de clorofilida (clorofila sem a calda de fitol), que apresenta coloração verde, mas maior solubilidade em água. A clorofilida tem mostrado efeitos antimutagênicos frente a aflatoxina-B1, hidrocarbonetos aromáticos policíclicos e algumas aminas heterocíclicas (MATOS, 2017), além de ação contra células de câncer de cólon humano (KOLLER, MUHR & BRAUNEGG, 2014).

A maioria dos carotenoides apresenta uma estrutura principal de 40 carbonos, constituída de unidades isopreno (2-metil-1,3-butadieno) e são divididos em dois grupos, os carotenos que são hidrocarbonetos insaturados e as xantofilas que tem um ou mais grupos funcionais contendo oxigênio em sua estrutura (VARELA et al., 2015). Os carotenoides são chamados de pigmentos acessórios na captura da energia luminosa, pois absorvem a energia eletromagnética em faixas espectrais onde as clorofilas não são capazes de absorver, 400 a 500 nm. Assim, absorvem o excesso de luz e mantém a integridade da clorofila (D'ALESSANDRO & ANTONIOSI FILHO, 2016).

Dentre as várias fontes naturais de carotenoides, as microalgas têm se destacado devido a facilidade de cultivo, adaptação a diversas condições ambientais e produção de uma variedade de metabólitos secundários (AHMED et al., 2014). Tradicionalmente, esses carotenoides são utilizados como corantes alimentares, entretanto devido a sua atividade antioxidante intrínseca e pesquisas estarem verificando ação anti-inflamatória, anticarcinogênica e neuroprotetiva, como também auxílio no controle da obesidade e diabetes, fizeram com que os pigmentos se tornassem populares suplementos alimentares (ZHANG et al., 2014).

3.4 EXTRAÇÃO DE METABÓLITOS MICROALGAIS

As células microalgais apresentam parede celular relativamente espessa e os metabólitos geralmente estão localizados em glóbulos ou ligados a membranas celulares, tornando sua extração um desafio. Ademais, a estrutura da parede celular das microalgas é complexa e sua composição é muito variável entre espécies (GÜNERKEN et al., 2015). O tratamento químico (solventes, ácidos) é muito utilizado para acelerar o processo de extração e recentemente algumas tecnologias como micro-ondas, ultrassom e fluido supercrítico tem sido empregadas (GRIMI et al., 2014).

Lipídios são compostos amplamente extraídos de células microalgais com solventes orgânicos. O método de Soxhlet é uma das referências, a extração é realizada por longos períodos de tempo utilizando grande volume de solventes como o éter de petróleo (GULDHE et al., 2014). Ademais, a mistura de metanol-clorofórmio é tida como a mais comum na extração de lipídios de biomassa tanto seca quanto úmida e por conter uma fase aquosa possibilita a extração simultânea de compostos polares livres (ácidos orgânicos, aminoácidos, carboidratos) (SHOW et al., 2014).

Os métodos clássicos de extração de pigmentos envolvem o uso de solventes orgânicos e maceração exaustiva. A extração de clorofilas em microalgas, por exemplo, é realizada principalmente pela combinação de solventes como metanol, etanol, acetona e dimetilformamida (DMF) (ZHANG et al., 2019). Enquanto carotenoides são extraídos convencionalmente com diversos solventes (diclorometano, hexano, metanol, etanol, éter dietílico, tolueno, propanol, butanol, heptano e acetonitrila) (MÄKI-ARVELA, HACHEMI & MURZIN, 2014).

Apesar de eficientes, atualmente métodos que utilizam solventes orgânicos e dependem de longos períodos de extração como os mencionados anteriormente têm sido substituídos por métodos que sejam menos nocivos ao meio ambiente. Essas modificações incluem redução ou eliminação do uso de solventes, assim como resíduos gerados, substituição por solventes menos tóxicos, economia de energia, redução no número de etapas e integração de processos (TOBISZEWSKI et al., 2015).

3.5 DETERMINAÇÃO DOS METABÓLITOS MICROALGAIS

Recentemente, a evolução da análise instrumental permitiu a detecção e quantificação de inúmeros compostos, principalmente pelo aumento da sensibilidade e da precisão. A determinação deve proporcionar baixos limites de detecção para todos os analitos que estejam presentes na amostra em um curto tempo de análise (GOLDANSAZ et al., 2017).

A GC é técnica mais utilizada para determinação de ácidos graxos de amostras lipídicas, devido sua alta resolução, sensibilidade e capacidade de separar isômeros. Tanto o detector de ionização em chama (FID) quanto o MS são utilizados para determinação desses compostos. Cerca de 99% dos ácidos graxos encontrados na natureza se apresentam na forma de triacilgliceróis, os quais não são suficientemente voláteis para serem analisados por GC (SHEN et al., 2016). Assim, esses compostos são submetidos a processos de derivatização para obtenção de derivados voláteis, como ésteres de ácidos graxos.

Vários métodos são empregados atualmente para conversão de triacilgliceróis a ésteres de ácidos graxos, os quais se baseiam nos princípios básicos de hidrólise ácida e alcalina (ALDAI et al., 2005):

- A hidrólise dos lipídios em ácidos graxos envolve a saponificação (hidrólise alcalina) em que a ligação éster entre o ácido graxo e o glicerol é clivada sob temperatura elevada na presença de um álcali forte. Posteriormente, ocorre a metilação na presença de um ácido forte em metanol.

- A transesterificação direta envolve catálise ácida ou alcalina em um processo em que o álcool (glicerol) do lipídio é deslocado da molécula e substituído por outro álcool em excesso sob condições anidras.

Diversas técnicas analíticas já foram descritas para análise de metabólitos polares como ácidos orgânicos e aminoácidos, mas as mais usuais são a LC e a eletroforese capilar (CE) (DA COSTA et al., 2016; OMAR, ELBASHIR & SCHMITZ, 2017). A GC surge como alternativa para a determinação destes compostos por apresentar alto poder de resolução, sensibilidade e fácil acoplamento a diferentes detectores (BEALE et al., 2018).

Entretanto, esses compostos polares não são suficientemente voláteis para serem analisados por GC, o que torna necessário sua conversão a derivados voláteis, principal requisito para o emprego da técnica. Em geral, as reações de derivatização de uma só etapa são preferidas, mas derivatizações de duas etapas, como de oximação (reação de um aldeído ou cetona com hidroxilamina, formado uma ligação dupla entre carbono e nitrogênio) seguida de sililação (o par de elétrons do heteroátomo mais eletronegativo da molécula, O, N ou S, ataca o átomo de silício do reagente de sililação), podem ser mais eficientes para posterior análise cromatográfica. Essas

reações são realizadas em temperaturas que variam de 30 a 90 °C por períodos de 0,5 a 6 h e consistem na substituição de grupos polares das moléculas para aumentar sua volatilidade e torná-las compatíveis a análise por GC (PHAN & BLANK, 2020).

Segundo estudos que realizaram a determinação de metabólitos em goiaba (LEE, et al., 2010), tomate (OMS-OLIU et al., 2011), *Saccharomyces cerevisiae* (KIM et al., 2013), abacate (HURTADO- FERNÁNDEZ, et al., 2014), amostras biológicas (sangue e plasma) (MOROS et al., 2017) e *Aspergillus niger* (ZHENG et al., 2018) a derivatização de duas etapas é eficiente para a determinação por GC de aminoácidos, carboidratos e ácidos orgânicos simultaneamente, visto que pode ser aplicada tanto para aldoses quanto cetoses, carbonilas e aminas e têm sido amplamente empregada na determinação de diversas misturas complexas, por ser eficiente e proporcionar cromatogramas com boa resolução.

Já pigmentos podem ser determinados por espectrofotometria, a quantificação ocorre pela medida da absorbância em um comprimento de onda pré-determinado para o pigmento ou a classe de interesse e o valor obtido é aplicado em uma equação (HENRIQUES, SILVA & ROCHA, 2007). Entretanto, essa técnica não é seletiva, podem haver outros pigmentos ou produtos de degradação absorvendo no mesmo comprimento de onda, superestimando a concentração, por exemplo (CASALE et al., 2012). Então, a cromatografia líquida de alta eficiência (HPLC) tem sido amplamente empregada para separação dos pigmentos antes da quantificação, pois faz uso de pequenas quantidades de amostra, as análises são consideradas rápidas, aliadas à precisão e sensibilidade (BORELLO & DOMENICI et al., 2019).

A separação de clorofilas é realizada em HPLC com fase reversa, uma vez que a fase normal não é compatível com amostras aquosas e a maioria dos solventes utilizados na extração de clorofilas são de base aquosa. Já a detecção é realizada por absorbância através de detector de arranjo de diodos (DAD) e com menor frequência por fluorescência (VIERA & ROCA, 2020). Os carotenoides antes da análise cromatográfica passam por uma saponificação com substâncias álcalis para remoção de clorofilas e de lipídios que são fontes de interferência (SINGH, AHMAD & AHMAD, 2015). A HPLC, tanto de fase normal quanto de fase reserva, é reportada para separação de carotenos e xantofilas, as quais são detectadas por absorbância (DAD). Para ambas classes de pigmentos, quando os métodos de detecção acima citados não são suficientes para identificação, é utilizada HPLC acoplada a MS (PÉREZ-GÁLVEZ & FONTECHA, 2020).

4 ARTIGOS CIENTÍFICOS INTEGRADOS

4.1 ARTIGO 1: ANALYTICAL STRATEGIES FOR USING GAS CHROMATOGRAPHY TO CONTROL AND OPTIMIZE MICROALGAE BIOPROCESSING

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Analytical strategies for using gas chromatography to control and optimize microalgae bioprocessing

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Microalgae are considered one of the most promising microorganisms for sustainable production of food products. In order to maximize the biotechnological potential of microalgae, further information on their metabolism is still needed. Gas chromatography (GC) is advantageous for the metabolites' analysis, since it allows the separation and detection of several organic compounds, among them volatiles, amino acids, organic acids, carbohydrates, fatty acids and sterols from different sample preparation, come under with high resolution and sensitivity. In addition to the monitoring and optimization of the microalgal bioprocesses, GC analyses also aid in the characterization and verification of the biomass quality obtained at the end of the cultivation. Thus, the use of GC shows great potential of monitoring of important metabolites for the development of microalgae bioprocesses.

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Introduction

Growing energy demand, shortages of fossil fuels and consequent price increases, environmental constraints, and a relatively poor social reputation of fossil fuels have stimulated research to replace traditional sources of energy with renewable sources [1]. Thus, agricultural crops are now used not only for human and animal consumption but also to produce fuels, materials, and chemicals. However, diverting agricultural production capacity to non-food purposes, given the energy crisis and population growth, reduces food security [2].

Biotechnology offers an alternative, competitive, and sustainable approach to obtaining several products, leveraging inherent strengths associated with biological processes [3]. Among microorganisms, microalgae are one of the most promising feedstocks for the sustainable supply of commodities for both food and non-food products. Microalgae are attractive, potentially lower-cost alternatives to microbial cell factories using bacteria or fungi due to microalgae's ability to utilize CO_2 and solar energy without the input of organic carbon [4].

To harness the biotechnological potential of microalgae, improved understanding of metabolic processes is needed. Our present knowledge is mostly limited to wild-type strains of different origins. Metabolite analysis has led to key insights into their physiology and the biochemical pathways that are potentially active in other marine microorganisms [5]. The analysis here complements these data, directly assessing active biochemical pathways by measuring the end products of biological metabolic activity to offer relevant information for the control and optimization of microalgal cultivation.

The main analytical techniques used for the metabolomic analysis of biological samples are nuclear magnetic resonance (NMR), gas chromatography (GC), and liquid chromatography (LC) coupled to different detectors, primarily mass spectrometry (MS) [6]. As an analytical tool, GC offers many advantages over other techniques. It allows the separation and detection of polar (organic acids, amino acids, sugars, and sugar alcohols) and non-polar (fatty acids and sterols) compounds with high resolution and sensitivity, besides allowing the simultaneous analysis of different chemical classes [7•].

This paper highlights the importance of microalgal bioprocesses and current perspectives thereon. Afterwards, GC is presented as an analytical tool of great importance for the prediction, control, optimization, and characterization of microalgal bioprocesses, allowing the analysis of several metabolites, such as volatile organic compounds, organic acids, amino acids, carbohydrates, lipids, fatty acids, and sterols. Finally, the main sample preparations for microalgal metabolites and their particularities are highlighted in order to support future research in this area.

Microalgae cultivation

Microalgae are a large and diverse group of photosynthetic organisms, both prokaryotes and eukaryotes, that

grow in various aquatic habitats (lakes, ponds, rivers, oceans, and wastewater). Prokaryotic microalgae have representatives in the *Cyanophyta* and *Prochlorophyta* groups, and the eukaryotes microalgae in the *Glaucophyta*, *Rhodophyta*, *Ochrophyta*, *Haptophyta*, *Cryptophyta*, *Dinophyta*, *Euglenophyta*, *Chlorarachniophyta* and *Chlorophyta* groups [8•]. However, cyanobacteria (*Cyanophyta*), chlorophytes (*Chlorophyta*), and diatoms (*Ochrophyta*) stand out in their promise for scientific research and biotechnological exploration.

Microalgae present high rates of cell growth and can be cultivated autotrophically, heterotrophically, or mixotrophically [9], factors that already suggest these microorganisms are useful in bioprocesses. In autotrophic cultivation, the microorganisms depend on luminous energy and inorganic carbon source to produce energy, whereas heterotrophic cultivation uses organic sources of carbon in the absence of luminosity. Mixotrophic microalgal cultivation uses both a supply of inorganic carbon to fix via photosynthesis and some organic source of carbon, such as glucose, glycerol, or acetate [8•].

The best conditions for cell growth vary greatly by species, and often the greatest production of biomass does not correspond to the greatest synthesis of the product of interest. Among the microalgal compounds relevant to the food industry are proteins, carbohydrates, lipids, fatty acids, sterols, pigments, volatile compounds, and vitamins [10]. Currently, the sale of microalgal biomass with these compounds occurs for food supplementation. Bioprocesses that target the extraction and purification of microalgal compounds for their addition to various foods to improve their sensory and nutritional qualities are still in their infancy.

During microalgae cultivation, external conditions (temperature, pH, luminosity, aeration) and cell growth are primarily monitored [11,12]. Because of the advances in and the availability of analytical tools, it would be greatly valuable to analyze metabolites that are synthesized during cultivation, since many are precursors to compounds of interest or markers of cellular physiology [13•]. It would thus be possible to verify the behavior of microalgal cells in different applied external conditions, in addition to obtaining information regarding the biomass obtained at the end of cultivation.

Analyses to verify the composition of the macromolecules present in biomass are still limited. The fatty acid profile present in the lipid fraction of microalgae is more widely diffused (despite some limitations that will be discussed later) than for proteins and carbohydrates [14•]. Many studies have indicated high production of proteins and carbohydrates by microalgae, but there is little information about their constituent monomers, which determine their nutritional quality. Table 1 outlines some recent studies with different objectives that have evaluated microalgal metabolites.

Metabolite extraction

Microalgal compounds are located intracellularly, whether in the cytoplasm, in internal organelles, or bound to cell membranes; in some cases, cell rupture is required before extraction for further analysis or to obtain product to market. Various methods for cell disintegration have been applied, including enzymatic treatment, alkali or acid treatment, high-pressure homogenization, bead milling, microwaves, pulsed electric field, and ultrasonication [40]. Most studies evaluating different methods of cell rupture have focused on the extraction efficiency of the lipid fraction, with fewer investigating the extraction of other constituents.

Santos *et al.* [41] evaluated the extraction of lipids in *Chlorella vulgaris* biomass using the solvents ethanol, hexane, and a mixture of chloroform and methanol in ratios of 1:2 and 2:1, along with other mechanisms of cell disruption, such as Potter homogenizer and ultrasound treatment. The mixture of chloroform and methanol (2:1) assisted by ultrasound was the most efficient method for lipid extraction. A study with *Spirulina* sp. evaluated lipid extraction by a mixture of chloroform and methanol (2:1) and by Soxhlet (hexane), along with the pre-treatments of milling, microwaves, and autoclaving [42]. The authors concluded that the chloroform and methanol (2:1) mixture was more efficient, and none of the pre-treatments had a positive effect on extraction.

Supercritical fluid extraction is an environmentally friendly and selective technique used in industrial applications and in preparing samples, since it reduces the use of organic solvents and increases yield [43]. Supercritical carbon dioxide ($SCCO_2$) was used to extract lipids from *Schizochytrium* sp. (240 min) [44], *C. vulgaris* (180 min) [45] and *Scenedesmus obliquus* and *Scenedesmus obtusiusculus* (540 min) [46]. $SCCO_2$ with ethanol as a co-solvent was also used for multicomponent extraction from *Chlorella* sp. (70 min) [47]. However, as seen above, the periods required for supercritical fluid extraction are quite long. In addition, this technology is not selective; all constituents with affinity for either the fluid or the co-solvent are extracted.

Pulsed electric field and high pressure homogenization treatments have emerged as promising methods for the mild and complete disruption of biological cells, respectively [48]. In *C. vulgaris*, pulsed electric field allowed the selective enhancement of the extraction yield of small ionic substances and carbohydrates, but the extraction efficiency of proteins, relatively low by comparison, did not exceed 5.2% of the protein total. High pressure homogenization favored instantaneous and efficient release of all intracellular material, including a large fraction of proteins [49]. For *Nannochloropsis*

Table 1

Recent studies evaluating microalgae metabolites

Strain	Compounds	Evaluation	Sample preparation	Tool	Ref.
Aphanothece microscopica Nägeli	CO_2, O_2	Photosynthesis control	Bioreactor headspace injection	GC	[15]
Tetraselmis chuii and *Nannochloropsis gaditana*	CO_2 and toluene	Removal efficiency	Bioreactor headspace injection	GC	[16]
Scenedesmus quadricauda, *Chlorella vulgaris* and *Botryococcus braunii*	CO_2	Removal efficiency	Bioreactor headspace injection	GC	[17]
Chlorella vulgaris	H_2, CO, CO_2, C2–C4	Biprocess control	Bioreactor headspace injection	GC	[18]
Chlamydomonas reinhardtii	VOCs	Release during cell death	Dynamic headspace	GC	[19]
Spirulina platensis, *Nostoc* spp. and *Anabaena* spp.	VOCs	Profile and influence of nitrogen level	Static headspace	GC	[20]
Phormidium autumnale	VOCs	Exogenous carbon sources	SPME	GC	[21]
Microcystis flos-aquae	VOCs	Nitrogen sources and toxic effects in *Chlorella vulgaris*	Dynamic headspace	GC	[22]
Microcystis flos-aquae	VOCs	Phosphours sources and toxic effects in *Chlamydomonas reinhardtii*	Dynamic headspace	GC	[23]
Nannochloropsis gaditana	AA; FA	Nutrients removal and biofuels intermediate production	Acid hydrolysis/Der	GC	[24]
Dunaliella salina	AA	Method development	Solvent extraction and electroporation/Der	CE	[25]
4 microalgae strains	AA; AO; FA	Development and method validation	Solvent extraction/Der	GC	[13]
Chlorella vulgaris	CHO	Method development	Acid hydrolysis/Der	GC	[26]
6 microalgae strains	CHO	Characterization	Acid hydrolysis/Der	LC	[27]
46 microalgae strains	CHO	Characterization and methods comparison	Acid hydrolysis	LC	[28]
Mychonastes homosphaera and *Chlorella* sp.	CHO	Method development	Acid hydrolysis	AEX	[29]
10 microalgae strains	CHO	Characterization and potential as functional food ingredients	Acid hydrolysis	AEX	[30]
5 microalgae strains	FA	Characterization and comparation	Solvent extraction/Der	GC	[31]
Phaeodactylum tricornutum, *Nannochloropsis oculata* and*Porphyridium cruentum*	FA	Extraction method and antioxidant activity	Supercritical fluid/Der	GC	[32]
Nannochloropsis oculata, *Tetraselmis suecica*, *Isochrysis galbana* and *Pavlova lutheri*	FA	Macro/micronutrients effects	Solvent extraction/Der	GC	[33]
Chlorella minutissima	FA	Production rate optimization	Solvent extraction/Der	GC	[34]
8 microalgae strains	FA	Temperature impact	Solvent extraction/Der	LC	[35]
Schizochytrium sp.	STE	Reducing Plasma Cholesterol Concentration	Solvent extraction/Sap	GC	[36]
Schizochytrium aggregatum	STE	Oxidative stability of microalgae oil and bioaccessibility and antioxidant ability of digested samples	Solvent extraction/Sap	GC	[37]
Pavlova lutheri	STE	Effects of UVeC radiation and hydrogen peroxide	Direct saponification/Der	GC	[38]
Phormidium autumnale	STE	Distinct sources of carbon	Direct saponification	GC	[39]

Ref., reference; CO_2, carbon dioxide; O_2, oxygen; CO, carbon monoxide; C2–C4, gases of two to four carbons; VOCs, volatile organic compounds; AA, amino acids; FA, fatty acids; CHO, carbohydrates; STE, sterols; GC, gas chromatography; LC, liquid chromatography; AEX, Anion exchange chromatography; Der, derivatization; Sap, saponification.

microalgae, pulsed electric field was also efficient in the selective extraction of water-soluble proteins [50].

The composition and resistance of microalgal cells varies greatly by species; *Chlorella* and *Scenedesmus* species usually possess several more robust components in their cell walls, so it is difficult to affirm which is the best method of cell disruption. *Chlorella* strains are known to contain cellulose, hemicellulose and chitin-like glycans within their cell walls. The cell wall of *S. obliquus* is reported to be rigid, since it also contains resilient sporopollenin biopolymers [51]. The method of cell rupture or extraction more suitable for each specie should be verified experimentally, taking into consideration the efficiency of obtaining the compounds of interest, availability of methods, and time and cost involved.

The extraction with organic solvents is a simple and efficient method to obtain microalgae compounds.

However, these extractions are currently condemned due to the environmental problems. A simple alternative to this is the reduction of the volume of organic solvents that are used in the extractions as well as the sample mass [13•]. Solvent extraction assisted by innovative techniques (e.g. high-pressure homogenization, microwaves, pulsed electric field, and ultrasonication) has shown good results in sample preparation procedures for the extraction of compounds from microalgae, besides being seen as environmentally friendly. However, the chosen method must be taken into account regarding to operational costs and equipment maintaining, since sometimes the long period required for extraction, number of steps, and any partition of solvents with non-selective methods where solvents are not completely eliminated.

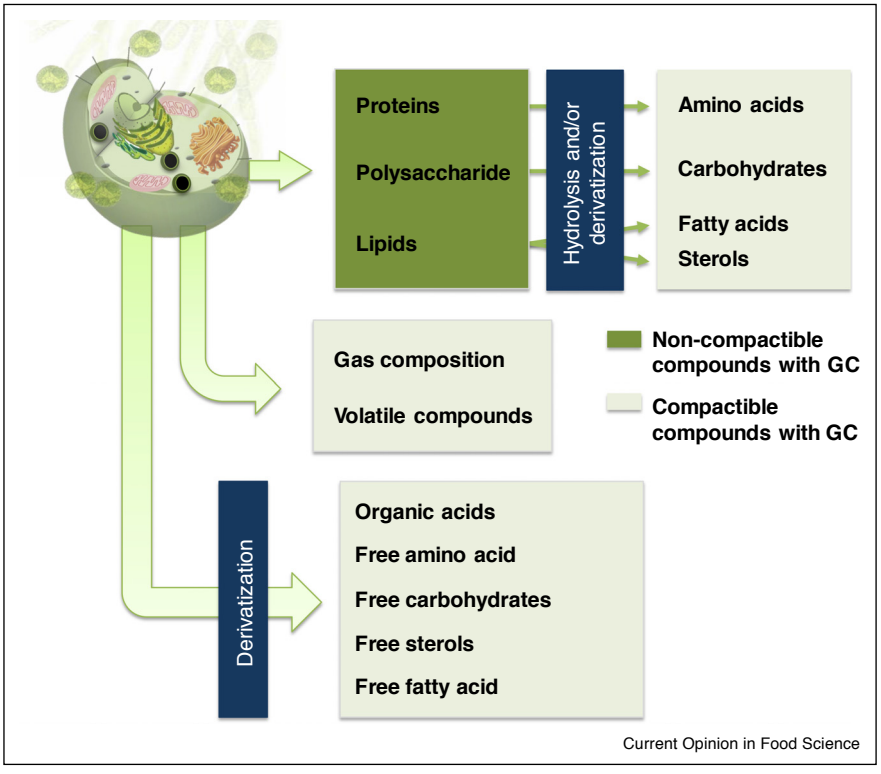
Gas chromatographic analysis

Metabolite analysis has greatly benefited from advances in analytical technologies, including NMR and MS, as well as separation techniques, such as LC and GC. GC/MS is a widely used analytical technique providing high sensitivity and reproducibility with unprecedented dynamic range and throughput [52•]. Unlike other separation techniques, GC has few limitations, primarily its restriction to semi-volatile analytes. This restriction is the reason for the requirement to convert some polar metabolites to volatile derivatives through chemical derivatization processes, which also require some analyses by LC.

GC/MS is the analytical reference technique for the analysis of gases and volatile compounds, but it is also possible to analyze several other compounds, both polar and non-polar (Figure 1). Among these compounds are organic acids, amino acids, carbohydrates, fatty acids, and sterols (Table 1). In addition, numerous polar compounds belonging to different chemical classes, can be analyzed in the same chromatographic run, which is advantage compared to analyses carried out with LC/MS. Currently, organic acids, amino acids, and carbohydrates have been analyzed simultaneously in plant samples [53,54], and organic acids and amino acids have been analyzed by GC-FID (flame ionization detector), GC/MS, or both, simultaneously in different species of microalgae [13•].

GC/MS has great relevance for metabolite analysis in biological systems due to the possibility of identification through mass spectra libraries, since biological samples can present hundreds or even thousands of compounds [55]. Identification by spectra library is due to the widespread use of the electrons impact (EI) standard of 70 eV. Unlike mild ionization techniques, such as electrolytic spray (ESI), which are widely used in LC/MS, EI is an extremely reproducible ionization process even on

Figure 1



Metabolites synthesized by microalgal cells and analyzed by gas chromatography.

different platforms [56•], which makes it possible to easily compare experimental mass spectra from laboratories with those in databases. Compounds identification is currently performed by the comparison of their mass spectra with spectra, since the fragmentation by IE is highly reliable to the chemical structure [52•].

There are general criticisms regarding the use of GC/MS for separation and identification of compounds from biological systems. These include the possibility of co-elution by complex samples and erroneous identification if only comparing mass spectra without the use of authentic standards. Positive identification using authentic standards would be the most appropriate procedure, but screening analyses have limitations in that regard due to the high number of compounds present in a sample, great variability between samples, and high cost of authentic standards due to their high purity [39,21].

In this sense, comprehensive two-dimensional gas chromatography, GC × GC, is a well-established and useful instrumental platform for analyzing biological samples. Compared to one-dimensional (one-column) chromatographic approaches, comprehensive two-dimensional gas chromatography–mass spectrometry (GC × GC/TOFMS) allows higher separation efficiencies, improved resolution, better identification, and quantitation of analytes, including co-eluting cases [57•]. The superior analytical performance of GC × GC/TOFMS compared to GC/MS is undisputed, but for some applications such performance is not required. Since higher performance comes at higher cost, the actual need to acquire and use the equipment required to obtain this performance should be studied in depth.

Analyses of gas composition

As mentioned previously, the microalgae cultivation may be autotrophic, heterotrophic or mixotrophic [9]. In these different cultivations, GC can be used to monitor the gas composition in microalgal bioprocess. In autotrophic cultivation of *Aphanothece microscopica* Nägeli a GC equipped with thermal conductivity detector (TCD) was used to determine CO_2 and O_2 concentration on the bioreactor headspace. Determination was made through the direct injection of the system gaseous phase with a syringe and the concentration determined by external calibration curves [15]. Similarly, a GC-TCD was used to verify the removal efficiency of CO_2 and toluene in a photobioreactor containing microalgae and bacteria [16].

In mixotrophic cultivation, using as the organic carbon source glucose and sewage and flue gas inputs as inorganic carbon source for the microalgae *Scenedesmus quadricauda*, *Chlorella vulgaris* and *Botryococcus braunii*, the monitoring of CO_2 removal was also performed by a GC-TCD [17]. In a mixotrophic cultivation of *C. vulgaris* a GC-TCD was used for the monitoring of CO_2, carbon monoxide (CO) and hydrogen. Besides these gases, analyses of hydrocarbon gases including methane and C2–C4 gases by GC with flame ionization detector (FID) were also carried out [18].

Analyses of volatile organic compounds

Volatile organic compounds (VOCs) from biological systems belong to different chemical classes and originate from primary and secondary metabolites [58•]. VOCs are generally substances with low molecular weights, low boiling points, and high vapor pressures under natural conditions, which are ideal for GC analysis. Although VOCs are broadly important in terrestrial biological systems, there is still little information regarding the production of these compounds in aquatic ecosystems. Santos *et al.* [21] evaluated the biogeneration of VOCs by microalgae, including their occurrence, behavior, ecological implications, and industrial applications. In seaweeds, one study showed that the production of these compounds is closely related to the physiology of the species [59].

The main extractive analytical techniques used to analyze VOCs are liquid extraction (LE), solid-phase extraction (SPE), solid-phase microextraction (SPME), and, most recently, stir bar sorptive extraction (SBSE) and its headspace variation, headspace sorptive extraction (HSSE) [58•]. The SPME technique applied to the headspace has been most used recently to analyze algae and microalgae, as applied, for example, to the green algae *Ulva prolifera*, *Ulva lynx*, and *Monostroma nitidum* [60], microalgae *Phormidium autumnale* [21], brown algae *Halopteris filicina* and *Dictyota dichotoma*, and green algae *Flabellia petiolata* [61]. Murati *et al.* [62] have already used the HSSE technique for analyzing VOCs from the green algae *Ulva* and *Enteromorpha* spp. and red *Gracillaria* sp. Studies with the microalga *Microcystis flos-aquae* have also used the dynamic headspace air-circulation method (Table 1).

The SPME technique essentially involves a fused silica fiber with a thin layer of extraction phase (polymer) mounted inside the needle of a syringe-like device. Analyte extraction is performed by the direct immersion of the fiber into the liquid sample or into the gaseous phase (headspace mode). Next, analyte desorption occurs directly on the GC injector [63]. SPME has the advantage that any sample preparation uses only one fiber and requires no solvents. However, SPME has little ability to detect low concentrations because the polymer coating fiber has low volume. In some cases, where higher sensitivity is required, to avoid using considerable volumes of solvent (e.g. LE and SPE), SBSE and HSSE techniques are recommended.

Analyses of polar compounds

Polar compounds include organic acids, amino acids, carbohydrates, and several other intermediates of metabolic pathways, which are synthesized by microalgae and

can be monitored during cultivation in their free forms. Characterizing polar compounds may elucidate the behavior of microalgal cells in their conditions of cultivation, and some of polar compounds may serve as physiological markers. Amino acids and carbohydrates may also be found in bound form constituting polymers; the characterization of these compounds then requires hydrolysis before the derivatization processes. Analysis of these metabolites is very important for indicating the nutritional quality of the microalgal proteins and polysaccharides. Table 1 shows recent studies that carried out the analysis of proteins and carbohydrates of microalgae by GC. The work for this sample is still limited, but it is possible to verify the possibility of using this tool for analysis as they are already found for other several matrices.

The most versatile extraction method for analyzing free metabolites is the chloroform and methanol mixture. From the aqueous phase, it is possible to simultaneously derivate and analyze organic acids, amino acids, and carbohydrates, while in organic phase, it is possible to determine the non-polar compounds (e.g. lipids and fatty acids) [64]. Because of their ability to form hydrogen bonds, polar compounds have low volatility, and GC analysis, chemical derivatization processes to increase volatility is required, mainly by substitution in the polar function using reactions like alkylation, acylation, and silylation [7•].

The most widely used derivatization procedure for analyzing metabolites by GC/MS is combining two steps, that is, oximation (reaction of aldehyde or ketone with hydroxylamine to form a double bond between carbon and nitrogen) followed by silylation (with the electron pair of the most electronegative heteroatom of the molecule — O, N, or S — attacking the silicon atom of the silylation reagent) [65]. It is worth mentioning that these reactions in sequence present good results for the simultaneous derivatization of organic acids, amino acids, and carbohydrates due to the attack of functional groups. In addition, these reactions have some practical advantages, such as low reagent volume (about 40 μL and 60 μL of the oximation and silylation agents, respectively) and mild reaction temperature.

To analyze amino acid and carbohydrate from microalgal proteins and polysaccharides, hydrolysis is required before derivatization. Protein hydrolysis is performed by heating the sample to 110°C with 6 M HCl for about 24 hours [66]. The exact required length of incubation varies with the sample; hydrolysis is complete when there is clear liquid without the presence of solids. To reach this condition, a vacuum condition in the hydrolysis tubes is essential. Polysaccharides hydrolysis is performed by the addition of trifluoroacetic acid 2 M along with heating at 110°C for about 2 hours [67].

Analyses of non-polar compounds

Much contemporary research has worked to understand the lipid composition of microalgal biomass (Table 1). Microalgal lipid composition can be modified during cultivation process and used for various distinct applications, from biodiesel production to nutrition for humans or animals, due to the presence of bioactive fatty acids. Microalgae lipids included both polar compounds, such as glycolipids and phospholipids, and non-polar compounds, such as triglycerides (TAGs), diglycerides (DAGs), monoglycerides (MAGs), and free fatty acids (FFAs). All of these metabolites may be evaluated by GC, a powerful tool for determining numerous molecules. Among lipid compounds, fatty acids in the form of methyl esters (FAMEs) are the most explored biomolecules by gas chromatography. Several methods for fatty acid determination have been described in the literature studying microalgae, with little consensus on the best method for FAME characterization and quantification. In fact, much research has obtained the fatty acid profile only by normalizing the GC peak areas, yet normalization does not express the real concentration of fatty acids in the lipid fraction and its results interdepend on an arbitrary area. To circumvent these issues, Visentainer [68] suggested determining fatty acid concentration by adding an internal standard of known mass, considering the theoretical chain size correction factor (applied for GC-FID) and ester-to-acid conversion factor as they relate to that internal standard. It should be noted that American Oil Chemists' Society also recommends the use of correction factors for quantification of long chain fatty acids (greater than 19 carbons) [69].

Although quantification with an internal standard is the best option, it can lead to low recoveries considering the total lipid in a sample. We hypothesize this aspect could be directly related to the presence of different molecules co-extracted in the fraction. A lipidomic analysis from *Nannochloropsis oceanica* showed a lipid profile with other molecules, such as phospholipids, that could influence the yield of fatty acids due to the presence of phosphate groups [70]. Also, besides co-extractives of triacylglycerol, chlorophyll may be another important molecule presenting interference [71]. In this regard, the choice of which fatty acid methodology to use for microalgal analysis should be made cautiously to report an accurate result.

Sterols are important metabolites for microalgal lipid metaboloma due to their importance to membrane fluidity. These compounds are tetracyclic triterpenic, specifically having a cyclopentaneperhydrophenanthrene ring with two methyl angular groups [72]. Some of these compounds are considered bioactive, and a few studies have identified in microalgae [73]. Recent studies have described different methods for sterol determination by GC, normally using a previous sample pretreated by saponification [39,74]. In microalgae, sterols may be

found in their free forms or bounded (esterified in fatty acids or acyl glycosylated); the saponification step is usually intended to obtain free sterols from their bonded forms. According to Montpetit *et al.* [75] the acyl glycosylated (SG) form did not derive free sterols, SG compounds can be analyzed by GC in their free form after separation in a silica gel bead.

Another possible problem with microalgal analysis by GC might involves the amount of sample involved in developing a biotechnological process at laboratory scale. Such small-scale work can produce low amounts of biomass, and in such cases, compatible methods must be chosen. Therefore, to obtain large amounts of lipid for sterol analysis requires performing saponification, which consequently requires a large amount of biomass. Thus, Pereira *et al.* [76] developed a method based on direct, ultrasound-assisted saponification, reaching great recovery from 99% to 102% with low limits of quantification. In contrast, Grasso *et al.* [77] developed a saponification method derivatizing sterols using N,O-bis(trimethylsilyl)trifluoroacetamide (BSTFA), a common reagent used to improve response in GC-FID. Microalgae lipid metabolomics are broad and present several other compounds, but for gas chromatography the mentioned classes are the most important to analyze, with their relevance for human nutrition and health.

Conclusions and future directions for research

Microalgae are promising microorganisms from which numerous products, many of interest to the food industry, may be obtained through bioprocesses. As the use of these microorganisms becomes increasingly common, the monitoring and optimization of cultivation and biomass characterization become essential. GC offers a viable and advantageous method to obtain results that meet the aforementioned requirements and that can highlight improvements to bioprocesses. Currently, analytical tools in general, and not just GC, are little explored for this purpose, despite its potential. We hope that promising results obtained through scientific research will stimulate their application to industrial processes.

Conflict of interest statement

Nothing declared.

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4.2 ARTIGO 2: *SCENEDESMUS OBLIQUUS* METABOLOMICS: EFFECT OF PHOTOPERIODS AND CELL GROWTH PHASES

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RESEARCH PAPER

Scenedesmus obliquus metabolomics: effect of photoperiods and cell growth phases

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Abstract
Environmental factors directly affect the growth and composition of microalgal biomass. Therefore, the present work analyzed the metabolomics (amino acids, organic acids, and fatty acids) of the microalga *Scenedesmus obliquus* cultivated in 24:0 and 12:12 (light:dark) photoperiods and different phases of cell growth. Furthermore, the metabolites were related to protein, lipid, and chlorophyll contents at the end of cultivation. The highest biomass concentration (4020 mg L^{-1}) and protein (47.3%) were obtained in culture under constant illumination. The cultivation 12:12 (light:dark) photoperiod triggered higher production of lipids (23.0%) and chlorophylls (26.4 mg g^{-1}) by *S. obliquus*. Microalgal metabolites were greatly affected by photoperiod and by phase of cell growth. Thus, metabolite production could be related to both the environmental conditions under which cultivation occurred and to the different concentrations of products (proteins, lipids, and chlorophylls) present in the *S. obliquus* biomass.

Keywords Microalgae · Metabolites · Protein · Lipids · Chlorophyll

Introduction

Microalgae are unicellular organisms that present chlorophyll and other auxiliary photosynthetic pigments, constituting one of the most efficient biological systems for transforming solar energy into organic compounds through photosynthesis [1]. Besides high efficiency of photosynthetic conversion, their ability to develop in diverse ecosystems, rapid rates of biomass production, and production of interesting compounds are among the advantages of microalgae compared to other photosynthetic organisms [2].

Microalgal biotechnology offers different commercial applications. Microalgal pigments are used in the food and cosmetic industries [3]. Compounds presenting bioactivity can have pharmaceutical importance, as well as antimicrobial, antioxidant, anti-inflammatory, and anticarcinogenic properties [4]. In addition, microalgal biomass can be used as a nutritional supplement due to its high protein, vitamin, lipid, and polysaccharide contents [5]. Some species with high lipid synthesis are used for biodiesel production [6].

Environmental parameters such as light intensity, photoperiod, temperature, and nutrients directly affect microalgal growth rates and biomass composition. In photosynthetic cultivation, light is the basic source of energy. Thus, intensity and photoperiods (light:dark) are the most critical parameters in biomass production, but the optimal conditions vary by species [7]. Natural light may not be favorable to microalgal growth due to a lack or an excess of energy supply, which leads to photoinhibition. Already, an artificial light source has the advantage of providing the intensity and exposure period appropriate to the development of each species [8].

Current research has focused on exploring techniques and processes to increase microalgal productivity rates and maximize the characteristics of interest of these microorganisms, such as protein, lipid, and pigment contents [9–11]. Doing so requires knowing the fundamental biosynthetic pathways and precursors of the target microalgae compounds, given pre-established conditions of cultivation [12].

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Determining metabolites in microalgal cultures could provide important data about their metabolism, about which the literature offers very little information. Such data could facilitate manipulation of the cultivation process to increase productivity and modify the composition of microalgal biomass. This study aimed to evaluate the metabolomics of *Scenedesmus obliquus* microalgae grown under different photoperiods at distinct stages of cell growth, in addition to correlating the produced metabolites with the protein, lipid, and chlorophyll contents obtained at the end of cultivation.

Materials and methods

Microorganisms and culture medium

Axenic cultures of *S. obliquus* CPCC05 were obtained from the Canadian Phycological Culture Centre. Stock cultures were propagated and maintained in a glass bubble column reactor with an internal diameter of 40 cm and height of 10 cm with synthetic BG-11 medium [13] with the following composition (mg L^{-1}): K_2HPO_4 (3.0), $MgSO_4$ (75.0), $CaCl_2{\cdot}2H_2O$ (36.0), ammonium citrate and iron (0.6), Na_2EDTA (1.0), NaCl (0.72), $NaNO_3$ (150.0), citric acid (0.6), Na_2CO_3 (15.0), H_3BO_3 (2.8), $MnCl_2{\cdot}4H_2O$ (1.8), $ZnSO_4{\cdot}7H_2O$ (0.22), $Na_2MoO_4{\cdot}2H_2O$ (0.39), and $CoSO_4{\cdot}6H_2O$ (0.04). Initial pH was adjusted to 8.0. Incubation conditions used were 25 °C, 30 µmol m^{-2} s^{-1} incident light intensity, continuous aeration of 1 VVM (volume of air per volume of culture per minute) without CO_2 enrichment and a photoperiod of 12 h. The inoculum was maintained until reaching the 200 mg L^{-1} concentration. The stock culture was used to perform the experiments with different photoperiods, and it was also one of the samples analyzed.

Photobioreactor design and obtaining kinetic data

The experiments were carried out in a bubble column photobioreactor, built with 4-mm-thick glass, an internal diameter of 7.5 cm and height of 75 cm. The bioreactors operated in batch mode with 2.0 L synthetic BG-11 medium. The experimental conditions were as follows: initial cell concentration 100 mg L^{-1}, isothermal reactor operating temperature 26 °C, incident light intensity 150 µmol m^{-2} s^{-1}, and continuous aeration of 1 VVM. The reactor was illuminated by 20 fluorescent lamps of 15 W in a photoperiod chamber located 8 cm from the photobioreactor. The evaluated photoperiods were (h:h) 24:0 and 12:12 (light:dark). The air dispersion system comprises a 1.5-cm-diameter diffuser located at the center of the column for continuous aeration of 1 VVM, with the injected air enriched with 15% CO_2. Gas supply was compressed air and industrial carbon dioxide (CO_2), with the CO_2/air mixture adjusted to achieve the desired concentration of CO_2 in the airstream through three rotameters that, respectively, measured the flow rates of CO_2, air, and the gas mixture. Cell concentration was monitored every 24 h during the growth phases of the microorganisms. All experiments were conducted until the decline phase was reached, where the cellular concentration began to reduce.

Kinetic parameters

Biomass data were used to calculate biomass productivity $\left[P_x = \left(X_\mathrm{i} - X_{\mathrm{i}-1}\right)/(t_\mathrm{i} - t_{\mathrm{i}-1})\right]$, where X_i is biomass concentration at time t_i (mg L^{-1}), $X_{\mathrm{i}-1}$ is biomass concentration at time $t_{\mathrm{i}-1}$ (mg L^{-1}), and t is residence time (h), defined as elapsed time to reach maximum cell concentration. Cell concentrations were evaluated gravimetrically as described in the following section. The specific maximum growth rate (μ_max) was calculated considering the exponential phase from the equation $\left[\ln\left(X_i/X_0\right) = \mu_\mathrm{max} \times t\right]$, where X_0 is the initial biomass concentration, X_i is biomass concentration at time t_i and t is residence time.

Analytical methods

Cell concentrations were evaluated gravimetrically by filtering a known volume of culture medium through a 0.45-µm filter (Millex FG, USA) and drying the result at 60 °C until constant weight was reached [14]. Luminous intensity was determined by a digital luxmeter (MLM-1010, Minipa, BR), which measured light incident on the external surface of the reactor. Temperature was controlled by thermostats, and flow rates of CO_2, air, and CO_2-enriched air were determined by rotameters (AFSG 100, Key Instruments, USA).

Protein determination

Nitrogen content was determined from 200 mg of dry biomass by the micro-Kjeldahl method, and a factor of 6.25 was used to obtain the protein content [15].

Chlorophyll determination

Chlorophylls were extracted exhaustively from 150 mg of dry biomass [16] as follows. Each sample was macerated with 0.5 g of glass beads (150–212 µm, Sigma-Aldrich, USA) and 3 mL ethyl acetate. After complete solvent evaporation, each sample was placed in a 10-mL test tube to which 7 mL ethyl acetate was added. Tubes were then homogenized and centrifuged to remove supernatant, which contained the pigments. This procedure was performed until color disappeared. The same process was performed with methanol as a solvent extractor. Extract was dried on a rotary evaporator at ≤ 30 °C, purged with N_2, and stored at − 20 °C in the dark until proceeding to determination of a, b, and total

chlorophyll in a UV–Vis spectrophotometer (Model 8453, Agilent Technologies, USA). For quantification, readings were taken at 652 and 665 nm, and equations following Porra et al. [17] were used.

Extraction of lipids, amino acids, and organic acids

Metabolites were extracted according to Vendruscolo et al. [18]. About 100 mg biomass was weighed, and 3 mL methanol, 1.5 mL chloroform, and 1.2 mL distilled water were added to 15-mL polypropylene tubes. Extraction occurred by agitating the samples (30 min at 250 rpm) on a shaker table (Q225M, Quimis, BR). Afterwards, 1.5 mL chloroform and 1.5 mL distilled water were again added into the tubes, which were then shaken (4 min) and centrifuged (MTD III PLUS, Metroterm, BR) (1006×*g* for 2 min) to separate phases. The aqueous phase was reserved for determining organic acids and amino acids, and the organic phase was reserved for determining lipids and fatty acids.

Determination of organic and free amino acids

Samples were derived according to the method described by Vendruscolo et al. [18]. The aqueous phase of biomass extracts (150 µL) and a norleucine internal standard (150 µL) were dried with a nitrogen stream and heating block (TE-021, Technal, BR) at 60 °C. Residue was reconstituted in 40 µL of 20 mg mL^{-1} methoxyamine hydrochloride in pyridine and derivatives at 38 °C for 90 min, followed by the addition of 60 µL MTBSTFA (*N*-methyl-*N*-tert-butyldimethylsilyltrifluoroacetamide) and 30 min at 38 °C. Samples were analyzed (1 µL) in splitless mode in a Varian 3400 gas chromatograph (USA) equipped with a flame ionization detector (GC-FID) and Varian 8100 autosampler (USA). The GC capillary column was an RTX-5MS (Restek, USA; 30 m × 0.25 mm × 0.25 µm). The column temperature was programmed isothermally at 100 °C for 2 min, afterwards increased to 165 °C at a rate of 2 °C min^{-1}, followed by an increase at 5 °C min^{-1} to 200 °C. Then temperature was increased at 3 °C min^{-1} to 300 °C, which was held for 5 min. Injector and detector temperatures were 320 °C. Metabolites found in samples were positively identified by comparing retention times and mass spectra with those of authentic standards. The spectra were obtained by a QP2010 Plus gas chromatography (Shimadzu, JPN) coupled to a mass spectrometer (GC/MS) under the same chromatographic conditions already described. The electron impact (EI) ionization source was operated at 70 eV, scanning from *m*/*z* 50 to 500. Quantification was performed by means of an external standard with previous area normalization, as described in the method validated by Vendruscolo et al. [18]. Results were expressed as µg g^{-1} of biomass.

Determination of lipids and fatty acids

The organic fraction (1 mL) was transferred to a pre-conditioned 10-mL beaker. For gravimetric determination of lipid content, after excess solvent evaporation, the beaker was placed in an oven with air circulation at 105 °C until constant weight was obtained. Next, 1.5 mL of organic phase containing the lipids was transferred to 10-mL tubes, and the solvent was dried with a nitrogen stream and heating block (TE-021, Technal, BR) at 40 °C. Derivatization was performed according to the method described by Hartman and Lago [19]. A methanolic solution of potassium hydroxide 0.4 mol L^{-1} (1 mL) was added to the lipid fraction, which was then kept in a water bath at 100 °C for 10 min. Next, a methanolic solution of sulfuric acid 1 mol L^{-1} (3 mL) was added and heated at 100 °C, again for 10 min. Tubes were then cooled, and 2 mL hexane was added, followed by homogenization by vortex mixer (MX-S, SCILOGEX, USA) for 30 s. After phase separation, the upper phase, containing fatty acid methyl esters (FAME) dissolved in hexane, was removed for chromatographic analysis. Samples were injected (1 µL) in splitless mode into a Varian 3400CX gas chromatograph (USA). The chromatograph was equipped with a flame ionization detector (GC-FID) and Varian 8200 autosampler (USA), and the GC column was a CP-Wax 52 CB (Middelburg, The Netherlands; 50 m × 0.32 mm × 0.20 µm). Column temperature was held at 50 °C for 1 min, after which temperature was increased to 180 °C at a rate of 20 °C min^{-1}, followed by an increase at 2 °C min^{-1} to 200 °C and an increase at 10 °C min^{-1} to 230 °C, where it was held for 8 min. Injector and detector temperatures were 250 °C and 240 °C, respectively. FAME were identified by comparison with standard certificate FAME Mix 37 (Supelco, USA). Results were expressed as g of fatty acid per 100 g of lipids.

Statistical analysis

Data normality was checked using the Shapiro–Wilk test and differences between treatments were evaluated by Student's *t* test ($p < 0.05$) and analysis of variance (ANOVA), followed by Tukey's test ($p < 0.05$), all performed using STATISTICA 7.0 (Statsoft Inc., USA). In addition, all data were submitted to Principal Component Analysis (PCA) using Pirouette 3.11 software (Infometrix Co., USA). Before this multivariate analysis, the data matrix was autoscaled for each variable so that each assumed the same weight during analysis.

Results and discussion

Kinetic parameters

The amount of light microalgal cells receive and store is directly related to their carbon-fixation capacity, determining biomass productivity, cell growth rate, and changes in biomass composition [7, 20]. Kinetic parameters and protein, lipid, and chlorophyll contents obtained by cultivation with 24:0 and 12:12 (light:dark) photoperiods are presented in Table 1.

Maximum final biomass (4020 mg L^{-1}) was obtained with 24:0 (light:dark) photoperiod, with a specific growth rate of 0.035 h^{-1}, residence time of 240 h, and biomass productivity of 16.3 mg L^{-1} h^{-1}. For the 12:12 (light:dark) photoperiod, the highest final biomass was 2560 mg L^{-1}, lower biomass concentration compared to 24:0 (light:dark) photoperiod cultivation. In addition, biomass productivity (10.0 mg L^{-1} h^{-1}) presented a statistical difference between the photoperiods (Student's *t* test, $p < 0.05$) (Table 1).

Table 1 Kinetic parameters, content of proteins, lipids and chlorophylls of *Scenedesmus obliquus* cultivated in different photoperiods

	24:0	12:12
X_{max} (mg L^{-1})	$4020^{a} \pm 60$	$2560^{b} \pm 34$
μ_{max} (h^{-1})	$0.035^{a} \pm 0.00$	$0.034^{a} \pm 0.00$
P_X (mg L^{-1} h^{-1})	$16.3^{a} \pm 0.3$	$10.0^{b} \pm 0.4$
RT (h)	$240^{a} \pm 0.00$	$240^{a} \pm 0.00$
Proteins (%)	$47.3^{a} \pm 1.1$	$41.1^{a} \pm 2.1$
Lipids (%)	$17.1^{b} \pm 0.7$	$23.0^{a} \pm 0.1$
Chlorophyll *a* (mg g^{-1})	$16.1^{b} \pm 0.3$	$20.7^{a} \pm 0.5$
Chlorophyll *b* (mg g^{-1})	$5.9^{a} \pm 0.2$	$5.7^{a} \pm 0.7$
Total chlorophyll total (mg g^{-1})	$21.9^{b} \pm 0.6$	$26.4^{a} \pm 0.1$

Results expressed in mean ± standard deviation. Different letters on the same line indicate difference by the Student's *t* test ($p < 0.05$)

X_{max} maximum cell biomass, μ_{max} maximum specific growth rate, *RT* cellular residence time, P_X productivity in biomass

Cell growth curves of *S. obliquus* microalgae grown in different photoperiods are presented in Fig. 1. The curves allow visualization of cell growth phases (adaptation, exponential, stationary and decline), from there allowing the collection of samples that characterize beginning of each phase. For the adaptation phase, the inoculum (stock culture) introduced to the photobioreactor at 0 h was used. A sample representing the exponential phase was collected at 72 h, while for the stationary and decline phases, collections were done at 168 and 240 h, respectively.

For *S. obliquus* cultivation, in 240 h cultivation, the literature reported values of final biomass concentration of 1500 mg L^{-1} and 500 mg L^{-1} for 24:0 and 12:12 (light:dark) photoperiods, respectively, lower values compared to this study that could be attributed to lower luminous intensity (60 µmol m^{-2} s^{-1}) and non-enrichment of CO_2 cultures in the earlier work [21]. Another study, which also used light intensity of 60 µmol m^{-2} s^{-1} but 10% enrichment with CO_2, reported a higher biomass concentration under constant illumination of 3510 mg L^{-1} [22], suggesting that all parameters involved in cultivation directly affect microalgal growth.

Lipids, proteins, and chlorophylls

The amount of product obtained from *S. obliquus* microalgae grown with different photoperiods is shown in Table 1. Lipid concentration was 17.1% for the 24:0 (light:dark) photoperiod, whereas 12:12 (light:dark) photoperiod yielded a 23.0% concentration, an increase of 34.1% in the obtained biomass

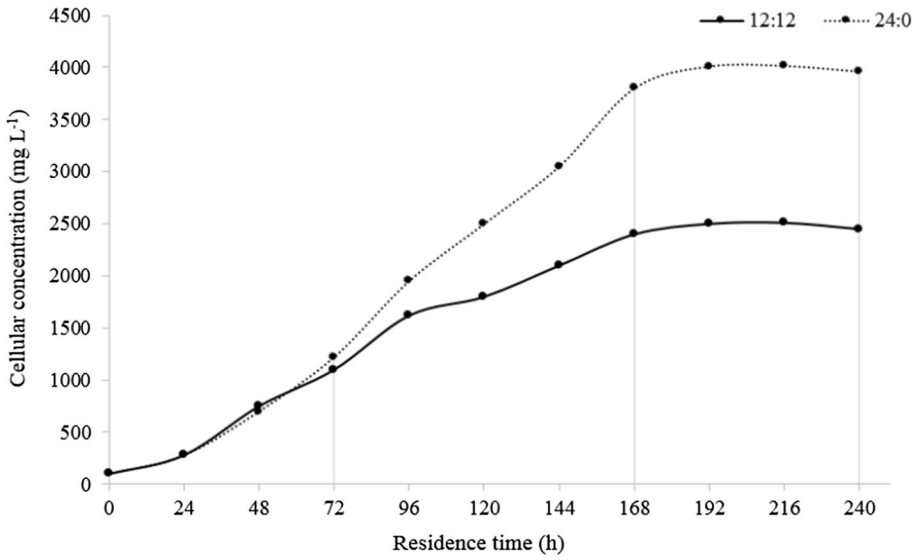

Fig. 1 Cell growth curves of *Scenedesmus obliquus* microalgae cultivated with 24:0 and 12:12 (light:dark) photoperiods and indication of sample collection times for metabolomics of the beginning adaptation (0 h), exponential (72 h), stationary (168 h) and decline (240 h) phases

for lipid. In *Scenedesmus* sp., Ma et al. [23] also reported obtaining higher lipid content with 12:12 (light:dark) photoperiod (28.1%) compared to 24:0 (light:dark) photoperiod (19.4%). The higher lipid content obtained with 12:12 (light:dark) photoperiod cultivation can be attributed to the storage of chemical energy in oil form, such as neutral lipids or triglycerides, by microalgae under stress conditions [24].

George et al. [25] obtained similar data for *Ankistrodesmus falcatus* microalgae, with the longer light incidence providing higher growth with less lipid accumulation. Finding conditions that allow maximum growth rates alongside maximum lipid productivities is difficult since higher lipid yields are not obtained under ideal growth conditions.

The protein content obtained for *S. obliquus*, however, did not differ statistically (Student's *t* test, $p < 0.05$) between the 24:0 and 12:12 (light:dark) photoperiods, at 47.3% and 41.1%, respectively (Table 1). Another study that evaluated the protein production of six strains of *S. obliquus* in photoautotrophic cultures using 60 μmol m^{-2} s^{-1} of light intensity with constant exposure and 20% of CO_2 enrichment obtained similar values to those reported here: 30–40% protein, varying by strain [22].

A study with *Chlorella vulgaris* microalgae evaluated different luminous intensities (37.5, 62.5, and 100 μmol m^{-2} s^{-1}) and different periods of exposure to light. In that study, the maximum light intensity and the highest irradiation period (24:0) positively affected protein concentrations [26]. Increase and duration of irradiance were also related to increased growth rate and protein concentration in the *S. obliquus* cultivations discussed in the present work.

The concentration of chlorophyll a was 20.66 mg g^{-1} for the 12:12 (light:dark) photoperiod and 16.1 mg g^{-1} for the 24:0 (light:dark) photoperiod. However, for chlorophyll *b*, which derives from chlorophyll *a*, no significant concentration differences were observed, with the 24:0 and 12:12 (light:dark) photoperiods measuring 5.9 and 5.7 mg g^{-1}, respectively. As can be seen in Table 1, due to the differences found for chlorophyll *a*, total chlorophyll also presented a significant difference at 21.89 mg g^{-1} for the 24:0 (light:dark) photoperiod and 26.39 mg g^{-1} for the 12:12 (light:dark) photoperiod.

Lower chlorophyll *a* concentrations were also observed in *Scenedesmus* sp. cultivated with a 24:0 (light:dark) photoperiod and attributed to molecular degradation because chlorophyll *a* is unstable and easily degrades under intense light exposure [23]. Chandra et al. [27] analyzed total chlorophyll of *Scenedesmus obtusus* biomasses grown with different photoperiods and also observed higher concentrations for the 12:12 (light:dark) photoperiod related to increased photosynthetic efficiency due to cultivation under 50% dark hours.

Several studies have evaluated chlorophyll production in microalga *C. vulgaris* grown in photobioreactors. Some found lower chlorophyll production than obtained for *S. obliquus*. For example, Mohsenpour et al. [9] measured 0.86 mg g^{-1} and 0.28 mg g^{-1} of chlorophylls *a* and *b*, respectively, under cultivation with light intensity 250 μmol m^{-2} s^{-1} and constant exposure. Sharma et al. [28] reported similar values as in this study: 15.7 mg g^{-1} and 4.2 mg g^{-1} chlorophylls *a* and *b*, respectively, for 24:0 (light:dark) photoperiod and 20.3 mg g^{-1} and 5.2 mg g^{-1} chlorophylls *a* and *b*, respectively, for 12:12 (light:dark) photoperiod with light intensity 50 μmol m^{-2} s^{-1}.

Microalgal metabolites

Principal component analysis (PCA) was used to visualize the relationship of polar and non-polar metabolites to cell growth phases and different photoperiods. Three principal components (PC) explained 87.6% of total data variance.

Figure 2a, b shows the scores (samples) and loadings (compounds), respectively, of the first two PCs. Regarding scores (Fig. 2a), PC1 discriminates those samples grown with a 12:12 (light:dark) photoperiod. Located in the right quadrants, these are mainly set apart by higher concentrations of amino acids, malic and succinic organic acids, and arachidonic acid (C20:4n6), as well as the presence of α-linolenic acid (C18:3n3) (Fig. 2b). On the left side are cultures under constant illumination, which showed higher concentrations of the saturated fatty acids, hexadecanoic acid (C16:0) and stearic acid (C18:0), as well as oleic acid (C18:1n9) and citric acid. However, the exponential phase of cultivation under constant illumination had higher concentrations of the fatty acids, undecanoic (C11:0), myristic (C14:0), pentadecanoic (C15:0), heptadecenoic (C17:1), and γ-linolenic (C18:3n6), differing from the other phases (stationary and decline), as shown in the lower quadrants of PC2.

Figure 3a, b presents the scores and loadings of PCs 1 and 3, respectively. These graphs show that PC3 allowed discrimination between cell growth phases in cultivation with the 12:12 (light:dark) photoperiod. The decline phase showed higher concentrations of the amino acids glycine, serine, aspartic acid, glutamic acid, and asparagine, as well as malic acid, linoleic acid (C18:2n6c), and C17:1 fatty acids, as shown in the upper quadrant. The exponential phase, in the lower quadrant, showed higher concentrations of proline, threonine, tyrosine, saturated fatty acids, eicosenoic acid (C20:1), and eicosatrienoic acid (C20:3n6). The stationary phase was located between the exponential and decline phases, indicating a metabolic profile similar to both the exponential and the decline phase with intermediate concentrations of the major compounds represented in these other phases, as a transitional period between these opposite phases.

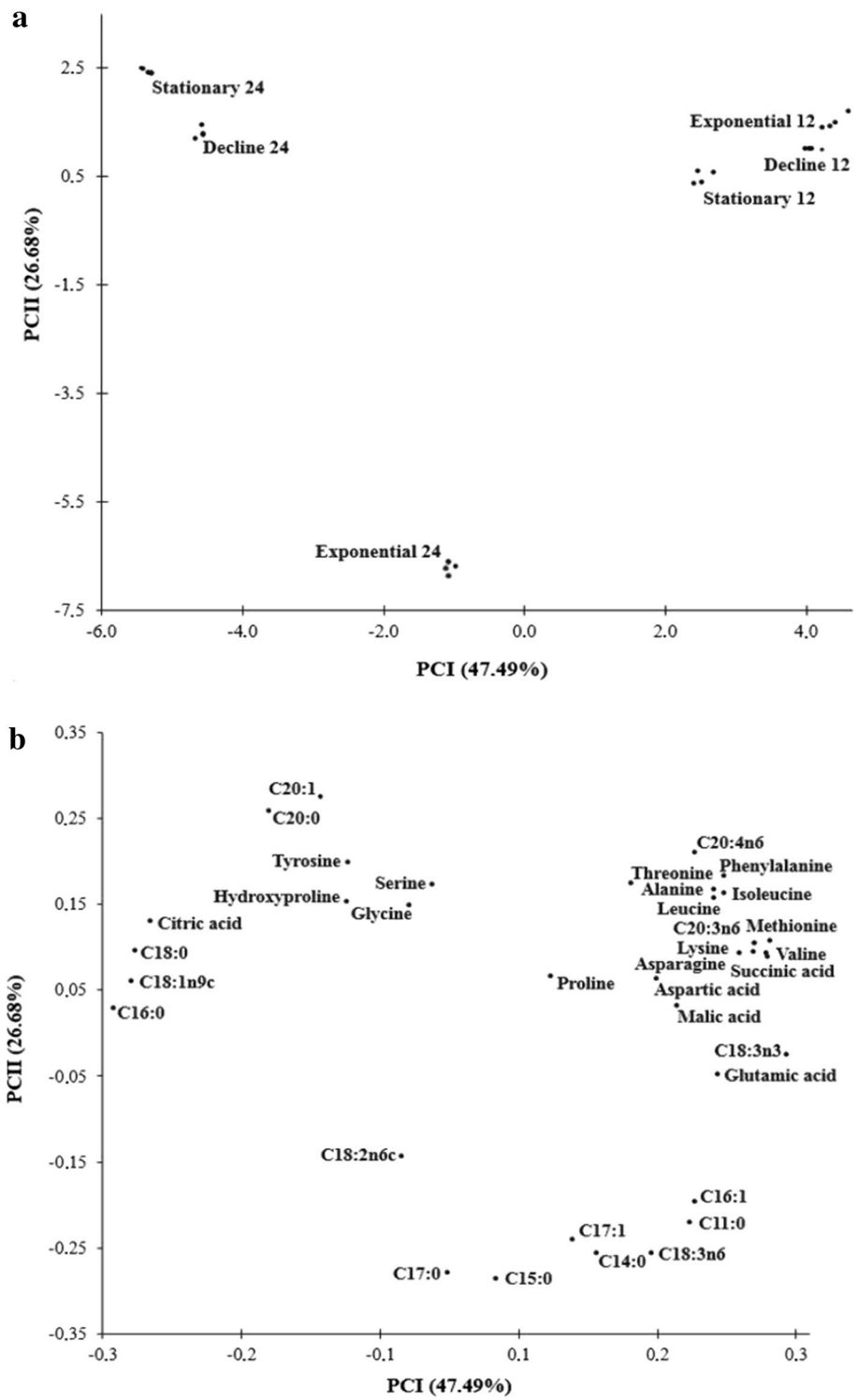

Fig. 2 **a, b** Plots of scores (samples) and loadings (variables) showing the principal components 1 and 2 from *Scenedesmus obliquus* metabolism in the phases of cell growth (exponential, stationary and decline) and different photoperiods (24:0 and 12:12 light:dark)

Organic acids

Three organic acids—citric, malic, and succinic—were detected in *S. obliquus* inoculum and in both photosynthetic cultures at different stages of cell development (Table 2). These acids participate in the tricarboxylic acid cycle (TCA). In addition to producing energy and other metabolites, as amino acids, they play a role in the osmotic adjustment of vacuoles [29].

It was observed an increase of the citric acid concentration of the inoculum to exponential phase in the two microalgal cultivation. Although luminosity conditions favor the

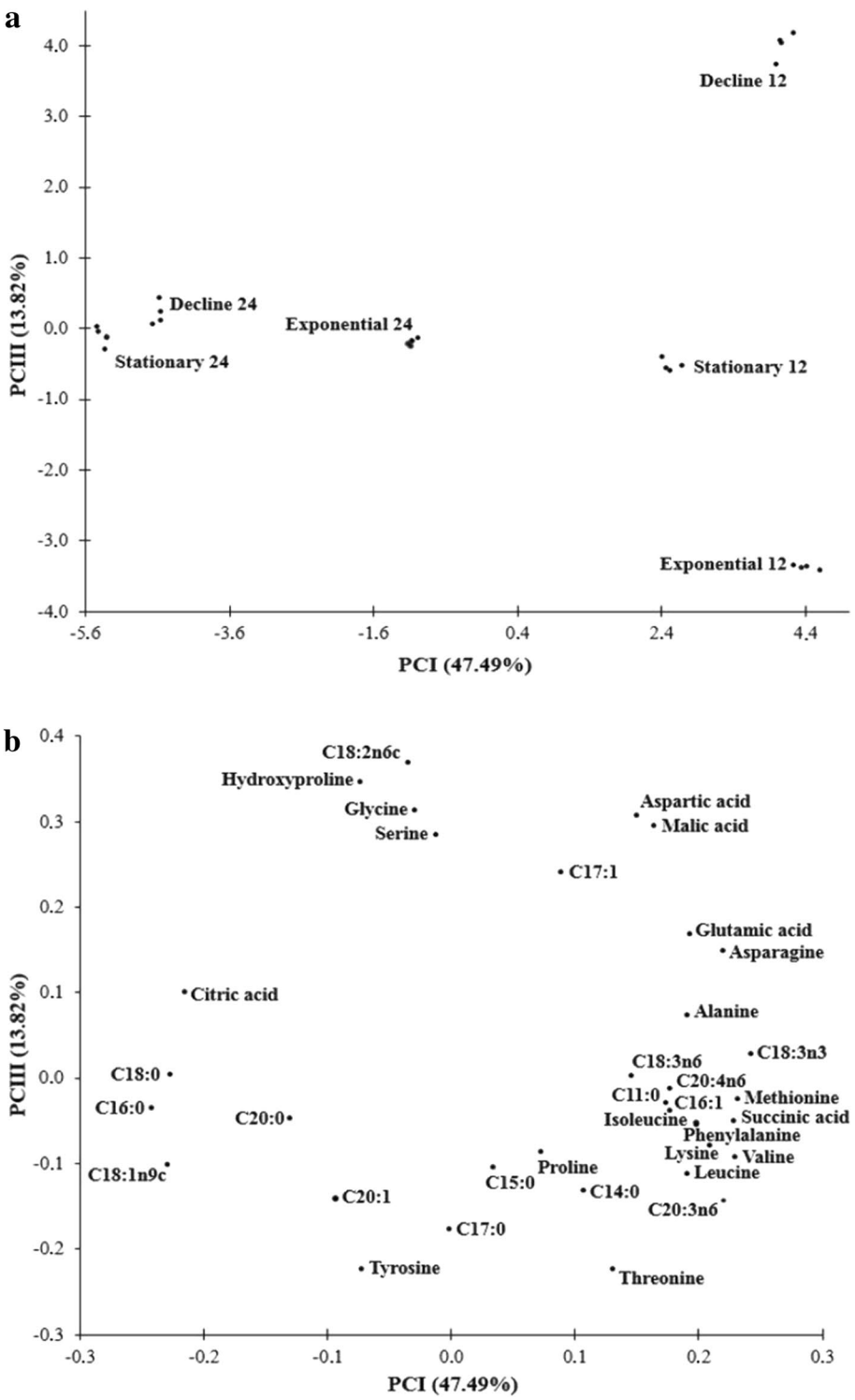

Fig. 3 **a, b** Plots of scores (samples) and loadings (variables) showing the principal components 1 and 3 from *Scenedesmus obliquus* metabolites (organic acids, amino acids and fatty acids) in the phases of cell growth cultivation (exponential, stationary and decline) with 24:0 and 12:12 (light:dark) photoperiods

photosynthetic metabolism, the carboxylation of phosphenol pyruvate is another CO_2 fixation pathway. In this case, oxaloacetate is easily converted into C4 dicarboxylic acids (e.g., citric acid), and subsequently into amino acids [18] indicating that the increase of the cell growth in the exponential phase propitiated the greater formation of this acid.

Regardless of photoperiod, lower concentrations of total organic acids were detected in the exponential phase, which involves greater cellular development that requires much energy and metabolites produced by the TCA. Thus, organic acids do not concentrate intracellularly, with only the necessary quantities of those acids participating in cell regulation

Table 2 Organic acids (μg g^{-1} of biomass) obtained in photosynthetic cultures of *Scenedesmus obliquus* throughout growth

Organic acids	Inoculum	Exponential phase		Stationary phase		Decline phase	
		24:0	12:12	24:0	12:12	24:0	12:12
Citric acid	$9.6^{e} \pm 0.4$	$222.4^{c} \pm 14.9$	$150.4^{d} \pm 8.4$	$702.6^{a} \pm 65.3$	$183.1^{cd} \pm 11.4$	$679.0^{a} \pm 52.1$	$308.7^{b} \pm 12.3$
Malic acid	$130.6^{b} \pm 10.4$	$82.6^{d} \pm 4.5$	$111.4^{c} \pm 8.7$	$51.1^{e} \pm 4.9$	$79.4^{d} \pm 6.2$	$79.9^{d} \pm 3.9$	$221.9^{a} \pm 3.5$
succinic acid	$70.0^{b} \pm 0.7$	$30.6^{e} \pm 0.5$	$77.4^{a} \pm 3.4$	$28.4^{e} \pm 1.3$	$50.2^{d} \pm 0.4$	$27.3^{e} \pm 0.3$	$66.0^{c} \pm 0.8$
∑AO	$210.2^{d} \pm 11.1$	$335.6^{c} \pm 14.1$	$339.2^{c} \pm 7.4$	$782.1^{a} \pm 57.6$	$312.7^{c} \pm 14.4$	$786.2^{a} \pm 54.8$	$596.5^{b} \pm 10.7$

Results expressed in mean ± standard deviation. Different letters on the same line indicate difference by Tukey test ($p < 0.05$)

AO organic acids

[29]. By contrast, higher concentrations were found in the stationary phase for the 24:0 (light:dark) photoperiod and in the decline phase for the 12:12 (light:dark) photoperiod. In these phases, metabolism is already less intense, and some organic acids accumulate intracellularly instead of being consumed to produce energy and metabolites.

Individually, the organic acids behaved differently in the inoculum, these differences may be related to the higher luminous intensity used in the cultivation and also to the 15% CO_2 enrichment. Malic acid presented the highest concentration at 130.6 μg g^{-1} of biomass, followed by succinic acid at 70.0 μg g^{-1} of biomass and citric acid at 9.6 μg g^{-1} of biomass. In cultivation, independent of photoperiod and cell development phase, citric acid was the major compound (150.4–702.6 μg g^{-1} of biomass), followed by malic acid (51.1–221.9 μg g^{-1} of biomass) and, in lower concentrations, succinic acid (27.4–77.4 μg g^{-1} of biomass). Higher concentrations of citric acid compared to the other TCA acids were also reported for cultures with different photoperiods of *Ectocarpus siliculosus* algae [30].

Concerning the different growth phases (Fig. 1), the concentration of citric acid increased significantly from the exponential phase to the stationary phase with 24:0 (light:dark) photoperiod cultivation but showed no difference between the stationary and declining phases. Malic acid reduced in the stationary phase, with its concentrations presenting no significant difference between the exponential and decline phases. The succinic acid concentration did not differ with *S. obliquus* growth.

With the 12:12 (light:dark) photoperiod, citric acid concentration did not differ between the exponential and stationary phases, but concentrations were higher in the decline phase. By contrast, malic and succinic acids both tended to reduce in concentration from the exponential to stationary phase and increase from the stationary to decline phase.

The photoperiods also influenced organic acid concentrations, as previously observed in *Chara australis* [29]. Citric acid concentrations were higher in culture under constant illumination, while higher concentrations of malic and succinic acids were detected with the 12:12 (light:dark) photoperiod, as visualized in PC1. In a study performed with *Ectocarpus siliculosus* algae, citric acid concentrations were also higher in cultures under constant illumination, but photoperiods did not alter the concentrations of succinic acid, unlike in this work [30].

Free amino acids

Fifteen amino acids (alanine, glycine, valine, leucine, isoleucine, proline, methionine, serine, threonine, phenylalanine, aspartic acid, glutamic acid, asparagine, lysine, and tyrosine) were detected in *S. obliquus* inoculum, totaling 1661.1 μg g^{-1} of biomass (Table 3). The major compounds were glutamic acid (541.3 μg g^{-1} of biomass), alanine (513.5 μg g^{-1} of biomass), serine (202.2 μg g^{-1} of biomass), and threonine (113.4 μg g^{-1} of biomass).

In the exponential phase of the 24:0 (light:dark) photoperiod, the concentration of amino acids was lower, compared to the inoculum, at 593.3 μg g^{-1} of biomass, and the amino acids methionine, asparagine, and lysine were not detected. In the stationary phase, the amino acid concentrations continued to decrease (to 549.9 μg g^{-1} of biomass), and the amino acid hydroxyproline was detected in that phase (Table 3).

The large reduction in the concentration of amino acids corresponds to accentuated microalgal growth between the exponential and stationary phases (Fig. 1), as amino acids were condensed to form the proteins that were the major constituent of the evaluated biomass (47.3%). In the decline phase, amino acid concentrations increased (to 663.8 μg g^{-1} of biomass). At this stage, microalgal growth is quite limited, so the amino acids synthesized by the microalgae are used with less intensity by the metabolic pathways for polymeric formation and some protein degradation may occur in this stage, therefore, accumulating its metabolites in the intracellular environment. This behavior was also observed in the inoculum, characterized by low cell growth and high accumulation of these components in cells.

With the 12:12 (light:dark) photoperiod, the same fifteen amino acids were detected in the inoculum, except that the amino acid hydroxyproline was found in the decline phase. As 24:0 (light:dark) photoperiod cultivation, amino acids

Table 3 Amino acids (μg g^{-1} of biomass) obtained in *Scenedesmus obliquus* cultures with different photoperiods at different stages of cell growth

Amino acids	Inoculum	Exponential phase		Stationary phase		Decline phase	
		24:0	12:12	24:0	12:12	24:0	12:12
Alanine	$513.5^{a} \pm 9.8$	$122.2^{f} \pm 1.9$	$276.0^{b} \pm 17.4$	$144.9^{e} \pm 10.7$	$226.3^{c} \pm 5.6$	$202.1^{d} \pm 1.6$	$295.3^{b} \pm 4.2$
Glycine	$30.7^{bc} \pm 1.9$	$9.4^{d} \pm 0.6$	$4.5^{e} \pm 0.0$	$32.8^{b} \pm 0.8$	$37.4^{a} \pm 2.6$	$26.6^{c} \pm 1.9$	$38.4^{a} \pm 3.4$
Valine	$38.8^{a} \pm 0.4$	$9.3^{e} \pm 0.3$	$21.1^{b} \pm 0.1$	$8.0^{f} \pm 0.2$	$15.1^{d} \pm 0.2$	$9.2^{e} \pm 0.3$	$16.8^{c} \pm 0.3$
Leucine	$22.5^{a} \pm 0.4$	$6.0^{e} \pm 0.2$	$15.3^{b} \pm 0.5$	$9.3^{d} \pm 0.4$	$15.2^{b} \pm 0.8$	$6.9^{e} \pm 0.2$	$12.1^{c} \pm 0.3$
Isoleucine	$14.5^{a} \pm 0.2$	$2.8^{f} \pm 0.1$	$6.7^{b} \pm 0.5$	$4.1^{e} \pm 0.3$	$4.9^{d} \pm 0.3$	$3.1^{f} \pm 0.1$	$5.9^{c} \pm 0.1$
Proline	$24.7^{c} \pm 0.7$	$9.4^{f} \pm 0.4$	$31.8^{b} \pm 0.4$	$32.5^{b} \pm 2.3$	$137.5^{a} \pm 1.2$	$15.7^{e} \pm 1.0$	$18.5^{d} \pm 1.1$
Methionine	$32.7^{a} \pm 2.3$	–	$4.8^{b} \pm 0.4$	–	$2.9^{b} \pm 0.2$	–	$4.1^{b} \pm 0.1$
Serine	$202.2^{a} \pm 15.8$	$19.5^{c} \pm 0.9$	$29.3^{c} \pm 1.3$	$32.1^{c} \pm 3.1$	$27.9^{c} \pm 0.3$	$51.6^{b} \pm 2.6$	$52.7^{b} \pm 0.6$
Threonine	$113.4^{a} \pm 8.8$	$3.6^{d} \pm 0.3$	$67.5^{b} \pm 2.8$	$21.5^{c} \pm 1.7$	$20.6^{c} \pm 1.3$	$22.0^{c} \pm 1.5$	$28.2^{c} \pm 0.5$
Phenylalanine	$9.9^{ab} \pm 0.8$	$2.7^{e} \pm 0.2$	$10.4^{a} \pm 0.8$	$5.2^{d} \pm 0.2$	$8.5^{c} \pm 0.2$	$4.6^{d} \pm 0.4$	$8.9^{bc} \pm 0.4$
Aspartic acid	$21.8^{b} \pm 1.6$	$2.4^{d} \pm 0.2$	$11.4^{c} \pm 1.0$	$1.7^{d} \pm 0.2$	$2.8^{d} \pm 0.2$	$2.7^{d} \pm 0.2$	$39.9^{a} \pm 1.9$
Hydroxyproline	–	–	–	$4.7^{b} \pm 0.3$	–	$4.6^{b} \pm 0.4$	$6.7^{a} \pm 0.3$
Glutamic acid	$541.3^{a} \pm 34.6$	$398.9^{c} \pm 23.0$	$455.4^{b} \pm 31.7$	$241.0^{e} \pm 18.9$	$314.2^{d} \pm 18.2$	$297.5^{d} \pm 6.3$	$567.2^{a} \pm 14.4$
Asparagine	$59.4^{a} \pm 2.7$	–	$10.1^{c} \pm 0.7$	–	$12.5^{c} \pm 0.8$	–	$17.6^{b} \pm 0.9$
Lysine	$9.2^{d} \pm 0.6$	–	$18.5^{b} \pm 1.1$	–	$24.1^{a} \pm 1.4$	–	$13.3^{c} \pm 1.0$
Tyrosine	$26.6^{a} \pm 2.1$	$7.1^{e} \pm 0.2$	$15.0^{c} \pm 0.9$	$12.3^{d} \pm 0.7$	$11.0^{d} \pm 0.7$	$17.3^{b} \pm 0.4$	$8.2^{e} \pm 0.4$
∑AA	$1661.1^{a} \pm 64.6$	$593.3^{ef} \pm 24.4$	$977.9^{c} \pm 42.3$	$549.9^{f} \pm 24.2$	$861.0^{d} \pm 25.6$	$663.8^{e} \pm 5.2$	$1133.7^{b} \pm 14.8$

Results are expressed as mean ± standard deviation. Different letters on the same line indicate difference by Tukey test ($p < 0.05$)

AA amino acids

were reduced in the exponential and stationary phases and increased in the decline phase, as was also observed for the 12:12 (light:dark) photoperiod cultivation (Table 3).

However, amino acid concentrations were higher in all phases for the 12:12 (light:dark) photoperiod than for compared to the 24:0 (light:dark) photoperiod. Concentrations of amino acids were inversely proportional to the protein content, which was higher in cultures under constant illumination, as has been observed in previous studies of *C. vulgaris* [28, 31, 32].

The higher concentration of amino acids under 12:12 (light: dark) photoperiod cultivation, one of the variables responsible for the separation of photoperiods in PC1, may be related to lower microalgal growth, such that the amino acids remained free in the intracellular environment. In addition, as the cultures were grown photosynthetically without an organic carbon source, dark is considered a stress factor for cells and growth limiting. This stress condition to which the microalgal culture was submitted meant metabolite synthesis was greater in periods of light exposure to compensate for the dark periods; notably, samples were always collected in the light periods for the 12:12 (light:dark) photoperiod treatment [28].

Previous studies of plants have mentioned the amino acid alanine as a universal biomarker of cell stress [33, 34]. Nehmé et al. [35] also verified increased alanine synthesis under stress conditions by microalga *Dunaliella salina*. This was observed in this study with *S. obliquus* microalgae. Alanine concentrations were higher in inoculum and with the 12:12 (light:dark) photoperiod, which presents a stress condition due to the absence of light compared to culture under constant illumination. Regarding the phases, in both cultivations the alanine concentrations were higher in the decline phase, when conditions began to restrict microalgal development; this also established a stress condition.

In addition to composing proteins, glutamic acid is a precursor of the C5 route to form chlorophylls, the major pigment class in photosynthetic organisms [36, 37]. This amino acid reduced in concentration during the exponential and stationary phases and increased in the decline phase. When *S. obliquus* grows, synthesized glutamic acid is probably directed to the protein formation route and the C5 pathway for chlorophyll synthesis, decreasing its concentration in the intracellular environment. In the declining phase, with limited growth, glutamic acid instead accumulated in the intracellular environment.

Glutamic acid concentrations were higher with the 12:12 (light:dark) photoperiod at all growth phases compared to the 24:0 (light:dark) photoperiod (Fig. 2). The higher synthesis of glutamic acid during growth may be related to the higher chlorophyll content obtained at the end of cultivation. The greatest difference was observed in the decline phase, in which 567.19 μg g^{-1} of biomass was detected for 12:12 (light:dark) photoperiod and 297.54 μg g^{-1} of biomass

was detected for 24:0 (light:dark) photoperiod. It occurs in the decline phase because the metabolic activity is reduced and consequently large amounts of glutamic acid previously directed to the production of chlorophyll and protein formation accumulate in the cells, same behavior observed and previously discussed for the amino acid content.

The amino acid hydroxyproline was only detected in the stationary phase with the 24:0 (light:dark) photoperiod and in the declining phase with the 12:12 (light:dark) photoperiod. Some species of microalgae may have cell walls constituted by glycoproteins rich in hydroxyproline [38–40]. The hydroxyproline's presence at low concentration in the intracellular environmental may indicate that *S. obliquus* is one of such species. A previous study reported the presence of hydroxyproline-rich glycoproteins in *S. obliquus*, although the hydroxyproline concentration found in the cell wall was considerably lower than that of *Chlamydomonas reinhardtii* microalgae, which are characterized by the presence of this constituent in its structure [41].

Aspartic acid is a precursor of five other amino acids that were detected in *S. obliquus* inoculum: asparagine, isoleucine, methionine, threonine, and lysine. However, asparagine was not observed in culture under constant illumination, detected instead only in the culture submitted to dark periods (12:12). Asparagine synthesis involves the transfer of an amino group from the amino acid glutamine to aspartic acid in the presence of ATP; this reaction is favored in dark conditions, corroborating with the obtained results [42]. In cultivation under constant illumination, the amino acids methionine and lysine were also not detected.

Fatty acids

Twelve fatty acids were detected in *S. obliquus* inoculum, of which 24.0% were saturated fatty acids (SFA), 12.0% were monounsaturated fatty acids (MUFA), and 64.0% were polyunsaturated fatty acids (PUFAs). The major fatty acids were C18:3n3 at 39.1%, linoleic acid (C18:2n6) at 20.6% and C16:0 at 19.3%. The two variables—different photoperiods and cell growth phases—influenced the composition of fatty acids compared to the inoculum (Table 4).

The 24:0 (light:dark) photoperiod had the same fatty acids as the inoculum in the exponential phase. Already detected in the stationary and declining phases of this same cultivation (24:0 light:dark) were eicosanoic acid (C20:0), C20:1, and arachidonic acid (C20:4n6), fatty acids that contributed to the separation of growth phases in PC2 (Fig. 2). The lowest SFA concentration in the 24:0 photoperiod (light:dark) was observed in the exponential phase, followed by a significant increase in the stationary and decline phases. The MUFA presented the highest concentrations in the stationary phase before reducing in

Table 4 Fatty acids (%) of *Scenedesmus obliquus* microalgae cultivated with different photoperiods during cell development

Fatty acids	Inoculum	Exponential phase		Stationary phase		Decline phase	
		24:0	12:12	24:0	12:12	24:0	12:12
C11:0	$0.7^{d} \pm 0.0$	$1.1^{a} \pm 0.0$	$1.0^{b} \pm 0.0$	$0.4^{e} \pm 0.0$	$0.8^{c} \pm 0.0$	$0.6^{d} \pm 0.0$	$0.9^{b} \pm 0.0$
C14:0	$0.63^{a} \pm 0.0$	$0.4^{b} \pm 0.0$	$0.4^{c} \pm 0.0$	$0.2^{e} \pm 0.0$	$0.3^{d} \pm 0.0$	$0.3^{d} \pm 0.0$	$0.3^{d} \pm 0.0$
C15:0	$0.4^{a} \pm 0.0$	$0.3^{b} \pm 0.0$	$0.2^{c} \pm 0.0$	$0.1^{e} \pm 0.0$	$0.2^{d} \pm 0.0$	$0.2^{cd} \pm 0.0$	$0.2^{d} \pm 0.0$
C16:0	$19.3^{c} \pm 0.2$	$25.5^{b} \pm 0.1$	$21.6^{c} \pm 1.0$	$30.3^{a} \pm 0.3$	$22.0^{c} \pm 0.9$	$31.5^{a} \pm 0.2$	$20.5^{c} \pm 0.8$
C16:1	$2.6^{a} \pm 0.0$	$0.5^{b} \pm 0.0$	$0.5^{bc} \pm 0.0$	$0.2^{f} \pm 0.0$	$0.4^{d} \pm 0.0$	$0.3^{e} \pm 0.0$	$0.4^{c} \pm 0.0$
C17:0	$0.9^{a} \pm 0.1$	$0.5^{b} \pm 0.0$	$0.4^{c} \pm 0.0$	$0.3^{d} \pm 0.0$	$0.3^{cd} \pm 0.0$	$0.3^{c} \pm 0.0$	$0.3^{d} \pm 0.0$
C17:1	$0.6^{f} \pm 0.0$	$5.6^{a} \pm 0.1$	$3.0^{de} \pm 0.0$	$2.8^{e} \pm 0.0$	$4.3^{c} \pm 0.2$	$3.2^{d} \pm 0.0$	$5.1^{b} \pm 0.1$
C18:0	$2.2^{d} \pm 0.1$	$4.0^{b} \pm 0.2$	$3.5^{bc} \pm 0.2$	$7.6^{a} \pm 0.1$	$2.7^{cd} \pm 0.2$	$7.7^{a} \pm 0.6$	$3.4^{bc} \pm 0.2$
C18:1n9c	$8.8^{f} \pm 0.0$	$17.8^{c} \pm 0.2$	$15.3^{d} \pm 0.2$	$32.4^{a} \pm 0.2$	$9.9^{e} \pm 0.0$	$26.8^{b} \pm 0.6$	$8.3^{f} \pm 0.0$
C18:2n6c	$20.6^{a} \pm 0.1$	$13.6^{b} \pm 0.1$	$9.9^{e} \pm 0.2$	$11.9^{d} \pm 0.1$	$12.9^{c} \pm 0.2$	$12.8^{c} \pm 0.1$	$13.8^{b} \pm 0.3$
C18:3n6	$4.3^{a} \pm 0.1$	$2.0^{b} \pm 0.1$	$1.4^{c} \pm 0.0$	$0.7^{e} \pm 0.0$	$1.5^{c} \pm 0.0$	$1.0^{d} \pm 0.1$	$1.5^{c} \pm 0.0$
C18:3n3	$39.1^{c} \pm 0.1$	$28.7^{d} \pm 0.1$	$41.0^{bc} \pm 1.3$	$12.0^{e} \pm 0.1$	$43.4^{ab} \pm 0.7$	$13.9^{e} \pm 0.2$	$44.1^{a} \pm 0.8$
C20:0	–	–	$0.4^{b} \pm 0.0$	$0.6^{a} \pm 0.0$	$0.3^{c} \pm 0.0$	$0.6^{a} \pm 0.0$	$0.3^{c} \pm 0.0$
C20:1	–	–	$0.4^{b} \pm 0.0$	$0.5^{a} \pm 0.0$	$0.3^{c} \pm 0.0$	$0.5^{ab} \pm 0.0$	$0.2^{c} \pm 0.0$
C20:3n6	–	–	$0.4^{a} \pm 0.0$	–	$0.3^{b} \pm 0.0$	–	$0.2^{c} \pm 0.0$
C20:4n6	–	–	$0.7^{a} \pm 0.0$	$0.2^{c} \pm 0.0$	$0.6^{a} \pm 0.0$	$0.4^{b} \pm 0.0$	$0.6^{a} \pm 0.0$
∑SFA	$24.0^{c} \pm 0.2$	$31.9^{b} \pm 0.4$	$27.4^{c} \pm 1.3$	$39.4^{a} \pm 0.4$	$26.5^{c} \pm 1.1$	$41.2^{a} \pm 0.8$	$25.8^{c} \pm 1.1$
∑MUFA	$12.0^{f} \pm 0.0$	$23.9^{c} \pm 0.1$	$19.1^{d} \pm 0.2$	$35.8^{a} \pm 0.2$	$14.8^{e} \pm 0.2$	$30.7^{b} \pm 0.6$	$14.1^{e} \pm 0.0$
∑PUFA	$64.0^{a} \pm 0.1$	$44.3^{d} \pm 0.3$	$53.4^{c} \pm 1.5$	$24.8^{e} \pm 0.2$	$58.7^{b} \pm 0.9$	$28.1^{e} \pm 0.2$	$60.2^{b} \pm 1.0$

Results are expressed as mean ± standard deviation. Different letters on the same line indicate difference by Tukey test ($p < 0.05$)

SFA saturated fatty acids, *MUFA* monounsaturated fatty acids, *PUFA* polyunsaturated fatty acids

the decline phase, while the PUFA presented the highest concentrations in the exponential phase before reducing significantly in stationary phase, which did not differ in PUFA concentration from the decline phase.

The highest diversity of fatty acids was detected with the 12:12 (light:dark) photoperiod. In addition to those already mentioned, C20:3n6 was found in all phases of cell growth. The concentrations of saturated fatty acids showed no significant difference between growth phases, while MUFA reduced in concentration across growth phases and PUFA increased.

In earlier work, *S. obliquus* cultivation with light intensity of 100 µmol m^{-2} s^{-1} and a photoperiod intermediate to this work, 16:8, showed the same trend in fatty acid concentrations between the exponential and stationary phases as observed here for the 24:0 (light:dark) photoperiod: saturated and monounsaturated fatty acids presented significant increases in concentration, while PUFA reduced in concentration. The earlier work did not evaluate the decline phase [43].

During cell development, the major fatty acids were the same in 24:0 (light:dark) photoperiod culture: C18:3n3, C16:0, and C18:1n9. In the exponential phase, the major fatty acid was C18:3n3 (28.7%), followed by C16:0 (25.5%) and C18:1n9 (17.8%). In the stationary phase, the fatty acid with the highest concentration was C18:1n9c (32.4%), followed by C16:0 (30.3%) and C18:3n3 (12.0%). In the decline phase, the most present fatty acid was C16:0 (31.5%), followed by C18:1n9c (26.8%) and finally C18:3n3 (13.9%).

Concerning the major fatty acids with 12:12 (light:dark) photoperiod cultivation, C18:3n3 had the highest concentration, ranging from 44.1 to 41.0%, followed by C16:0 at concentrations of 22.1–20.5%, independent of cell growth phase. In the exponential phase, C18:1n9c (15.3%) was among the most present fatty acids, while in the stationary and declining phases, C18:2n6 was found in higher concentrations (at 12.9% and 13.8%, respectively).

Light is a critical factor in the synthesis and composition of lipids, and the amount of light required by each species varies greatly. Thus, there is much variation in the composition of fatty acids between different species, as well as for the same species exposed to different intensities and periods of light [27].

For the different photoperiods, independent of cell growth phase, cultures under constant illumination presented higher concentrations of saturated and monounsaturated fatty acids and lower polyunsaturated fatty acids compared to the cultivation with the 12:12 (light:dark) photoperiod. Higher concentrations of PUFA in culture with the 12:12 (light:dark) photoperiod, compared to cultures under constant illumination, were also observed for *Scenedesmus* sp [23] and *Chlorella minutissima* [44].

The biosynthesis of fatty acids in microalgae occurs mainly in the chloroplast with the production of C16:0 and C18:0-ACP (acyl carrier protein); later elongation and desaturation reactions occur. These AGs will be the precursors of lipid synthesis. For the synthesis of unsaturated AGs, a double bond is introduced into the acyl group esterified to ACP by the enzyme acyl-ACP desaturase [45]. As the 12:12 photoperiod (light: dark) showed higher PUFA values, indicating greater activity of the acyl-ACP desaturase enzyme.

In summary, at the end of cultivation with constant illumination, *S. obliquus* biomass had lower lipid content (17.1%), comprising 41.2% SFA, 30.7% MUFA, and 28.1% PUFA. With the 12:12 (light:dark) photoperiod, more lipid content was obtained (23.0%), comprising 60.2% PUFA, 25.8% SFA, and 14.1% MUFA. The cultures provided different concentrations and lipid compositions that could be targeted by application, such as biodiesel production or food supplementation.

Fatty acid profiles with lower levels of PUFA, such as that absorbed in the phase of decline of the 24:0 (light:dark) photoperiod, favor the biodiesel production, since these fatty acids contribute negatively to important properties of the product, such as ignition quality and oxidative stability [46]. On the other hand, the fatty acids composition in the decline phase of the 12:12 (light dark) photoperiod with a higher proportion of PUFA is suitable for application in dietary supplements [47].

Conclusion

Different photoperiods directly affected *S. obliquus* microalgal growth, as well as protein, lipid, and chlorophyll content. Cultivation under constant illumination was favorable to cell development and protein production, while cultivation with dark periods (12:12) induced higher lipid and chlorophyll production. The concentration of the evaluated metabolites, organic acids, amino acids, and fatty acids was influenced by different photoperiods and cell growth phases. The concentrations of these compounds could be related to the conditions under which cultivation occurred, such as stress conditions, and also to the biomass constituents obtained at the end of cultivation (proteins, lipids, and chlorophylls), because they are metabolic precursors.

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Compliance with ethical standards

Conflict of interest The authors declare that there is no conflict of interests.

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4.3 ARTIGO 3: DEVELOPMENT OF A NEW METHOD FOR SIMULTANEOUS EXTRACTION OF CHLOROPHYLLS AND CAROTENOIDS FROM MICROALGAL BIOMASS

Artigo em fase de revisão na revista Journal of Applied Phycology.

DEVELOPMENT OF A NEW METHOD FOR SIMULTANEOUS EXTRACTION OF CHLOROPHYLLS AND CAROTENOIDS FROM MICROALGAL BIOMASS

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Abstract

Microalgae are microorganisms with high biotechnological importance, including for the food sector, being the source of several valuable inputs such as pigments. There are still limitations in the methods for extracting these compounds. This work aimed to develop simple method for simultaneous extraction of chlorophylls and carotenoids from the microalgae *Spirulina* sp. and *Scenedesmus obliquus*. The extraction was performed using a methanol:chloroform (2:1 v/v) mixture with different homogenization times (30, 60 and 120 min); after exhaustive extraction with chloroform, acetone, ethyl acetate and ethanol were tested and compared with the reference maceration method (MM). Satisfactory recovery values, mostly between 101.11- 124.18%, were obtained with 30 min of homogenization and extraction with ethanol after chloroform (PM30E). In general, the chlorophyll and carotenoid profiles obtained by the PM30E presented fewer isomers and possible degradation compounds. PM30E showed a reduction of about five times the volume of organic solvents and was four times faster than the MM. In addition, this method provided greater analytical frequency and security for the analyst, since PM30E is performed in a closed system of mechanical homogenization.

Keywords: Pigments, Chlorophyceae, Cyanobacteria, Quantification, High Performance Liquid Chromatography

1. Introduction

Microalgae is a term without taxonomic value, including more than 70,000 consistently cataloged species of prokaryotic and eukaryotic microorganisms with photosynthetic capacity (Jacob-Lopes et al. 2019). Through photosynthesis mechanism, microalgae have the capacity to accumulate large amounts of biomass composed of polysaccharides, proteins, lipids, pigments, sterols, vitamins and others bioactive compounds (Barsanti and Gualtieri 2018). The production of these organic compounds combined with the versatility of these microorganisms developing in wide ranges of temperature, salinity, pH and different light intensities, has aroused the interest for their commercial exploitation by several industrial segments, including food (Khan et al. 2018).

At the cellular level, microalgal pigments are associated with light harvesting, fixation of CO_2 and protection from damage caused by excessive lighting (Matos 2017). The most common pigments in microalgae generally belong to the group of chlorophylls, and carotenoids, in some cases phycobiliproteins (synthesized by prokaryotic microalgae). First, microalgal pigments found a market in the food industry as colorants and stood out compared to synthetic pigments for being non-toxic and non-cancerous (Galasso et al. 2019). As humans do not synthesize them, microalgae pigments have been employed both as natural colorants and food supplements, considering their anti-inflammatory, anti-hypertensive, anti-cancer, antioxidant, antidepressant and anti-aging activities has been proven (Camacho et al. 2019).

Chlorophylls and carotenoids are usually fat-soluble molecules or soluble in organic solvents, while phycobiliproteins are water-soluble pigments (Begum et al. 2016). Chlorophylls are greenish pigments, having in their structure a phytol tail and porphyrin macrocycle of four pyrrole rings linked in a central atom of magnesium responsible for converting solar energy into chemical energy in photosynthesis (D'Alessandro and Antoniosi Filho 2016). So far, five types of chlorophylls are known *a*, *b*, *c*, *d* and *f*, which are structurally similar differing in peripheral groups of the macrocycle. Chlorophyll *a* is the most abundant in photosynthetic organisms and is the precursor to structures *b* and *d*, already the synthesis of chlorophylls *c* and *f* from chlorophyll *a* is not entirely clear (Da Silva Ferreira and Sant'Anna 2017). The chlorophylls extraction can be made from plant species, but microalgae are more productive, reaching values above 2.5% in dry mass (Vendruscolo et al. 2019).

Concerning carotenoids, the chemical basic structure is a tetraterpene formed from eight five-carbon isoprenoid units (Ambati et al. 2019). They can be classified as cyclic or acyclic by the

presence or absence of rings at the structural ends, and also regarding the presence of oxygen, if formed exclusively by carbons, are carotenes, while presenting oxygenated structures are called xanthophylls (Rodriguez-Concepcion et al. 2018). In photosynthesis, carotenoids act as accessory pigments absorbing energy in different spectral bands than chlorophylls and in protecting the photosynthetic system (Mussagy et al. 2019). The use of microalgae as source of carotenoids is related to the great diversity found in these microorganisms, high content in cells and the possibility of modulation through external conditions (Galasso et al. 2019). Among the carotenoids identified in microalgae, β-carotene, astaxanthin and lutein have the greatest market potential (Novoveská et al. 2019), however violaxanthin, antheraxanthin, zeaxanthin and neoxanthin are also commonly found (Paliwal et al. 2016).

Homogenization for several hours with organic solvents or exhaustive maceration are the main form to carry out the extraction of lipophilic pigments (chlorophylls and carotenoids) in microalgae (Zhang et al. 2019). In order to reduce the time and maximize the pigments recovery, extractions can be performed assisted by ultrasound or microwave, however it has already been verified that application of high energies can lead to the decomposition of pigments due to the thermal effect (Zhu et al. 2017). Furthermore, these technologies present an additional cost to analytical procedure in acquisition, operation and maintenance (Kapoore et al. 2018).

Green extraction technologies can also be cited, for example deep eutectic solvents (DES), ionic liquid solvents (ILS), selectable solvents (SS) and extraction by supercritical fluid (SFE). However, not many works are found in the literature reporting DES, ILS and SS for pigment extraction in microalgae due to their poor transfer properties (Esquivel-Hernández et al. 2017). Despite having SFE potential, it extracts has many co-products with pigments and high cost and limitations for extractions with low sample masses even in pilot scale equipment, disadvantages that depreciate this technology (Kapoore et al. 2018).

Extraction methods that use reduced sample mass are necessary to solve problems found in laboratorial-scale microalgae cultivation designs, for example. Microalgal cultivation for obtaining bioproducts, such as pigments, are initially built on a laboratory scale to define more suitable cultivation conditions and to verify their viability for further scale up. Laboratory reactors generally have 1 to 2 L of total volume; the mass obtained at the end of the cultivation is limited and must be used to evaluate all the parameters. Thus, reduced or miniaturized methods make it possible to carry out all the necessary analyzes.

As noted, there are still several limitations on pigment extraction. Therefore, the aim of this work was to develop a method for simultaneous extraction of chlorophylls and carotenoids in low microalgae biomass amount using a mixture of organic solvents, due to its high extractive potential. The method to be developed required to reduce the volume of organic solvents used and the extraction time, in addition to not using maceration and high-cost technologies. In view of commercial interest, the method was applied to samples of *Spirulina* sp. and *S. obliquus*, since Cyanobacteria and Chlorophyta are among those with the greatest market potential (Jacob-Lopes et al. 2019).

2. Materials and Methods

2.1 Samples

The method development was carried out with commercial biomass of *Spirulina* sp. due needing for larger amount of test sample. Subsequently, the method was applied to *S. obliquus* biomass obtained experimentally under phototrophic conditions. Axenic cultures of *S. obliquus* (CPCC05) obtained from the Canadian Phycological Culture Center were propagated and maintained in synthetic medium BG-11 (Rippka et al. 1979). Cultivation was carried out in a column photobioreactor operating in batch mode, using 2.0 L of synthetic medium BG-11, 100 mg L^{-1} of initial inoculum concentration, temperature of 26 °C, constant lighting of 150 µmol m^{-2} s^{-1} and continuous aeration of 1 VVM (volume of air per volume of culture per minute) without CO_2 enrichment. The experiment was carried out until the decline phase, indicated by the reduction of cell concentration. *S. obliquus* biomass were freeze-drying in lyophilizer (L101, Liotop, BR) for 24 h with 0.200-0.300 µHg of vacuum and condenser temperature at −37 °C.

2.2 Pigments extraction by maceration

Chlorophylls and carotenoids extraction were performed by the exhaustive maceration method (MM) to obtain reference concentration values (Mandelli et al. 2012; Rodrigues et al. 2015; do Nascimento et al. 2020). About 100 mg of biomass with 0.5 g of glass beads (150-212 µm, Sigma-Aldrich, USA) and 3 mL of ethyl acetate were macerated until complete solvent evaporation. Afterwards, the biomass was placed in a 10 mL test tube with 9 mL of ethyl acetate, vigorously homogenized in a vortex mixer (MX-S, Scilogex, USA) for 1 min, and then centrifuged (80-2B, Centrilab, BR) at 1790 ×g for 7 min to remove the supernatant containing the pigments.

The procedure was performed exhaustively, until the biomass color disappeared. Subsequently, the same was done with the methanol solvent. The extract obtained was dried on a rotary evaporator at ≤ 30 °C and suspended in petroleum ether and diethyl ether mixture (1:1 v/v). The concentration of chlorophyll *a*, *b* and total chlorophylls was determined by UV-Vis diode array spectrophotometer (Model 8453, Agilent Technologies, USA) and calculated according to Porra et al. (1989). To determine total carotenoids, extracts were submitted to saponification procedure with 10% (w/v) KOH methanolic solution for 16 h at room temperature (do Nascimento et al. 2020). After removing the alkali by washing with distilled water, the total carotenoids concentration was determined by UV-Vis spectrophotometer and calculated according to Davies (1976).

2.3 Method development for pigment extraction

The extraction was carried out by homogenization with a mixture of organic solvents methanol and chloroform (Vendruscolo et al. 2018; Vendruscolo et al. 2019). About 100 mg of biomass was placed in a 15 mL Falcon-type tube with 20 borosilicate glass beads (2 mm diameter), 3 mL of methanol, 1.5 mL of chloroform and 1.2 mL of distilled water. Tubes were homogenized on orbital shaker table (Q225M, Quimis, BR) at 150 rpm, and different times (30, 60 and 120 min) of shaking were tested. Then, 1.5 mL of chloroform and 1.5 mL of distilled water were added; the tubes were shaken vigorously in a vortex mixer (MX-S, Scilogex, USA) for 1 min and centrifuged (80-2B, Centrilab, BR) at 1790 ×g for 7 min for phase separation. The organic phase containing the pigments was removed and then 3 mL of chloroform were added again, homogenized in a vortex mixer for 1 min and centrifuged in the same way to remove the pigments. This procedure was repeated until the color disappeared. Afterwards, the methanolic phase was removed and the solvents acetone, ethyl acetate and ethyl ether were tested to extract the pigments remaining in the biomass. Thus, 3 mL of the test solvents were added, homogenization and centrifugation were performed as previously described, the procedure was also performed exhaustively. The determination of chlorophylls and total carotenoids (including the saponification) were performed as described in the previous section.

2.4 High-performance liquid chromatography coupled with photo diode array detector (HPLC-PDA) analysis

The carotenoids and chlorophylls were analyzed by HPLC (Shimadzu, JPN) equipped with binary pumps (model LC-20AD), online degasser, and automatic injector (Rheodyne, USA). The equipment was connected in series to a PDA detector (model SPD-M20A). The UV-Vis spectra were processed at 450 nm for carotenoids and at 660 nm for chlorophylls. Prior to HPLC-PDA analysis, the carotenoids and chlorophylls extracts were solubilized in methanol (MeOH): methyl tert-butyl ether (MTBE) (70:30) and filtered through Millipore membranes (0.22 μm). The pigments separation was performed on a C30 YMC column (5 μm, 250 × 4.6 mm) (Waters, USA) using as mobile phase a linear gradient of MeOH: MTBE from 95:5 to 70:30 in 30 min, to 50:50 in 20 min. The flow rate was 0.9 mL min^{-1} and a column temperature set at 29 °C. HPLC-PDA parameters were set as previously described by De Rosso and Mercadante (2007).

The tentative identification of carotenoids was performed according to the following combined information: elution order on C30 HPLC column, co-chromatography with authentic standards of all-*trans*-lutein and all-*trans*-β-carotene (Sigma-Aldrich, USA), and UV-Vis spectrum (λ max, spectral fine structure, peak *cis* intensity), compared with data available in the literature (Britton et al. 1995; De Rosso and Mercadante 2007; Rodrigues et al. 2015; Patias et al. 2017). The combined results of the following parameters also were considered for chlorophylls identification: elution order on C30 HPLC column, co-chromatography with authentic standards of chlorophyll *a* and chlorophyll *b* (Sigma-Aldrich, USA), UV-Vis spectrum, and data available in the literature (Lanfer-Marquez et al. 2005; Huang et al. 2008; Loh et al. 2012; Fernandes et al. 2017).

2.5 Statistical analysis

Data normality was checked using the Shapiro-Wilk test and differences between treatments were evaluated by analysis of variance (ANOVA), followed by Tukey's test ($p < 0.05$), all performed using STATISTICA 7.0 (Statsoft Inc., USA).

3. Results and Discussion

3.1 Development of method and quantitative analysis

Spirulina sp. commercial biomass was employed to method development due to the greater availability of samples. Time and organic solvents variables levels were evaluated on recovery of the main pigments. The reference values of pigments concentration were obtained by MM, which it is widely used in the literature for several matrices, including microorganisms and specifically

microalgae (Mandelli et al. 2012; Rodrigues et al. 2015; do Nascimento et al. 2020). To select the most efficient variables of the proposed extraction method in comparison to MM, the quantitative values of chlorophylls and carotenoids obtained by the extracts absorbances in a spectrophotometer were taken into account. 652 and 665 nm were used to determine the concentration of chlorophyll *a*, chlorophyll *b* and total chlorophylls (Porra et al. 1989) and 450 nm for total carotenoids (Davies 1976). Although quantitative values of chlorophyll *a*, chlorophyll *b* and total chlorophylls are obtained, it is known that spectrophotometry is not a selective analytical technique (Casale et al. 2012), allowing several molecules of the chlorophylls class to absorb in this visible spectrum region (652 and 665 nm) and not just chlorophyll *a* and chlorophyll *b*, for example. In addition, few Cyanobacteria, such as *Spirulina* sp., are naturally capable of synthesizing chlorophyll *b*, so far it is known that this compound is found only in the genera *Prochlorococcus*, *Prochloron*, *Prochlorothrix* and *Acaryochloris* (Lim et al. 2019). Thus, the quantitative data of chlorophylls will be reported as chlorophyll *a* (most abundant pigment in all photosynthetic organisms), "other chlorophylls" and total chlorophylls (Da Silva Ferreira and Sant'Anna 2017).

The reference values for chlorophylls obtained from the extraction by MM for *Spirulina* sp. were 7.22 mg g^{-1} of dry biomass of chlorophyll *a*, 1.43 mg g^{-1} of dry biomass of "other chlorophylls", 8.66 mg g^{-1} of dry biomass of total chlorophylls and 0.91 mg g^{-1} of dry biomass of total carotenoids (Table 1). For exhaustive extraction of these pigments, six macerations and extractions with methanol were necessary, followed by three times with ethyl acetate. Considering that in each maceration 3 mL of organic solvent were used, and 9 mL for each extraction, 72 mL of methanol and 36 mL of ethyl acetate were spent, totaling 108 mL of organic solvent for each analytical repetition. Nine extractive cycles were performed (six with methanol and three with ethyl acetate) lasting about 30 min, since each cycle includes the processes of maceration, extraction, centrifugation and removal of the pigments (described in the material and methods), resulting in 4.5 h of extraction per sample. This brief survey shows that the MM is expensive in time and chemical consumables, besides the intense of manual activity and analyst exposure to toxic chemicals.

Chemical methods presented high efficiency for pigments extraction, organic solvents such methanol, ethanol, acetone, chloroform, isopropanol, petroleum ether, ethyl acetate, dimethyl sulfoxide (DMSO) and hexane are used (Zhu et al. 2017; Mussagy et al. 2019; Zhang et al. 2019); for method development, these solvents and results of previous works were considered. The

methanol: chloroform mixture allowed high cell permeation and extraction of various metabolites from the microalgae strains *Chlorella vulgaris*, *S. obliquus*, *Phormidium autumnale* and *Aphanothece microscopica* Nägeli (Vendruscolo et al. 2018; Vendruscolo et al. 2019), so, the mixture methanol:chloroform was selected for proposed method (PM). Glass beads were added to the extraction tubes along with the biomass and solvents, in order to simulate a ball mill during homogenization. Different homogenization times were evaluated on an orbital shaking table (30, 60 and 120 min); the results and recovery values of PM in relation to the MM are shown in Table 1.

The homogenization times evaluated in the extraction by the PM did not show significant difference in the values of chlorophyll *a* and total chlorophylls (Table 1). In concern to the concentration of "other chlorophylls" increased significantly with homogenization for 120 min (PM120), when compared to homogenization performed for 30 and 60 min (PM30 and PM60, respectively). Regarding carotenoids, it was observed that the increase in homogenization time resulted in a proportional reduction in the concentration of these compounds (Table 1). In the longest time of homogenization, PM120 (2 h), a total carotenoids concentration of 0.94 mg g^{-1} of dry biomass was observed, statistically equivalent to 0.91 mg g^{-1} of dry biomass obtained by MM that lasted 4.5 h.

Carotenoid degradation occurs due to the presence of oxygen and light and is accelerated by heating (Gong and Bassi 2016), as the experiments were conducted in the absence of light and at room temperature (24 °C), possibly the degradation was the longest exposure time of the pigments to oxygen, since the extractions were not carried out in a modified atmosphere. Saini and Keum (2018) listed five variables that lead to carotenoid degradation in extractive methods and two of them are extraction for long periods of time and non-inert atmosphere. The organic solvents used in the extraction could also have affected the carotenoids stability. However, a study evaluated the stability of lutein and β-carotene in different solvents, it was found that even after exposure for 24 h in methanol and chloroform both compounds maintained 97% of their initial absorbance (Craft and Soares 1992). Thus, PM30 was chosen, which with less homogenization time prevented the carotenoids degradation, maintained the values of chlorophyll *a* and total chlorophylls, in addition to meeting the requirement to develop ~~a~~ faster method.

The PM30 presented as a disadvantage a lower "other chlorophylls" concentration, 0.94 mg g^{-1} of dry biomass, a value that represents only 65.73% of recovery in relation to the MM, when

the expected values are above 80% (Simon et al. 2019). Then, in order to increase the "other chlorophylls" extraction, some solvents were evaluated after exhaustive extraction with chloroform, since most pigments extraction methods use the solvents combination to maximize the results. So, after using chloroform exhaustively, in the same way, ethyl acetate (used in MM), acetone and ethanol were used, which unlike chloroform showed negative hydrophobicity (Zhang et al. 2019), allowing the extraction of more polar compounds, in addition to having already shown good results in pigment extraction (Kapoore et al. 2018). The results obtained by PM30 with a combination of solvents can be seen in Table 2. The evaluated solvents showed satisfactory performance in the extraction of chlorophyll *a*, total chlorophylls and total carotenoids, with recovery values above 90% in relation to MM. However, the "other chlorophylls" values were 0.98 and 1.09 mg g^{-1} of dry biomass for exhaustive extraction with chloroform followed by ethyl acetate (PM30EA) and acetone (PM30A), respectively. Although the concentration of "other chlorophylls" increased, the recovery values still remained low, 68.53% for PM30EA and 76.22% for PM30A. Better recovery values for the pigments evaluated were found in the exhaustive chloroform extraction method followed by ethanol (PM30E). The concentrations obtained by PM30E in mg g^{-1} of dry biomass followed by recovery values were: 7.84 chlorophyll *a* (108.59%), 1.23 "other chlorophylls" (86.01%), 9.05 total chlorophylls (104.50%) and 1.13 total carotenoids (124.18%).

Ethanol, solvent with intermediate polarity between ethyl acetate and acetone, was the solvent selected for exhaustive extraction after chloroform, as it had the highest quantitative and recovery values in relation to MM for all pigments. Despite being considered lipophilic, chlorophylls and carotenoids are classes of pigments that include numerous compounds with different functional groups and carbon chains sizes and therefore different polarities (Saini and Keum 2018). Thus, extraction with a solvents combination expands the polarity range of the extractive system and allows the complete extraction of chlorophyll and carotenoid compounds. Ramluckan et al. (2014) also evaluated the pigments extraction from the *Chlorella* sp. microalgae with solvents mixture and concluded that among the solvents of greater polarity, ethanol was that provided the best chlorophylls recovery values. Amorim-Carrilho et al. (2014) also lists the ethanol as the most used polar solvent in solvents mixture for total carotenoids extraction.

The selected method PM30E was performed by initial homogenization on an orbital shaking table for 30 min with 3 mL of methanol and 1.5 mL of chloroform, after another 1.5 mL

of chloroform were added, totaling 6 mL of organic solvents in the first extractive cycle. Afterwards, two more extractive cycles were performed with 3 mL of chloroform and duration of 10 min (homogenization in a vortex shaker for 1 min, centrifugation for 7 min and pigments removal) to achieve exhaustive extraction. Likewise, two more extraction cycles were performed with the ethanol solvent. In summary, after the initial expenditure (6 mL of organic solvents and 30 min) four extractive cycles were carried out with 3 mL of solvent each and 10 min in duration, totaling 18 mL of organic solvents and 70 min for PM30E application. It should be noted that in PM30E mechanical homogenization is used for extraction and not manual maceration, allowing several samples to be extracted simultaneously by the same analyst.

Then, with all the extraction variables defined, the PM30E was applied to the *S. obliquus* biomass to verify whether the results obtained would be reproducible for different strains of microalgae. In addition to being eukaryotic microalgae of commercial importance, the genus *Scenedesmus* presenting additional compounds of greater robustness in the cell wall, which is a challenge for extraction methods. *S. obliquus* in particular has a cell wall with high rigidity because together with the cellulose it contains resilient sporopollenin (Klassen et al. 2015). Sporopollenin is a highly cross-linked biopolymer that is among the most resistant organic materials of biological origin (Faegri et al. 1964; Mackenzie et al. 2015); sporopollenin molecules have already been found intact in sedimentary rocks about 500 million years old (Wellman et al. 2003). Due to the complexity of the cell wall, it was concluded that *S. obliquus* would be an adequate reference to verify the method efficiency among microalgae samples.

S. obliquus was cultivated under controlled and favorable conditions to provide high amount of pigments in produced biomass, which was freeze-dried to avoid pigment degradations; 24.94 mg g^{-1} of chlorophyll *a*, 13.67 mg g^{-1} of "other chlorophylls", 38.61 mg g^{-1} of total chlorophylls and 16.77 mg g^{-1} of total carotenoids were obtained by MM (values expressed in relation to dry biomass) (Table 3). The MM was carried out in the same way as for *Spirulina* sp. biomass, but due to the higher pigment concentrations for exhaustive extraction, nine cycles of maceration-extraction with ethyl acetate and four with methanol were necessary (12 mL and 30 min each). Thus, 156 mL of organic solvents were spent and 6.5 hours were required for each extraction of the pigments from the *S. obliquus* biomass by the MM.

The quantitative results obtained by the PM30E for *S. obliquus* biomass and the recovery values in relation to the MM are shown in Table 3. PM30E showed recovery values above 80%,

108.86% for chlorophyll *a*, 86.91% for "other chlorophylls", 101.11% for total chlorophylls and 108.95% for total carotenoids, confirming the extraction efficiency even in microalgae with complex cell walls and with high concentrations of pigments. In addition to the 30 min initial homogenization with 6 mL of solvents mixture (methanol:chloroform) on an orbital shaker table, four extractive cycles with chloroform and three with ethanol (3 mL and 10 min each) were performed, totaling 27 mL and 100 min. It was possible to obtain satisfactory quantitative results of chlorophylls and carotenoids from the microalgae *S. obliquus* with a significant reduction in the volume of organic solvents and time of analysis.

3.2 Chlorophyll and carotenoid profiles

The requirements for quantitative analysis of chlorophylls and carotenoids from the *Spirulina* sp. and *S. obliquus* biomasses by PM30E were reached, so the qualitative profiles of these pigments were checked in comparison to MM (Table 4).

The major compounds in the chlorophyll profile of the microalgae *Spirulina* sp. were hydroxychlorophyll *a* (63.53%) and chlorophyll *a* (29.72%) for MM and for PM30E were chlorophyll *a* (54.85%) and pheophytin *a* (23.51%). Hydroxychlorophyll *a* is a chlorophyll *a* derivative, this compound may be a natural consequence of the microorganisms metabolism or be related to the extraction method used, which can cause the formation of this hydroxylated derivative (Chen et al. 2017; Fernandes et al. 2017). Since the hydroxychlorophyll *a* was not found in the PM30E chlorophyll profile, it can be concluded that it is a product formed by MM and not by the *Spirulina* sp. In the profile obtained by MM, pheophytin *a* and pheophytin *a′* were also found, in addition to those in the PM30E extract, chlorophyll *a′*, hydroxypheophytin *a* and pheophorbide *a* were detected. The MM profile showed ~~a~~ smaller variety of chlorophyll compounds that can be considered to come from degradation, but in a higher concentration (70.28%) than PM30E (45.15%).

The chlorophylls profile of *S. obliquus* obtained by MM presented as major compounds chlorophyll *a* (49.75%) and hydroxychlorophyll *a* (34.27%), whereas in the PM30E profile were chlorophyll *a* (78.32%) and chlorophyll *a′* (12.38%), chlorophyll *b* was also detected in both extracts, 0.58% in MM and 1.06% in PM30E. The chlorophyll *a* epimer, chlorophyll *a′*, has already been produced by microalgae species, but its appearance may be related to degradation reactions (Louda et al. 1998), so it is not possible to determine its origin because this epimer was found in

both profiles *S. obliquus*. In addition to chlorophyll *a* and chlorophyll *b*, which represent 50.34% of the total chlorophylls obtained by MM and 79.38% by PM30E, all other chlorophyll compounds can come from degradation caused by the extractive process or from the harvesting, drying and storage processes of biomass. Possible degradation compounds are 49.66% and 20.62% of the extracts obtained by MM and PM30E, respectively. This same behavior was observed in the two microalgae, *Spirulina* sp. and *S. obliquus*; the extracts obtained by PM30E have lower percentages of chlorophyll-derived compounds.

The carotenoid profiles of the microalgae *Spirulina* sp. were similar in both methods; the majority compounds in MM were all-*trans*-*β*-carotene (44.75%), 9-*cis*-*β*-carotene (17.00%) and all-*trans*-echinenone (9.74%) and in PM30E were all-*trans*-*β*-carotene (34.18%), 9-*cis*-*β*-carotene (14.04%) and all-*trans*-echinenone (7.73%). The lowest percentages of all-*trans*-*β*-carotene and 9-*cis*-*β*-carotene in PM30E may be related to the 13-*cis*-*β*-carotene isomer, in this extraction method its concentration in area was 12.22%, while in MM was 3.16%; PM30E favors the formation of 13-*cis* isomers. Another difference observed was in relation to all-*trans*-*α*-carotene, only detected in PM30E (6.26%). In addition to the all-*trans* carotenoids and *cis* isomers, an epoxide isomer, all-*trans*-5,8-epoxy-*β*-carotene, was found, but in similar percentages 2.44% and 2.64% in MM and PM30E, respectively.

Already for microalgae *S. obliquus*, carotenoid profiles showed more differences, mainly related to the formation of *cis* and hydroxylated isomers. All-*trans* forms represent 50.88% of the profile obtained by MM and 84.25% by PM30E, *cis* isomers 16.70% in MM and 7.96% in PM30E and hydroxylated isomers 31.52 and 6.62% in MM and PM30E, respectively. These differences are reflected in the majority compounds and their percentages, in MM were 2-hydroxy-*β*-carotene (31.52%), all-*trans*-*α*-cryptoxanthin (16.47%) and all-*trans*-lutein (15.75%); and PM30E were all-*trans*-lutein (60.43%), all-*trans*-*β*-carotene (8.19%) and 2-hydroxy-*β*-carotene (6.62%). MM favors the *cis* isomers formation and, mainly, hydroxylates, possibly due to the high extraction time necessary for exhaustive extraction of the pigments from the *S. obliquus* biomass (6.5 h), which influences the quantification and also the carotenoid profiles (Saini and Keum 2018). It was also verified the isomers presence in carotenoid extracts obtained by exhaustive maceration of several fruits (Biazotto et al. 2019).

In applications terms, the isomers formed from all-*trans*-carotenoids have different biological activity, as lower antioxidant activity (Khalid and Barrow 2018; Zhang et al. 2017) and,

in general, low pro-vitamin activity. In addition, feature distinct colors and *cis* isomers are thermodynamically less stable than their all-*trans* because their lower melting points (Ribeiro et al. 2018; Nabavi et al. 2020).

3.3 Extraction methods comparison

The variables and basic characteristics of the MM and the PM30E were compared and can be seen in Table 5. Regarding the sample mass used, 100 mg of microalgal biomass were used in both methods to facilitate direct comparison between them in terms of necessary extractive cycles. However, PM30E can be performed with a reduction of up to 50% of the sample mass, 50 mg, without prejudice to the quantitative and qualitative results of chlorophylls and carotenoids in microalgae samples. Organic solvents were replaced, MM uses ethyl acetate and methanol and PM30E chloroform, methanol and ethanol; based on the principles of green analytical chemistry (GAC) it was not possible to meet the requirement of not using organic solvents (Gałuszka et al. 2013), mainly toxic, the chloroform use was necessary to maintain the method efficiency.

Although the PM30E still uses chloroform in conjunction with ethanol, volumes have been significantly reduced, at least five times; as well as the extraction time, which was reduced by approximately four times. Regarding frequency, in MM the analyst can extract only one sample in the same period, while in PM30E several samples can be extracted simultaneously. In addition to the improvement of these variables predicted by the GAC (Marcinkowska et al. 2020), the analyst's safety was also taken into account, in the MM the extraction system was open with intense manipulation by the analyst for long periods, in PM30E the system is closed and minimal direct handling with organic solvents. Thus, the PM30E presents a reduction in costs and waste, and consequently, less environmental damage, combined with greater analyst safety.

4. Conclusion

Proposed method (PM30E) with reduction of time and volume of organic solvents allowed the pigments extraction from the microalgae *Spirulina* sp. and *S. obliquus*. The quantification of chlorophylls and carotenoids with adequate recovery values was obtained by exhaustive extraction with chloroform followed by ethanol. The pigment profiles were also evaluated, in the PM30E there was less formation of isomers and possible chlorophyll degradation compounds than in the reference method (MM) for both microalgae. In relation to carotenoids, the *Spirulina* sp. profile

was quite similar regardless of the extraction method, but for *S. obliquus*, the extract obtained by PM30E presented more than 80% of all-*trans* compounds and less isomerization than MM. It is worth mentioning that in addition to the advantages already presented, the PM30E is also safer method for analysts because it is performed in a closed system.

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Conflict of Interest

The authors declare that there is no conflict of interests.

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Table 1. Pigments from the *Spirulina* sp. biomass extracted from the maceration method (MM) and the proposed method (PM) with different homogenization times, followed by the recovery values.

Methods	Chlorophyll *a*		"Other chlorophylls"		Total chlorophylls		Total carotenoids	
	mg g^{-1}	RC (%)	mg g^{-1}	RC (%)	mg g^{-1}	RC (%)	mg g^{-1}	RC (%)
MM	$7.22^{a} \pm 0.18$		$1.43^{a} \pm 0.01$		$8.66^{a} \pm 0.17$		$0.91^{b} \pm 0.03$	
PM30	$7.41^{a} \pm 0.11$	102.63	$0.94^{c} \pm 0.02$	65.73	$8.35^{a} \pm 0.13$	96.42	$1.10^{a} \pm 0.05$	120.88
PM60	$7.45^{a} \pm 0.10$	103.19	$0.97^{c} \pm 0.02$	67.83	$8.43^{a} \pm 0.11$	97.34	$1.06^{a} \pm 0.02$	116.48
PM120	$7.62^{a} \pm 0.17$	105.54	$1.16^{b} \pm 0.02$	81.12	$8.78^{a} \pm 0.15$	101.39	$0.94^{b} \pm 0.01$	103.30

*Average ± standard deviation and values were expressed in mg g^{-1} of dry biomass.
*Different letters in the same column express significantly differences between the means ($P < 0.05$).
*PM30- PM with 30 min homogenization; PM60- PM with 60 min homogenization; PM120- PM with 120 min homogenization; RC- PM recovery in relation to the MM.

Table 2. Pigments from the *Spirulina* sp. biomass extracted by maceration method (MM) and method proposed by homogenization with the methanol:chloroform for 30 min (PM30), followed by different organic solvents.

Methods	Chlorophyll *a*		"Other chlorophylls"		Total chlorophylls		Total carotenoids	
	mg g^{-1}	RC (%)	mg g^{-1}	RC (%)	mg g^{-1}	RC (%)	mg g^{-1}	RC (%)
MM	$7.22^{b} \pm 0.18$		$1.43^{a} \pm 0.01$		$8.66^{b} \pm 0.17$		$0.91^{c} \pm 0.03$	
PM30EA	$7.05^{b} \pm 0.02$	97.65	$0.98^{d} \pm 0.02$	68.53	$8.02^{c} \pm 0.01$	92.61	$1.01^{b} \pm 0.01$	110.99
PM30A	$7.71^{a} \pm 0.05$	106.79	$1.09^{c} \pm 0.01$	76.22	$8.80^{ab} \pm 0.05$	101.62	$1.04^{b} \pm 0.02$	114.29
PM30E	$7.84^{a} \pm 0.13$	108.59	$1.23^{b} \pm 0.02$	86.01	$9.05^{a} \pm 0.15$	104.50	$1.13^{a} \pm 0.00$	124.18

*Average ± standard deviation and values were expressed in mg g^{-1} of dry biomass.
*Different letters on the same column express significantly differences between the means ($P < 0.05$).
*PM30EA- PM with 30 min homogenization and ethyl acetate; PM30A- PM with 30 min homogenization and acetone; PM30E- PM with 30 min homogenization and ethanol; RC- PM recovery in relation to the MM.

Table 3. Pigments from the *Scenedesmus obliquus* biomass extracted from the maceration method (MM) and the proposed method (PM30E), followed by the recovery values.

Pigments	MM	PM30E	RC (%)
Chlorophyll *a*	$24.94^{b} \pm 0.52$	$27.15^{a} \pm 0.47$	108.86
"Other chlorophylls"	$13.67^{a} \pm 0.70$	$11.88^{b} \pm 0.39$	86.91
Total chlorophylls	$38.61^{a} \pm 1.18$	$39.04^{a} \pm 0.63$	101.11
Total carotenoids	$16.77^{a} \pm 0.99$	$18.27^{a} \pm 0.33$	108.95

*Average ± standard deviation and values were expressed in mg g^{-1} of dry biomass.
*Different letters on the same line express significantly differences between the means ($P < 0.05$).
*PM30E- PM with 30 min homogenization and ethanol; RC- PM recovery in relation to the MM.

Table 4. Chlorophylls and carotenoids profiles from *Spirulina* sp. and *Scenedesmus obliquus* microalgae.

Peak	Pigment	t_R (min)[a]	UV-vis characteristics			*Spirulina* sp.		*S. obliquus*	
			λmax (nm)[b]	III/II[c] (%)	AB/II[d] (%)	MM (%)	PM30E (%)	MM (%)	PM30E (%)
Chlorophylls*									
1	Pheophorbide *a*	7.7	409, 666	na[e]	na	-	5.13	-	-
2	Chlorophyll *b*	9.5	462, 649	na	na	-	-	0.58	1.06
3	Hydroxychlorophyll *a*	12.6-13.6	430, 664	na	na	63.53	-	34.27	5.14
4	Chlorophyll *a*	17.6-18.5	431, 664	na	na	29.72	54.85	49.75	78.32
5	Chlorophyll *a*'	19.3-19.5	431,665	na	na	-	6.34	15.39	12.38
6	Hydroxypheophytin *a*	25.5	407, 666	na	na	-	7.23	-	-
7	Pheophytin *a*	33.1-33.9	409, 666	na	na	2.58	23.51	-	3.10
8	Pheophytin *a*'	41.3-41.9	408, 666	na	na	4.17	2.94	-	-
Carotenoids**									
1	All-*trans*-loroxanthin	7.6	418, 445, 471	43	0	-	-	0.32	2.05
2	All-*trans*-neoxanthin	8.0	417, 439, 468	84	0	-	-	0.50	1.76
3	9-*cis*-neoxanthin	8.5	329, 412, 435, 464	83	6	-	-	1.08	2.56
4	15-*cis*-lutein	11.2	332, 418, 441, 467	25	42	-	-	4.49	2.22
5	13-*cis*-lutein	12.1	333, 414, 438, 466	35	39	-	-	0.90	1.17
6	All-*trans*-lutein	13.3	420, 444, 472	57	0	5.35	5.00	15.75	60.43
7	All-*trans*-zeaxanthin	15.3	424, 447, 474	18	0	4.78	4.73	1.00	3.86
8	9-*cis*-lutein	16.3	331, 418, 439, 467	50	nc[f]	-	-	0.31	1.82
9	15 or 13-*cis*-cantaxanthin	16.4	459/466	nc	8	6.48	6.11	-	-
10	All-*trans*-canthaxanthin	17.4	471	nc	0	1.11	0.83	-	-
11	9-*cis*-zeaxanthin	18.2-19.6	338, 419, 444, 469	14	33	1.58	1.25	3.45	-
12	All-*trans*-zeinoxanthin	20.1	423, 442, 470	20	0	-	0.97	-	-
13	All-*trans*-*β*-cryptoxanthin	22.4	418, 451, 474	50	0	2.02	2.25	-	-

14	All-*trans*-*α*-cryptoxanthin	23.7	420, 445, 473	52	0	-	-	16.47	-
15	9-cis-*β*-cryptoxanthin	24.0-24.1	339, 420, 446, 471	44	nc	1.59	1.79	-	-
16	9-*cis*-*α*-cryptoxanthin	25.0	418, 440, 468	48	nc	-	-	0.53	-
17	All-*trans*-echinenone	25.4	462/464	nc	0	9.74	7.73	-	-
18	All-*trans*-crocoxanthin	26.3	420, 446, 473	66	0	-	-	-	6.41
19	All-*trans*-5,8-epoxy-*β*-carotene	26.4	408, 428, 451	70	0	2.44	2.64	-	-
20a	Di-*cis*-*β*-carotene	28.7	341, 419, 442, 467	12	13	-	-	0.59	-
20b	13-*cis*-*β*-carotene	28.7	341, 419, 450, 474	16	nc	3.16	12.22	-	-
21a	2-hydroxy-*β*-carotene	29.6-31.4	426, 451, 476	23	0	-	-	31.52	6.62
21b	All-*trans*-*α*-carotene	30.9-31.3	419, 445, 473	55	0	-	6.26	4.76	1.55
22	9-*cis*-*α*-carotene	32.0	330, 420, 445, 471	30	4	-	-	4.71	-
23	All-*trans*-*β*-carotene	34.6-34.7	421, 451, 476	16	0	44.75	34.18	12.08	8.19
24	9-*cis*-*β*-carotene	36.3-36.4	338, 420, 447, 471	25	41	17.00	14.04	1.54	1.35

[a]t_R: Retention time on the C30 column.
[b]Linear gradient Methanol:MTBE.
[c]Spectral fine structure: Ratio of the height of the longest wavelength absorption peak (III) and that of the middle absorption peak (II).
[d]Ratio of the cis peak (AB) and the middle absorption peak (II).
[e]Na: not applied.
[f]Not calculated.
*The identification was compared with data available in the literature (Lanfer-Marquez et al., 2005; Huang et al., 2008; Loh et al., 2012; Fernandes et al., 2017).
**The identification was compared with data available in the literature (De Rosso & Mercadante, 2007; Britton et al., 2012; Patias et al., 2017).

Table 5. Comparation of maceration method (MM) and proposed method (PM30E).

	Spirulina sp.		*Scenedesmus obliquus*	
	MM	PM30E	MM	PM30E
Sample mass (mg)	~ 100	~ 100	~ 100	~ 100
Organic solvents	EtOAc and MeOH	$CHCl_3$ and EtOH	EtOAc and MeOH	$CHCl_3$ and EtOH
Solvents volume (mL)	108	18	156	27
Extraction time (min)	270	70	390	100
Extraction frequency	One sample	Several samples	One sample	Several samples
Extraction system	Open	Closed	Open	Closed
Analyst exposure	Greater	Lowest	Greater	Lowest

*EtOAc- ethyl acetate; MeOH- methanol; $CHCl_3$- chloroform; EtOH- ethanol.

4.4 ARTIGO 4: THE EFFECT OF CO_2 ON SCENEDESMUS OBLIQUUS BIOMASS CHEMICAL COMPOSITION

Artigo será submetido para revista Bioresource Technology.

THE EFFECT OF CO_2 ON S*CENEDESMUS OBLIQUUS* BIOMASS CHEMICAL COMPOSITION

Abstract

This work evaluated the *S. obliquus* composition biomass obtained from photosynthetic cultures enriched with 3, 5, 10, 15, 20 and 25% CO_2 (v/v). Cultivations with 3, 5 and 10% CO_2 showed the greater synthesis of amino acids and proteins; the protein content reached values above 60% of the dry biomass. The highest concentrations of chlorophylls were found in cultures with 15%, 20% and 25% CO_2, 24.23, 23.06 and 30.79 mg g^{-1}, respectively, but the profiles have higher percentages of degradation compounds. Carotenoid concentrations were three times higher in cultures with 3, 5, and 10% CO_2 and all-*trans*-β-carotene was the major compound. Lipid synthesis was intensified with the increase in CO_2 percentages, as well as the accumulation of polyunsaturated fatty acids. CO_2 treatments significantly altered all the compounds concentration in *S. obliquus* biomasses.

Keywords: free polar compounds, proteins, amino acids, chlorophylls, carotenoids, lipids, fatty acids.

1 Introduction

Microalgae have been recognized in biotechnology due to their great diversity, development in various habitats, photosynthetic capacity and high biomass productivity (Maeda et al., 2018). The ubiquitous nature combined with the ability to synthesize compounds with applicability in human and animal food, chemical feed, pharmaceuticals and bioenergy encourages biotechnological research around microalgae species (estimated 200,000- 800,000 in total) (Koyande et al., 2019; Kumar et al., 2019). Due to the countless morphological, physiological and genetic differences, there is a wide variation between microalgae in terms of growth rate, productivity, biomass composition and nutrient and light requirements (Rastogi et al., 2017). Thus, researches are limited to species with rapid growth and considerable accumulation of interest compounds.

In this sense, diatoms (*Bacillariophyceae*) and green algae (*Chlorophyceae*), both eukaryotic, and cyanobacteria (*Cyanophyceae*) with prokaryotic cell structure are more biotechnologically explored (Jacob-Lopes et al., 2019). *Chlorophyceae* is among the phyla with

the largest number of species and geographic distribution (Kumar et al., 2017). These microalgae are characterized by having significant concentrations of chlorophylls, mainly chlorophyll *a*, in their chloroplasts and are common to be found in places with abundant light, such as shallow water (Makkar et al., 2016; Morais et al., 2020). The intrinsic characteristics of *Chlorophyceae* make them microorganisms with high photosynthetic capacity and carbon dioxide (CO_2) assimilation (Soares et al., 2018).

CO_2 generation in energy production and other industrial processes is a significant contributor to the emission of greenhouse gases (GHG). Carbon capture and use, as well as alternative and renewable energies, are strategies for reducing CO_2 emissions (Vuppaladadiyam et al., 2018). The biological capture and use of CO_2 by microalgae such as *Chlorophyceae* have demonstrated potential, and some advantages have been listed: solar energy can be used (savings in the face of chemical and physical methods); high photosynthetic efficiency (10-50 times higher than plants); rapid growth rate (multiplication in a few hours); ability to adapt to the most diverse environments; biomass and compounds obtained with commercial applicability (Xu et al., 2019).

Chlorophyceae *S. obliquus* was used in research to mitigate CO_2, photosynthetic cultivations enriched with values of up to 50% (Tang et al., 2011) and 70% (Ho et al., 2010) (v/v of air) were evaluated. Both studies verified the biomass and lipid productivity and the fatty acid profiles, despite concluding that the better performance of *S. obliquus* in the evaluated parameters was with 10% CO_2 enrichment, it was observed that the strain performed well with values in the 20% CO_2 range. In a recent study, researchers collaborating in this work evaluated the biomass productivity of *S. obliquus* photosynthetically cultivated with values of 1 to 25% CO_2 (v/v air), the values obtained in the cultivations with 20 and 25% were significantly higher than those with lower CO_2 percentages (Deprá et al., 2020). The highest percentages of CO_2 were favorable to the biomass accumulation, but studies on the chemical composition and its better applicability are still needed.

The external conditions, such CO_2 percentages, directly imply in the biomass productivity and the production of various metabolites by microalgae (Kumar et al., 2019). Thus, the objective of this work was to evaluate the effect of CO_2 concentrations (3, 5, 10, 15, 20 and 25%) on free polar compounds, total protein and protein amino acid profile, total lipids and fatty acids profile, total chlorophylls and carotenoids, as well as their profiles. Thus combining the possibility of biological capture of CO_2 by *S. obliquus* and obtaining various compounds of commercial interest.

2 Material and Methods

2.1 Microorganism and culture medium

Axenic cultures of *S. obliquus* (CPCC05) from Canadian Phycological Culture Center were maintained and propagated in synthetic BG-11 medium (Rippka et al. 1979) with the following conditions: initial pH 8.0, 25 °C, continuous aeration of 1 VVM (volume of air per volume of culture per minute) without CO_2 enrichment, the incident light intensity of 30 μmol m^{-2} s^{-1}.

2.2 Bioreactor design and experimental conditions

The microalgal cultivations were carried out in a bubble column photobioreactor with a nominal working volume of 2.0 L (Tecnal, BR) as described by Maroneze et al., 2016. The photobioreactor was operated in batch mode with synthetic BG-11 medium, initial cell concentration 100 mg L^{-1}, isothermal temperature 28 °C, incident light intensity 150 μmol m^{-2} s^{-1} and continuous aeration of 1 VVM. The injected air was enriched with 3, 5, 10, 15, 20 and 25% of industrial CO_2. Cell concentration was monitored every 24 h during the microalgae growth. All experiments were conducted until the decline phase was reached, where the cellular concentration began to reduce, according to Deprá et al. 2020. At the end of the experiments, the *S. obliquus* biomasses were freeze-drying in lyophilizer (L101, Liotop, BR) for 24 h with 0.200-0.300 μHg of vacuum and condenser temperature at −37 °C.

2.3 Extraction of free polar compounds and total lipids

The free polar compounds and the total lipids were extracted with methanol:chloroform mixture according to the method described by Vendruscolo et al. 2018. 80 mg of freeze-dried biomass were added 3 mL of methanol, 1.5 mL of chloroform and 1.2 mL of distilled water. The extraction occurred by homogenization on an orbital shaking table (Q225M, Quimis, BR) for 30 min. Afterwards, another 1.5 mL of chloroform and 1.5 mL of distilled water were added and centrifuged (80-2B, Centrilab, BR) for 2 min (1006 ×*g*), where the separation of the aqueous phase with the free polar compounds and organic phase with de total lipids occurred. Extractions were performed in triplicate.

2.4 Free polar compounds determination

The solvents from the aqueous fraction (100 µL) and the norleucine (P/N N1398, Supelco, USA) internal standard (100 µL, at 20 µg mL^{-1}) were evaporated at 60 °C in a heating block (CE-350/25, Cienlab, BR) with nitrogen flow. To the residue were added 40 µL of pyridine and kept at 38 °C for 90 min, then 60 µL of MTBSTFA (N-Methyl-N-tert-butyldimethylsilyltrifluoroacetamide) were added, and maintained for another 30 min at 38 °C to occur the silylation reaction. After derivatization, 1 µL of the samples was injected in splitless mode (1 min split-valve off, then split 20:1) in a gas chromatograph (3400, Varian, USA) equipped with a flame ionization detector (GC-FID) and an autosampler (8100, Varian, USA). The free polar compounds were separated in RTX-5MS capillary column (Restek, USA; 30 m × 0.25 mm × 0.25 µm). The initial column temperature was 100 °C for 2 min, increasing to 165 °C at a rate of 2 °C min^{-1}, then increased to 200 °C at a rate of 5 °C min^{-1} and then at a rate of 3 °C min^{-1} reached 300 °C, where it remained for 5 min. Injector and detector were kept at 320 °C. The compounds were identified by comparing the retention times with the authentic standards of 22 amino acids (P/N LAA21, Supelco, USA), citric acid (P/N 251275, Sigma Aldrich, USA), malic acid (P/N 02300, Sigma Aldrich, USA) and tartaric acid (P/N 251380, Sigma Aldrich, USA). Quantification was performed by external calibration (0.1-80 µg mL^{-1}) with areas previously normalized by the internal standard. Results were expressed as µg g^{-1} of dry biomass. The determinations were carried out in triplicate.

2.5 Total lipids determination and fatty acids profile

Part of the organic fraction containing the lipids was placed in a beaker previously tared, and the content of total lipids was determined by gravimetry on an analytical balance with 0.01 mg minimum display (AUW220D, Shimadzu, JP) after solvent evaporation in air circulation oven (B5AFD, DeLeo, BR) at 105 °C. The rest of the organic fraction was derivatized with 1 mL of methanolic KOH (0.4 M) followed by 3 mL of methanolic H_2SO_4 (1 M), after each addition, the samples were kept for 10 min in a heating block (CE-350/25, Cienlab, BR) at 100 °C (Hartman and Lago, 1973) to obtain fatty acid methyl esters (FAME). The FAME were recovered hexane and analyzed using a Varian 3600 gas chromatograph equipped with a flame ionization detector (GC-FID) (USA) and Varian 8200 autosampler (USA). 1 µL was injected in splitless mode (0.8 min split-valve off, then split 20:1), and the FAME were separated in an HP-88 capillary column (Agilent Technologies, USA) (100 m × 0.25 mm × 0.20 µm). The column temperature was held at

50 °C for 1 min; then it was increased to 185 °C at a rate of 15 °C min^{-1}, followed by an increase at 0.5 °C min^{-1} to 190 °C and finally at a rate of 15 °C min^{-1} until reaching 230 °C, remaining for 10 min. Injector and detector temperature was 250 °C. FAME were identified by comparison with authentic standard FAME Mix 37 (P/N 47885-U, Supelco, USA). Results were expressed as a percentage of the total area of the chromatograms, considering the correction factors of the flame ionization detector and conversion of the ester to acid (Visentainer, 2012). The determinations were carried out in triplicate.

2.6 Total protein determination and protein amino acids profile

The protein content was calculated by determining the total nitrogen; 80 mg of microalgal biomass and a correction factor of 6.25 were used (Horowitz and Latimer, 2000). To determine the protein amino acid profile, 50 mg of biomass were subjected to acid hydrolysis with 6 M hydrochloric acid (HCl) at 110 °C (Heating Block CE-350/25, Cienlab, BR) for 24 h under vacuum (Do Nascimento et al., 2019). After hydrolysis, the samples were derivatized and analyzed in GC-FID as previously described in item 2.4. The determinations were carried out in triplicate.

2.7 Chlorophylls and carotenoids determination

Chlorophylls and carotenoids were extracted from 80 mg of sample, first 3 mL of methanol, 1.5 mL of chloroform and 1.2 mL of distilled water were added, and the whole was homogenized on an orbital shaking table (Q225M, Quimis, BR) at 150 rpm for 30 min. Then another 1.5 mL of chloroform and 1.2 mL of distilled water were added to the tubes and subjected to centrifugation (80-2B, Centrilab, BR) at 1790 ×g for 5 min. The organic phase containing the pigments was removed, and an additional 3 mL of chloroform was added, the tubes homogenized in a vortex mixer (MX-S, Scilogex, USA) for 1 min, centrifuged and the removal was performed again. This process was carried out until the total color disappeared. After exhaustive extraction with chloroform, the same procedure was done with the ethanol solvent. The total chlorophylls concentration was determined by UV-Vis diode array spectrophotometer (8453, Agilent Technologies, USA) and calculated according to Porra, Thompson and Kriedemann (1989). To determine total carotenoids, extracts were submitted to saponification procedure with 10% (w/v) KOH methanolic solution for 16 h at room temperature (do Nascimento et al., 2020). After removing the alkali by washing with distilled water, the total carotenoids concentration was

determined by UV-Vis spectrophotometer and calculated according to Davies (1976). Afterwards, pigments were analyzed by high performance liquid chromatography HPLC (Shimadzu, JP) equipped with binary pumps (LC-20AD), online degasser, and automatic injector (SIL-20A HT). The equipment was connected in series to a Diode Array Detector (PDA) (SPD-M20A) and a mass spectrometry was performed with a Shimadzu 8040 triple quadrupole mass spectrometer and atmospheric pressure chemical ionization (APCI) source (Shimadzu America, USA). The pigments separation was performed on a C30 YMC column (5 μm, 250 × 4.6 mm) (Waters, USA). HPLC-PDA-APCI(+)QqQ analysis was performed according to De Rosso and Mercadante (2007) and Murillo et al. (2013), with adaptations. Prior to HPLC-PDA-APCI(+)QqQ analysis, the pigments extracts were solubilized in methanol (MeOH): methyl-tercbutyl-ether (MTBE) (70:30) and filtered through Millipore membranes (0.22 μm). The mobile phases A (MeOH) and phase B (MTBE), using a linear gradient program as follows: from 0 to 30 min 5% B; from 30 to 40 min, 5 to 30% B; from 40 to 41 min, 30 to 50% B, from 41 to 50 min, 50 to 5% B. The flow rate was set at 0.9 mL min^{-1}, the injection volume was 20 μL, the column temperature was maintained at 29 °C, the UV/vis spectra were acquired between 220 and 700 nm, and the chromatograms were processed at 450 nm for carotenoids and 660 nm for chlorophylls. The MS/MS detection was achieved through an APCI interface operated in positive (+) mode; detector voltage: 4.5 kV; interface temperature: 350 °C; desolvation line (DL) temperature: 200 °C; heat block temperature: 200 °C; nebulizing gas flow (N_2): 3.0 L min^{-1}; drying gas flow (N_2): 5.0 L min^{-1}; collision induced dissociation (CID) gas: 23 kPa (argon); event time: 0.5 s. The identification was performed according to the following combined information: elution order on C30 HPLC column, co-chromatography with authentic standards, UV-visible spectrum (λ max, spectral fine structure, peak cis intensity), and mass spectra characteristics (protonated molecule ([M + H]+) and MS/MS fragments), compared with data available in the literature (Rodrigues et al., 2014, 2015; Chen et al., 2015; Patias et al., 2017; Fernandes et al., 2017; Maroneze et al., 2019, 2020; Fernandes et al., 2020). Results were expressed as a percentage of the total area of the chromatograms.

2.8 Statistical analysis

Before performing the analysis of variance (ANOVA) was tested the normality of the distribution using Shapiro-Wilk and the homoscedastic using Cochran test. Differences between treatments were evaluated by ANOVA, followed by Tukey's test ($p < 0.05$), all performed using

STATISTICA 7.0 (Statsoft Inc., USA). In addition, all data were submitted to Principal Component Analysis (PCA) using Pirouette 3.11 software (Infometrix Co., USA). Before this multivariate analysis, the data matrix was autoscaled for each variable so that each assumed the same weight during analysis.

3 Results and Discussion

Microalgae can absorb inorganic carbon in different ways, direct assimilation of CO_2 by the plasma membrane, conversion of bicarbonates into CO_2 through extracellular carbonic anhydrase or direct capture of bicarbonates by active membrane transporters. These strategies are intrinsic to each strain, the microalgae *S. obliquus*, for example, can perform the three forms of assimilation. After absorption, inorganic carbon fixation can occur through autotrophic (inorganic carbon only) or mixotrophic (presence of inorganic and organic carbon) metabolism (Vuppaladadiyam et al., 2018). *S. obliquus* cultivation were conducted exclusively with inorganic carbon (CO_2). Briefly, in autotrophic metabolism through the Calvin-Benson cycle (CBC), two molecules of 3-phosphoglycerate (3PG) are formed from a molecule of ribulose 1,5-bisphosphate (RuBP), CO_2 and water. When six 3PG molecules are formed, five are directed to restore RuBP, and one is used to create the most diverse metabolites necessary for the cellular activity (Xu et al., 2019). Following, the results that show the influence of the CO_2 concentration in the formation of *S. obliquus* metabolites will be presented and discussed.

3.1 Free polar compounds

Free polar metabolites are precursors to microalgal compounds, formed from the CBC and Tricarboxylic Acid Cycle (TCA) and can be used as markers of the cellular activity of these microorganisms. Several free amino acids and organic acids, malic and citric important TCA intermediates, were detected (Table 1). The detected amino acids can be synthesized from 3PG, intermediates between CBC and TCA, such as phosphoenolpyruvate and pyruvate, and TCA intermediates, α-ketoglutarate and oxalate. The presence of these compounds indicates that all routes were being performed regardless of the percentage of CO_2 enrichment in *S. obliquus* cultivations, but at different intensities, as significant differences were detected in the concentrations of both organic acids and free amino acids (Table 1).

Higher concentrations of free polar metabolites were found in cells in cultivations with 3, 5 and 10% CO_2, possibly, the polymer formation routes had been reduced activities at the time of obtaining the biomass causing this accumulation. The values decreased significantly with CO_2 enrichment (15, 20 and 25%) (Table 1). Despite the reduction, the free amino acid in the highest concentration remained glutamic acid, possibly because it is also a precursor to chlorophylls, the main photosynthetic pigment, through the C5 route (Kang et al., 2011; Nehmé et al., 2017).

The free polar metabolites, mainly free amino acids, showed a behavior directly proportional to protein synthesis. Cultivations with 3, 5 and 10% CO_2 that showed the highest accumulation of these compounds inside *S. obliquus* cells were also the ones that showed the highest concentration of proteins (Table 2). The cells remained to synthesize high amounts of amino acids even with high protein content. In these cultivations the 3PG was directed, preferably, the routes of formation of amino acids and proteins. In addition, in cases of low CO_2, oxygen can compete in the CBC for RuBP, 3PG and 2-phosphoglycolate (2PG) are obtained as products, and 2PG is metabolized directly to amino acids. Photorespiration, as this process is called, occurs mainly in the presence of light, as in these *S. obliquus* cultivation, performed with constant lighting. (Zeng et al., 2011).

Glutamic acid, major free amino acid, presented an inversely proportional behavior to the synthesis of chlorophylls in these cultivations, higher chlorophyll concentrations were found in biomasses with less accumulation of this free amino acid inside the cells (Table 3). The most significant synthesis of chlorophyll compounds occurred in cultures with 15, 20 and 25% CO_2, in which greater photosynthetic efficiency was required for gas assimilation and absorption, so the glutamic acid was directed to the C5 route to obtain chlorophylls.

3.2 Protein and protein amino acids

Protein synthesis in microalgae is the most important and complex; it begins with the synthesis of amino acids that condense into peptides and then into primary proteins (Zeng et al., 2011). In the *S. obliquus* biomasses cultured with 3, 5 and 10% CO_2, high synthesis of free amino acids was observed (Table 1), as well as protein synthesis, values of 62.31, 61.61 and 60.74% of total proteins were found, respectively (Table 2). The increase in CO_2 levels in cultivation affected inversely proportional to protein content, in culture with 15% CO_2 the protein value was 33.36%, with 20% CO_2 was 29.10% and with 25% CO_2, it was 31.12% (Table 2). Previous studies have

also observed this relationship for *S. obliquus*, reduction of total proteins in cultivation with an increase in CO_2 from 0.03 to 13.8% (Basu et al., 2013) and for *Chlorella pyrenoidosa* cultivated with 3, 5 and 10% of CO_2 (Huang et al., 2017). In these cases, it is suggested that in cultivation with less CO_2 enrichment, 3PG is directed at the production routes of free amino acids and these in turn in proteins, in addition to photorespiration as mentioned earlier.

Besides total protein contents, the protein amino acid profiles were also modified significantly by the CO_2 enrichment percentages. In the 3% CO_2 cultivation, the major protein amino acids were leucine (60.42 mg g^{-1}), alanine (53.28 mg g^{-1}) and glutamic acid (52.46 mg g^{-1}). With 5% CO_2 were lysine (65.58 mg g^{-1}), leucine (56.07 mg g^{-1}) and glutamic acid (55.14 mg g^{-1}) and with 10% CO_2 were leucine (52.59 mg g^{-1}), glutamic acid (52.40 mg g^{-1}) and phenylalanine (50.09 mg g^{-1}). With an increase to 15% of CO_2, there was a significant reduction in the concentrations of major amino acids, in this culture values of 36.87 mg g^{-1} of phenylalanine, 31.06 mg g^{-1} of lysine and 28.62 mg g^{-1} of leucine were found. In the cultivation of *S. obliquus* with 20% CO_2, the highest concentrations were 37.16 mg g^{-1} of phenylalanine, 25.30 mg g^{-1} of leucine and 25.10 mg g^{-1} of glutamic acid. Cultivation with 25% CO_2 showed the same major compounds as cultivation with 20% CO_2, but different concentrations, 39.23 mg g^{-1} of phenylalanine, 29.82 mg g^{-1} of leucine and 28.37 mg g^{-1} of glutamic acid (Table 2).

S. obliquus biomass has a high protein content, though the percentages of CO_2 applied in the cultivation, values above 60% were obtained. In addition, what attracts attention for applications in the area of food and supplements is also related to the microalgal protein quality (Ejike et al., 2017). The essential amino acids for human nutrition, which include valine, leucine, isoleucine, methionine, threonine, phenylalanine, lysine, histidine and tryptophan, represent more than 53% of the amino acid profile, regardless of treatment. In order to combine protein quantity and quality, *S. obliquus* biomass cultivated with 5% CO_2 stands out, with 61.61% of total proteins, and the highest concentration of essential amino acids 322.84 mg g^{-1} of biomass dry, more than 32% of the biomass content (Table 2).

The limitation about the protein quality of the *S. obliquus* biomass is related to the deficiency of two or three specific essential amino acids, as can be seen in this study, lysine that was detected only in the cultures with 5 and 15% CO_2 and histidine only in the 10% CO_2 (Table 2). The other essential amino acids were found in concentrations necessary or higher than those recommended by the Food and Agriculture Organization (FAO) of the United Nations and the

World Health Organization (WHO) (Amorim et al., 2020), even in the treatment with a lower concentration of essential amino acids, supplementation with 20% CO_2. Through the data obtained, it is highlighted that the biomass of *S. obliquus* is an alternative protein source of quality, resembling matrices such as eggs and soy.

3.3 Total pigments and profiles of chlorophylls and carotenoids

Total chlorophylls concentrations in the biomasses of *S. obliquus* were upper in the highest percentages of CO_2 (Table 3). The values in mg g^{-1} were 19.52 (3% CO_2), 18.45 (5% CO_2), 18.04 (10% CO_2), 24.23 (15% CO_2), 23.06 (20% CO_2) and 30.79 (25% CO_2). Previous studies have also found an increase in chlorophylls in *S. obliquus* with an increase in CO_2 from 0.03 to 13.8% (Basu et al., 2013) and *Scenedesmus* sp. in cultivations with 0 to 15% CO_2 (Tripathi et al., 2015). Swarnalatha et al. (2015) evaluated the total chlorophyll content of different microalgae strains grown with 0, 10, 20 and 30% CO_2, *Desmodesmus* sp. and *Acutodesmus* sp. increased the synthesis of chlorophylls up to 20% CO_2 and showed a reduction with 30% CO_2. In contrast, *Kirchneriella commuta* showed an increasing concentration up to 30% CO_2.

Chlorophylls are composed of phytol tail and a macrocycle with four pyrrole rings joined by a magnesium molecule (D'Alessandro and Antoniosi Filho, 2016). In microalgal cells, the phytol tail is found next to the chloroplast membrane lipids. At the same time, the double bonds of the pyrrole rings act as antennas for capturing light and the magnesium atom charge the electrons for photosynthesis reactions to occur. Chlorophyll-*a* is the most important, which is found in the reaction center. However other pigments act as accessories, like as other chlorophylls compounds absorbing light at other wavelengths and passing energy to chlorophyll-*a* and carotenoids absorbing the excess (Carvalho et al., 2011). As in the *S. obliquus* cultivation constant lighting was used, and there was an increase in the CO_2 present in the medium, the cells synthesized higher chlorophylls concentrations to intensify the photosynthetic process.

The chlorophyll profiles in cultures with 3, 5 and 10% CO_2 showed more than 80% chlorophyll-*a*, 88.10, 85.01 and 86.53%, respectively. The other minority compounds are derived from chlorophyll-*a*, 15^1-hydroxy-lactone-chlorophyll-*a*, 13^2-hydroxy-chlorophyll-*a* and chlorophyll-*a'*. In cultivations with 20 and 25% CO_2, the compound with the highest concentration was also chlorophyll-*a*, 58.96 and 45.74%, respectively. In addition to the derivatives already mentioned, pheophytin-*a*, the accessory pigment chlorophyll-*b* and its derivative 13^2-hydroxy-

chlorophyll-*b* were also detected. The cultivation with 15% CO_2 showed the same compounds present in those with 20 and 25% CO_2 enrichment, however the compound with the highest concentration was 15^1-hydroxy-lactone-chlorophyll-*a*. All derivatives are products of chemical reactions, 15^1-hydroxy-lactone-chlorophyll-*a* is an extreme oxidation product of chlorophyll-*a* by peroxidase enzyme and not from synthesis by *S. obliquus* (Kao et al., 2011). Thus, the chlorophylls from *S. obliquus* biomasses grown 3, 5 and 10% CO_2, despite the lower concentration, are more viable for commercial applications due to the higher proportion of chlorophyll-*a* in relation to derivatives that are degradation products (Table 3).

The carotenoids, in turn, showed a behavior inversely proportional to the increase in CO_2 percentages. The cultivations with the highest concentration were those with 3, 5 and 10% CO_2, 25.27, 22.70 and 18.12 mg g^{-1}, respectively. In the other cultures, there was a significant reduction in carotenoids; 7.25 mg g^{-1} (15% CO_2), 5.68 mg g^{-1} (20% CO_2) and 7.70 mg g^{-1} (25% CO_2) were found. Carotenoids are accessory pigments and have the function of absorbing excess light so that chlorophyll degradation does not occur. As there was a high synthesis of chlorophylls in treatments 15, 20 and 25% of CO_2 and they are main responsible for the absorption of light for the photosynthetic process, there was a low production of carotenoids. However, the proportion of carotenoids to chlorophylls in these biomasses was low, causing oxidative damage and formation of more derivatives and in greater proportion.

CO_2 treatments also changed the carotenoid profiles; in cultures with 3, 5 and 10% CO_2 the significant compounds were all-*trans*-β-carotene with more than 40% of the total chromatogram area, 9-*cis*-echinenone above 15%, all-*trans*-echinenone with more than 10% and all-*trans*-zeaxanthin in the range of 10%. The biomass of *S. obliquus* cultivated with 15% CO_2 showed the major compounds all-*trans*-lutein (73.04%), all-*trans*-β-carotene (6.43%) and all-*trans*-neoxanthin (4.83); with 20% CO_2 were all-*trans*-lutein (76.78%), all-*trans*-neoxanthin (5.35%) and all-*trans*-β-carotene (3.71%). In the 25% CO_2 cultivation, the compounds in the highest percentage were all-*trans*-lutein, all-*trans*-β-carotene and 15-*cis*-lutein, with 67.93, 5.45 and 5.25%, respectively. UV-vis characteristics and fragment ions used in the identification of chlorophyll and carotenoid compounds are presented in the Supplementary Material 1.

The glyceraldehyde 3-phosphate (GAP) formed in the CBC from the 3PG is conducted through the methylerythritol phosphate (MEP) pathway for carotenoids synthesis. The synthesis begins with the condensation of two molecules of geranylgeranyl pyrophosphate (GGPP), giving

rise to the phytoene that is converted into lycopene. α-carotene and ɣ-carotene are formed from lycopene; the α conformation gives rise to lutein, a major compound in cultivations with 15, 20 and 25% CO_2. Ɣ-carotene originates β-carotene, and from it, echinenone and zeaxanthin are formed, compounds in greater concentration in cultures with 3, 5 and 10% CO_2. The highest CO_2 percentages intensified the derivatives formation from α-carotene. While in the lowest gas concentrations, a greater proportion of compounds formed from ɣ-carotene was observed (Mander and Liu, 2010; Zhang et al., 2015). However, all carotenoid profiles obtained have market potential, due to the high concentration of β-carotene and lutein (Novoveská et al., 2019).

3.4 Total lipids and fatty acids profiles

The different CO_2 levels had a significant influence on the total lipids content of the biomasses (Table 4). The highest values were 12.87 and 13.08% in treatments with 15 and 20% CO_2, respectively, followed by cultivation with 25% CO_2 that presented 11.91% lipids. The other values were 10.66% of lipids in the cultivation with 3% of CO_2, 9.97% with 5% of CO_2 and 10.90% with 10% of CO_2. Despite not being linear values, it was possible to observe a trend of more significant lipids accumulation in the cells of *S. obliquus* with the increase in the percentages of CO_2 employed. This same behavior has been observed in previous studies of CO_2 mitigation with *S. obliquus* (Ho et al., 2010; Tang et al., 2011; Basu et al., 2013), *Scenedesmus dimorphus* (Vidyashankar et al., 2013) and *Scenedesmus bajacalifornicus* (Patil and Kaliwal, 2017). Higher levels of lipids in microalgal cells are related to stress conditions, and the studies cited found that enrichment above 15% of CO_2 could be considered one of these conditions.

The fatty acid (FA) profiles were also modified by the different levels of CO_2 enrichment (Table 4) and, as well as for the total lipid content, the results obtained for treatments with a lower percentage of enrichment (3, 5 and 10% CO_2) showed similarities. Changes in FA concentrations were observed, but in these treatments, the major compounds were palmitic acid (C16:0), oleic acid (C18:1n9c), linoleic acid (C18:2n6c) and ɣ-linolenic acid (C18:3n6). In the cultivation with 3% of CO_2 the concentrations of the majority FA were 59.01% (C16:0), 10.94% (C18:3n6), 10.09% (C18:2n6) and 6.49% (C18:1n9c); in the sample that was grown with 5% CO_2, 62.06% (C16:0), 8.68% (C18:3n6), 7.56% (C18:2n6c) and 7.20% (C18:1n9c); and in cultivation with 10% CO_2 enrichment, C16:0 showed a concentration of 56.92%, C18:2n6 of 9.02%, C18:3n6 of 8.49% and C18:1n9c of 7.61%.

The biomasses obtained with 15, 20 and 25% CO_2 also showed C16:0, C18:1n9c and C18:2n6 as major FA, however, instead of C18:3n6, one of the compounds with the highest concentration was α-linolenic acid (C18:3n3), also called ALA and known extensively for its health benefits. In the 15% CO_2 cultivation the highest concentrations were 43.31% (C16:0), 21.20% (C18:1n9c), 10.24% (C18:2n6c) and 9.01 (C18:3n3); with 20% CO_2 they were 38.82% (C16:0), 22.84% (C18:1n9c), 11.86% (C18:3n3) and 10.28 (C18:2n6); and with 25% CO_2 they were 36.86% (C16:0), 17.87% (C18:3n3), 16.28% (C18:1n9c) and 12.41% (C18:2n6c). The greatest enrichment with CO_2 showed a clear tendency to reduce saturated FA C16:0 and increase the synthesis of FA omega-3 C18:3n3.

In addition to the differences observed in the majority FA, stearic acid (C18:0) which was present in concentrations of 2.55 to 3.88% in cultivations with 3, 5 and 10% CO_2, in the other treatments (15, 20 and 25% CO_2) presented concentrations of 5.32 to 7.87%. Arachidonic acid (C20:4n6) was detected only in the three cultures with the lowest CO_2 content, and docosapentaenoic acid (DPA) (C22:5n3) showed concentrations five to ten times (0.77-0.94%) higher in these cultures. On the other hand, lignoceric (C24:0) and docosahexaenoic (DHA) acids (C22:6n3) were only detected in cultivations with higher percentages of CO_2 (15, 20 and 25% CO_2). Through these compounds, there was a proportional increase in the percentage of higher carbon chain compounds caused by the increase in CO_2.

The Calvin-Benson cycle forms the 3PG, the first stable molecule of photosynthesis, phosphoenolpyruvate (PEP) is formed from it by enzymatic reactions, followed by pyruvate (PYR) and acetyl coenzyme A (acetyl-CoA). For FA synthesis, specifically, acetyl-CoA and bicarbonate give rise to malonyl-CoA, a reaction catalyzed by acetyl-CoA carboxylase. Afterwards, malonyl-CoA ACP (acyl carrier protein) transacylase catalyzes the reaction of malonyl-CoA to malonyl-ACP (carbon donor molecule for elongation reactions). Saturated FAs are formed from malonyl-ACP, mainly C16:0 and C18:0-ACP, precursors of the other compounds due to carbon chain elongation and desaturation reactions (Rismani-Yazdi et al., 2011; Blatti et al., 2013). The increase in CO_2 percentages in *S. obliquus* cultivation intensified the FA elongation and desaturation reactions, mainly in the C18:3n3 pathway. In this case, C18:0 (Also C16: 0), through Δ9 and Δ12 (or ω6) desaturases originate C18:2n6, which is again desaturated by Δ15 (or ω3) desaturase, originating C18:3n3 (Monroig et al., 2013).

By altering the metabolism of *S. obliquus*, the FA profiles were modified, as well as their applicability (Sun et al., 2019). In the lowest percentages of CO_2 enrichment, mostly saturated profiles were found, reaching up to 70.55% saturated fatty acids (SFA) in the treatment with 5% CO_2, 11.19% monounsaturated fatty acids (MUFA) and 18.26% polyunsaturated fatty acids (PUFA), profile suitable for biodiesel production. As the CO_2 levels increased, the profiles changed and proved to be more attractive for applications in food and supplementation, with 25% CO_2 found 49.35% SFA, 19.11% MUFA and 31.54% PUFA. As well as the n6/n3 ratio used to check the oil healthiness level, the lower the ratio, the greater the benefit provided, in the cultivation with 3% CO_2 the n6/n3 ratio was 13.56, in the cultivation with 25% CO_2 reduced to 0.75 (Table 4).

3.5 Exploratory multivariate analysis

Principal component analysis (PCA) was used to better visualize the effect of different CO_2 treatments on the composition of *S. obliquus* biomasses. The three main components (PC) explained 89.18% of the general variance of the results obtained. Figures 1a and 1b show the scores (samples) and loadings (compounds) of principal components 1 and 2. In PC1, the samples were separated into two groups, those obtained with a lower percentage of CO_2 enrichment (3, 5 and 10%) and those with the highest percentages (15, 20 and 25%). Samples with 3, 5 and 10% CO_2 were located in the right quadrant, mainly due to the higher concentrations of free amino acids, protein amino acids, total proteins, SFA, chlorophyll-*a*, total carotenoids and all-*trans*-β-carotene. On the left side, samples that were grown with 15, 20 and 25% CO_2 were placed, with higher levels of total lipids, MUFA, PUFA, C18:3n3, total chlorophylls, hydroxylated chlorophyll derivatives, chlorophyll-*b* and derivatives and all-*trans*-lutein. CO_2 levels significantly altered the metabolic routes of all compounds evaluated in this study.

In PC2, it was possible to verify a differentiation of the sample grown with 10% CO_2, located in the upper quadrant, in relation to those grown with 3 and 5% CO_2. The distinction of cultivation with 10% CO_2 is related to the high concentrations of alanine in the free form and C22:0 and also to compounds that only appeared in this treatment, proline in the free form and histidine in the protein form. The separation of the 10% CO_2 treatment also appears as the beginning of the microalgal metabolism transition that was performed in the 15, 20 and 25% CO_2 treatments, compounds such as valine, leucine and phenylalanine (all in free form) and malic acid became detected from this CO_2 concentration.

Figures 2a and 2b show the scores and loadings of PCs 1 and 3. PC3 allowed the differentiation between treatments with a higher percentage of CO_2 enrichment, in the lower quadrant the samples obtained in cultures with 15 and 20% CO_2, while in the upper quadrant is the treatment with 25% CO_2 enrichment. The biomass of the cultivation with 25% CO_2 showed the highest significant concentrations of PUFA, C18:3n3, total chlorophylls, chlorophyll-*a'*, pheophytin-*a* and only in this treatment the chlorophyll-*b'* was detected.

4 Conclusion

The enrichment with 3, 5, 10, 15, 20 and 25% of CO_2 in the photosynthetic cultures of *S. obliquus* modified or intensified the metabolic routes, causing alterations in the compounds evaluated in this work. In general, treatments with lower CO_2 percentages (3, 5 and 10%) showed a total composition of proteins, chlorophylls and carotenoids, as well as their respective profiles, with high potential for applications in the area of food and supplementation. However, there was less lipid synthesis and the FA profiles, mostly saturated, proved to be more suitable for biofuel production. With the increase in CO_2 percentages, there was an increase in the lipids production by *S. obliquus*, as well as significant changes in the FA profile, with high concentrations of C18:3n3 and PUFA, as well as low n6/n3 ratios. These changes in relation to the lipid fraction showed an apex in the culture that was enriched with 25% CO_2.

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Conflict of Interest

The authors declare that there is no conflict of interests.

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Fig. 1a, b Plots of scores (samples) and loadings (variables) showing the principal component 1 (PCI) and 2 (PCII) from *Scenedesmus obliquus* cultivated with different percentages of CO_2.

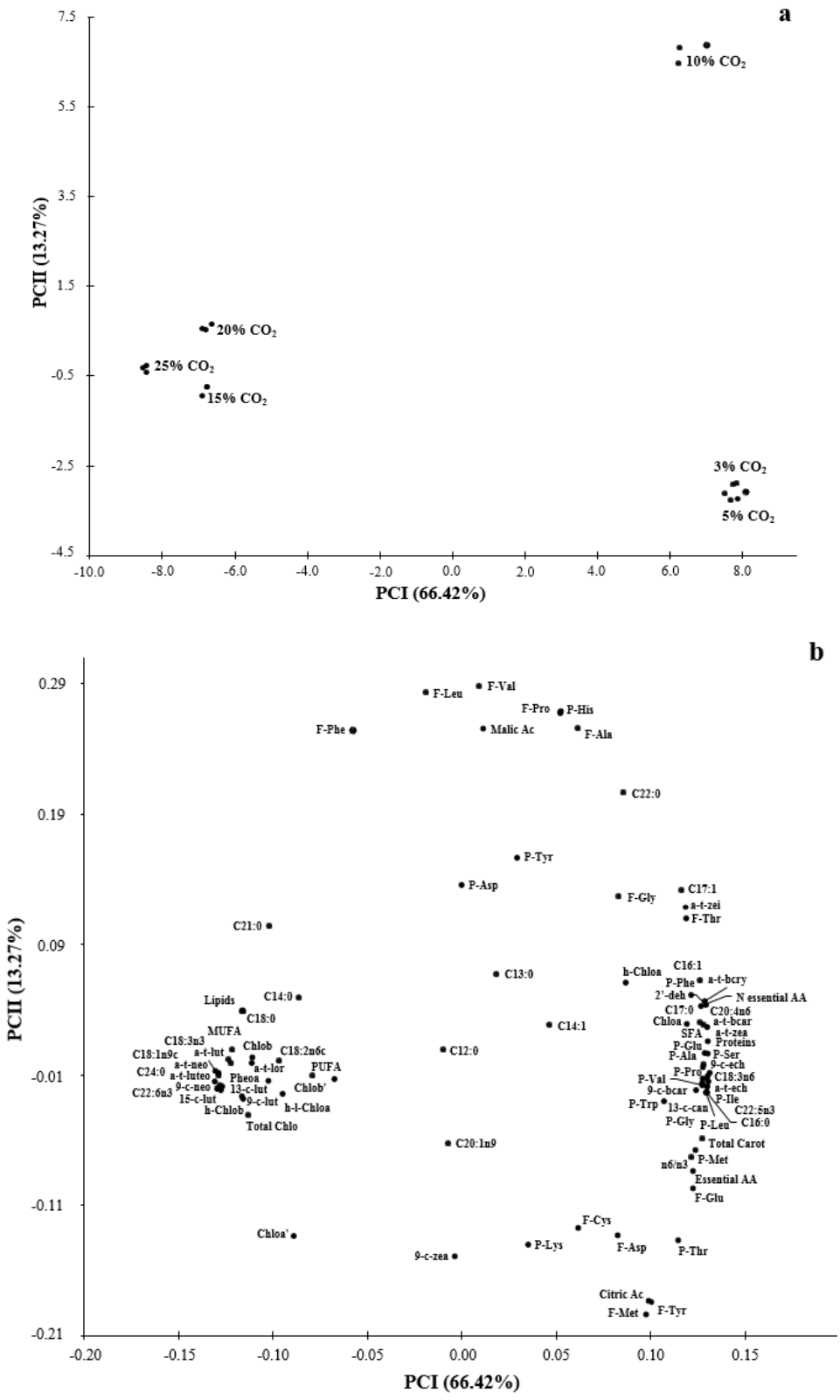

Fig. 2a, b Plots of scores (samples) and loadings (variables) showing the principal component 1 (PCI) and 3 (PCIII) from *Scenedesmus obliquus* cultivated with enrichment levels of CO_2.

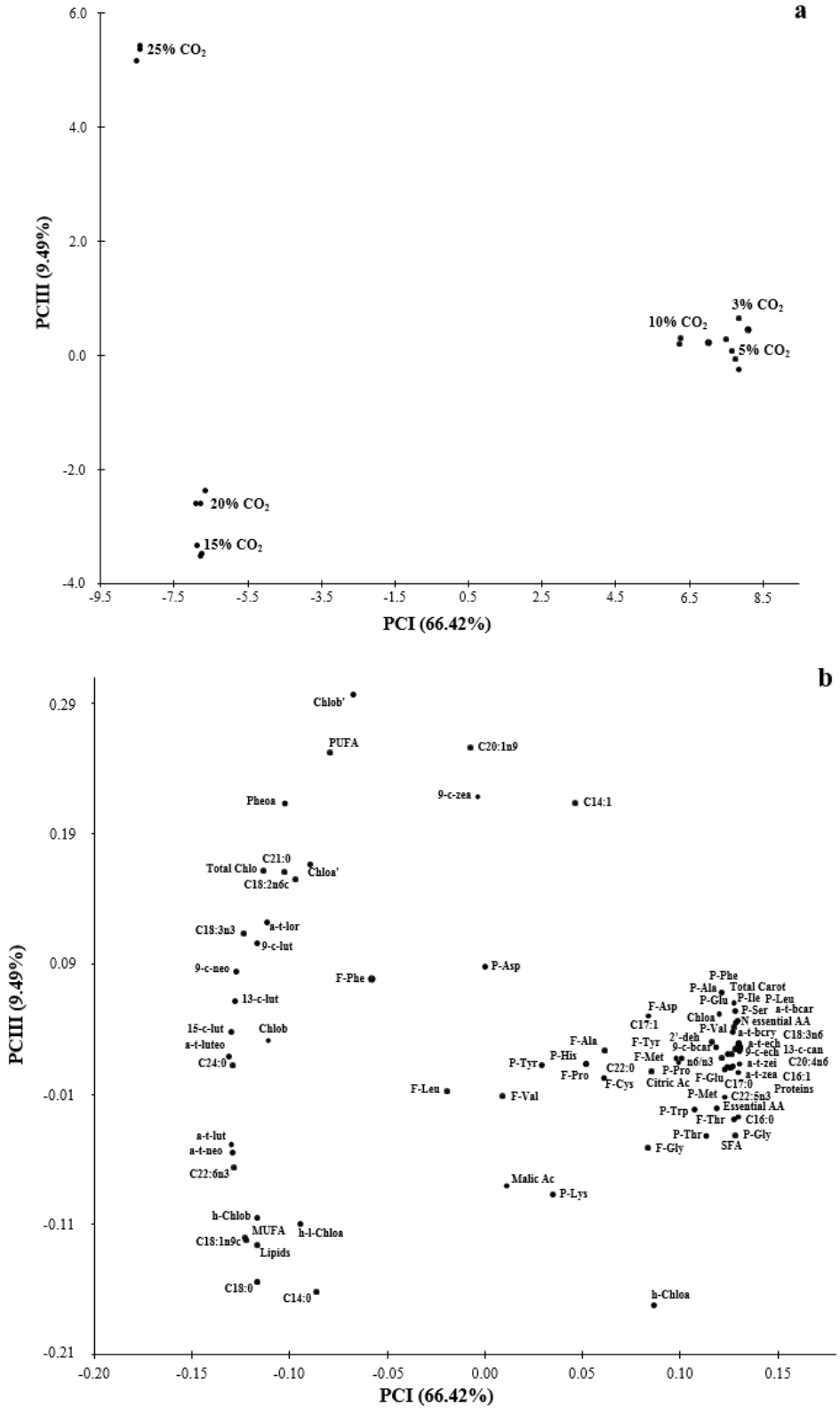

Figure Abbreviations

h-Chlob- 13^2-hydroxy-chlorophyll-*b*; h-l-Chloa- 15^1-hydroxy-lactone-chlorophyll-*a*; Chlob- chlorophyll-*b*; Chlob'- chlorophyll-*b*'; h-Chloa- 13^2-hydroxy-chlorophyll-*a*; Chloa- chlorophyll-*a*; Chloa'- chlorophyll-*a*'; Pheoa- pheophytin-*a*; a-t-lor- all-*trans*-loroxanthin; a-t-neo- all-*trans*-neoxanthin; 9-c-neo- 9-*cis*-neoxanthin; a-t-luteo- all-*trans*-luteoxanthin; 15-c-lut- 15-*cis*-lutein; 13-c-lut- 13-*cis*-lutein; a-t-lut- all-*trans*-lutein; a-t-zea- all-*trans*-zeaxanthin; 9-c-lut- 9-*cis*-lutein; 9-c-zea- 9-*cis*-zeaxanthin; 13-c-can- 13-*cis*-cantaxanthin; 2'-deh- 2'-dehydrodeoxymyxol; a-t-zei- all-*trans*-zeinoxanthin; a-t-bcry- all-*trans*-β-cryptoxanthin; a-t-ech- all-*trans*-echinenone; 9-c-ech- 9-*cis*-echinenone; a-t-bcar- all-*trans*-β-carotene; 9-c-bcar- 9-*cis*-β-carotene.

Table 1. Free polar compounds (μg g^{-1}) from *Scenedesmus obliquus* biomass obtained with enrichment of CO_2 (%).

	3%	5%	10%	15%	20%	25%
Amino Acids						
Alanine	$33.65^{cd} \pm 2.74$	$48.12^{b} \pm 4.47$	$149.61^{a} \pm 0.59$	$27.99^{d} \pm 0.75$	$35.63^{c} \pm 2.24$	$34.56^{cd} \pm 1.76$
Glycine	$27.05^{ab} \pm 0.73$	$19.30^{c} \pm 0.64$	$28.30^{a} \pm 1.65$	$15.37^{d} \pm 1.32$	$23.89^{b} \pm 1.79$	$15.60^{d} \pm 0.46$
Valine	-	-	$42.05^{a} \pm 0.57$	$12.66^{b} \pm 0.87$	$9.66^{c} \pm 0.49$	$9.31^{c} \pm 0.47$
Leucine	-	-	$47.05^{a} \pm 0.03$	$21.39^{b} \pm 1.60$	$16.06^{c} \pm 0.93$	$18.18^{c} \pm 0.87$
Isoleucine	-	-	$32.21^{a} \pm 1.16$	$9.74^{c} \pm 0.35$	-	$28.82^{b} \pm 2.23$
Proline	-	-	$69.17^{a} \pm 2.17$	-	-	-
Methionine	$118.95^{b} \pm 5.29$	$127.94^{a} \pm 6.15$	-	-	-	-
Threonine	$21.60^{b} \pm 1.74$	$24.47^{b} \pm 1.63$	$29.78^{a} \pm 1.00$	$11.43^{cd} \pm 0.29$	$14.56^{c} \pm 0.64$	$9.94^{d} \pm 0.75$
Phenylalanine	-	-	$29.28^{a} \pm 2.57$	$11.97^{d} \pm 1.09$	$19.06^{c} \pm 1.10$	$23.25^{b} \pm 0.56$
Aspartic Acid	$54.23^{a} \pm 3.44$	$16.50^{b} \pm 1.02$	$7.25^{c} \pm 0.65$	-	$5.13^{c} \pm 0.34$	$6.67^{c} \pm 0.40$
Cysteine	-	$25.05^{a} \pm 1.62$	-	-	-	-
Glutamic Acid	$426.02^{a} \pm 31.63$	$471.75^{a} \pm 10.77$	$246.09^{b} \pm 18.21$	$55.48^{c} \pm 1.71$	$93.59^{c} \pm 5.78$	$52.93^{c} \pm 2.21$
Tyrosine	$168.24^{a} \pm 16.75$	$156.11^{a} \pm 7.57$	$19.29^{b} \pm 0.84$	$11.11^{b} \pm 0.18$	$13.93^{b} \pm 0.32$	$11.84^{b} \pm 0.33$
Total FAA	$849.74^{a} \pm 31.65$	$889.25^{a} \pm 27.85$	$700.08^{b} \pm 22.64$	$177.13^{c} \pm 5.11$	$231.51^{c} \pm 11.24$	$211.10^{c} \pm 6.07$
Organic Acids						
Malic Acid	-	-	$25.28^{a} \pm 0.78$	-	$18.61^{b} \pm 0.12$	-
Citric Acid	$108.10^{a} \pm 5.76$	$84.07^{b} \pm 6.04$	$6.24^{c} \pm 0.42$	-	$8.38^{c} \pm 0.41$	$1.46^{c} \pm 0.12$
Total OA	$108.10^{a} \pm 5.76$	$84.07^{b} \pm 6.04$	$31.52^{c} \pm 1.10$	-	$26.99^{c} \pm 0.51$	$1.46^{d} \pm 0.12$

*Results expressed in Mean ± Standard Deviation.
* Different letters on the same line indicate difference by Tukey test ($p > 0.05$).

Table 2. Protein amino acids (mg g^{-1}) and total protein (%) *Scenedesmus obliquus* cultivated photosynthetically with different percentages of CO_2.

	3%	5%	10%	15%	20%	25%
Alanine	$53.28^{a} \pm 2.49$	$47.10^{b} \pm 1.33$	$46.41^{b} \pm 3.63$	$23.31^{c} \pm 0.60$	$21.28^{c} \pm 1.90$	$26.25^{c} \pm 1.63$
Glycine	$40.00^{a} \pm 3.44$	$35.07^{b} \pm 0.78$	$34.70^{b} \pm 2.35$	$22.41^{b} \pm 1.97$	$21.77^{bc} \pm 0.45$	$18.18^{c} \pm 0.35$
Valine	$43.57^{a} \pm 1.26$	$39.08^{b} \pm 2.73$	$37.49^{b} \pm 0.20$	$19.58^{c} \pm 0.48$	$17.14^{c} \pm 0.56$	$18.41^{c} \pm 0.20$
Leucine	$60.42^{a} \pm 0.62$	$56.07^{b} \pm 3.57$	$52.59^{c} \pm 1.21$	$28.62^{d} \pm 0.32$	$25.30^{e} \pm 0.45$	$29.82^{d} \pm 0.21$
Isoleucine	$41.58^{a} \pm 0.70$	$37.40^{b} \pm 1.67$	$35.36^{c} \pm 0.41$	$15.77^{d} \pm 0.22$	$13.69^{e} \pm 4.37$	$15.21^{de} \pm 4.41$
Proline	$21.05^{a} \pm 2.00$	$19.81^{a} \pm 0.99$	$19.06^{a} \pm 1.78$	$12.58^{b} \pm 0.28$	$10.83^{b} \pm 0.61$	$11.26^{b} \pm 0.78$
Methionine	$19.16^{a} \pm 1.88$	$16.88^{ab} \pm 0.60$	$14.70^{b} \pm 0.94$	$10.28^{c} \pm 0.41$	$9.28^{c} \pm 0.60$	$9.42^{c} \pm 0.63$
Serine	$15.68^{a} \pm 0.25$	$14.27^{b} \pm 0.43$	$14.32^{b} \pm 0.84$	$7.81^{c} \pm 0.18$	$6.21^{d} \pm 0.46$	$7.80^{c} \pm 0.63$
Threonine	$36.46^{a} \pm 1.38$	$33.13^{a} \pm 1.33$	$19.48^{b} \pm 1.72$	$15.97^{c} \pm 0.60$	$14.37^{c} \pm 0.64$	$9.88^{d} \pm 0.19$
Phenylalanine	$46.85^{a} \pm 3.28$	$48.94^{a} \pm 2.27$	$50.09^{a} \pm 1.97$	$36.87^{b} \pm 1.27$	37.16b ± 0.39	$39.23^{b} \pm 0.94$
Aspartic Acid	-	$3.11^{a} \pm 0.25$	$3.08^{a} \pm 0.29$	1.40d ± 0.05	$1.95^{c} \pm 0.14$	$2.53^{b} \pm 0.06$
Glutamic Acid	$52.46^{a} \pm 2.74$	$55.14^{a} \pm 3.25$	$52.40^{a} \pm 4.61$	$24.67^{b} \pm 0.54$	$25.10^{b} \pm 0.35$	$28.37^{b} \pm 1.81$
Lysine	-	$65.58^{a} \pm 1.51$	-	$31.06^{b} \pm 1.33$	-	-
Histidine	-	-	$10.05^{a} \pm 0.70$	-	-	-
Tyrosine	$20.85^{a} \pm 0.56$	-	$21.71^{a} \pm 2.06$	$11.07^{b} \pm 0.41$	$10.25^{b} \pm 0.36$	$10.48^{b} \pm 0.84$
Tryptophan	$12.82^{a} \pm 0.70$	$8.88^{b} \pm 0.78$	$9.48^{b} \pm 0.89$	$5.99^{c} \pm 0.39$	$7.96^{b} \pm 0.23$	$5.84^{c} \pm 0.38$
∑Essential AA	$280.01^{b} \pm 6.62$	$322.84^{a} \pm 9.37$	$233.89^{c} \pm 5.04$	$174.42^{d} \pm 2.59$	$134.18^{e} \pm 2.33$	$137.24^{e} \pm 3.21$
∑Non-essential AA	$203.32^{a} \pm 3.48$	$174.51^{b} \pm 5.40$	$201.73^{a} \pm 15.12$	$103.24^{c} \pm 2.29$	$97.38^{c} \pm 3.08$	$104.86^{c} \pm 5.08$
Total Protein	$62.31^{a} \pm 2.42$	$61.61^{a} \pm 0.26$	$60.74^{a} \pm 0.97$	$33.36^{b} \pm 0.22$	$29.10^{c} \pm 1.29$	$31.12^{bc} \pm 0.06$

*Results expressed in Mean ± Standard Deviation.
*Essential AA- essential amino acids; Non-essential AA- non-essential amino acids.
* Different letters on the same line indicate difference by Tukey test (p> 0.05).

Table 3. Total pigments (mg g^{-1}) and chlorophylls and carotenoids profiles (%) from *Scenedesmus obliquus* cultivated with different levels of CO_2 enrichment.

Compounds	3% CO_2	5% CO_2	10% CO_2	15% CO_2	20% CO_2	25% CO_2
Chlorophylls						
13^2-hydroxy-chlorophyll-*b*	-	-	-	$6.65^{a} \pm 0.04$	$3.43^{b} \pm 0.59$	$3.52^{b} \pm 0.19$
15^1-hydroxy-lactone-chlorophyll-*a*	$3.29^{e} \pm 0.29$	$4.07^{de} \pm 0.09$	$5.44^{d} \pm 0.23$	$53.39^{a} \pm 1.12$	$14.04^{c} \pm 0.23$	$23.20^{b} \pm 0.46$
chlorophyll-*b*	-	-	-	$3.20^{c} \pm 1.14$	$11.95^{a} \pm 0.11$	$9.04^{b} \pm 0.60$
chlorophyll-*b'*	-	-	-	-	-	$4.60^{a} \pm 0.01$
13^2-hydroxy-chlorophyll-*a*	$3.69^{a} \pm 0.36$	$4.21^{a} \pm 0.12$	$4.49^{a} \pm 0.27$	$1.63^{b} \pm 0.01$	$4.41^{a} \pm 0.53$	-
Chlorophyll-*a*	$88.10^{a} \pm 0.80$	$85.01^{b} \pm 0.09$	$86.53^{ab} \pm 0.46$	$26.93^{e} \pm 0.04$	$58.96^{c} \pm 1.33$	$45.74^{d} \pm 0.41$
Chlorophyll-*a'*	$4.91^{e} \pm 0,15$	$6.71^{c} \pm 0.11$	$3.55^{f} \pm 0.04$	$7.23^{b} \pm 0.03$	$5.90^{d} \pm 0.10$	$9.92^{a} \pm 0.02$
Pheophytin-*a*	-	-	-	$0.98^{b} \pm 0.01$	$1.31^{b} \pm 0.23$	$3.97^{a} \pm 0.45$
Total Chlorophylls (mg g^{-1})	$19.52^{d} \pm 0.22$	$18.45^{e} \pm 0.19$	$18.04^{e} \pm 0.11$	$24.23^{b} \pm 0.54$	$23.06^{c} \pm 0.54$	$30.79^{a} \pm 0.35$
Carotenoids						
all-*trans*-loroxanthin	-	-	-	$0.93^{c} \pm 0.03$	$3.40^{b} \pm 0.01$	$4.08^{a} \pm 0.02$
all-*trans*-neoxanthin	$0.06^{e} \pm 0.01$	$0.04^{e} \pm 0.01$	$0.18^{d} \pm 0.00$	$4.83^{b} \pm 0.03$	$5.35^{a} \pm 0.01$	$4.44^{c} \pm 0.03$
9-*cis*-neoxanthin	-	-	-	$1.72^{b} \pm 0.01$	$1.60^{c} \pm 0.00$	$2.67^{a} \pm 0.09$
all-*trans*-luteoxanthin	-	-	-	$1.98^{c} \pm 0.03$	$2.16^{b} \pm 0.03$	$2.49^{a} \pm 0.05$
15-*cis*-lutein	-	-	-	$4.34^{b} \pm 0.09$	$3.55^{c} \pm 0.09$	$5.25^{a} \pm 0.04$
13-*cis*-lutein	-	-	-	$2.01^{b} \pm 0.03$	$1.56^{c} \pm 0.02$	$2.63^{a} \pm 0.07$
all-*trans*-lutein	$0.82^{f} \pm 0.04$	$1.97^{e} \pm 0.04$	$4.03^{d} \pm 0.00$	$73.04^{b} \pm 0.28$	$76.78^{a} \pm 0.30$	$67.93^{c} \pm 0.16$
all-*trans*-zeaxanthin	$9.25^{c} \pm 0.00$	$10.49^{a} \pm 0.25$	$9.95^{b} \pm 0.00$	$1.13^{d} \pm 0.03$	$0.35^{e} \pm 0.00$	$0.30^{e} \pm 0.00$
9-*cis*-lutein	-	-	-	$2.03^{b} \pm 0.10$	$0.83^{c} \pm 0.06$	$2.73^{a} \pm 0.08$
9-*cis*-zeaxanthin	$1.67^{b} \pm 0.00$	$1.56^{c} \pm 0.02$	$1.06^{d} \pm 0.01$	$1.56^{bc} \pm 0.01$	$0.71^{e} \pm 0.02$	$2.02^{a} \pm 0.09$
13-*cis*-cantaxanthin	$4.68^{a} \pm 0.01$	$3.90^{b} \pm 0.44$	$3.44^{b} \pm 0.01$	-	-	-
2'-dehydrodeoxymyxol	$3.74^{c} \pm 0.01$	$4.31^{b} \pm 0.05$	$4.64^{a} \pm 0.01$	-	-	-

all-*trans*-zeinoxanthin	$0.45^{b} \pm 0.00$	$0.30^{c} \pm 0.00$	$0.64^{a} \pm 0.00$	-	-	-
all-*trans*-β-cryptoxanthin	$1.54^{c} \pm 0.01$	$1.82^{b} \pm 0.04$	$1.96^{a} \pm 0.02$	-	-	-
all-*trans*-echinenone	$13.46^{a} \pm 0.02$	$11.95^{b} \pm 0.05$	$10.70^{c} \pm 0.01$	-	-	-
9-*cis*-echinenone	$16.96^{b} \pm 1.11$	$18.09^{a} \pm 0.91$	$15.40^{c} \pm 0.00$	-	-	-
all-*trans*-β-carotene	$44.22^{a} \pm 1.11$	$40.17^{b} \pm 1.06$	$44.50^{a} \pm 0.01$	$6.43^{c} \pm 0.07$	$3.71^{d} \pm 0.21$	$5.45^{c} \pm 0.08$
9-*cis*-β-carotene	$3.15^{c} \pm 0.01$	$5.40^{a} \pm 0.06$	$3.50^{b} \pm 0.00$	-	-	-
Total Carotenoids (mg g^{-1})	$25.27^{a} \pm 0.45$	$22.70^{b} \pm 0.14$	$18.12^{c} \pm 0.99$	$7.25^{d} \pm 0.44$	$5.68^{e} \pm 0.27$	$7.70^{d} \pm 0.39$

*Results expressed in Mean ± Standard Deviation.
* Different letters on the same line indicate difference by Tukey test ($p > 0.05$).

Table 4. Fatty acids composition and total lipids (%) from *Scenedesmus obliquus* cultivated with different CO_2 concentrations.

Compounds	3%	5%	10%	15%	20%	25%
C12:0	$1.29^{b} \pm 0.07$	$1.17^{bc} \pm 0.08$	$1.24^{b} \pm 0.03$	$0.86^{d} \pm 0.03$	$1.09^{c} \pm 0.01$	$1.75^{a} \pm 0.01$
C13:0	$1.59^{bc} \pm 0.14$	$1.43^{cd} \pm 0.13$	$1.71^{b} \pm 0.02$	$1.06^{e} \pm 0.09$	$1.26^{de} \pm 0.07$	$2.00^{a} \pm 0.04$
C14:0	$0.24^{d} \pm 0.00$	$0.29^{cd} \pm 0.02$	$0.34^{bc} \pm 0.01$	$0.54^{a} \pm 0.04$	$0.36^{b} \pm 0.01$	$0.35^{b} \pm 0.01$
C14:1	$0.59^{b} \pm 0.05$	$0.87^{a} \pm 0.08$	$0.77^{a} \pm 0.05$	$0.55^{b} \pm 0.02$	$0.58^{b} \pm 0.03$	$0.79^{a} \pm 0.02$
C16:0	$59.01^{b} \pm 0.13$	$62.06^{a} \pm 1.04$	$56.92^{c} \pm 0.68$	$43.31^{d} \pm 0.75$	$38.82^{e} \pm 0.48$	$36.86^{f} \pm 0.28$
C16:1	$1.44^{b} \pm 0.03$	$1.15^{c} \pm 0.10$	$1.58^{a} \pm 0.04$	$0.34^{d} \pm 0.00$	$0.23^{d} \pm 0.01$	$0.26^{d} \pm 0.02$
C17:0	$0.81^{b} \pm 0.06$	$1.01^{a} \pm 0.08$	$0.97^{a} \pm 0.06$	$0.44^{c} \pm 0.03$	$0.34^{c} \pm 0.02$	$0.37^{c} \pm 0.01$
C17:1	$0.54^{b} \pm 0.03$	$0.52^{b} \pm 0.01$	$0.81^{a} \pm 0.01$	$0.21^{c} \pm 0.00$	$0.18^{c} \pm 0.01$	$0.21^{c} \pm 0.01$
C18:0	$2.55^{f} \pm 0.11$	$2.91^{e} \pm 0.10$	$3.88^{d} \pm 0.01$	$7.87^{a} \pm 0.20$	$7.32^{b} \pm 0.15$	$5.32^{c} \pm 0.03$
C18:1n9c	$6.49^{e} \pm 0.08$	$7.20^{de} \pm 0.25$	$7.61^{d} \pm 0.14$	$21.20^{b} \pm 0.66$	$22.84^{a} \pm 0.12$	$16.28^{c} \pm 0.15$
C18:2n6c	$10.09^{b} \pm 0.10$	$7.56^{d} \pm 0.38$	$9.02^{c} \pm 0.27$	$10.24^{b} \pm 0.13$	$10.28^{b} \pm 0.31$	$12.41^{a} \pm 0.07$
C18:3n6	$10.94^{a} \pm 0.29$	$8.68^{b} \pm 0.73$	$8.49^{b} \pm 0.24$	$1.05^{c} \pm 0.01$	$0.99^{c} \pm 0.07$	$1.08^{c} \pm 0.02$
C18:3n3	$0.79^{e} \pm 0.01$	$0.93^{e} \pm 0.03$	$1.87^{d} \pm 0.06$	$9.01^{c} \pm 0.31$	$11.86^{b} \pm 0.49$	$17.87^{a} \pm 0.19$
C20:1n9	$1.29^{bc} \pm 0.09$	$1.45^{ab} \pm 0.12$	$1.26^{bc} \pm 0.06$	$1.15^{c} \pm 0.10$	$1.31^{abc} \pm 0.11$	$1.57^{a} \pm 0.09$
C21:0	$1.28^{d} \pm 0.04$	$1.28^{d} \pm 0.08$	$1.72^{c} \pm 0.05$	$1.60^{c} \pm 0.02$	$2.00^{b} \pm 0.05$	$2.38^{a} \pm 0.01$
C22:0	$0.17^{c} \pm 0.01$	$0.40^{b} \pm 0.03$	$0.90^{a} \pm 0.05$	$0.10^{cd} \pm 0.00$	$0.06^{d} \pm 0.00$	$0.06^{d} \pm 0.00$
C20:4n6	$0.11^{b} \pm 0.00$	$0.15^{a} \pm 0.01$	$0.14^{a} \pm 0.00$	-	-	-
C24:0	-	-	-	$0.19^{b} \pm 0.00$	$0.26^{a} \pm 0.02$	$0.26^{a} \pm 0.01$
C22:5n3	$0.77^{b} \pm 0.05$	$0.94^{a} \pm 0.07$	$0.76^{b} \pm 0.04$	$0.15^{c} \pm 0.00$	$0.09^{c} \pm 0.01$	$0.07^{c} \pm 0.00$
C22:6n3	-	-	-	$0.15^{a} \pm 0.00$	$0.13^{b} \pm 0.01$	$0.12^{c} \pm 0.00$
∑SFA	$66.94^{b} \pm 0.19$	$70.55^{a} \pm 0.88$	$67.69^{b} \pm 0.64$	$55.96^{c} \pm 0.91$	$51.52^{d} \pm 0.57$	$49.35^{e} \pm 0.32$
∑MUFA	$10.36^{f} \pm 0.16$	$11.19^{e} \pm 0.24$	$12.03^{d} \pm 0.12$	$23.45^{b} \pm 0.55$	$25.13^{a} \pm 0.18$	$19.11^{c} \pm 0.17$
∑PUFA	$22.70^{b} \pm 0.34$	$18.26^{d} \pm 1.07$	$20.28^{c} \pm 0.53$	$20.59^{c} \pm 0.37$	$23.35^{b} \pm 0.74$	$31.54^{a} \pm 0.25$
n6/n3	$13.56^{a} \pm 0.60$	$8.75^{b} \pm 0.87$	$6.72^{c} \pm 0.15$	$1.21^{d} \pm 0.04$	$0.93^{d} \pm 0.02$	$0.75^{d} \pm 0.01$
Total Lipids	$10.66^{c} \pm 0.04$	$9.97^{d} \pm 0.29$	$10.90^{c} \pm 0.15$	$12.87^{a} \pm 0.42$	$13.08^{a} \pm 0.17$	$11.91^{b} \pm 0.12$

* Results expressed in Mean ± Standard Deviation.
* SFA- saturated fatty acids; MUFA- monounsaturated fatty acids; PUFA- polyunsaturated fatty acids; n6/n3- omega 6 and omega 3 ratio.
* Different letters on the same line indicate difference by Tukey test ($p > 0.05$).

Supplementary Material 1**.** UV-vis characteristics and fragment ions used in the identification of chlorophyll and carotenoid compounds from *Scenedesmus obliquus* biomasses.

Compounds	UV-vis characteristics			Fragment ions (positive mode) (*m/z*)	
	$\lambda_{máx}$ (nm)[a]	III/II[b] (%)	A_B/II[c] (%)	$[M + H]^+$	MS/MS
Chlorophylls					
13[2]-hydroxy-chlorophyll-*b*	465, 650	na[e]	na	923	645 $[M + H - 278]^+$
15[1]-hydroxy-lactone-chlorophyll-*a*	430, 664	na	na	925	647 $[M + H - 278]^+$, 615 $[M + H - 278 - 32]^+$
chlorophyll-*b*	466, 651	na	na	907	629 $[M + H - 278]^+$, 569 $[M + H - 278 - 60]^+$
chlorophyll-*b'*	466, 655	na	na	907	629 $[M + H - 278]^+$, 569 $[M + H - 278 - 60]^+$
13[2]-hydroxy-chlorophyll-*a*	421, 655	na	na	909	631 $[M + H - 278]^+$
chlorophyll-*a*	431, 665	na	na	893	615 $[M + H - 278]^+$, 583 $[M + H - 278 - 31]^+$, 555 $[M + H - 278 - 59]^+$
chlorophyll-*a'*	430, 665	na	na	893	615 $[M + H - 278]^+$, 583 $[M + H - 278 - 31]^+$, 555 $[M + H - 278 - 59]^+$
pheophytin-*a*	407, 666	na	na	871	593 $[M + H - 278]^+$; 533 $[M + H - 278 - 60]^+$
Carotenoids					
all-*trans*-loroxanthin	410, 441, 468	57	0	585	567 $[M + H - 18]^+$, 479 [M + H - 106]
all-*trans*-neoxanthin	415, 438, 468	80	0	601	583 $[M + H - 18]^+$, 565 $[M + H - 18 - 18]^+$, 547 $[M + H - 18 - 18 - 18]^+$, 509 $[M + H - 92]^+$, 491 $[M + H - 18 - 92]^+$
9-*cis*-neoxanthin	327, 413, 434, 463	81	12	601	583 $[M + H - 18]^+$, 565 $[M + H - 18 - 18]^+$, 547 $[M + H - 18 - 18 - 18]^+$, 509 $[M + H - 92]^+$, 491 $[M + H - 18 - 92]^+$
all-*trans*-luteoxanthin	400, 420, 446	100	0	601	583 $[M + H - 18]^+$, 565 $[M + H - 18 - 18]^+$, 509 $[M + H - 92]^+$
15-*cis*-lutein	332, 410, 438, 464	22	47	569	551 $[M + H - 18]^+$
13-*cis*-lutein	332, 410, 437, 464	44	43	569	551 $[M + H - 18]^+$
all-*trans*-lutein	419, 444, 471	60	0	569	551 $[M + H - 18]^+$, 533 $[M + H - 18 - 18]^+$, 463 $[M + H - 106]^+$
all-*trans*-zeaxanthin	425, 449, 476	28	0	569	551 $[M + H - 18]^+$, 533 $[M + H - 18 - 18]^+$, 477 $[M + H - 92]^+$
9-*cis*-lutein	336, 417, 438, 465	66	22	569	551 $[M + H - 18]^+$
9-*cis*-zeaxanthin	338, 419, 442, 471	62	30	569	551 $[M + H - 18]^+$, 533 $[M + H - 18 - 18]^+$
13-*cis*-cantaxanthin	357, 449	nc[d]	43	565	547 $[M + H - 18]^+$, 509 $[M + H - 56]^+$, 459 $[M + H - 106]^+$

2'-dehydrodeoxymyxol	445, 472, 504	50	0	567	549 $[M + H - 18]^+$, 475 $[M + H - 92]^+$
all-*trans*-zeinoxanthin	423, 442, 471	70	0	553	535 $[M + H - 18]^+$, 461 $[M + H - 92]^+$
all-*trans*-β-cryptoxanthin	420, 451, 473	37	0	553	535 $[M + H - 18]^+$, 461 $[M + H - 92]^+$
all-*trans*-echinenone	463	nc	0	551	533 $[M + H - 18]^+$
9-*cis*-echinenone	342, 456	nc	20	551	533 $[M + H - 18]^+$
all-*trans*-β-carotene	425, 451, 476	28	0	537	481 $[M + H - 56]^+$, 444 $[M + H - 92]^+$
9-*cis*-β-carotene	343, 420, 443, 471	42	25	537	481 $[M + H - 56]^+$, 444 $[M + H - 92]^+$

[a]Linear gradient MeOH:MTBE.
[b]Spectral fine structure: Ratio of the height of the longest wavelength absorption peak (III) and that of the middle absorption peak (II).
[c]Ratio of the *cis* peak (A_B) and the middle absorption peak (II).
[d]Not calculated.
[e]Not applied.

5 DISCUSSÃO GERAL

As microalgas são uma fonte renovável de compostos que podem ser utilizados como insumos na indústria de alimentos e as condições de cultivos influenciam diretamente no crescimento celular desses microrganismos, bem como na sua composição (MATOS, 2017). Assim, o monitoramento dos metabólitos que são formados ao longo dos cultivos microalgais são de extrema importância para o controle, a otimização e a caracterização do bioprocesso. Atualmente, várias técnicas analíticas estão disponíveis para análises de metabólitos, como ressonância magnética nuclear (NMR), cromatografia gasosa (GC) e cromatografia líquida (LC) acoplada a diferentes detectores, principalmente espectrometria de massa (MS) (LONGNECKER et al., 2015).

A GC pode ser empregada, por exemplo, para análise direta da composição gasosa dos cultivos microalgais, principalmente teores de O_2 e CO_2, com a finalidade de verificar a eficiência do processo fotossintético (JACOB-LOPES & FRANCO, 2013). Ademais, permite a análise direta de compostos orgânicos voláteis, que podem ser utilizados como marcadores de metabolismo e da eficiência de desodorização em cultivos de tratamento de efluentes por microalgas, além da aplicação comercial dessas moléculas (SANTOS et al., 2016). Vários outros compostos microalgais não voláteis podem ser analisados por GC, porém são necessários métodos de preparo de amostra específicos para que se tornem compatíveis com a técnica.

A análise dos constituintes não voláteis por GC requer que a biomassa seja submetida a uma etapa de extração ou hidrólise e derivatização. Para análise do perfil de proteínas e carboidratos, por exemplo, deve ser realizada hidrolise, já para lipídios e compostos polares livres, extração, e após derivatizações para todos os compostos (GARRETA et al., 2016). Apesar de necessárias etapas de preparo de amostra, a GC é uma plataforma analítica viável para análises metabólicas e de caracterização de biomassas microalgais. A técnica possibilita a separação de vários compostos pertencentes a diferentes classes químicas simultaneamente com elevada resolução e sensibilidade (FARAJZADEH, NOURI & KHORRAM et al., 2014).

Em face disso, a GC foi empregada para monitorar a síntese de metabólitos durante as fases do crescimento celular, exponencial, estacionário e de declínio, de *S. obliquus* cultivada fotossinteticamente com diferentes fotoperíodos 24:0 e 12:12 (claro:escuro). Ao final dos cultivos também foi verificada a influência dos metabólitos no conteúdo total de proteínas, lipídios e clorofilas. A síntese de todos os metabólitos avaliados, ácidos orgânicos, aminoácidos livres e

ácidos graxos, durantes os cultivos microalgais foi afetada significativamente pelas duas variáveis, fase do crescimento celular e fotoperíodo, o que possibilitou a diferenciação das amostras estudadas através da Análise de Componente Principal (PCA).

O crescimento celular e a composição da biomassa apresentaram diferenças em relação aos fotoperíodos. O cultivo 24:0 (luz:escuro) apresentou a maior concentração de biomassa, 4020 mg L^{-1}, a iluminação constante se mostrou uma variável favorável ao desenvolvimento da *S. obliquus*. Já o cultivo com fotoperíodo 12:12 (claro:escuro), apresentou menor concentração de biomassa (2560 mg L^{-1}), mas maior síntese de lipídios (23,00%), que é maximizada em condições de estresse como os períodos de escuro desse cultivo (HEXIN et al., 2013), e também de clorofilas totais (26.40 mg g^{-1}). Os teores de proteínas não diferiram entre os cultivos com diferentes fotoperíodos.

Esses estudos evidenciaram a aplicabilidade e a versatilidade da GC para monitoramento e caracterização de cultivos microalgais, porém verificou-se a necessidade de avaliar uma fração importante que não é abrangida por essa técnica, os pigmentos. Na literatura os métodos reportados para extração simultânea de clorofilas e carotenoides de microalgas para posterior análise por espectrofotômetro ou LC, apresentam várias desvantagens e limitações. O mais usual é o método de maceração com elevado volume de solventes orgânicos por longos períodos de tempo, mas também podem ser citadas extrações com fluído supercrítico ou extrações assistidas por ultrassom e micro-ondas, tecnologias que fornecem custos adicionais para o procedimento analítico (KAPOORE et al. 2018).

Nos estudos anteriores os metabólitos microalgais foram extraídos com a mistura metanol:clorofórmio através da homogeneização mecânica e foi observado também a extração de pigmentos, que permaneceram na fração orgânica (VENDRUSCOLO et al., 2018; VENDRUSCOLO et al., 2019). Então, foi avaliada a extração exaustiva dos pigmentos utilizando clorofórmio após homogeneização em mesa agitadora orbital por 30, 60 e 120 min. Os maiores tempos de extração evidenciaram degradação dos carotenoides e apesar de apresentarem influência significativa na extração de clorofilas, não foram obtidos valores de recuperação satisfatórios (acima de 80%) para todos os compostos clorofilados. Após extração exaustiva com clorofórmio, foram utilizados os solventes acetona, acetato de etila e etanol. Os melhores resultados foram obtidos com 30 min de homogeneização e extração exaustiva com clorofórmio seguida de etanol (PM30E), devido, possivelmente, a ampliação da faixa de polaridade do sistema extrativo (RAMLUCKAN, MOODLEY & BUX, 2014).

Em comparação com o método de maceração (MM) que utiliza acetato de etila e metanol e foi utilizado como referência, o PM30E apresentou redução de aproximadamente cinco vezes o volume total de solventes empregados e extração cerca de quatro vezes mais rápida. Por fazer uso de homogeneização mecânica, o PM30E ampliou a frequência analítica, aliada a maior segurança do analista que não fica exposto diretamente ao solvente por horas como no MM.

Após a definição das estratégias analíticas para determinação de vários compostos microalgais, foi realizada a caracterização de biomassas de *S. obliquus* também cultivadas fotossinteticamente, mas com diferentes percentuais de enriquecimento de CO_2 (3, 5, 10, 15, 20 e 25%). Assim foram avaliados os compostos polares livres, proteínas totais e aminoácidos proteicos, lipídios totais e ácidos graxos, clorofilas e carotenoides totais e seus respectivos perfis. Através dos metabólitos polares livres (ácidos orgânicos e aminoácidos), foram observadas intensidades metabólicas distintas entre os cultivos, os com 3, 5 e 10% apresentaram acúmulo superior desses compostos nas células microalgais, com redução significativa na concentração nos cultivos com maiores teores de CO_2 (15, 20 e 25%).

A maior síntese de aminoácidos detectada nos cultivos com 3, 5 e 10% de CO_2 foi diretamente proporcional ao conteúdo de proteínas desses cultivos, que atingiram valores superiores a 60%, enquanto nos demais cultivos (15, 20 e 25%) os valores permaneceram na faixa dos 30%. Possivelmente, os menores percentuais de CO_2 intensificaram as rotas de síntese de aminoácidos e proteínas, juntamente com o fenômeno de fotorrespiração que prioriza a síntese de aminoácidos (ZENG et al., 2011). Já a síntese de clorofilas foi intensificada com o aumento do CO_2, máximo de 30,79 mg g^{-1} no cultivo com 25% de CO_2. Com a iluminação constante presente e aumento das concentrações de CO_2 as células passaram a sintetizar mais clorofilas para maximizar o processo fotossintético, o que não ocorreu com os carotenoides, pigmentos acessórios, suas concentrações foram inversamente proporcionais as de clorofilas (CARVALHO et al., 2011). Um aumento na síntese de lipídios totais foi observado nos cultivos com 15, 20 e 25% de CO_2, assim como de ácidos graxos poli-insaturados pela ação de enzimas dessaturases (MONROIG et al., 2013).

6 CONCLUSÃO GERAL

O monitoramento e a caracterização dos cultivos microalgais é de suma importância para obtenção de biomassas com as características necessárias, principalmente, em estudos em fase

experimental em que se busca a otimização do processo biotecnológico. Assim, o desenvolvimento de métodos de preparo de amostra integrados, mais simples e rápidos e com um menor consumo de solventes orgânicos, se tornam mais viáveis para aplicação posterior de uma ferramenta analítica como a GC para mensuração de diversos metabólitos e espectrofotômetro ou LC para pigmentos.

Ademais, foi verificada a capacidade de modulação do metabolismo das microalgas através de condições externas, como diferentes fotoperíodos e percentuais de CO_2. Mantendo o enriquecimento de CO_2 constante e variando o fotoperíodo, foi possível maximizar a produção de biomassa empregando iluminação constante, 24:0 (claro:escuro) e com períodos de escuro, 12:12 (claro:escuro), intensificar a síntese de lipídios e clorofilas totais. Já em cultivos com iluminação constante e variação dos teores de CO_2, foi observado aumento da produção de clorofilas e lipídios nos cultivos com maior concentração do gás. Enquanto a maior síntese de proteínas e carotenoides foi observada nas biomassas cultivadas com menores percentuais de CO_2.

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ANEXO A – PERMISSÃO DIVULGAÇÃO ARTIGO 1

Analytical strategies for using gas chromatography to control and optimize microalgae bioprocessing

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