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PRODUÇÃO DE BIOCOMPOSTOS MICROALGAIS EM DIFERENTES CONDIÇÕES DE CULTIVO

Santa Maria, RS
2021

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Dissertação 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 **Mestre em Ciência e Tecnologia dos Alimentos.**

Orientadora: Prof.ª Dr.ª Leila Queiroz Zepka

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Uma mente que se abre ao conhecimento,
jamais voltará ao seu tamanho original.

(Albert Einstein)

RESUMO

PRODUÇÃO DE BIOCOMPOSTOS MICROALGAS EM DIFERENTES CONDIÇÕES DE CULTIVO

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Existe um consenso mundial na indústria de alimentos que visa a substituição de ingredientes sintéticos por ingredientes naturais para promoção da saúde. Ao mesmo tempo, está em constante crescimento a relevância industrial das microalgas como fontes de um extensivo espectro de bioprodutos e como promissoras matérias-primas para produção de aditivos naturais. O interesse se justifica, fundamentalmente, na composição química da biomassa, como os carotenoides e o limoneno, devido suas atividades bioativas com efeitos benéficos à saúde. Esses metabólitos por terem uma ótima perspectiva para o crescimento e desenvolvimento industrial, busca-se uma produção com maior potencial através de sistemas de cultivo e condições otimizadas, sendo um desafio subsequente o desenvolvimento de bioprocessos que vinculem os resultados científicos às necessidades comerciais. Nesse contexto, este estudo teve como objetivo avaliar o perfil de carotenoides produzidos pela microalga *Desertifilum* spp. em cultivo fototrófico, heterotrófico e mixotrófico, e, investigar a formação do composto volátil limoneno, derivado das microalgas *Desertifilum* spp., *Chlorella sorokiniana, Chlorella vulgaris, Scenedesmus bijuga, Scenedesmus obliquus*e e *Spirulina* sp., a partir de cultivo fotoautotrófico, além de identificar suas propriedades de aroma. Pelo estudo, constatou-se que a microalga *Desertifilum* spp. exibe forte potencial para a produção de carotenoides, sendo majoritária as formas de all-*trans*-β-caroteno, all-*trans*-zeaxantina e all-*trans*-equinenona, respectivamente. Nesse sentido, o cultivo mixotrófico apresentou melhores resultados quantitativos quando comparado ao cultivo fotoautotrófico e heterotrófico. Paralelamente, identificou-se a presença do composto bioativo limoneno na fração volátil das seis microalgas, contribuindo de forma positiva para o aroma cítrico e menta, sendo a microalga *Scenedesmus bijuga* aquela com maior potencial quantitativo. Dessa forma, o estudo possibilitou demonstrar que as microalgas são fontes de bioprodutos não voláteis (carotenoides) e voláteis (limoneno), compostos que, segundo a literatura, apresentam importantes propriedades bioativas e/ou nutracêuticas, cuja importância econômica está associada a uma ampla gama de aplicações nas indústrias de alimentos e biomédicas. Adicionalmente, será publicado o capítulo de livro “Avanços recentes na bioacessibilidade e biodisponibilidade de compostos bioativos de algas” (Capítulo 4), como pesquisa complementar a este trabalho.

Palavras-chave: Compostos bioativos. Cultivo microalgal. Produção de carotenoides. Produção de limoneno.

ABSTRACT

PRODUCTION OF MICROALGAE BIOCOMPOUNDS IN DIFFERENT CULTIVATION CONDITIONS

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There is a worldwide consensus in the food industry that aims to replace synthetic ingredients with natural ingredients for health promotion. At the same time, the industrial relevance of microalgae as sources of an extensive spectrum of bioproducts and as promising raw materials for the production of natural additives is constantly growing. The interest is fundamentally justified in the chemical composition of biomass, such as carotenoids and limonene, due to their bioactive activities with beneficial effects on health. Since these metabolites have an excellent perspective for industrial growth and development, production with greater potential is sought through optimized cultivation systems and conditions, with the subsequent challenge being the development of bioprocesses that link scientific results to commercial needs. In this context, this study aimed to evaluate the profile of carotenoids produced by the microalgae *Desertifilum* spp. in phototrophic, heterotrophic and mixotrophic cultivation, and to investigate the formation of the volatile compound limonene, derived from the microalgae *Desertifilum* spp., *Chlorella sorokiniana*, *Chlorella vulgaris*, *Scenedesmus bijuga*, *Scenedesmus obliquus* and *Spirulina* sp., and its aroma properties. Through the study, it was found that the microalgae *Desertifilum* spp. exhibits a strong potential for the production of carotenoids, with the majority being the forms of all-*trans*-β-carotene, all-*trans*-zeaxanthin and all-*trans*-equinenone, respectively. In this sense, the mixotrophic cultivation showed better quantitative results when compared to the photoautotrophic and heterotrophic cultivation. At the same time, the presence of the bioactive compound limonene was identified in the volatile fraction of the six microalgae, contributing positively to the citrus and mint aroma, with the microalgae *Scenedesmus bijuga* being the one with the greatest quantitative potential. Thus, the study made it possible to demonstrate that microalgae are sources of non-volatile (carotenoids) and volatile (limonene) bioproducts, compounds that, according to the literature, have important bioactive and/or nutraceutical properties, whose economic importance is associated with a wide range applications in the food and biomedical industries. Additionally, the book chapter “Recent advances in the bioaccessibility and bioavailability of algal bioactive compounds” (Chapter 4) will be published as complementary research to this work.

Keywords: Bioactive compounds. Microalgal cultivation. Production of carotenoids. Production of limonene.

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INTRODUÇÃO

O mercado consumidor atual tem demonstrado interesse cada vez maior em hábitos alimentares saudáveis. Buscando adequação, há um consenso crescente na indústria mundial de alimentos que visa a substituição de ingredientes sintéticos por ingredientes naturais para prevenção e promoção da saúde, investindo assim em novas tecnologias. Apesar de pouco exploradas, está em constante crescimento a relevância industrial das microalgas como fontes de um amplo espectro de bioprodutos e como promissoras matérias-primas para sistemas de produção de aditivos naturais (FERNANDES et al., 2020b).

As microalgas apresentam grande potencial para atender às necessidades dietéticas da população, gerando amplo interesse por esses micro-organismos pela sua composição nutricional e pela presença de compostos bioativos, como os carotenoides (compostos orgânicos não voláteis) e o limoneno (composto orgânico volátil). Esses biocompostos apresentam propriedades funcionais e/ou nutracêuticas que previnem e promovem a saúde, detendo inúmeras oportunidades para o desenvolvimento de produtos inovadores e sustentáveis nas indústrias de alimentos, farmacêuticas e também de cosméticos (FERREIRA et al., 2021; IBÁÑEZ; SANCHEZ-BALLESTER; BLÁZQUEZ, 2020; MOLINO et al., 2020; RU et al., 2020; TANG et al., 2020; SOARES et al., 2019; VIEIRA et al., 2018; MATOS et al., 2017).

Estudos sobre carotenoides e limoneno tem demonstrado efeitos preventivos e protetores das moléculas em várias doenças crônicas, como diabetes mellitus, síndrome metabólica, câncer e doenças cardiovasculares (SATHASIVAM; KI, 2018; VIEIRA et al., 2018).

Esses metabólitos derivados das microalgas por terem uma ótima perspectiva para o crescimento e desenvolvimento industrial (BILAL et al., 2017), busca-se uma produção com maior potencial através de sistemas de cultivo e condições otimizadas. Nesse sentido, estratégia mais eficientes ainda estão em andamento, sendo necessários estudos para identificar a composição e a concentração de bioprodutos presentes em microalgas cultivadas em comparação a diferentes condições de cultivo para que atinja características de escala proporcionais às demandas industriais desses biocompostos naturais.

OBJETIVOS

OBJETIVO GERAL

Considerando que a biomassa microalgal possui um elevado teor de compostos bioativos, podendo ser otimizado através de novas tecnologias, este estudo teve como objetivo geral avaliar o perfil de carotenoides produzidos em diferentes condições de cultivo e explorar o potencial de diferentes microalgas na produção do composto orgânico volátil limoneno, visando uma maior produtividade para fins industriais.

OBJETIVOS ESPECÍFICOS

Identificar e quantificar os carotenoides presentes na microalga *Desertifilum* spp. submetida a diferentes condições de cultivo microalgal: fototróficas, heterotróficas e mixotróficas;

Identificar e quantificar o composto orgânico volátil limoneno nas microalgas *Desertifilum* spp., *Chlorella sorokiniana, Chlorella vulgaris, Scenedesmus bijuga, Scenedesmus obliquus*e e *Spirulina* sp., e, avaliar suas propriedades de aroma.

CAPÍTULO 1

REVISÃO BIBLIOGRÁFICA

1. MICROALGAS

As microalgas abrangem um grupo diverso de micro-organismos com cerca de 72.500 espécies catalogadas de forma consistente (JACOB-LOPES et al., 2019). São micro-organismos fotossintéticos que podem crescer rapidamente e viver em condições adversas devido à sua estrutura unicelular ou multicelular simples (MATA; MARTINS; CAETANO, 2010).

Nesse sentido, são classificadas de acordo com suas estruturas celulares, estando divididas em dois grupos: micro-organismos procariontes, como uma das representantes as Cyanophyta (cianobactérias), que incluem a *Spirulina* spp. e *Desertifilum* spp.; e as microalgas eucariontes, tendo como exemplo as Chlorophyta (algas verdes), que incluem a *Chlorella* spp. e *Scenedesmus* spp., dentre outras, nas quais as Chlorophyta e Cyanophyta são as que se destacam em termos de exploração biotecnológica (HAMIDI et al., 2020; MUTANDA et al., 2011; MATA; MARTINS; CAETANO, 2010).

Tendo em vista essa diversidade, há um interesse global na exploração dos processos e produtos baseados em microalgas, fundamentalmente apoiado na composição química da biomassa microalgal como os pigmentos (carotenoides) (JACOB-LOPES et al., 2019), devido suas atividades bioativas importantes (NASCIMENTO et al., 2020), além de serem consideradas as melhores fontes comerciais de carotenoides naturais (SALBITANI et al., 2020), utilizados em indústrias alimentícias, farmacêuticas, nutracêuticas, cosméticas, de suplementos e corantes biotecnológicos (HAMIDI et al., 2020; KHAN; SHIN; KIM, 2018).

Além dos carotenoides, outro importante coproduto microalgal é o composto orgânico volátil limoneno, também amplamente aplicado em alimentos, produtos farmacêuticos, cosméticos, biomateriais e biocombustíveis, devido à sua fragrância agradável, propriedades físico-químicas favoráveis e atividades biológicas especiais (REN et al., 2020).

O interesse biotecnológico de muitos desses organismos autotróficos foi reconhecido, sendo um desafio subsequente o desenvolvimento de bioprocessos que vinculem os resultados científicos às necessidades comerciais. Ainda são necessárias tecnologias inovadoras para superar seu desempenho satisfatório, a exemplo de questões relacionadas ao desenvolvimento de linhagens de microalgas e sistemas de cultivo. Nesse contexto, o uso de fotobiorreatores para realizar processos baseados em microalgas impulsionou o desenvolvimento de diferentes configurações de reatores para atingir características de escala proporcionais às demandas industriais (DEPRÁ et al., 2019).

Visando esta produção e sabendo que diferentes tipos de cultivo de microalgas contem diferentes componentes químicos e, portanto, podem afetar o crescimento das células das microalgas e o acúmulo dos seus coprodutos, biotecnologias, incluindo fotobiorreatores de alta eficiência e condições de crescimento otimizadas, são aplicadas no cultivo microalgal e podem ser realizadas em diferentes condições de cultivo gerando modificações metabólicas (XIE et al., 2019; GONG; BASSI, 2016).

2. SISTEMAS E CONDIÇÕES DE CULTIVO MICROALGAL

O cultivo microalgal em fotobiorreatores (FBR’s) - sistema fechado, é o único método que pode ser projetado e otimizado para fornecer as melhores condições de crescimento para cepas específicas de micro-organismos e assim, melhorar a eficiência da produção de biomassa (BOROWIAK et al., 2021). Segundo os mesmos autores, um fator diferencial nesse sistema é a capacidade de alterar e controlar com precisão os parâmetros operacionais (temperatura, intensidade de iluminação e faixa de comprimento de onda e o pH do meio) do processo de cultivo, sendo a luz e a temperatura os fatores mais importantes, uma vez que são vitais para o crescimento de microalgas e produção de biomassa.

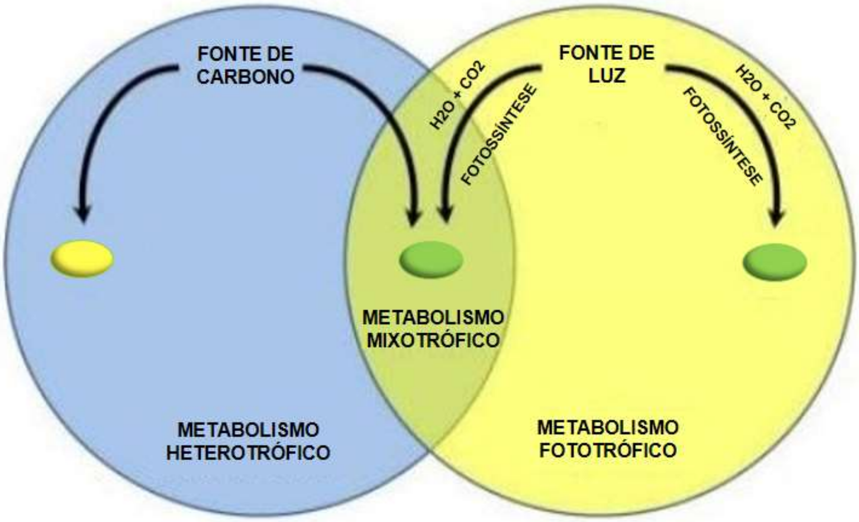
O controle dos parâmetros operacionais é bastante lucrativo quando o estresse fisiológico dos micro-organismos induz a produção ou aumento de metabólitos desejados. Em comparação com os sistemas abertos, os FBR’s requerem menos espaço, reduzem as chances de contaminação e produzem a cultura independente das condições ambientais, com maior homogeneidade e produtividade da biomassa microalgal. Por esse motivo, o uso de sistemas fechados em maior escala estão se tornando cada vez mais utilizados, especialmente na produção de produtos de alto valor agregado, como biofármacos, cosméticos ou componentes de produtos alimentares saudáveis. Sendo assim, as biotecnologias, incluindo fotobiorreatores de alta eficiência e condições otimizadas de crescimento, são aplicadas no cultivo microalgal (BOROWIAK et al., 2021; GONG; BASSI, 2016), podendo ser realizado sob condições fototróficas, heterotróficas e mixotróficas (XIE et al., 2019), conforme ilustrado na Figura 1.

O procedimento mais comum para o cultivo de microalgas é o fotoautotrófico, o qual envolve a conversão de CO_2 e luz em componentes orgânicos via fotossíntese (VERMA et al., 2020). Porém, apresenta limitações na produção de biomassa pelo autossombreamento celular que dificulta a disponibilidade de luz no final do crescimento (LIN; WU, 2015; CHEIRSILP; TORPEE, 2012).

Como alternativa, o cultivo heterotrófico de microalgas possibilita o crescimento com ausência de luz, com uso de carbono orgânico por vias respiratórias e fermentativas combinadas (CHEN; WANG; WANG, 2020; VERMA et al., 2020).

Enquanto, o cultivo mixotrófico é uma variante do cultivo heterotrófico, em que o CO_2 e o carbono orgânico são assimilados simultaneamente, para produzir biomassa e metabólitos via metabolismos respiratório e fotossintético (CHEN; WANG; WANG, 2020).

Figura 1 - Metabolismo de microalgas em diferentes condições de cultivo: fototrófico, heterotrófico e mixotrófico



Fonte: Adaptação de PINA et al. (2021)

Pesquisas com diferentes cepas de microalgas indicam que o cultivo mixotrófico promove níveis ainda mais elevados de biomassa e, consequentemente, maior produtividade dos seus coprodutos (VERMA et al., 2020; ZHANG et al., 2017), como carotenoides e compostos orgânicos voláteis. Assim, outra estratégia para melhorar o uso eficiente da luz ou eliminar sua necessidade pelas células, reduzindo os custos de produção de biomassa microalgal, seria o cultivo mixotrófico (LIN; WU, 2015; CHEIRSILP; TORPEE, 2012), com intuito de gerar uma maior produção de compostos bioativos microalgais.

3. COMPOSTOS BIOATIVOS MICROALGAIS

Compostos bioativos são constituintes extras nutricionais e ocorrem tipicamente em pequenas quantidades nos alimentos. Apresentam influências biológicas decorrida de efeitos proporcionados por sua estrutura química. O estudo desses biocompostos inspirou o conceito de alimentos funcionais, sendo um alimento natural ou processado, que, além de seus nutrientes, possua componentes que atuem no metabolismo e na fisiologia humana, promovendo efeitos benéficos à saúde, devendo ser seguro para consumo sem supervisão médica. São capazes de influenciar nas atividades celulares que modificam e reduzem o risco de diversas doenças crônicas-degenerativas associadas ao estresse oxidativo ou desregulação do metabolismo lipídico plasmático, melhorando a qualidade e a expectativa de vida (COUCH et al., 2017; GARCÍA-BLANCO et al., 2017; BHAT et al., 2015; MARTINEZ-FLORES et al., 2015; COSTA-SINGH; BITENCOURT; JORGE, 2012; SCHWINGSHACKL; HOFFMANN, 2012; FERNÁNDEZ et al., 2011; ARON; KENNEDY, 2008).

Esses alimentos também vêm sendo denominados de nutracêuticos quando apresentam a capacidade de prevenção e cura de enfermidades, enquanto os funcionais apenas reduzem o risco do aparecimento de doenças (TRIPATHI, 2014). O reconhecimento científico dos nutracêuticos vem crescendo cada vez mais, com interesse em encontrar novas opções terapêuticas, utilizando novas tecnologias (ALALI et al., 2021).

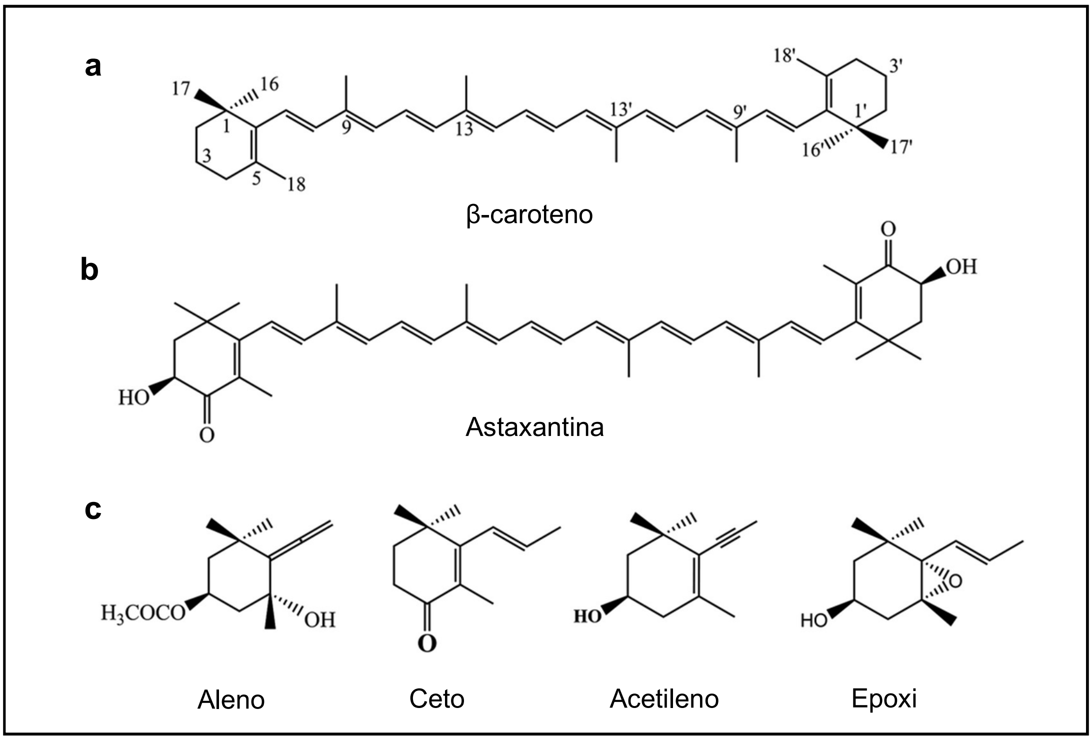
Recentemente, as microalgas receberam atenção devido a capacidade, tanto em produzir novos metabólitos bioativos, incluindo uma ampla gama de carotenoides diferentes e de compostos orgânicos voláteis, como o limoneno, que podem proporcionar benefícios à saúde, quanto ao seu funcionamento como agentes bioativos e nutracêuticos, pois apresentam atividade antioxidante, anti-inflamatória, anticarcinogênica, dentre outros efeitos benéficos à saúde (HAMIDI et al., 2020; IBÁÑEZ; SANCHEZ-BALLESTER; BLÁZQUEZ, 2020; JACOB-LOPES et al., 2019; SATHASIVAM; KI, 2018; ZHANG et al., 2014; GUEDES et al., 2011).

3.1. Carotenoides

Os carotenoides são compostos bioativos responsáveis pelas tonalidades amarela, laranja e vermelha dos alimentos. Esse efeito decorre de sua estrutura básica que consiste em um esqueleto linear e simétrico com uma série de duplas ligações conjugadas (DLC), denominado cromóforo de absorção de luz, sendo necessário sete DLC para apresentarem coloração. A mudança de cor ocorre à medida que o número de duplas ligações aumenta, pois há um deslocamento no espectro de absorção da molécula, o que caracteriza cada carotenoide (RODRIGUEZ-AMAYA, 2015; IRAZUSTA et al., 2013; JOMOVA; VALKO, 2013; MALDONADE; RODRIGUEZ-AMAYA; SCAMPARINI, 2008; RODRIGUEZ-AMAYA, 2001).

A estrutura desses compostos são geralmente tetraterpenos (C_{40}) constituídos por 8 unidades isoprenoides (C_5) e podem ser classificados com base nos grupamentos funcionais; xantofilas são carotenoides que contem oxigênio como grupo funcional tais como epoxi (violaxantina, neoxantina e fucoxantina), hidroxi (luteina e zeaxantina), ceto (astaxantina, cantaxantina e equinenona) e metoxi (spiriloxantina); e os carotenoides, que contém apenas cadeia de hidrocarboneto com grupo funcional em suas estruturas, tais como α-caroteno, β-caroteno e licopeno (Figura 2). São pigmentos lipossolúveis produzidos como metabólitos secundários e possuem importantes propriedades fisiológicas, com efeitos benéficos para saúde, estando entre os fitoquímicos bioativos creditados na redução do risco de doenças degenerativas (LAFARGA; CLEMENTE; GARCIA-VAQUERO, 2020; GILLE et al., 2018; MAGOSSO et al., 2016; GUL et al., 2015; ZAGHDOUDI et al., 2015; BRITTON; LIAAEN-JENSEN; PFANDER, 2008).

Figura 2 - Estruturas químicas: a. Caroteno. b. Xantofila. c. Grupos terminais cíclicos exclusivos de microalgas



Fonte: Adaptação de ZHANG et al. (2014)

Na classe dos carotenos, destaca-se o β-caroteno que oferece uma gama de benefícios à saúde, incluindo a redução do risco de doenças cardíacas e alguns tipos de câncer, aumento do desempenho do sistema imunológico, prevenção de fibrose hepática e de cegueira noturna, propriedades anti-neurodegenerativas, fotoproteção da pele contra a luz ultravioleta (UV), prevenção de síndrome coronariana aguda e crônica, no crescimento e desenvolvimento embrionário, devido à sua atividade como provitamina A (fornecida por alguns carotenoides) (HAMIDI et al., 2020; GILLE et al., 2018; KHAN; SHIN; KIM, 2018; MAGOSSO et al., 2016; GUL et al., 2015; RODRIGUEZ-AMAYA, 2015; MALDONADE; RODRIGUEZ-AMAYA; SCAMPARINI, 2008).

Todavia, outros mecanismos são também conhecidos, como a absorção de luz e a filtração de luz azul na saúde ocular. A bioatividade dos carotenoides maculares dietéticos (classe das xantofilas: luteína e zeaxantina) oferece meios de fortificar as defesas antioxidantes da mácula, ao se acumularem, reduzindo assim o risco de catarata e degeneração macular relacionada à idade e/ou sua progressão

(EISENHAUER et al., 2017; KOUSHAN et al., 2013; SABOUR-PICKETT et al., 2012; SENTANI; RODRIGUEZ-AMAYA, 2007).

A proteção dos compostos bioativos (carotenoides) contra algumas doenças está associada especialmente pelo seu elevado potencial antioxidante, pela capacidade de sequestrar o oxigênio singlete e reagir com radicais livres, visto que estes podem causar efeitos prejudiciais, como o desencadeamento de muitas doenças, incluindo câncer, doença das artérias coronárias, obesidade, diabetes, acidente vascular cerebral isquêmico, doença de Alzheimer, entre outras (KHAN; SHIN; KIM, 2018; TREJO-SOLÍS et al., 2013).

Estudos sugerem que os carotenoides microalgais apresentem maior atividade antioxidante em relação às fontes convencionais, devido à presença de carotenoides exclusivos, que possuem efeito bato crômico, como é o caso da equinenona e da cantaxantina com 12 e 13 DLC respectivamente, em sua estrutura química, uma vez que a extensão do cromóforo está intimamente relacionada ao aumento da atividade antioxidante (SATHASIVAN; KI, 2018; KLASSEN; FOGHT, 2011; UENOJO; MARÓSTICA JUNIOR; PASTORE, 2007; ALBRECHT et al., 2000).

O corpo humano possui seu próprio sistema antioxidante enzimático que impede o estresse oxidativo e protege o corpo dos efeitos danosos dos radicais livres. No entanto, quando os radicais livres superam os antioxidantes naturais do corpo, ocorre o estresse oxidativo, que é uma das principais causas de várias outras doenças, inclusive, com risco de vida. Em tais casos, a captação de antioxidantes externos é de importância crucial. Por isso, muitos compostos antioxidantes naturais têm sido relatados como forte atividade antioxidande, como os carotenoides e vitaminas, que podem prevenir danos oxidativos às células e tecidos (KHAN; SHIN; KIM, 2018).

Um carotenoide bioativo de origem em microalgas, que pode ter diversas aplicações industriais, é a equinenona, considerada um antioxidante ainda mais potente que o β-caroteno. Esses carotenoides secundários não participam da fotossíntese e são caracterizados pela localização extra-tilacóide. Essa característica metabólica contribui para a grande variedade estrutural de carotenoides de microalgas (PATIAS et al., 2017).

Nesse contexto, as microalgas são micro-organismos promissores, pois podem ser isolados diversos carotenoides, como β-caroteno, α-caroteno, zeaxantina, luteína, violaxantina, mixoxantofila, equinenona, astaxantina, cantaxantina, dentre outros, alguns dos quais são produzidos exclusivamente por microalgas, com habilidades bioativas potencializadas (FERNANDES et al., 2020a, NASCIMENTO et al., 2020; SATHASIVAM et al., 2019).

Muitas empresas multinacionais começaram a produzir vários tipos de carotenoides para uso em diferentes aplicações. Por esta razão, a produção de carotenoides é considerada uma importante oportunidade de negócio para aplicações biomédicas e industriais (SATHASIVAM; KI, 2018).

Segundo dados de produtividade de carotenoides, o mercado global está estimado em US $ 1,7 bilhão em 2022. Carotenoides como β-caroteno, astaxantina e luteína apresentam as maiores participações de mercado, as projeções demonstram que no ano de 2022 a astaxantina venha atingir o valor de US$ 426,9 milhões, o β-caroteno US$ 572,78 milhões e a luteína US$ 357,7 milhões (FERNANDES et al., 2020a).

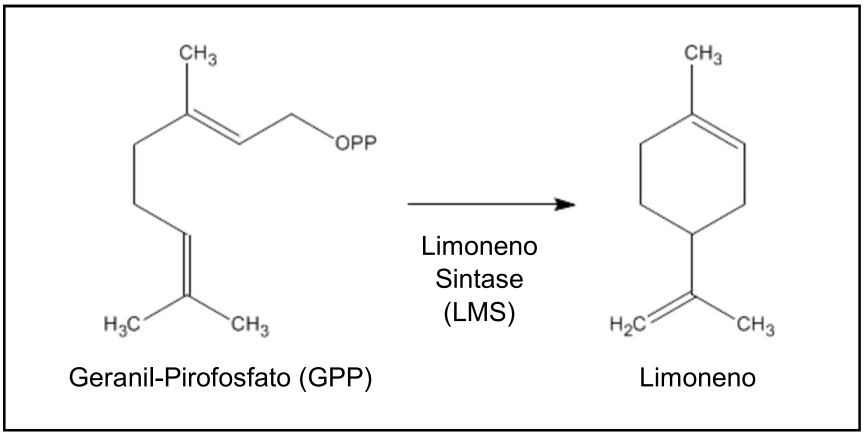
Essas perspectivas demonstram que as microalgas são matérias-primas apropriadas e lucrativas para a produção comercial de carotenoides naturais, visto que a produção comercial está aumentando, de β-caroteno, bem como de outros pigmentos menos estabelecidos, que também estão ganhando impulso no mercado global, como por exemplo, luteína, zeaxantina e fucoxantina (NOVOVESKÁ et al., 2019).

3.2. Compostos Orgânicos Voláteis

Os compostos orgânicos voláteis (COV’s) são metabólitos secundários que podem ser utilizados como importante fonte de insumos na indústria de química fina, com a obtenção de diferentes classes de compostos (SANTOS et al., 2016). Dependendo da espécie, cultura e condições ambientais, as microalgas são capazes de produzir uma variedade de COV’s, incluindo os terpenóides, um dos principais tipos de COV’s de plantas e algas (YE et al., 2018).

Algumas espécies de microalgas produzem limoneno, um hidrocarboneto monoterpeno monocíclico volátil ($C_{10}H_{16}$), a partir da via isoprenóide pela ação da enzima limoneno sintase (LMS) do geranil-pirofosfato (GPP) que é ciclizado, formando o limoneno (KIYOTA et al., 2014; ANTONELLI et al., 2020), ilustrado na Figura 3.

Figura 3 - Formação do Limoneno a partir da via isoprenóide pela ação da enzima Limoneno Sintase (LMS) do Geranil-Pirofosfato (GPP) ciclizado



Fonte: Adaptação de UNKEFER et al. (2017)

Embora a química conceda acesso a uma ampla gama de aromas sintéticos, a tendência atual dos consumidores é escolher produtos naturais, considerados mais saudáveis e ecologicamente corretos. Algumas destas fragrâncias e sabores naturais podem ser encontrados em plantas (RAPINEL et al., 2020), cianobactérias e algas (SINGH et al., 2017).

Sabores e fragrâncias são misturas complexas de um grande número de compostos voláteis responsáveis pelas propriedades organolépticas de um produto. É importante destacar que os sabores não têm nenhum valor nutricional, mas continuam a ser essenciais na alimentação, por exemplo, para estimular o apetite, além de permitir a distinção entre alimentos frescos e impróprios para consumo (RAPINEl et al., 2020).

O limoneno pela segurança comprovada, tem sido amplamente explorado como agente aromatizante e adjuvante nas indústrias de alimentos e bebidas, bem como em cosméticos para a formulação de perfumes e outros produtos de higiene pessoal (IBÁÑEZ; SANCHEZ-BALLESTER; BLÁZQUEZ, 2020).

O aroma do limoneno pode ser classificado como cítrico, de menta (CHIŞ et al., 2020), frutado e floral, podendo contribuir positivamente para o aroma da biomassa microalgal (ZUO, 2019), mesmo que tenham valores de limiar relativamente baixos (EKPA; FOGLIANO; LINNEMANN, 2020). Em razão da descrição de aroma atraente, é utilizado na indústria de alimentos, como por exemplo, para o aroma de bebidas de limão ou lima (TETALI, 2019), tradicionalmente utilizado como composto aromatizante em produtos com sabor cítrico (JONGEDIJK et al., 2016).

Além disso, também demonstra um amplo espectro de benefícios à saúde. Estudos *in vivo* e *in vitro* abordam os efeitos de seu potencial bioativo, como redução da inflamação alérgica do trato respiratório, efeito gastroprotetor, aumento da produção de muco gástrico, no tratamento de úlcera gástrica, de colite, na diminuição dos níveis de colesterol LDL e aumento do HDL, ação anti-inflamatória, redução da quimiotaxia de neutrófilos, antidiabético, com antiglicante, atividade antioxidante, com níveis reduzidos de peróxido de hidrogênio em baixas concentrações, além de ter efeito quimiopreventivo contra vários tipos de câncer, entre outras atividades, contribuindo assim para novos ensaios clínicos, visto que é crescente o interesse pelas atividades biológicas do limoneno (VIEIRA et al., 2018), além da produção comercial.

As microalgas desempenham um papel importante na economia mundial atualmente (DEPRÁ et al., 2019). A produção global de compostos aromáticos como o limoneno está aumentando a uma taxa significativa e o tamanho do mercado está projetado para ultrapassar 1,9 bilhão (USD) até 2024. Considerando a aplicação generalizada e as crescentes demandas do mercado, há uma necessidade urgente de fornecimento estável e em massa do limoneno e seus derivados (REN et al., 2020). Sendo assim, estudos com diferentes microalgas são fundamentais para analisar a produção de seus biocompostos com vistas a aplicações industriais e biomédicas.

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CAPÍTULO 2

CAROTENOIDS PROFILE OF *DESERTIFILUM* spp. IN MIXOTROPHIC CONDITIONS

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Carotenoids profile of *Desertifilum* spp. in mixotrophic conditions

Perfil de carotenoides de *Desertifilum* spp. em condições mixotróficas

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ABSTRACT

The aim of this study was to identify and quantify the carotenoids present in the biomass of the microalgae *Desertifilum* spp. grown under mixotrophic conditions, as well as to compare the concentration of major carotenoids with other cultivation methods, aiming at the production of these compounds for industrial purposes. The carotenoid profile was evaluated by high performance liquid chromatography coupled to a matrix of photodiodes and mass spectrometry detectors. A total of eleven carotenoids were identified in the biomass of myxotrophic cultivation (1645.92 $\mu g \cdot g^{-1}$). All-*trans*-β-carotene (338.71 $\mu g \cdot g^{-1}$), all-*trans*-zeaxanthin (269.13 $\mu g \cdot g^{-1}$) and all-*trans*-echinenone (216.93 $\mu g \cdot g^{-1}$) were the majority. As for productivity, the mixotrophic cultive was 2.3 times greater than the photoautotrophic cultivation (714.3 $\mu g \cdot g^{-1}$) and 9 times greater than the heterotrophic cultivation (183.03 $\mu g \cdot g^{-1}$). The observed results show that the microalgae *Desertifilum* spp. it has great potential for the production of carotenoids, the mixotrophic cultivation being the one that provides the highest yield.

Keywords: Mixotrophic cultivation, Mass spectrometry, Microalgae, Carotenoids production.

RESUMO

O objetivo deste estudo foi identificar e quantificar os carotenoides presentes na biomassa da microalga *Desertifilum* spp. cultivada em condições mixotróficas, bem como comparar a concentração dos carotenoides majoritários com outros métodos de cultivo, visando a produção desses compostos para fins industriais. O perfil de carotenoides foi avaliado por cromatografia líquida de alta performance acoplada a uma matriz de fotodiodos e detectores de espectrometria de massa. Um total de onze carotenoides foram identificados na biomassa do cultivo mixotrófico (1645.92 $\mu g \cdot g^{-1}$). O all-*trans*-β-caroteno (338.71 $\mu g \cdot g^{-1}$), all-*trans*-zeaxantina (269.13 $\mu g \cdot g^{-1}$) e all-*trans*-equinenona (216.93 $\mu g \cdot g^{-1}$) foram os majoritários. Quanto a produtividade, o cultivo mixotrófico foi 2,3 vezes maior que o cultivo fotoautotrófico (714.3 $\mu g \cdot g^{-1}$) e 9 vezes maior que o cultivo heterotrófico (183.03 $\mu g \cdot g^{-1}$). Os resultados observados mostram que a microalga *Desertifilum* spp. possui grande potencial para produção de carotenoides, sendo o cultivo mixotrófico aquele que proporciona maior rendimento.

Palavras chave: Cultivo mixotrófico, Espectrometria de massas, Microalgas, Produção de carotenoides.

1 INTRODUCTION

Biotechnologies including high efficiency photobioreactors and optimized growth conditions are applied in microalgal cultivation (Gong & Bassi, 2016), and can be carried out under phototrophic, heterotrophic and mixotrophic conditions (Xie et al., 2019).

The most common procedure for cultivating microalgae is the photoautotrophic, which involves the conversion of CO_2 and light into organic components via photosynthesis (Verma et al., 2020). However, it has limitations in the production of biomass due to cellular self-shading, which hinders the availability of light at the end of growth (Cheirsilp & Torpee, 2012).

As an alternative, the heterotrophic cultivation of microalgae enables growth in the absence of light, with the use of organic carbon through combined respiratory and fermentative routes (Chen, Wang, & Wang, 2020; Verma et al., 2020).

Meanwhile, mixotrophic cultivation is a variant of heterotrophic cultivation, where CO_2 and organic carbon are assimilated simultaneously, to produce biomass and metabolites via respiratory and photosynthetic metabolisms (Chen, Wang, & Wang, 2020).

Research indicates that the mixotrophic cultivation of microalgae promotes higher levels of biomass (Verma et al., 2020; Zhang et al., 2017). Thus, another strategy to improve the efficient use of light or eliminate its need for cells by reducing the costs of producing microalgal biomass would be myxotrophic cultivation (Cheirsilp & Torpee, 2012).

In this sense, a more efficient cultivation strategy is still in progress investigation. This way, the objective of this study is to identify the composition and the concentration of the carotenoids present in the microalgae *Desertifilum* spp. grown under mixotrophic conditions, as well as to compare the production of the main carotenoids with other cultivation methods (phototrophic and heterotrophic), with the purpose of obtaining a larger production of these compounds for industrial purposes.

2 MATERIAL AND METHODS

2.1 MICROALGAE CULTURE AND BIOMASS PRODUCTION

Axenic cultures of *Desertifilum* spp. (MK307822) were originally isolated from the lakes at the Cuatro Cienegas desert in Mexico (26°59′ N, 102°03′ W). The biomass productions were made in mixotrophic conditions. The cultivations were performed in a bubble column photobioreactor operating under a batch regime, fed on 1.5 L of BG-11 medium supplemented with 5 $g.L^{-1}$ of fructose (Braun-Grunow medium) (Rippka et al., 1979). The experimental conditions were as follows: initial cell concentration of 100 $mg.L^{-1}$, isothermal reactor operating at a temperature of 25 °C, photon flux density of 25 $\mu mol.m^{-2}.s^{-1}$, and a light cycle of 24:0h (light:dark) (Patel et al., 2019). The CO_2/air mixture was adjusted to achieve the 5% concentration of carbon dioxide in the airstream, through three rotameters that measured the flow rates of carbon dioxide, air and the mixture of gases, respectively. The biomass was separated from the culture medium by centrifugation (10000 rpm, 10 min, 10 °C), the supernatant was discarded, and the remaining biomass was freezing at -18 °C for 24 hours.

After, the biomass was freeze-dried for 24 h at -50 °C above -175 µm Hg and then stored at -18 °C until analysis.

2.2 CAROTENOIDS EXTRACTION

The carotenoids were exhaustively extracted from the freeze-dried sample (0.1 ± 0.02 g) first with ethyl acetate and then with methanol in a mortar with a pestle followed by centrifugation (Hitachi, Tokyo, Japan) for 7 min at 1500×g (Mandelli et al., 2012). The extraction procedure was repeated until the supernatant becomes colorless. The homogenized sample suspension was filtered through a 0.22 µm polyethylene membrane, concentrated in a rotary evaporator (T < 30 °C), suspended in a mixture of petroleum ether/diethyl ether [1:1 (v/v)], and saponified overnight (16 h) with 10% (w/v) methanolic KOH (potassium hydroxide) at room temperature. The alkali was removed by washing with distilled water, and each extract was once again concentrated in a rotary evaporator, flushed with N_2 and kept at -37 °C in the dark until chromatographic analysis. All extractions were performed in triplicate.

2.3 HPLC-PDA-MS/MS CAROTENOIDS ANALYSIS

The carotenoids were analyzed by high performance liquid chromatography HPLC (Shimadzu, Kyoto, Japan) equipped with binary pumps (model LC-20AD), online degasser, and automatic injector (Rheodyne, Rohnert Park-CA, USA). The chromatograph with photodiode array detection (PDA) (model SPD-M20A) was connected in series to an atmospheric pressure chemical ionization (APCI) source (Shimadzu America, Columbia, MD, USA), and a mass spectrometer (MS) Shimadzu 8040 triple quadrupole. The pigments separation was performed on a C30 YMC column (5 µm, 250 × 4.6 mm) (Waters, Wilmington-DE, USA).

HPLC-PDA analysis was performed according to Murillo et al. (2013) with some minor modifications. The mobile phase consisted of a binary solvent mixture system. Solvent A consisted of MeOH:MTBE:H_2O (81:15:4) and solvent B MeOH:MTBE:H_2O (16:80:4), using a linear gradient program as follows: from 0 to 20 min 0% B; from 20 to 140 min, 0–100% B; from 140 to 141 min, 100 to 0% B, from 141 to 150 min, 0% B. The flow rate was set at 0.8 $mL.min^{-1}$, the injection volume was 20 µL, the column temperature was maintained at 35 ºC, the UV/Vis spectra were acquired between 220 and 700 nm, and the chromatograms were processed at 450 nm.

The MS/MS analysis was achieved according to Giuffrida, Zoccali, Giofrè, Dugo, & Mondello (2017) with adaptations, the APCI interface operated in positive (+) mode; detector voltage: 4.5 kV; interface temperature: 350 ºC; DL temperature: 250 ºC; heat block temperature: 200 ºC; 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. To improve the quality of identification, MS/MS was used simultaneously in SIM (Select Ion Monitoring) and MRM (Multiple Reaction Monitoring) modes.

The identification was performed according to the following combined information: elution order on C30 HPLC column, co-chromatography with authentic standards, UV-Vis spectrum (Spectral fine structure ($\lambda_{máx}$), ratio of the height of the longest wavelength absorption peak (III) and that of the middle absorption peak (II), ratio of the *cis* peak (AB) and the middle absorption peak (II)), and mass characteristics (protonated molecule ($[M+H]^+$) and MS/MS fragments), compared with data available in the literature (Rodrigues et al., 2014; Rodrigues, Menezes, Mercadante, Jacob-Lopes, & Zepka, 2015; Fernandes et al., 2020; Nascimento et al., 2020). Carotenoids were quantified by HPLC-PDA using five-point calibration curves.

3 RESULTS AND DISCUSSION

The separated carotenoids were identified based on the combined information obtained from chromatographic elution, co-chromatography with standards, UV-Visible, and mass spectra characteristics (Table 1).

A detailed description of the carotenoids analyzed identification has already been demonstrated for microalgae and can be found in the following studies: Rodrigues et al. (2014), Rodrigues, Menezes, Mercadante, Jacob-Lopes, & Zepka (2015), and Maroneze et al. (2020).

Table 1 describes the carotenoid profile of *Desertifilum* spp. (MK307822) grown under mixotrophic conditions. In its biomass, 11 different carotenoids were identified (4 all-*trans* carotenoids and 6 *cis* carotenoids), totaling 1645.92 $\mu g \cdot g^{-1}$ (dry wt), a substantial value when compared with the carotenoid content from other sources such as fruits and vegetables (Rodriguez-Amaya et al., 2008).

Among these, the majoritary carotenoids found were all-*trans*-β-carotene (338.71 $\mu g \cdot g^{-1}$ ± 30.44), all-*trans*-zeaxanthin (269.13 $\mu g \cdot g^{-1}$ ± 3.31) and all-*trans*-echinenone (216.93 $\mu g \cdot g^{-1}$ ± 7.87). In addition to all-*trans*-echinenone, *cis* isomers were also identified as 13-*cis*-echinenone (161.49 $\mu g \cdot g^{-1}$ ± 0.52) and 15-*cis*-echinenone (74.19 $\mu g \cdot g^{-1}$ ± 0.76), respectively.

Table 1. Characterization of carotenoids in *Desertifilum* spp. biomass obtained by HPLC-PDA-MS/MS

Peak	**Carotenoids**	**t_R** (min)[a]	**UV-vis characteristics**			**Fragment ions *(m/z)***		**Pigment content**[e]
			$\lambda_{\text{máx}}$ (nm)[b]	III/II[c] (%)	A_B/II[d] (%)	[M + H]$^+$	MS/MS	
1	15-*cis*-zeaxanthin	14.3	339, 419, 442, 468	28	60	569	551 [M + H - 18]$^+$, 533 [M + H - 18 - 18]$^+$, 477 [M + H - 92]$^+$	61.08±6.13
2	all-*trans*-zeaxanthin	16.9	425, 450, 476	37	0	569	551 [M + H - 18]$^+$, 533 [M + H - 18 - 18]$^+$, 495, 477 [M + H - 92]$^+$, 459	269.13±3.31
3	2'-dehydrodeoxymyxol	21.2	445, 473, 504	69	0	567	549 [M + H - 18]$^+$	105.25±5.92
4	all-*trans*-zeinoxanthin	25.2	423, 442, 471	44	0	553	535 [M + H-18]$^+$, 461 [M + H-92]$^+$, 361	46.64±6.79
5	15-*cis*-echinenone	36.4	330, 449	nc[f]	23	551	533 [M + H - 18]$^+$, 427, 203	74.19±0.76
6	13-*cis*-echinenone	38.0	330, 450	nc	12	551	533 [M + H - 18]$^+$, 427, 203	161.49±0.52
7	all-*trans*-echinenone	41.1	460	nc	0	551	533 [M + H - 18]$^+$, 427, 203	216.93±7.87
8	15-*cis*-β-carotene	45.5	333, 420, 443, 473	25	31	537	444 [M + H - 92]$^+$, 399, 355	71.28±0.04
9	13-*cis*-β-carotene	47.8	338, 420, 444, 471	11	46	537	444 [M + H - 92]$^+$, 399, 355	105.93±6.48
10	all-*trans*-β-carotene	58.0	425, 451, 476	28	0	537	444 [M + H - 92]$^+$, 399, 355	338.71±30.44
11	9-*cis*-β-carotene	60.6	345, 420, 446, 472	37	14	537	444 [M + H - 92]$^+$, 399, 355	195.29±2.79
	Total							1645.92

[a] t_R: Retention time on the C30 column.
[b] Linear gradient MeOH: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 (A_B) and the middle absorption peak (II).
[e] μg·g^{-1} dry wt; n = 3
[f] Not calculated.

Echinenone is considered to be stronger antioxidant than β-carotene, a bioactive carotenoid of microalgae origin, which may have diverse industrial applications. Secondary carotenoids do not participate in photosynthesis and are characterized by extra-thylakoid localization. This metabolic characteristic contributed for the great structure variety of carotenoids from microalgae (Patias et al., 2017).

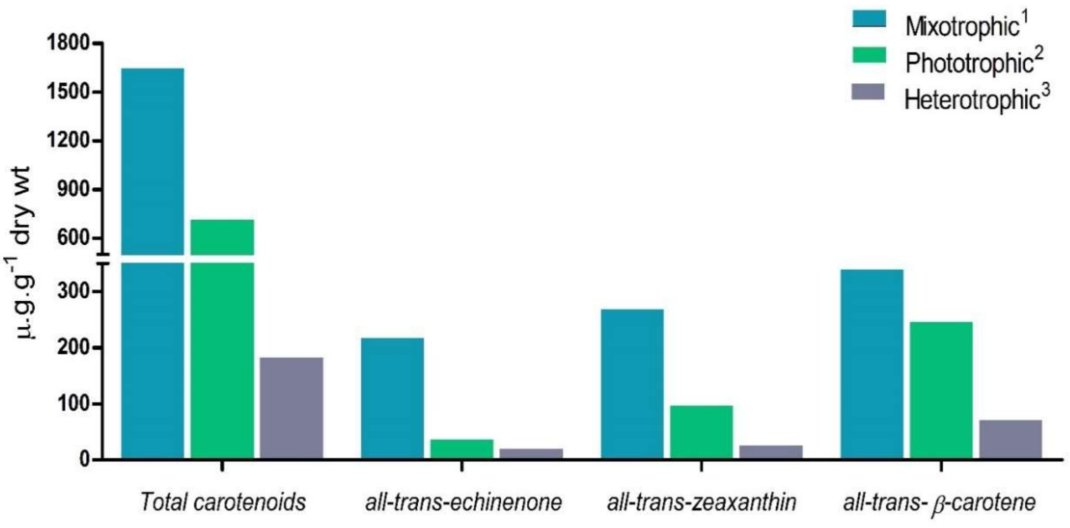
Carotenoids, including β-carotene and zeaxanthin are also valorized for a wide range of nutraceutical activities (antioxidant, prevention of age-associated macular degeneration – DMLA, among many other beneficial health effects) and functional properties such as food coloring (Lobo, Brandão, & Assis, 2021; Nascimento et al., 2021; Nascimento et al., 2020; Rumin et al., 2020; Hamidi et al., 2020; Nascimento et al., 2019), being among the main types of carotenoids used commercially in the global market (Sathasivam & Ki, 2018).

For these reasons, the worldwide demand for carotenoids has been markedly increasing. In terms of safety, people give preference to carotenoids obtained from natural sources (Zhang et al., 2014), with microalgae being considered one of the best commercial sources of natural carotenoids (Salbitani et al., 2020).

In this sense, the microalgae *Desertifilum* spp. has a characteristic carotenoid profile for this species under specific growing conditions, with the potential to be used as a source of bioactive compounds.

A detailed description of carotenoid identification was already reported by Rodrigues et al. (2015) and Rodrigues et al. (2014) in studies of our research group with the same cyanobacteria (*Desertifilum* spp., which is phylogenetically similar to *Phormidium*) (Maroneze et al., 2020), grown under phototrophic and heterotrophic conditions. These data were utilized as comparative with the mixotrophic cultivation carried out in this study to investigate obtain major carotenoid production (Figure 1).

Figure 1. Comparison between *Desertifilum* spp. carotenoids obtained from different types of cultivation



[1]Data obtained in the present study
[2]According to Rodrigues et al. (2015)
[3]According to Rodrigues et al. (2014)

Differents types of cultivation of microalgae contain different chemical components, and can thus affect both microalgae cell growth and carotenoids accumulation (Xie et al., 2019). As shown in Fig. 1, the myxotrophic cultivation displayed the highest productivity of total carotenoids in dry biomass. Among the major carotenoids, myxotrophic cultivation was 2.3 times lager than photoautotrophic cultivation (total 714.3 μg·g^{-1}) and 9 times lager than heterotrophic cultivation (total 183.03 μg·g^{-1}).

Regarding the production of majoritary carotenoids, all-*trans*-echinenone in myxotrophic cultivation (216.93 μg·g^{-1}) was 6 times lager than photoautotrophic cultivation (36.06 μg·g^{-1}) and 10.9 times lager than heterotrophic cultivation (19.87 μg·g^{-1}). The all-*trans*-zeaxanthin in myxotrophic cultivation (269.13 μg·g^{-1}) was 2.8 times lager than photoautotrophic cultivation (96.54 μg·g^{-1}) and 10.3 times lager than heterotrophic cultivation (26.25 μg·g^{-1}). And all-*trans*-β-carotene in myxotrophic cultivation (338.71 μg·g^{-1}) was 1.4 times lager than photoautotrophic cultivation (246.54 μg·g^{-1}) and 4.8 times lager than heterotrophic cultivation (70.72 μg·g^{-1}).

The other carotenoids identified in common, also presented smaller amounts in the photoautotrophic and heterotrophic cultures, when compared to the mixotrophic cultivation. Similar results were described by Gong & Huang (2020) for the same mixotrophic, phototrophic and heterotrophic crops, in different strains of microalgae. The dry weight of myxotrophic and heterotrophic cells increased progressively faster than photoautotrophic cells.

Zhang et al. (2017) also concluded that the biomass productivity of mixotrophic cultivation were higher than the sum of photoautotrophic and heterotrophic cultivation, which indicating the synergistic effects between photosynthesis and aerobic respiration.

To achieve the high productivity of compounds, such as carotenoids, the high growth rate of microalgae must be guaranteed to obtain high biomass productivity, mixotrophic cultivation being considered a promising model because it has a high growth rate and biomass yield, shortening the growth cycle and loss of biomass and reduced photoinhibition (Zhang et al., 2020).

Therefore, a strategy where the microalgae cells are cultivated under mixotrophic conditions to promote cell growth and for carotenoids accumulation, will likely effectively improve productivity, achieving sustainable carotenoids production.

4 CONCLUSION

The microalgae *Desertifilum* spp. (MK307822) exhibits strong potential for carotenoids production. In this sense, mixotrophic cultivation presents better results when compared to cultivation photoautotrophic and heterotrophic, respectively. The findings of this study will help to improve microalgae-based carotenoids production to be explored and applied for industrial purposes to better meet the current demand.

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CAPÍTULO 3

LIMONENE PRODUCTION IN MICROALGAL PHOTOAUTOTROPHIC CULTIVATION

O artigo será submetido para a revista “Brazilian Journal of Development”

Limonene production in microalgal photoautotrophic cultivation

Produção de limoneno em cultivo fotoautotrófico microalgal

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ABSTRACT

There is a worldwide consensus in the food industry that aims to replace synthetic ingredients with natural ingredients for health promotion. The industrial relevance of microalgae as sources of extensive spectrum of bioproducts and as promising raw materials for the production of natural additives is constantly growing. Limonene, due to its proven safety, makes it widely exploitable in the food, cosmetics and pharmaceutical industries, for exhibiting vast biological activity, with beneficial effects on health. Some microalgae produce this biocompound from the isoprenoid pathway through the action of the enzyme limonene synthase (LMS) from geranyl-pyrophosphate (GPP). Therefore, the aim of the study was to investigate the formation of the volatile compound limonene, derived from six microalgae, from photoautotrophic cultivation, as well as to evaluate its odor properties. The experiment was carried out in a bioreactor with BG11 medium. The incubation conditions used were 25°C, photon flux density of 25 μmol.m^{-2}.s-^{1}, aeration of 1VVM (air volume per volume of medium per minute) and light cycle of 24:0h (bright:dark). Volatiles were isolated by headspace solid phase microextraction, separated by gas chromatography, identified by mass spectrometry (SPME-GC-MS) and their odor significance was evaluated based on the NIST spectrum library. Limonene was identified in the volatile fraction of the six microalgae, contributing positively to the aroma citrus and mint, demonstrating high biotechnological capacity to obtain an alternative of this natural biocompound with commercial viability, with the microalgae *Scenedesmus bijuga* being the one with the greatest quantitative potential.

keywords: Aroma; Bioactive compounds; Microalgae; Monoterpene; Volatile organic compounds

RESUMO

Existe um consenso mundial na indústria de alimentos que visa a substituição de ingredientes sintéticos por ingredientes naturais para promoção da saúde. Está em constante crescimento a relevância industrial das microalgas como fontes de um extensivo espectro de bioprodutos e como promissoras matérias-primas para produção de aditivos naturais. O limoneno pela segurança comprovada, o torna amplamente explorável nas indústrias de alimentos, cosméticos e farmacêuticos, por exibir vasta atividade biológica, com efeitos benéficos à saúde. Algumas microalgas produzem esse biocomposto a partir da via dos isoprenóides pela ação da enzima limoneno sintase (LMS) a partir do geranil-pirofosfato (GPP). Diante disso, o objetivo do estudo foi investigar a formação do composto volátil limoneno, derivado de seis microalgas, a partir de cultivo fotoautotrófico, bem como avaliar suas propriedades de odor. O experimento foi realizado em biorreator com meio BG11. As condições de incubação usadas foram 25°C, densidade de fluxo de fótons de 25 μmol.m^{-2}.s-^{1}, aeração de 1VVM (volume de ar por volume de meio por minuto) e ciclo de luz de 24:0h (claro:escuro). Os voláteis foram isolados por microextração de fase sólida por *headspace*, separados por cromatografia gasosa, identificada por espectrometria de massa (SPME-GC-MS) e sua significância de odor foi avaliada com base na biblioteca de espectro NIST. O limoneno foi identificado na fração volátil das seis microalgas, contribuindo de forma positiva para o aroma cítrico e menta, demonstrando alta capacidade biotecnológica para obtenção alternativa desse biocomposto natural com viabilidade comercial, sendo a microalga *Scenedesmus bijuga* aquela com maior potencial quantitativo.

Palavras-chave: Aroma; Compostos bioativos; Microalgas; Monoterpeno; Compostos orgânicos voláteis

1 INTRODUCTION

The current consumer market has shown increasing interest in healthy and ecologically correct eating habits (Rapinel et al., 2020). Seeking adequacy, there is a growing consensus in the global food industry that aims to replace synthetic ingredients with natural ingredients for prevention and health promotion, thus investing in new technologies. Although little explored, is constantly growing the industrial relevance of microalgae as sources of an extensive spectrum of bioproducts, fundamentally based on chemical composition, and as promising raw materials for production systems of natural additives (Fernandes et al., 2020; Jacob-Lopes et al., 2019).

Thinking of providing better growth conditions for specific strains of these microorganisms and thus improving the efficiency of biomass production, the only method that can be projected and optimized is microalgal cultivation in photobioreactors. For this reason, the use of closed systems on a larger scale is becoming increasingly used, especially in the production of high value-added products (Borowiak et al., 2021; Gong & Bassi, 2016).

In addition to these biotechnologies, different cultivation strategies have been studied, such as photoautotrophic cultivation (considered the most common), due to the high photosynthetic efficiency that involves the conversion of CO_2 and light into valuable organic compounds. These metabolic forms aim, in addition to optimizing the production of microalgal biomass, the generation of its co-products, such as volatile organic compounds (VOC's) (Nörnberg et al., 2021; Abiusi; Wijffels & Janssen, 2020; Chenebault et al., 2020; Verma et al., 2020; Caetano et al., 2019; Verma & Srivastava, 2018).

The VOC's are secondary metabolites that can be utilized as important source of inputs in fine chemical industries, with obtaining different classes of compounds (Santos et al., 2016). Depending on species, culture and environmental conditions, microalgae are capable of producing a variety of VOC's, including the terpenoids, one of the main kinds of VOC's from plants and algae (Ye et al., 2018).

Some species of microalgae produce limonene, volatile monocyclic monoterpene hydrocarbon ($C_{10}H_{16}$), from the isoprenoid pathway by the action of the enzyme limonene synthase (LMS) from the geranyl-pyrophosphate (GPP) which is cyclized to give limonene (Antonelli et al., 2020; Kiyota et al., 2014).

Limonene for its proven safety, holds numerous opportunities for the development of innovative and sustainable products. It has been widely explored as a flavoring agent and adjuvant in the food and beverage, pharmaceutical, cosmetic, biomaterial and biofuel industries, due to its pleasant fragrance, favorable physicochemical properties and special biological activities, which include activity antioxidant, anti-inflammatory, anti-cancer, anxiolytic, analgesic, antidiabetic, antiallergic, among others (Ibáñez; Sanchez-Ballester; Blázquez, 2020; Ren et al., 2020).

These microalgae-derived metabolites for having a great perspective for industrial growth and development (Bilal et al., 2017), production with greater potential is sought through optimized cultivation systems and conditions. In this sense, studies are needed to identify the composition and concentration of bioproducts present in microalgae in order to achieve scale characteristics proportional to the industrial demands of these natural biocompounds.

Given the above, the aim of this study was to investigate the formation of the volatile compound limonene derived from the microalgae *Scenedesmus obliquus*, *Chlorella vulgaris*, *Chlorella sorokiniana, Spirulina* sp., *Scenedesmus bijuga* and *Desertifilum* spp., in photoautotrophic cultivation, as well evaluate its odor properties based NIST spectrum library.

2 MATERIAL AND METHODS

2.1. MICROALGAE CULTURE AND BIOMASS PRODUCTION

Axenic cultures of *Scenedesmus obliquus* (CPCC05), *Chlorella vulgaris* (CPCC90), of *Chlorella sorokiniana* (CPCC138) and *Spirulina* sp. (CPCC695) were obtained from the Canadian Phycological Culture Center. Axenic of *Scenedesmus bijuga* (UTEX2980) was obtained from the Algae Cultures Collection. Axenic cultures of *Desertifilum* spp. (MK307822) were originally isolated from the lakes at the Cuatro Cienegas desert in Mexico (26°59′ N, 102°03′ W). The biomass productions were made in phototrophic conditions. The cultivations were performed in a bubble column photobioreactor (Maroneze et al., 2019) operating under a batch regime, fed on 2.0 L of BG-11 medium (Braun-Grunow medium) (Rippka et al., 1979). The experimental conditions were as follows: initial cell concentration of 100 $mg.L^{-1}$, isothermal reactor operating at a temperature of 25°C, photon flux density of 25 $\mu mol.m^{-2}.s^{-1}$, continuous aeration of 1VVM (volume of air per volume of culture per minute), and a light cycle of 24:0h (light:dark). The biomass was separated from the culture medium by centrifugation (10000 rpm, 10 min, 10°C), the supernatant was discarded, and the remaining biomass was freezing at -18°C for 24 hours. After, the biomass was freeze-dried for 24 h at -50°C above -175 µm Hg and then stored at -18°C until analysis.

2.2. EXTRACTION, IDENTIFICATION AND QUANTIFICATION OF VOLATILE COMPOUNDS

2.2.1. Isolation of the Volatile Organic Compounds

The volatile compounds were isolated using solid-phase microextraction (HS-SPME) with a 50/30 µm divinylbenzene/carboxen/polydimethylsiloxane (DVB/CAR/PDMS) fibre (Supelco, Bellefonte, USA). The SPME fibre was inserted into the headspace of the vial containing the microalgae biomass for 60 min at 40°C temperature, with agitation provided by a magnetic stir bar. After this period, the fibre was removed from the vial and immediately desorbed into the injector of the GC equipment (Zepka et al., 2014).

2.2.2. Analysis of Gas Chromatography - Mass Spectrometry (GC-MS)

The volatile compounds were analyzed according to Zepka et al. (2014), separated on DB-5 and DB-Wax fused silica capillary columns, both 30 m in length, 0.25 mm id and 0.25 µm film thickness (J & W, Folsom, USA) in a Varian model CP 3380 gas chromatograph. The splitless mode injector was maintained at 230°C and the flame ionization detector (FID) at 250°C. Hydrogen was the carrier gas at a flow rate of 1.5 mL/min. The oven temperature for the DB-5 column was set at 50°C, held for 8 min, programmed to 260°C at 4°C/min, then to 280°C at 20°C/min and finally held at that temperature for 5 min. The initial oven temperature for the DB-Wax column was 40 °C for 15 min, followed by a linear increase at 4°C/min to 210°C, and held at this temperature for 17 min.

For identification, the volatile compounds were analysed using a Shimadzu (model QP 2010) GC with a mass spectrometer (MS), applying an electron-impact ionization voltage of 70 eV and using the same columns and oven conditions as described above for the GC-FID analysis, with the exception of using helium as the carrier gas. The volatile compounds were identified by a comparison of their MS spectra with those provided by the computerized library (NIST 2021 MS Library) and, when available, with those obtained from standards analysed under the same GC–MS conditions. In addition, to assist with the identification, each volatile linear retention index (LRI) was calculated using the retention times of a standard mixture of paraffin homologues prepared in hexane, and compared with the LRI values published in the literature for columns with the same polarity (Acree & Arn, 2021). Co-injection of the sample and the standard mixture provided experimental linear retention indices (LRI) for the compounds, which were compared with those of standards analysed under similar conditions, confirming the identification of the volatiles. The formation and degradation of each volatile compound was estimated comparing the volatile electric signal (mVolts) provided by FID for the control sample (model solution with no heating) with that obtained for the heated samples. Analytes were quantified based on relative peak areas.

2.3. STATISTICAL ANALYSIS

The statistical analysis was performed using "Statistica 8.0" software (Statsoft, Tulsa-OK, USA). The statistical differences among treatments were estimated by analysis of variance (ANOVA). The significance of the experimental data was determined using the Tukey test ($p < 0.01$).

3 RESULTS AND DISCUSSION

Some natural fragrances and flavors can be found in cyanobacteria and algae (Singh et al., 2017). These are complex mixtures of a large number of volatile compounds responsible for the organoleptic properties of a product. It is important to note that flavors have no nutritional value, but remain essential in the diet, to stimulate the appetite, in addition to allowing the distinction between fresh and unfit for consumption foods (Rapinel et al., 2020).

In this perspective, using the GC-MS technique, the volatile organic compound limonene was identified through chromatographic data in six microalgae, cultivated in photoautotrophic medium. To aid in the identification, the Linear Retention Index (LRI) was calculated for the limonene compound of each microalgae, using retention times (tR) and mass spectra, as well as identifying the odor description based on the NIST spectrum library (Acree & Arn, 2021).

GC-MS is the conventional method for analyzing volatiles in food matrices due to reliable identification and quantification of compounds (Ekpa; Fogliano & Linnemann, 2020), while the complementation with data from the Linear Retention Index (LRI) makes the identification process even more reliable (Rigano et al., 2019).

The quantification of the limonene volatile compound produced by the different microalgae was performed based on the relative peak areas, shown in Table 1, in elution order.

Table 1. Limonene - Volatile organic compound detected by GC-MS in photoautotrophic cultivation of microalgae with retention index (LRI) and odor descriptor.

Microalgae	**tR (min)**[1]	**LRI DB-Wax (cps)**[2]	**Relative Peak Area (%)**[3]	**Odor description (NIST)**[4]
Desertifilum spp.	14.0	1209	0.43±0.01^{b}	citrus, mint
Spirulina sp.	14.1	1207	0.33±0.01^{c}	citrus, mint
Scenedesmus obliquus	14.1	1208	0.31±0.01^{d}	citrus, mint
Chlorella vulgaris	14.2	1207	0.27±0.00^{e}	citrus, mint
Chlorella sorokiniana	14.2	1208	0.21±0.00^{f}	citrus, mint
Scenedesmus bijuga	14.5	1208	0.82±0.01^{a}	citrus, mint

[1] Retention time on the column.
[2] Linear Retention Indices in the DB-Wax column.
[3] Mean and standard deviation often independent experiments. Different letters in the columns indicate different means ($p < 0.01$).
[4] Description of the odor compared to the name of the compound, chromatographic column and retention index (Acree & Arn, 2021).

The presence of limonene was detected in all samples. According to the peaks in retention times (tR) observed in the GC-MS chromatogram of limonene, the results were similar. The initial peak occurred with tR of 14.0 minutes related to microalgae *Desertifilum* spp., and the last peak with tR of 14.5 minutes, related to microalgae *Scenedesmus bijuga*, however, in different amounts ($p < 0.01$).

Among the microalgae analyzed, the one that presented the compound limonene in the majority, based on the relative areas of the peaks, was *Scenedesmus bijuga* (0.82%), approximately twice superior to *Desertifilum* spp. (0.43%), the second largest producer. The other microalgae also presented the compound limonene, however, in a minority form: *Spirulina* sp. (0.33%), *Scenedesmus obliquus* (0.31%), *Chlorella vulgaris* (0.27%), *Chlorella sorokiniana* (0.21%), respectively.

Studies on the potentiality of limonene production in microalgae are scarce, with evaluations being found in studies carried out by Caetano et al. (2019), Nass et al. (2019) and Nascimento et al. (2020). In this context, Nascimento et al. (2020) reported the relative area of limonene of 0.43% in microalgae *Phormidium autumnale* (phylogenetically similar to *Desertifilum* spp.) (Nörnberg et al., 2021; Maroneze et al.,

2020) and 0.32% in the microalgae *Scenedesmus obliquus,* result similar to this study for the microalgae *Desertifilum* spp. in the first case and for the microalgae *Spirulina* sp. and *Scenedesmus obliquus* in the second, however, all values are clearly inferior to the production presented by the microalgae *Scenedesmus bijuga*, which demonstrates high potential of production of the microalgal limonene.

Caetano et al. (2019), described the relative area of limonene of 0.18% in the microalgae *Chlorella vulgaris*, a value lower than that observed in the present study for the same microalgae, under photoautotrophic conditions. The difference can be explained by different cultivation conditions, such as photon flux and photoperiod. Previous studies have reported that limonene develops citrus and mint olfactory notes (Bonneau at al., 2016; Chiş et al., 2020), which corroborates the results of the odor description of the six microalgae analyzed, in which the LRI ranged from 1207 cps to 1209 cps, indicating citrus and mint odor, according to the NIST spectrum library (Acree & Arn, 2021). Nascimento et al. (2020), also identified the compound limonene with the same aroma (Acree & Arn, 2021) in the microalgae *Scenedesmus obliquus* and *Phormidium autumnale*, despite having a slightly higher LRI (1230 cps).

According to data from this research, the microalgae *Chlorella vulgaris* and *Spirulina* sp. were the ones that registered the lowest peaks of the volatile compound limonene (LRI of 1207 cps), however, they presented the same odor descriptors (citrus, mint) as the other microalgae analyzed. The aroma of the volatile compound limonene can be classified in addition to citrus and mint, as fruity and floral, and can contribute positively to the aroma of microalgal biomass (Zuo, 2019), even if they have relatively low threshold values (Ekpa; Fogliano; Linnemann, 2020).

Caetano et al. (2019) and Nass et al. (2019), reported that in the microalgae *Chlorella vulgaris* and *Phormidium autumnale*, the aroma of limonene was identified as lemon, citrus and orange, since the LRI was 1182 cps, lower than that presented in this study. The lower value of the LRI of the microalgae *Phormidium autumnale* analyzed by Nass et al. (2019), can be justified by the different form of microalgal cultivation, carried out under heterotrophic conditions, however, both forms presented an attractive odor. In reason to the description of the attractive aroma of limonene, this compound is widely used in the food industries, for example, for the aroma of lemon or lime beverages (Tetali, 2019), traditionally used as a flavoring compound in citrus flavored products (Jongedijk et al., 2016).

Due to its special biological activities, it is also applied in pharmaceutical products (Ren et al., 2020). In vivo and in vitro studies address the effects of its bioactive potential, with beneficial results for health, such as anti-inflammatory, antioxidant, anti-diabetic, gastroprotective action, decrease in LDL cholesterol levels and increase in HDL, in addition to presenting chemopreventive effects against several types of cancer, among other activities, thus contributing to new clinical trials, as interest in the biological activities of limonene is growing (Vieira et al., 2018), in addition to commercial production.

Microalgae play an important role in the world economy currently (Deprá et al., 2019). Global production of aromatic compounds such as limonene is increasing at a significant rate and the market size is projected to exceed 1.9 billion (USD) by 2024. Considering the widespread application and growing market demands, there is an urgent need for a stable and mass supply of limonene and its derivatives (Ren et al., 2020), with the analyzed microalgae being an appropriate alternative for commercial production. Overall, microalgae can convert 183 G tons of CO_2 to produce 100 G tons of biomass showing better growth rate with photosynthetic capacity than higher plants (Kumar; Ghosh & Pal, 2019).

Finally, the biotechnological interest of many of these autotrophic microorganisms was recognized, with the subsequent challenge being the development of bioprocesses that link scientific results to commercial needsInnovative technologies are still needed to overcome their satisfactory performance, such as issues related to the development of microalgae strains and cultivation systems. In this context, the use of photobioreactors to carry out processes based on microalgae boosted the development of different reactor configurations to achieve scale characteristics proportional to industrial demands (Deprá et al., 2019).

4 CONCLUSION

The bioactive compound limonene was identified in the volatile fraction of the six microalgae evaluated from the photoautotrophic cultivation, contributing positively to the aroma citrus and mint, demonstrating high biotechnological capacity for obtaining an alternative of this natural compound with commercial viability, contributing to the growth and development of several industries, the microalgae *Scenedesmus bijuga* being the one with the greatest quantitative potential.

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CONCLUSÃO GERAL

No presente estudo, constatou-se que a microalga *Desertifilum* spp. exibe forte potencial para a produção de carotenoides, sendo majoritária as formas de all-*trans*-β-caroteno, all-*trans*-zeaxantina e all-*trans*-equinenona, respectivamente. Nesse sentido, o cultivo mixotrófico apresenta melhores resultados quantitativos quando comparado ao cultivo fotoautotrófico e heterotrófico.

Paralelamente, identificou-se a presença do composto bioativo limoneno na fração volátil das seis microalgas avaliadas (*Desertifilum* spp., *Chlorella sorokiniana, Chlorella vulgaris, Scenedesmus bijuga, Scenedesmus obliquus e Spirulina* sp.), a partir do cultivo fotoautotrófico, contribuindo de forma positiva para o aroma cítrico e menta, sendo a microalga *Scenedesmus bijuga* aquela com maior potencial quantitativo desse composto.

Por fim, pode-se concluir que as microalgas são fontes de bioprodutos não voláteis (carotenoides) e voláteis (limoneno), compostos que, segundo a literatura, apresentam importantes propriedades bioativas e/ou nutracêuticas, cuja importância econômica em todo o mundo está associada a uma ampla gama de aplicações nas indústrias de alimentos e biomédicas.

CAPÍTULO 4

ANEXO A - RECENT ADVANCES IN THE BIOACCESSIBILITY AND BIOAVAILABILITY OF ALGAL BIOACTIVE COMPOUNDS

O capítulo será publicado no livro “Books on Algae. Book 6 – Algal Metabolites: Biotechnological Applications” pela editora “AAP/CRC Press, USA”.

Chapter 6

RECENT ADVANCES IN THE BIOACCESSIBILITY AND BIOAVAILABILITY OF ALGAL BIOACTIVE COMPOUNDS

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Abstract: The growing demand for natural inputs has boosted the biotechnological exploration of microalgal metabolites, with emphasis on carotenoids. Carotenoids can behave as active ingredients, minimizing the risk of developing cardiovascular disease, cancer, and age-related macular degeneration due to its antioxidant potential. Despite the beneficial properties of carotenoids, their effectiveness at preventing or treating of diseases depends on their bioaccessibility and bioavailability. Thus, models that simulate the digestion process have been described, seeking to contribute to a better understanding of the potential availability of that compound. In that context, this chapter provides a comprehensive description of carotenoids from microalgae, remarkable biological properties, and the bioaccessibility and bioavailability of these metabolites.

Keywords: Bioaccessibility, bioavailability, bioactive compounds, biological properties, carotenoids.

1. INTRODUCTION

Microalgae are a source of valuable natural ingredients due to their content of bioactive compounds (Jacob-Lopes et. 2019). Of particular interest are the carotenoids, which represent a model of success in terms of commercial carotenoid production, through the cultivation of *Dunaliella salina* and *Haematococcus pluvialis*, with a focus on β-carotene and astaxanthin. Besides, the global carotenoid market is estimated to be valued at USD 1.7 Billion in 2022 (Fernandes et al. 2020; Rammuni et al. 2018; Mcwilliams 2018).

In addition, carotenoid metabolites have also gained interest, due to exceptional bioactive properties, including antioxidant activity, pro-vitamin A precursors, immune activators, anti-neurodegenerative, anti-cancer, anti-inflammatory agents, bone health, and protection of macular degeneration (Saini, and Keum 2020; El-Akabawy and El-Sherif 2019; Patias et al. 2017).

However, the bioactive properties of carotenoids are extremely dependent on their bioaccessibility and bioavailability. Numerous factors affect the carotenoid bioaccessibility and bioavailability, the matrix and its release, changes during digestion, uptake, metabolism, and biodistribution to target tissues following ingestion (Xavier and Mercadante 2019; Kopec and Failla 2018; Bohn 2018).

Thus, the goal of this chapter is to provide provides an overview of the microalgal carotenoid profile, its importance in human health, and finally, recent advances in the bioaccessibility and bioavailability of these structures.

2. CHARACTERISTICS OF MICROALGAL CAROTENOIDS

Carotenoids are responsible for the yellow, orange, and red tones of foods. This effect stems from its basic structure, a polyene backbone that contains a variable number of conjugated double bonds, called a light-absorbing chromophore, requiring seven conjugated double bonds to be colored. The color change occurs as the number of double bonds increases, as there is a shift in the absorption spectrum of the molecule, which characterizes each carotenoid (Rodriguez-Amaya 2015; Irazusta et al. 2013; Jomova and Valko 2013; Maldonade et al. 2008; Rodriguez-Amaya 2001).

The structure of these compounds are generally tetraterpenes (C40) consisting of 8 isoprenoid units (C5) can be classified as xanthophylls are carotenoids that contain oxygen as a functional group such as epoxy (violaxanthin and fucoxanthin), hydroxy (lutein and zeaxanthin), and keto (astaxanthin and canthaxanthin). Carotenes, which contain only by carbon and hydrogen atoms (α-carotene and β-carotene) (Lafarga et al. 2020; Gille et al.

2018; Magosso et al. 2016; Gul et al. 2015; Zaghdoudi et al. 2015). The structures of xanthophylls and carotenes shown in Fig. 1a.

Microalgae can synthesize large quantities of carotenoids that have shown different biotechnological explorations, in addition to physiological properties, discussed in Table 6.1. Furthermore, they have become an attractive option as a natural source of inputs for the industry due to the emerging of health safety issues caused by synthetic products such as allergic reaction and hyperactivity in humans (Khoo et al. 2019). The main commercial sources of natural carotenoids with β-carotene and astaxanthin are *Dunaliella salina* and *Haematococcus pluvialis* and represent a successful model (up to 7%-14% in dry weight) (Fernandes et al. 2020; Rammuni et al. 2018).

The *Dunaliella salina* produces β-carotene in quantities that represent about 10-14% of its dry mass, being the world leader in the production of β-carotene (Khan et al. 2018; Mulders et al. 2014; Borowitzka 2013). The carotenoids provitamin-A of *D. salina* widely used in pharmaceutical products and as a food color (Jacob-Lopes et al. 2019; Chen et al. 2015; Guedes et al. 2011).

Moreover, astaxanthin produced by *Haematococcus pluvialis* applications are important in the pharmaceutical, nutraceutical, cosmetic, food industries (dye, food additive or dietary supplement), and in animal feed as a source of pigmentation (Hamidi et al. 2020; Jacob-Lopes et al. 2019; Sathasivam and Ki 2018; Panis and Carreon 2016; Shah et al. 2016; Zhang et al. 2014; Xia et al. 2013; Vílchez et al. 2011).

Besides, other microalgae species represent a potential dietary source of carotenoids as lutein and its structural isomer zeaxanthin. The *Muriellopsis* sp. produces a high lutein content and a high growth rate; therefore, it has been exploited for commercial production (Sathasivam et al. 2019; Guedes et al. 2011). Lutein can be used as a food additive in the nutraceutical field and as cosmetics. It also has been used as a pigment source for birds. In addition, it has been marketed as a food supplement for its beneficial effects on vision (Mehariya et al. 2019; Sathasivam et al. 2019).

The microalgae used for the production of zeaxanthin are the *Chlorella vulgaris*, *Phaeodactylum tricornutum*, *Scenedesmus almeriensis*, and *Nannochloropsis oculata*. Zeaxanthin is mainly used in pharmaceutical products, applications in the cosmetics and food industry, also used in aquaculture and poultry as a pigment source (Singh et al. 2020; Sathasivam and Ki 2018; Gille et al. 2018; Dufossé 2016; Chen et al. 2015; Mulders et al. 2014).

Some carotenoids are found mainly in microalgae, such as crocoxanthin, fucoxanthin, echinenone, and canthaxanthin (Fig. 1b.). Taking into account the structures, suggest a higher antioxidant activity for microalgal carotenoids about conventional sources, due to the presence of exclusive carotenoids, which acetylenic bond and the greater number of conjugated double bonds are characteristic associated with the high antioxidant action of microalgae carotenoids (Nascimento et al. 2019; Sathasivam and Ki 2018; Patias et al. 2017; Klassen and Foght 2011; Uenojo et al. 2007).

These compounds are highly active against reactive oxygen species and free radicals and gained great interest (Nascimento et al. 2020). Thus, microalgae represent natural resources of antioxidants due to their vast biodiversity. On the other hand, not all groups of microalgae can be applied as sources of antioxidants due to their widely diversified product content and to unfavorable growth, among other factors. The main types of microalgae in industrial production are *Arthrospira* sp., *Chlorella* sp., *Nitzschia* sp., *Porphyridium cruentum*, *Nannochloropsis* sp, *Crypthecodinium cohnii, Phaeodactylum tricornutum*, and *Schizochytrium* sp., sources of antioxidants and natural bioactive compounds that exhibit beneficial effects on human health (Hamidi et al. 2020; Khan et al. 2018).

3. BIOACTIVE CAROTENOIDS AND THEIR PROPERTIES PHYSIOLOGICAL

Scientific evidence has shown that microalgae to their ability both to produce bioactive metabolites, including a wide range of different carotenoids that can provide health benefits and to their functioning as bioactive and nutraceutical agents, as they have antioxidant, anti-carcinogenic, anti-inflammatory effects, among many other beneficial health effects, as Table 6.1 (Hamidi et al. 2020; Jacob-Lopes et al. 2019; Sathasivam and Ki 2018; Zhang et al. 2014; Guedes et al. 2011).

The protection of bioactive compounds present in carotenoids against some diseases is associated especially with its high antioxidant potential, the ability to sequester singlet oxygen and react with free radicals, as these can cause harmful effects, such as the triggering of many diseases, including cancer, coronary artery disease, obesity, diabetes, ischemic stroke, Alzheimer's disease, among others (Khan et al. 2018; Trejo-Solís et al. 2013).

3.1. β-carotene

The β-carotene, in addition to its antioxidant properties, has other benefits to human health, such as prevention of liver fibrosis, prevention of night blindness, anti-neurodegenerative properties, photoprotection of the skin against ultraviolet (UV) light, prevention of acute coronary syndrome and chronic, as well as some types of cancer. It also acts on vision, on increasing the performance of the immune system, on embryonic growth and development, due to its activity as provitamin A (Hamidi et al. 2020; Gille et al. 2018; Khan et al. 2018; Magosso et al. 2016; Gul et al. 2015; Rodriguez-Amaya 2015; Maldonade et al. 2008).

Studies *in vitro* and *in vivo* showed that β-carotene is involved in reducing angiogenesis by suppressing cell proliferation and migration (Goff et al. 2019). Research with mice showed that a biomass-based diet of powdered microalgae *Dunaliella*, containing 0.6% β-carotene, can reduce cholesterol levels in blood plasma and inhibit the progression of atherosclerosis in diets rich in fat (Harari et al. 2013).

β-carotene have substantial effects on the enzymatic antioxidant defense system, preventing oxidative stress by eliminating free radicals, protecting lipids from peroxidation, factors linked to severe and lethal diseases, such as cardiovascular, Parkinson, atherosclerosis, and cancer (Goff et al. 2019; Khan et al. 2018). The anti-cancer activity of these molecules involves several mechanisms, including the induction of cell apoptosis and suppression of cell proliferation. In particular, an in vivo study showed that β-carotene helps to reduce liver neoplasms (Sathasivam and Ki 2018).

Second, Goff et al. (2019), serum β-carotene levels are inversely correlated with glycated hemoglobin levels A1c (HbA1c) associated with decreased insulin sensitivity. Sluijs et al. (2015) investigated the association of a carotenoid-rich diet with type 2 diabetes. The carotenoids included in the diet were: β-carotene, α-carotene, β-cryptoxanthin, lycopene, lutein, and zeaxanthin; and the sum of all these carotenoids. According to this study, intake of β-carotene and α-carotene reduced type 2 diabetes, both in men and women.

3.2. Astaxanthin

In a comparative study of the antioxidant activities of some carotenoids, Naguib (2000) reported that astaxanthin has higher activity when compared to α-carotene, β-carotene, lutein, and lycopene, in the elimination of reactive oxygen species (ROS), which makes it a powerful antioxidant and anti-inflammatory (Yeh et al. 2016).

The oxidative stress is a causal factor or at least an aid in the pathogenesis of the main neurodegenerative diseases (Alzheimer's, Huntington, Parkinson, and amyotrophic lateral sclerosis (ALS). Natural astaxanthin can pass the blood-brain barrier and thus extend its antioxidant effect. Therefore, astaxanthin can work by reducing the effects of Alzheimer's and other neurological diseases (Shah et al. 2016).

Anti-inflammatory effects, enhanced by astaxanthin, preserve essential fatty acids and lymphocyte proteins. Astaxanthin works by inducing actions of the enzymes superoxide dismutase and catalase. Other studies have shown that astaxanthin protects against inducing liver damage (CCl4), inhibiting lipid peroxidation, stimulating the cellular antioxidant system and modulating the inflammatory process (Vílchez et al. 2011), reduces the occurrence of cancer, antiproliferative activity, and reduction of metastases (induced by stress) (Goff et al. 2019).

In addition to the anti-cancer effect, astaxanthin can be used in the prevention and treatment of chronic diseases like cardiovascular disorders, diabetes, and diabetic nephropathy (Zhang et al. 2014), as well as can prevent inflammation in biological systems. It can fight ulcerative diseases by *Helicobacter pylori*, protecting against gastric lesions (ulcers), and improving gastrointestinal health and discomfort (Shah et al. 2016).

Studies have shown that astaxanthin is effective and safe in controlling body weight, being considered as an anti-obesity agent. The administration of astaxanthin, in addition to significantly reducing body weight and adipose tissue, can also reduce hepatic, plasma, and total cholesterol triglycerides (Zhang et al. 2014).

Astaxanthin supplementation may be beneficial for people at increased risk for heart attacks. It is carried by VLDL, LDL, and HDL in the blood and protects LDL cholesterol against oxidation (Shah et al. 2016). In addition, another study carried out in humans, aged 25 to 60 years, showed that 12 weeks of administration of astaxanthin significantly decreased serum triglyceride levels, while significantly increasing high-density lipoprotein (HDL) (Sathasivam and Ki 2018).

Research has also reported anti-hypertensive effects of astaxanthin in spontaneously hypertensive rats (SHRs). In the study, oral administration of astaxanthin, at a concentration of 50 mg/kg, for 14 days, promoted a significant reduction in blood pressure in SHRs (Sathasivam and Ki 2018).

3.3. Lutein

Lutein is considered a functional nutrient because it has benefits to human health, acting in the prevention of age-related macular degeneration (AMD), cataracts, reduction of oxidative stress in the retina and inhibition of the pathological activity of diabetic retinopathy, improvement of the early stages of atherosclerosis, protection from cardiovascular disease and some types of cancer (Mehariya et al. 2019; Dufossé 2016).

Hayashi et al. (2014) conducted a study with forty patients, all with identical degrees of cataracts in both eyes. To analyze changes in antioxidant capacity and oxidative state, they collected aqueous humor before and after taking an oral antioxidant supplement, based on lutein. The results showed that the consumption of antioxidant supplements effectively reduces oxidation in aqueous humor, inhibiting the oxidation of surrounding tissues. In another study, the use of *Chlorella* extracts containing lutein reduced the incidence of cancer and prevented macular degeneration. Similarly, carotenoids specifically extracted from *Chlorella ellipsoidea* and *Chlorella vulgaris* inhibited the development of colon cancer (Guedes et al. 2011).

Lutein can also delay the spread of chain reactions such as those initiated by the degradation of polyunsaturated fatty acids known to contribute to the deterioration of lipid membranes, impairing their integrity. One example is the decline in cognitive ability that accompanies Alzheimer's disease, apparently caused by persistent oxidative stress in the brain. Using transgenic mice fed with *Chlorella* sp. containing, the authors found that significant prevention of cognitive impairment occurred (Guedes et al. 2011).

Lutein, in addition to antioxidant, protect diabetic, anticancer, also shows antimicrobial action, being able to prevent gastric infection by *Helicobacter pylori* (Hamidi et al. 2020). Studies of antibacterial products reported with microalgal lutein have shown inhibition of the growth of Gram-positive and Gram-negative bacteria. Some microalgae, such as *Dunaliella*, also produce compounds with antifungal activities (Khan et al. 2018).

3.4. Zeaxanthin

As oxidative stress induced by reactive oxygen species is the main reason for inflammatory events that lead to many different medical conditions, such as cancer, diabetes, neurodegenerative and cardiovascular diseases, the quest to understand the mechanisms of oxidative stress and also to find new antioxidant compounds have become of great interest to medical scholars. Epidemiological investigations have shown a strong correlation between antioxidants, such as zeaxanthin, and a decreased risk of these chronic diseases such as cardiovascular disease and cancer (Hamidi et al. 2020).

The anti-cancer activity of carotenoids involves a variety of mechanisms, including the induction of cell apoptosis and suppression of cell proliferation. An in vivo study showed that β-carotene, astaxanthin, canthaxanthin, and zeaxanthin help to reduce the size and number of liver cancers (Sathasivam and Ki 2018; Vílchez et al. 2011).

In addition to the antioxidant capacity of carotenoids, other mechanisms are also known, such as the absorption and filter of blue light in eye health. The bioactivity of dietary macular carotenoids (class of xanthophylls: lutein and zeaxanthin) offers a means of strengthening the antioxidant defenses of the macula, as they accumulate, thereby reducing the risk of age-related macular degeneration and/or progression (Sathasivam and Ki 2018; Eisenhauer et al. 2017; Dufossé 2016).

Recent findings show that carotenoids such as lycopene, lutein, and zeaxanthin can protect against diabetic retinopathy (Hamidi et al. 2020; Sathasivam and Ki 2018). Besides, studies report that oral administration of zeaxanthin is effective in managing inflammatory responses induced by ultraviolet irradiation (Zhang et al. 2014).

3.5. Fucoxanthin

Recently, fucoxanthin and its derivatives have been shown to have many beneficial health effects. This carotenoid has been reported to exhibit strong potential as an antioxidant, anti-cancer, anti-obesity, anti-diabetic, anti-inflammatory, anti-hypertensive agent, in addition to the bone protection effect (Gille et al. 2018; Khan et al. 2018; Sathasivam and Ki, 2018; Petrushkina et al. 2017; Xia et al. 2013).

Fucoxanthin also has cardioprotective activity. The administration of this carotenoid in an in vivo study showed a reduction in blood triglyceride levels. Rats fed 2 mg/kg of fucoxanthin, showed a significant reduction in the absorption of triglycerides when receiving a diet with 10% soy oil (Sathasivam and Ki 2018).

Obesity is considered a multifactorial metabolic disorder associated with many complications and diseases such as cancer, cardiovascular diseases, diabetes mellitus, and aging. As obesity occurs due to excessive deposition of adipose tissue, it can be controlled by reducing adipogenesis. Research has revealed several anti-hyperlipidemic agents from natural sources, such as medicinal plants. Currently, microalgae are being studied as potential sources of these agents. Thus, obesity can be controlled by protecting cells against adipogenesis (Khan et al. 2018). Promising results, in this sense, were obtained in research with obese rats supplemented with fucoxanthin (Maeda et al. 2006).

Fucoxanthin has been used in the treatment of osteoclastic diseases, which suggest that fucoxanthin suppresses osteoclastogenesis, inhibiting osteoclast differentiation and inducing apoptosis in osteoclasts. Also, fucoxanthin food supplements may be useful in preventing bone diseases, such as osteoporosis and rheumatoid arthritis, known to be related to bone resorption (Sathasivam and Ki 2018).

Another property reported in animal studies is that fucoxanthin in algae is effective in chemoprevention of cancer (Xia et al. 2013). Many reports indicate these natural products' potential to treat cancer and tumors by inhibiting angiogenesis (Khan et al. 2018).

Fucoxanthin also showed therapeutic effects on diabetes and arachidonic acid synthesis, and the DHA content in the liver of mice, inhibition of skin melanogenesis by the negative regulation of the transcription factors involved. Besides, it showed protection from photooxidation DNA (Khan et al. 2018).

3.6. Canthaxanthin

Carotenoids also can stimulate the immune system, being potentially involved in more than 60 life-threatening diseases, including various forms of cancer, coronary heart disease, premature aging, and arthritis; this is specifically the case for canthaxanthin and astaxanthin, and other non-provitamin carotenoids (Hamidi et al. 2020; Guedes et al. 2011).

According to studies, the effects of canthaxanthin on breast tissue chemically induced carcinogenesis in mice shows that ingesting canthaxanthin for three weeks, before cancer induction with dimethylbenzanthracene, can reduce the occurrence of cancer by 65% (Sathasivam and Ki 2018). canthaxanthin also has antioxidant, anti-inflammatory, and neuroprotective properties (Sathasivam et al. 2019). Canthaxanthin has therapeutic action as an antitumor agent, suppressing the growth of cancer cells through the induction of apoptosis (Gao et al. 2020).

The data on the bioaccessibility and bioavailability process of microalgae carotenoids are little limited; therefore, a more detailed assessment of the metabolism, biotransformation, and bioactivity of the uptake carotenoids is necessary.

4. BIOAVAILABILITY AND BIOACCESSIBILITY OF MICROALGAL CAROTENOIDS

Microalgae are a potential source of bioactive metabolites, including carotenoids, that show many bioactive functions. However, in order to mediate such activities, this metabolite must be bioavailable (Gille et al. 2016; Gille et al. 2019). The term bioavailability is defined as the fraction of the ingested carotenoids that is available for utilization in physiological functions (Wood 2005). A prerequisite for these structures bioavailability is its bioaccessibility. Bioaccessibility can be set as the portion of the ingested compound released from the matrix that gets incorporated into mixed micelles and is available for absorption (Parada and Aguilera 2007).

The application of whole microalgal biomass in food systems might have some implications for the bioactive efficacy of carotenoids in the human body

(Niccolai et al. 2019). The data generated by previous researchers on the bioaccessibility of carotenoids in microalgae had been compiled in Table 6.2.

The extent to which carotenoids are released upon digestion in the gastrointestinal tract depends on several factors, including the physical entrapment in the matrix, chemical structure (isomeric forms), physicochemical properties of the carotenoid the physiological conditions, and the digestive enzymes (Colle et al. 2016; Donhowe and Kong 2014; Lemmens et al. 2014).

The bioaccessibility of carotenoids of untreated microalgae biomass after in vitro digestion seems to be species-dependent. Generally, low bioaccessibility values (0-7%) have been reported for carotenoids in *Chlorella vulgaris and Nannochloropsis sp.* (1-6%) (Gille et al. 2016; Bernaerts et al. 2020). In contrast, higher values were observed for carotenoids in *Phaeodactylum tricornutum* (27-52%) and *Chlamydomonas reinhardtii* (10-20%) (Gille et al. 2016; Gille et al. 2018).

A possible explanation might be the distinct composition of the cell walls of these microalgae species. While *C. vulgaris* contain cellulose polymers in their cell wall, these are absent in *P. tricornutum* and *C. reinhardtii*. However, more research is required to conclude a direct relation between the cell wall composition and carotenoid bioaccessibility. As a matter of fact, while the fragile cell wall of *Isochrysis galbana* would suggest a high bioaccessibility, low values (8 -13%) have been reported in this microalga (Bernaerts et al. 2020).

Another factor the higher efficiency of micellarization of isomers compared to form all-*trans*. Possibly it is a consequence of the acyclic and rigid structure of all-*trans* that limits its incorporation into the micellar fraction during digestion. While, the *cis*-configuration presents bent to shape to the isomers, which improved their incorporation in micelles. Ferruzzi et al. (2006), in an *in vitro* study with carotenoids from microalgae *Dunaliella salina* showed that micellarization of *cis*-β-carotene exceeded that of all-*trans*-β-carotene.

Micellarization of carotenoids also dependent on lipophilicity. Thus, xanthophylls are generally more bioaccessible than carotenes due to the least hydrophobic character that facilitates transfer to micelles during digestion. Gille et al. (2016), in an *in vitro* study, showed that micellarization of lutein larger that of β-carotene. Gille et al. (2019) have reported that epoxy carotenoids fucoxanthin showed a good bioaccessibility. Further, they presumed that the absorption and metabolism of epoxy carotenoids are different from the other carotenoids.

Among the strategies to modulate the bioavailability and bioaccessibility of carotenoids are the protocols *in vivo* and *in vitro*. *In vivo* studies provide more specific information about the bioavailability of carotenoids, but these studies are more expensive, involve ethical constraints and until now, there is no optimal animal model to assess carotenoid metabolism (Failla et al. 2008). Therefore, *in vitro* methods may simulate gastrointestinal digestion, and the final absorption process is usually assessed using the Caco-2 cell. Finally, *in vitro* bioaccessibility assay can help with a preliminary assessment of the carotenoid metabolism and estimation of their bioavailability. However, the use of a standardized *in vitro* digestion protocol would aid the production of more comparable data in the future.

5. CONCLUSION

Microalgae are excellent sources of bioactive carotenoids applied in various areas of the industry. However, the bioaccessibility and bioavailability of carotenoids require a better understanding and will aid in achieving more desirable high-value compounds and thereby creating noteworthy progress towards the future of microalgae-based biotechnology.

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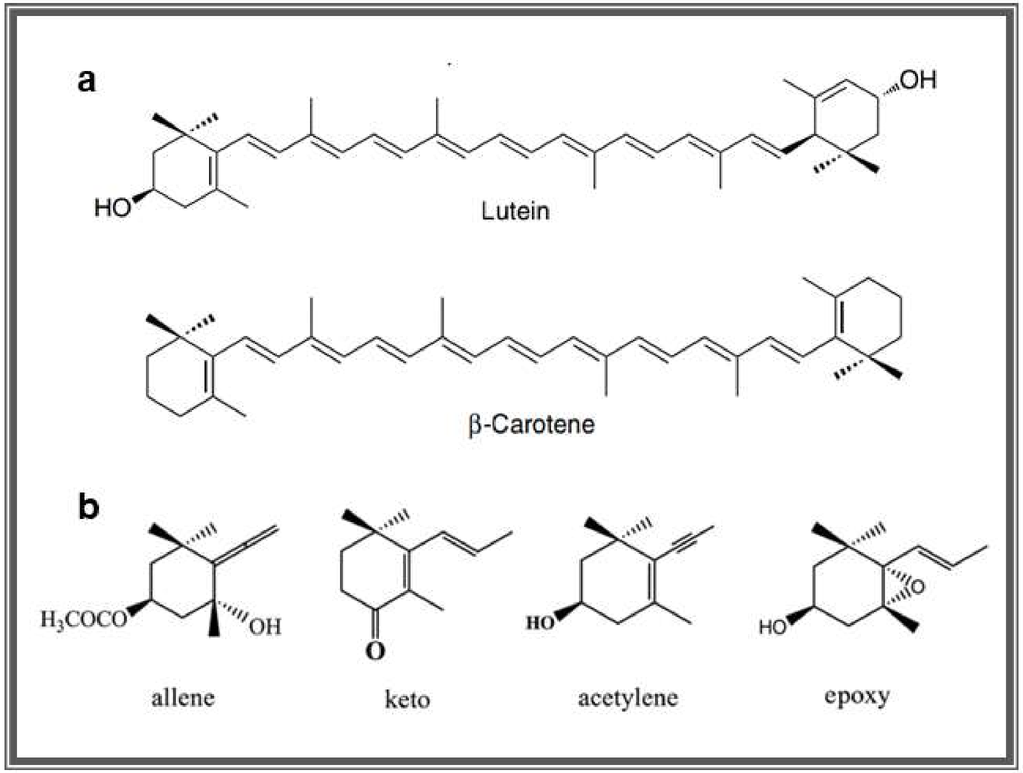

Figure 6.1. Chemical structures: a. Xanthophylls and carotenes. b. Unique cyclic end groups from microalgae.

Table 6.1. Some carotenoid-producing microalgae, their biotechnological explorations and its physiological properties.

Microalgae source	Main carotenoid	Biotechnological exploration	Physiological properties	References
Botryococcus braunii *Chlorella vulgaris* *Chlorella zofingiensis* *Dunaliella salina* *Galdieria sulphuraria* *Haematococcus pluvialis*	Astaxanthin	aquaculture pharmaceuticals nutrition cosmetics health food feed additives farming of salmon and trout nutraceuticals food ingredientes food coloring agente food supplement	benign prostatic hyperplasia and prostate and liver tumors anti-inflammatory properties active against liver neoplasms strong antioxidant property cardiovascular health hepatoprotective effect anti nephropathy diabetic anti hypertension anti stroke anti-cancer anti-neurodegenerative diseases photoprotection of eyes and skin	Hamidi et al. 2020; Goff et al. 2019; Sathasivam and Ki 2018; Sathasivam et al. 2019; Dufossé 2016; Shah et al. 2016; Yeh et al. 2016; Cuellar-Bermudez et al. 2014; Koller et al. 2014; Mulders et al. 2014; Zhang et al. 2014; Guedes et al. 2011; Vílchez et al. 2011; Naguib 2000.
Botryococcus braunii *Chlorella pyrenoidosa* *Chlorella saccharophila* *Chlorella zofingiensis* *Chlorella vulgaris* *Coccomyxa acidófila* *Dunaliella bardawil* *Dunaliella salina* *Dunaliella tertiolecta*	β-carotene	aquaculture pharmaceuticals nutrition cosmetics health food food supplement feed feed additive food colorant	provitamin A colorectal cancer prevention of acute and chronic coronary syndromes photoprotection of skin against uv light antioxidant property prevention liver fibrosis night blindness prevention	Hamidi et al. 2020; Nascimento et al. 2020; Maroneze et al. 2019; Nascimento et al. 2019; Gille et al. 2018; Jacob-Lopes et al. 2019; Khan et al. 2018; Patias et al. 2017; Dufossé 2016; Rodrigues et al. 2015;

Haloferax alexandrinus *Halorubrum sp.* *Phormidium autumnale* *Porphyridium cruentum* *Scenedesmus almeriensis* *Scenedesmus obliquus* *Spirulina máxima* *Spirulina platensis*			anti-neurodegenerative diseases Immune function Embryonic development	Cuellar-Bermudez et al. 2014; Koller et al. 2014; Mulders et al 2014; Zhang et al. 2014; Guedes et al. 2011; Vílchez et al. 2011.
Chlorella vulgaris *Chlorella zofingiensis* *Dietzia natronolimnaea* *Gordonia jacobaea* *Haematococcus pluvialis* *Haloferax alexandrinus* *Micrococcus roseus* *Nannochloropsis gladitana* *Nannochloropsis oculata* *Nannochloropsis salina* *Phormidium autumnale*	Canthaxanthin	pharmaceuticals nutrition cosmetics health food food additive farming of salmonids and chicken tanning pills food coloring agent animal feed additive	antioxidant property prevention diseases cardiovascular antitumoral activity anti-cancer immune system stimulation anti-inflammatory neuroprotective effects	Gao et al. 2020; Hamidi et al. 2020; Sathasivam et al. 2019; Sathasivam and Ki 2018; Rodrigues et al. 2015; Cuellar-Bermudez et al. 2014; Koller et al. 2014; Mulders et al. 2014; Guedes et al. 2011.
Chaetoceros gracilis *Cylindrotheca closterium* *Isochrysis aff. Galbana* *Isochrysis galbana* *Nitzschia closterium* *Nitzschia sp.* *Odontella aurita* *Phaeodactylum tricornutum*	Fucoxanthin	pharmaceuticals nutrition cosmetics animal nutrition baby food	anti-obesity antioxidant property anti-cancer bone-protective effects anti-inflammatory effects anti-hepatotoxicity effects anti-diabetic antihypertensive	Hamidi et al. 2020; Sathasivam and Ki 2018; Cuellar-Bermudez et al. 2014; Zhang et al. 2014; Xia et al. 2013.

Botryococcus braunii *Chlorella fusca* *Chlorella protothecoides* *Chlorella pyrenoidosa* *Chlorella sorokiniana* *Chlorella vulgaris* *Chlorella zofingiensis* *Chlorococcum citriforme* *Coccomyxa acidófila* *Coccomyxa onubensis* *Coelastrum proboscideum* *Dunaliella salina* *Galdieria sulphuraria* *Haematococcus pluvialis* *Muriella aurantiaca* *Muriella decolor* *Muriellopsis sp.* *Phormidium autumnale* *Porphyridium cruentum* *Scenedesmus almeriensis* *Scenedesmus obliquus* *Tetracystis aplanosporum* *Tetracystis intermedium* *Tetracystis tetrasporum*	Lutein	aquaculture pharmaceuticals nutrition health food feed additives food supplement cosmetics pigmentation of animal tissues food coloring agent poultry feeding	prevention of acute and chronic coronary syndromes and stroke helps to maintain a normal visual function cataract and age-related macular degeneration prevention prevention of retinitis to avoid gastric infection by *H. pylori* anti-cancer antioxidant properties ocular protective effects anti-diabetic retinopathy prevention diseases cardiovascular	Hamidi et al. 2020; Nascimento et al. 2020; Mehariya et al. 2019; Nascimento et al. 2019; Sathasivam and Ki 2018; Patias et al. 2017; Dufossé 2016; Rodrigues et al. 2015; Cuellar-Bermudez et al. 2014; Koller et al. 2014; Mulders et al. 2014; Zhang et al. 2014; Xia et al. 2013; Guedes et al. 2011; Vílchez et al. 2011.

Botryococcus braunii *Chlorella pyrenoidosa* *Chlorella saccharophila* *Dunaliella salina* *Gramella oceani* *Gramella planctonica* *Kordia aquimaris* *Mesoflavibacter zeaxanthinifaciens* *Muricauda lutaonensis* *Nannochloropsis gladitana* *Nannochloropsis oculata* *Phormidium autumnale* *Porphyridium cruentum* *Scenedesmus almeriensi*	Zeaxanthin	aquaculture food additive animal feed pharmaceutical food coloring agente poultry feeding feed cosmetics food supplement	active against liver neoplasms prevention of acute and chronic coronary syndromes helps maintain normal visual function cataract prevention prevents age-associated macular degeneration anti colon cancer antioxidant properties anti-diabetic retinopathy prevention diseases cardiovascular	Hamidi et al. 2020; Singh et al. 2020; Nascimento et al. 2020; Maroneze et al. 2019; Sathasivam and Ki 2018; Dufossé 2016; Rodrigues et al. 2015; Koller et al. 2014; Mulders et al. 2014; Zhang et al. 2014; Vílchez et al. 2011

Table 6.2. Bioaccessibility and bioavailability assays of microalgal carotenoids.

Microlgae	Main carotenoid	Assay	Processing methods	References
D. salina	β-carotene and *cis*-β-carotene	*In vitro* digestion and cells Caco-2	emulsions	Ferruzzi et al. 2006
S. almeriensis	lutein, zeaxanthin and β-carotene	*In vitro* digestion	extract	Granado-Lorencio et al. 2009
N. oculata	β-carotene and lycopene	*In vitro* digestion	extract	Goh et al. 2009
C. calcitrans	β-carotene and lycopene	*In vitro* digestion	extract	Goh et al. 2009
C. vulgaris	Lutein	*In vitro* digestion and cells Caco-2	microfluidization	Cha et al. 2011
C. vulgaris	lutein and β-carotene	*In vitro* digestion	sonication	Gille et al. 2016
C. reinhardtii	lutein and β-carotene	*In vitro* digestion	sonication	Gille et al. 2016
C. vulgaris	lutein and β-carotene	*In vitro* digestion	maceration biomass	Gille et al. 2018
P. tricornutum	fucoxanthin, zeaxanthin and β-caroten	*In vitro* digestion	maceration biomass	Gille et al. 2018
C. vulgaris	lutein and β-carotene	*In vivo* (C57BL/6J)	maceration biomass	Gille et al. 2018
P. tricornutum	fucoxanthin, zeaxanthin	*In vivo* (C57BL/6J)	maceration biomass	Gille et al. 2018
P. tricornutum	zeaxanthin, fucoxanthin and β-caroten	*In vitro* digestion and cells Caco-2	sonication and cellulase digestion	Gille et al. 2019
Nannochloropsis sp.	antheraxanthin, zeaxanthin and β-carotene	*In vitro* digestion	high pressure	Bernaerts et al. 2020
Nannochloropsis sp.	antheraxanthin, zeaxanthin and β-carotene	*In vitro* digestion	extract	Bernaerts et al. 2020