UNIVERSIDADE FEDERAL DE SANTA MARIA
CENTRO DE CIÊNCIAS RURAIS
PROGRAMA DE PÓS-GRADUAÇÃO EM CIÊNCIA E TECNOLOGIA DOS ALIMENTOS

Luciana Dapieve Patias

CARACTERIZAÇÃO DE CAROTENOIDES E ATIVIDADE ANTIOXIDANTE DA BIOMASSA MICROALGAL

Santa Maria, RS
2017

Luciana Dapieve Patias

CARACTERIZAÇÃO DE CAROTENOIDES E ATIVIDADE ANTIOXIDANTE DA BIOMASSA MICROALGAL

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

Orientador: Prof. Dr. Eduardo Jacob Lopes

Santa Maria, RS
2017

Ficha catalográfica elaborada através do Programa de Geração Automática
da Biblioteca Central da UFSM, com os dados fornecidos pelo(a) autor(a).

Patias, Luciana Dapieve
Caracterização de carotenoides e atividade antioxidante da biomassa microalgal / Luciana Dapieve Patias.- 2017.
101 f.; 30 cm

Orientador: Eduardo Jacob Lopes
Tese (doutorado) - Universidade Federal de Santa Maria, Centro de Ciências Rurais, Programa de Pós-Graduação em Ciência e Tecnologia dos Alimentos, RS, 2017

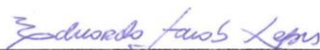
1. Microalga 2. Cianobactéria 3. Pigmento 4. Carotenoides 5. Antioxidante I. Lopes, Eduardo Jacob II. Título.

Luciana Dapieve Patias

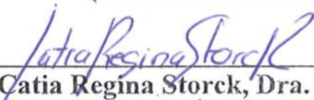
CARACTERIZAÇÃO DE CAROTENOIDES E ATIVIDADE ANTIOXIDANTE DA BIOMASSA MICROALGAL

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

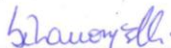
Aprovado em 29 de setembro de 2017:



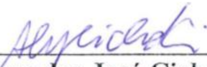
Eduardo Jacob Lopes, Dr. (UFSM)
(Presidente/Orientador)



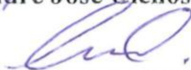
Catia Regina Storck, Dra. (UNIFRA)



Silvana Maria Michelin Bertagnolli, Dra. (UNIFRA)



Alexandre José Cichoski, Dr. (UFSM)



Cristiano Ragagnin de Menezes, Dr. (UFSM)

Santa Maria, RS
2017

DEDICATÓRIA

Dedico este trabalho a minha família, em especial aos meus pais Ivone e Valmor, pela educação recebida, apoio, sacrifico e exemplo de luta, humildade e honestidade.

A minha irmã Naiana que é minha inspiração.

E ao meu amor Marciel.

AGRADECIMENTOS

A Deus, por estar sempre presente em minha vida, e tornar tudo possível.

Ao meu orientador, Prof. Eduardo pela oportunidade de realizar o tão sonhado doutorado. Por ter acreditado em mim, antes mesmo de me conhecer. Obrigada pelo privilégio da tua orientação, dedicação, incentivo, sempre compartilhando tuas experiências e conhecimentos. Que Deus continue te iluminando.

A minha co-orientadora, Prof. Leila pela paciência, empenho e cooperação. Obrigada por ter sido tão prestativa.

Aos meus pais Valmor e Ivone, a minha irmã Naiana e ao meu cunhado Guilherme, pelo amor incondicional e pela força diária que necessitei em todos os momentos de dificuldades nessa caminhada. Foi por vocês que eu não desisti.

Ao meu esposo Marciel Redin, por todo carinho, companheirismo, incentivo e dedicação de sempre. Te amo!

À amiga Tatiéli, não só por ter me auxiliado nas intermináveis extrações durante os finais de semana e feriados, mas principalmente por ter doado seu tempo para que o meu trabalho fosse realizado. Você terá sempre um lugarzinho reservado no meu coração. Obrigada, que Deus possa te recompensar.

A colega Andressa, por ter sido peça fundamental no desenvolvimento e conclusão deste trabalho. Sem você esse sonho não seria possível. Não tenho palavras para te agradecer.

As colegas Gabriela Nogara e a Bruna Tischer pelo auxilio durante as análises antioxidantes.

A colega Mariany Deprá, que me recebeu de braços abertos no NTA, me orientou nos primeiros passos e sempre esteve disposta a me escutar.

A colega que se tornou uma grande amiga Graciele, obrigada por ter compartilhado minhas angustias e ter me motivado a continuar.

As colegas Mariana e Stefânia, pela contribuição na escrita do nosso capítulo.

Aos amigos que o doutorado me possibilitou conhecer, companheiros de chimarrão que dividiram muitos bons momentos: Aline, Alberto e Ihana. Obrigada pelo carinho.

A Dona Lucia, funcionária do NTA que na sua humildade sempre esteve solícita em nosso ambiente de trabalho.

Agradeço a todos, que de uma maneira ou de outra, participaram e colaboraram para que esse trabalho fosse desenvolvido.

“ Humilde é aquela pessoa que sabe
que não sabe tudo, que sabe que outra pessoa
sabe o que ela não sabe,
que ela e a outra pessoa saberão muitas coisas juntas,
que ela e outra pessoa nunca saberão tudo que pode ser sabido.”

(Mário Sérgio Cortella)

RESUMO

CARACTERIZAÇÃO DE CAROTENOIDES E ATIVIDADE ANTIOXIDANTE DA BIOMASSA MICROALGAL

AUTORA: Luciana Dapieve Patias
ORIENTADOR: Eduardo Jacob Lopes

Este trabalho teve por objetivos: (i) identificar o perfil de carotenoides de três espécies de microalgas: *Chlorella vulgaris*, *Scenedesmus obliquus* e *Aphanotece microscopica Nägeli;* (ii) quantificar os carotenoides das três espécies de microalgas; (iii) avaliar a atividade antioxidante dos extratos de carotenoides obtidos das biomassas microalgais; (iv) identificar o perfil de carotenoides do extrato da microalga com maior potencial antioxidante em diferentes tempos de cultivo e (v) quantificar os carotenoides dos extratos da microalga com maior potencial antioxidante em diferentes tempos de cultivo. Os resultados obtidos demonstraram que a três espécies de microalgas apresentaram capacidade de produzir um conteúdo significativo de carotenoides sob condições fotoautotróficas, indicando seu potencial como fonte renovável desses pigmentos. Estes extratos provaram ser potente eliminador de radical peroxil. A maior atividade antioxidante foi da microalga *Chlorella vulgaris*, favorecido pelo seu perfil de carotenoides, que contém um grande número de ligações duplas conjugadas. É importante ressaltar que este foi o primeiro trabalho relacionado ao perfil de carotenoides e a capacidade antioxidante de *Scenedesmus obliquus* e *Aphanotece microscopica Nägeli*. O estudo também mostrou que o tempo de cultivo pode ser importante para definir em qual fase podemos acumular carotenoides de interesse, e prever o desempenho e otimização de condições operacionais. Os resultados encontrados nesse trabalho são promissores do ponto de vista de que a microalga deve ser considerada como uma matéria-prima potencial para a produção de uma grande diversidade de carotenoides naturais.

Palavras chave: Microalga. Cianobactéria. Pigmento. Carotenoides. Antioxidante.

ABSTRACT

CHARACTERIZATION OF CAROTENOIDS AND ANTIOXIDANT ACTIVITY OF MICROALGAL BIOMASS

AUTHOR: LUCIANA DAPIEVE PATIAS
ADVISOR: EDUARDO JACOB LOPES

This study has the folloing objectives: (i) to identify the carotenoid profile of these three species of microalgae: *Chlorella vulgaris, Scenedesmus obliquus and Aphanotece microscopica Nägeli*; (ii) to quantify the carotenoids of those three microalgae species; (iii) to evaluate the antioxidant activity of carotenoid extracts obtained from microalgal biomasses; (iv) to identify the carotenoid profile of the microalga extract with the highest antioxidant potential at different culture times and (v) to quantify the carotenoids of the microalgae extracts with higher antioxidant potential at different culture times. The obtained results have showed that the three species of microalgae have presented the capacity to produce a significant carotenoid content under photoautotrophic conditions, indicating their potential as a renewable source of these pigments. These extracts have proved to be a potent peroxil radical scavenger. The highest antioxidant activity has been noticed in the microalgae *Chlorella vulgaris*, favored by its carotenoid profile, which contains a large number of conjugated double bonds. It is important to emphasize that this has been the first research related to the carotenoid profile and the *Scenedesmus obliquus* and *Aphanotece microscopica Nägeli* antioxidant capacity. The study has also showed that the cultivation time may be important to define in which phase one can accumulate specific carotenoids, and predict the performance and the optimization of operating conditions. The results achieved from this study in the sense that microalgae must be considered a potential feedstock to a great diversity of natural carotenoids production.

Keywords: Microalgae. Cyanobacteria. Pigment. Carotenoids. Antioxidant.

LISTA DE ILUSTRAÇÕES

CAPÍTULO

Figure 1. Fundamental principles of energy balance 31

Figure 2. Chemical structure of typical microalgal biomass fatty acids 40

Figure 3. Chemical structure of a xanthophyll (lutein) and a carotene (β-carotene) 41

Figure 4. Chemical structure of fucoxanthin 42

Figure 5. Chemical structure of astaxanthin 43

ARTIGO

Figure 1. Chromatogram, obtained by HPLC-DAD, of the carotenoid extract from *Scenedesmus obliquus* (A), *Chlorella vulgaris* (B), and *Aphanothece microscopic Nägeli* (C). See text for chromatographic conditions. Peak identification and characterization are given in table 1. Chromatogram was processed at 451 nm 73

Figure 2. Chemistry mechanism for biotechnology production of carotenoids from *Scenedesmus obliquus*, *Chlorella vulgaris*, and *Aphanothece microscopic Nägeli.* Description of the illustration: 1 Cyclization; 2 Ketolation; 3 *Cis*-isomerization; 4 Hydroxylation; 5 Acetylation; 6 Epoxidation; 7 Epoxide-furanoxide rearrangement; 8 Glycosylation; *Intermediate compound not identified (All-*trans*-neoxanthin) between the peak one and peak two 75

Figure 3. (A) Fluorescense decay of C11-BODIPY581/591 induced by peroxyl radicals in the presence of concentrations at 70 μM of extract microalgae. Inset: linear relationship between the standards concentrations and net AUC values from the fluorescence decay curves of C11–BODIPY581/591 oxidation. (B) Fluorescence decay of C11–BODIPY581/591 induced by peroxyl radicals in the presence of different concentrations of α-tocopherol. Inset: linear relationship between α-tocopherol concentrations (62;126;252;504;756;1260 and blank in 0 μM) and net AUC values from the fluorescence decay curves of C11–BODIPY581/591 oxidation 76

MANUSCRITO

Figure 1. Chromatogram, obtained by HPLC–DAD, of the carotenoid extract from *Chlorella vulgaris* – 0 h (A), 72 h (B), and 168 h (C). See text for chromatographic conditions. Peak identification and characterization are given in Table 1. Chromatogram was processed at 451 nm 84

LISTA DE TABELAS

CAPÍTULO

Table 1. Average different compositions of the main groups of microorganisms (% dry weight) 35
Table 2. Biometric evaluation, glycemic index and total cholesterol values 45
Table 3. Products and ingredients from microalgae commercially applied 54

ARTIGO

Table 1. Chromatographic, UV-vis spectrum and mass characteristics, obtained by HPLC- PDA-MS/MS of microalgal carotenoids 74
Table 2. Quantitative characterization of carotenoids in microalgal extracts (µg/g dry weight) 75
Table 3. ROO• scavenging capacity of carotenoids from microlgal extracts 75

MANUSCRITO

Table 1. Chromatographic, UV–vis spectrum and mass characteristics, obtained by HPLC–PDA–MS/MS of *Chlorella vulgaris* carotenoids 85
Table 2. Quantitative characterization of carotenoids in microalgal extracts of *Chlorella vulgaris* (µg/g dry weight) 88

SUMÁRIO

1. INTRODUÇÃO **13**
2. REVISÃO DA LITERATURA **15**
2.1 MICROALGAS 15
2.2 FORMAS DE CULTIVO DE MICROALGAS 16
2.3 METABOLISMO DAS MICROALGAS 18
2.4 POTENCIAL DE USO DA BIOMASSA MICROALGAL 20
2.5 PROTEÍNA UNICELULAR 21
2.6 PIGMENTOS 22
2.7 CAROTENOIDES 23
2.8 BIOPRODUTOS DE ATIVIDADE BIOLÓGICA 25
3. OBJETIVOS **27**
3.1 OBJETIVO GERAL 27
3.2 OBJETIVOS ESPECÍFICOS 27
4. CAPÍTULO: SINGLE-CELL PROTEIN AS A SOURCE OF BIOLOGICALLY ACTIVE INGREDIENTS FOR THE FORMULATION OF ANTI-OBESITY FOODS **28**
5. ARTIGO: CAROTENOID PROFILE OF THREE MICROALGAE / CYANOBACTERIA SPECIES WITH PEROXYL RADICAL SCAVENGER CAPACITY **70**
6. MANUSCRITO: CAROTENOID PROFILE OF MICROALGAE Chlorella vulgaris AT DIFFERENT HARVESTING TIMES **78**
7. CONCLUSÃO **92**
REFERÊNCIAS **93**

1. INTRODUÇÃO

Atualmente, uma das principais áreas de pesquisa em ciência dos alimentos é a tecnologia de obtenção e caracterização de novas fontes naturais com atividade biológica. Estas substâncias são preferidas pelos consumidores por terem uma origem natural, sendo comumente extraídas a partir de plantas e microrganismos (GOUVEIA et al., 2010; WIJFFELS et al., 2013).

Neste contexto, microrganismos como as microalgas, constituem um grupo com ampla distribuição no globo terrestre e elevada relevância ecológica. Recentemente, esse grupo vem sendo considerado promissor para produzir diferentes compostos, incluindo produtos químicos especiais, inclusive bioquimicamente ativos, que podem ser incorporados a uma infinidade de aplicações industriais (GAFFNEY et al., 2014; HLAVOVA et al., 2015; GONG & BASSI, 2016).

Além disso, a imensa biodiversidade e consequentemente variabilidade da bioquímica da biomassa obtida a partir das culturas microalgais, fazem destas um importante campo de pesquisa, principalmente no que diz respeito à identificação de metabólitos com alto valor agregado, em função da atividade biológica que estes compostos desempenham (IBANEZ-CIFUENTES, 2013; GIMPEL et al., 2015).

Neste âmbito, existe uma tendência emergente no sentido da identificação destas substâncias como alternativa às fontes convencionas. Sabe-se que dentre estas substâncias, os pigmentos naturais, mais especificamente os carotenoides, estão abundantemente presentes na biomassa microalgal, especialmente por sua alta demanda como compostos bioativos. Assim, os pigmentos antioxidantes são atualmente os produtos de microalgas mais comercializados, renovando o interesse em aumentar a pesquisa e desenvolvimento nessa área (POOJARY et al., 2016).

Sabe-se que, já foram caracterizados cerca 600 carotenoides, esse número inclui a enorme variedade destes compostos em microalgas (BRITTON et al., 2004). Entretanto, os carotenoides de microalgas possuem características estruturais muito diferentes das encontradas comumente em frutas e vegetais, o que os torna importante para pesquisa (RODRIGUES et al., 2015).

O mercado global de carotenoides foi de US $ 1,2 bilhão em 2010 e deverá aumentar para US $ 1,4 bilhão em 2018. As maiores quotas de mercado são β-caroteno e astaxantina, com um preço médio próximo a US $ 2500 / kg (SUGANYA et al., 2016). Atualmente, os

carotenoides microalgais de *Dunaliella salina* e *Haematococcus pluvialis* são os mais produzidos. No entanto, existem desvantagens na produção destas cepas, como crescimento lento, rendimento insuficiente e requisitos nutricionais rigorosos (GUO et al., 2016). Por outro lado, as microalgas *Scenedesmus obliquus, Chlorella vulgaris* e *Aphanothece microscopica Nägeli* têm alto teor de carotenoides, crescem rapidamente e de forma robusta em biorreatores (FRANCISCO et al., 2010; MARONEZE et al., 2016). Em face disto, o presente trabalho teve por objetivo caracterizar os carotenoides e avaliar a atividade antioxidante da biomassa microalgal.

2. REVISÃO DA LITERATURA

2.1 MICROALGAS

As microalgas correspondem a um grupo diverso de microrganismos, apresentando duas formas celulares básicas: procariotas, como as cianobactérias (*Cyanophyceae)* e eucariotas como as algas verdes (*Chlorophyta)* e diatomáceas *(Bacillariophyta) (*CHACON-LEE & GONZÁLEZ-MARIÑO, 2010; KUMAR et al., 2014; AL HATTAB & GHALY, 2015). As células procariotas apresentam estrutura interna simplificada desprovida de organelas envoltas por membranas. Por outro lado, os eucariotas possuem células maiores e com maior complexidade, apresentando estruturas delimitadas por membranas, denominadas organelas (MADIGAN et al., 2004; LEE, 2008).

O número exato de espécies de microalgas não é conhecido, no entanto, são encontradas citações que relatam a existência de 200.000 até alguns milhões de representantes deste grupo (NORTON et al., 1996; PULZ & GROSS, 2004; GIMPEL et al., 2015).

As microalgas são microrganismos fotossintéticos, unicelulares e pigmentados que usualmente, porém não necessariamente, estão em ambientes aquáticos marinhos e de água doce, como também podem ser encontradas na superfície de alguns tipos de solos (CHEN et al., 2011). Entretanto, devido a sua grande diversidade, as microalgas podem ser encontradas em praticamente todos os nichos terrestres, inclusive em localidades que apresentam grandes variações físicas e químicas (TIRICHINE & BOWLER, 2011; BLUNT et al., 2012; HILDEBRAND et al., 2013).

As principais vantagens para a produção de biomassa de microalgas estão relacionadas à rápida reprodução e a utilização de fontes baratas de energia e de nutrientes para sua multiplicação. Evidentemente que isto se faz à custa do meio em que os microrganismos se desenvolvem, podendo ser constituído de substratos dos mais variados, alguns de baixo custo, incluindo resíduos industriais, os quais resolveriam problemas de ordem ambiental, servindo ainda para produção de insumos (RAVINDRA & ANUPAMA, 2000).

Das diferentes formas de obtenção de biomassa para geração de energia, as microalgas têm se destacado, principalmente devido a sua maior atividade fotossintética, quando comparadas com plantas superiores e a possibilidade de serem cultivadas ao longo de todo ano em áreas impróprias para a agricultura (COUTO, 2016).

A grande diversidade das microalgas e suas características fisiológicas tornam este grupo uma fonte potencialmente rica para aplicação em diferentes setores da economia (OLAIZOLA, 2003; GOUVEIA et al., 2010; GAFFNEY et al., 2014). Desta forma, alguns usos potencias das microalgas são possíveis, como na alimentação humana (fonte de proteína unicelular e uso na suplementação de produtos como massas, sopas e bebidas) (GUIL-GUERREIRO et al., 2004), na alimentação animal (algumas espécies são frequentemente empregadas na alimentação de peixes e crustáceos) (OLVERA-NOVOA et al., 1999); na produção de biomoléculas (fonte de Clorofila *a*, ficocianina, β-caroteno, ácido γ–linolênico, e ácido eicosapentanóico) (SPOLAORE et al., 2006; JACOB-LOPES et al., 2007; FRANCISCO et al., 2014); em fertilizantes (uso de biomassa como fonte de nitrogênio e fósforo em terras agriculturáveis) (CHAE et al., 2006); na produção de biogás (produzindo CH_4 em fermentadores a partir da digestão de biomassa) (YEN & BRUNE, 2007); na produção de biocombustíveis (produzindo biodiesel através da fração lipídica da células) (MIAU & WU, 2006; XU et al., 2006; HLAVOVA et al., 2015; KOSINKOVA et al., 2015) e em menor quantidade, compostos fenólicos (MAADANE et al., 2015).

2.2 FORMAS DE CULTIVO DE MICROALGAS

O crescimento de microalgas é resultado da interação entre fatores biológicos, físicos e químicos. A composição química da biomassa das microalgas é determinada pela natureza de cada espécie de microalga. Os cultivos de microalgas podem ser classificados em: fotoautotrófico, utilizando a luz como única fonte de energia, a qual é convertida em energia química através das reações fotossintéticas; heterotrófico, usando compostos orgânicos como energia e fonte de carbono; ou ainda em sistema de cultivo mixotrófico, quando a fotossíntese e a oxidação de compostos orgânicos acontecem concomitantemente (FERREIRA & SOARES, 2013).

Os estudos de cultivos de microalgas em grande escala têm dado ênfase ao metabolismo fotoautotrófico, uma vez que algumas espécies apresentam altas taxas fotossintéticas, bioconvertendo eficientemente a energia solar. Além disso, apresentam grande versatilidade metabólica, a qual reflete as diversas condições dos habitats onde podem ser encontrados. Em muitas ocasiões, os habitats naturais das microalgas apresentam condições inadequadas para a fotossíntese, principalmente em relação à luminosidade, sendo capaz de

crescer também heterotroficamente, na ausência de energia luminosa (WEN & CHEN, 2003; BRUTEMARK & GRANÉLI, 2011; JEONG et al., 2010; PEREIRA et al., 2012).

O crescimento heterotrófico no escuro, apoiado por uma fonte de carbono exógena, é uma habilidade importante de algumas espécies de organismos fotossintéticos (FRANCISCO et al., 2014). Além disso, os cultivos heterotróficos também são caracterizados por serem baratos, simples para a construção de instalações e fáceis de manter em grande escala, uma vez que requer apenas um fermentador convencional como biorreator (PEREZ-GARCIA et al., 2011). No entanto, as culturas heterotróficas apresentam algumas limitações, como o número limitado de espécies de microalgas que podem crescer heterotroficamente, contaminação e competição com outros microrganismos, incapacidade de produzir metabólitos induzidos pela luz e alta dependência de substratos de carbono. As microalgas podem assimilar uma variedade de fontes orgânicas de carbono (como glicose, acetato, glicerol, frutose, sacarose, lactose, galactose e manose) para o crescimento. Estima-se que cerca de 80% do custo total do meio de cultura seja atribuído a uma fonte de carbono orgânico exógeno em cultivo heterotrófico. Nesse sentido, alguns estudos têm como objetivo buscar fontes de carbono orgânicas mais baratas, como resíduos agroindustriais e amido (FRANCISCO et al., 2014; FRANCISCO et al., 2015).

Além de atender a demanda por carbono orgânico, o uso de águas residuais nesses cultivos também contribui para o gerenciamento de resíduos agroindustriais, tornando economicamente viável a produção de proteínas monocelulares (MARONEZE et al., 2014).

O cultivo mixotrófico é quando as microalgas sofrem fotossíntese e utilizam compostos orgânicos e carbono inorgânico (CO_2) como fonte de carbono para o crescimento. A capacidade de mixotrofos para processar substratos orgânicos significa que o crescimento celular não é estritamente dependente da fotossíntese, portanto, a energia da luz não é um fator absolutamente restritivo para o crescimento, pois os substratos de carbono leve ou orgânico podem suportar o crescimento (CHOJNACKA & MARQUEZ-ROCHA, 2004).

Devido à presença de compostos orgânicos no meio, a cultura mixotrófica pode ser facilmente contaminada por microrganismos heterotróficos. Além disso, o custo do meio aumenta o custo da produção de biomassa, como no cultivo heterotrófico. Outra questão que deve ser levada em consideração é o *design* do biorreator, para ter um melhor controle sobre as condições de cultura, aumentando a produtividade da biomassa.

A produção de microalgas em larga escala pode ser realizada em diferentes tipos de sistemas, que podem ser abertos ou fechados. A forma de cultivo de microalgas mais utilizada

são as lagoas aeradas abertas e os fotobiorreatores fechados (RAZZAK et al., 2013). No entanto, o uso de ambas as técnicas de cultivos sofre influências pelas características do local de cultivo, pela espécie utilizada, pela quantidade de luz necessária e o processo de recuperação da biomassa do meio de cultura (centrifugação, floculação e filtração), que se pretende utilizar (CARDOSO & VIEIRA, 2011).

De forma geral, o cultivo através de lagoas aeradas abertas é realizado em bacias de pouca profundidade aberta para o meio ambiente. É um cultivo considerado relativamente barato e de fácil construção, desde que a área seja plana, projetados em locais que tenham disponibilidade de área e radiação solar. Este cultivo pode ser feito diretamente sobre o solo ou ainda pode-se utilizar uma cobertura na superfície simples para minimizar a perda de água devido ao escoamento. Porém, esse tipo de sistema apresenta como maior desvantagem os riscos de contaminação, onde uma espécie, por exemplo, com elevada produtividade de lipídios pode ser facilmente invadida por uma espécie selvagem de crescimento rápido (MATA et al., 2010; LEITE et al., 2013).

Os fotobiorreatores fechados são sistemas que funcionam em recipientes transparentes onde são colocadas as culturas de microalgas, podendo ter diferentes tamanhos e formas, e serem de sacos de plástico, placas planas e tubos. Como principais vantagens de seu uso são a resistência à contaminação por espécies de algas selvagens ou herbívoras, alta produtividade por unidade de área, e a possibilidade de controlar parâmetros como o pH, temperatura e a intensidade de luz. O fotobiorreator pode ser colocado no interior das instalações de fábricas ou no exterior, usando a luz solar, luz artificial ou uma mistura de ambas (MATA et al., 2010; LEITE et al., 2013).

2.3 METABOLISMO DAS MICROALGAS

Algumas espécies de microalgas possuem versatilidade no que se refere à manutenção de suas estruturas, usufruindo de diferentes tipos de metabolismo energético, como: a respiração, a fotossíntese, e a fixação de nitrogênio (GROBBELAAR, 2004; JEONG et al., 2010; BRUTEMARK & GRANÉLI, 2011; QUEIROZ et al., 2013). O modo mais comum de cultivo destes microrganismos é o crescimento autotrófico, através da utilização de CO_2 como fonte de carbono inorgânico, nutrientes e luz. Segundo Beck et al. (2012), estes simples requerimentos potencializam o interesse na exploração tecnológica destes organismos.

A fotossíntese é a forma de obtenção de energia mais utilizada pelas microalgas; porém, em casos que não há contato com a luz, algumas obtêm energia através da respiração. Entretanto, quando o nitrogênio é escasso no meio, existem microalgas que desenvolvem organelas capazes de fixá-lo (LOURENÇO, 2006).

A fotossíntese é um processo no qual, compostos inorgânicos e energia luminosa são convertidos em matéria orgânica. As microalgas são organismos fotossintéticos que produzem oxigênio, o que significa que eles usam a energia luminosa para extrair prótons e elétrons da água para reduzir o CO_2, a fim de formar moléculas orgânicas. O processo de fotossíntese pode ser dividido em duas fases de reação, que diferem pelo fato da primeira ser dependente de luminosidade. Na fase da luz, a energia luminosa é capturada na forma de fótons por um complexo sistema coletor de luz ligado a pigmentos (clorofilas e carotenoides que são componentes lipofílicos, e as ficobilinas que são os componentes hidrofílicos). As ficobilinas estão covalentemente ligadas a proteínas específicas, formando ficobiliproteínas, as quais se associam em complexos altamente ordenados chamados ficobilissomas, que constituem as principais estruturas de captação de luz nestes microrganismos (MASOJÍDEK et al., 2004; MARKOU & GEORGAKAKIS, 2011). Esta energia é utilizada pelo fotossistema II na oxidação da água, liberando prótons, elétrons e moléculas de O_2. Os elétrons são transferidos através da cadeia transportadora de elétrons até o fotossistema I, e levam à redução da ferredoxina para a formação do intermediário redutor NADPH. Um gradiente eletroquímico é formado devido à liberação de prótons após a oxidação da água para o lúmen do tilacóide, o qual é utilizado para conduzir a produção de ATP via ATP sintase. Na fase seguinte, os produtos das reações com luz são subsequentemente consumidos pela redução de CO_2 a carboidratos. Os produtos fotossintéticos NADPH e ATP são os substratos para o ciclo de Calvin-Benson, onde o CO_2 é fixado em moléculas de três átomos de carbono que são assimilados em açúcares, amido, lipídios, ou outras moléculas exigidas para o crescimento celular. Já o substrato para a hidrogenase, íon hidrogênio e elétron, são supridos tanto via cadeia de transporte de elétrons fotossintéticos como via fermentação do carboidrato armazenado (BEER et al., 2009).

O oxigênio gerado neste processo é então facilmente ativado, e pode gerar espécies reativas de oxigênio (ERO's). Microalgas desenvolveram mecanismos de adaptação e de proteção no sentido de evitar danos oxidativos e um deles consiste na produção de compostos antioxidantes capazes de minimizar a concentração de ERO's (RODRIGUEZ-GARCIA &

GUIL-GUERRERO, 2008). Desta forma, a biomassa de microalgas tem aparecido como uma fonte natural alternativa de compostos com atividade antioxidante (KUMAR et al., 2014).

A respiração é outra via metabólica importante utilizada por microalgas, sendo que o metabolismo das microalgas é semelhante as plantas superiores. A utilização desta via metabólica, suportada por uma fonte de carbono exógeno, supera grandes limitações na produção de compostos úteis a partir de microalgas, principalmente a dependência de luz, que complica significativamente o processo, aumenta os custos e diminui o rendimento de produção (PEREZ-GARCIA et al., 2011).

As características metabólicas das microalgas fazem com que estes microrganismos apresentem uma importante fonte de recursos a serem explorados (TRAJUDDIN & SUBRAMANIAM, 2005).

2.4 POTENCIAL DE USO DA BIOMASSA MICROALGAL

O cultivo de microalgas é praticado há quase 140 anos, porém nas últimas décadas com o avanço e aprimoramento de tecnologias e ciências como a fisiologia, microbiologia e as engenharias de maneira geral, houve um avanço considerável na compreensão do potencial biotecnológico destes microrganismos (RICHAMOND, 2004; LOURENÇO, 2006).

O potencial biotecnológico e comercial das microalgas representa um recurso ainda inexplorado, uma vez que das possíveis espécies existentes, relativamente poucas foram estudadas em detalhe, tanto do ponto de vista bioquímico quanto fisiológico (WATERBURY, 2006). A partir da manipulação das condições de cultivo e notadamente a presença ou ausência de determinados nutrientes, estimula a biossíntese de compostos que vão desde enzimas até fármacos com elevados valores comerciais (DONG & ZAO, 2004).

As microalgas vêm sendo reconhecidas, por sua versatilidade, principalmente devido à identificação de diversas substâncias com componentes valiosos, sintetizadas por esses organismos, com uma ampla gama de aplicação (METTING & PYNE, 1986; FERREIRA et al., 2013; PRAVEENKUMAR et al., 2015). Ainda, as microalgas são microrganismos clorofilados capazes de converter através do processo da fotossíntese o CO_2 em uma grande variedade de metabólitos e produtos químicos, incluindo proteínas, polissacarídeos, hidrogênio e lipídeos (MARKOU & NERANTZIS, 2013; GONG & BASSI, 2016).

Uma vez que a produtividade destes organismos é muito elevada, quando comparada a processos convencionais de produção de nutrientes, estes microrganismos constituem uma

importante reserva de proteínas e outras substâncias celulares que podem ser utilizadas, desde que bem exploradas tecnologicamente (JACOB-LOPES et al., 2006). Neste sentido, as características destes organismos, como alta capacidade fotossintetizante, alto potencial de absorção de CO_2, rápido crescimento e valor agregado da biomassa resultante, tem despertado grande interesse para fins comerciais (KHAN et al., 2009).

Os compostos de particular interesse comercial, produzidos a partir de microalgas incluem ácidos graxos, carboidratos, pigmentos, proteínas e vitaminas (SPOLAORE, 2006; STENGEL et al., 2011; LÓPEZ-GONZÁLEZ et al., 2015) o que as torna uma fonte importante de bioprodutos, muitos dos quais apresentam múltiplas bioatividades com aplicações.

2.5 PROTEÍNA UNICELULAR

O termo proteína unicelular foi definido pelo Instituto de Tecnologia de Massachusetts em 1966, referindo-se à biomassa de microrganismos, tais como: leveduras, bactérias, fungos e microalgas, cultivados em sistemas de cultura em larga escala para uso na alimentação humana e animal (JACOB-LOPES et al., 2006; SUMAN et al., 2015). No entanto, não só as proteínas, mas também os aminoácidos, lipídios, carboidratos, vitaminas e minerais são frequentemente incluídos no termo da proteína unicelular (NALEGE et al., 2016).

A produção de proteína unicelular por microrganismos apresentam algumas vantagens, como: alta taxa de crescimento e tempo de produção; capacidade de crescer em diversos substratos; utilização de fontes baratas de energia e nutrientes para sua multiplicação; resistência à contaminação e condições adversas; possibilidade de modificação genética; alto conteúdo nutricional; proteínas, gorduras e carboidratos de alta qualidade; baixo teor de ácido nucleico; alta digestibilidade; ausência de toxicidade; boas propriedades sensoriais e funcionais (GOLDBERG, 1985; NALAGE et al., 2016).

Dentre os microrganismos utilizados para a produção de proteína unicelular, destacam-se as microalgas devido aos altos teores de proteínas (40-70%) e gorduras (7-30%), além de possuírem elevados níveis de aminoácidos essenciais e ácidos graxos. Somando-se a isso, contém uma alta porcentagem de proteínas digeríveis (60-75%), vitaminas, aminoácidos e outros componentes com propriedades funcionais (GOLDBERG, 1985).

O teor proteico das microalgas associado ao balanço de aminoácidos indica o alto valor nutricional destas proteínas, que em muitos casos é compatível e até mesmo superior a fontes proteicas convencionais como a carne, ovos e farinha de trigo. A composição bioquímica destes microrganismos também apresenta ácidos graxos com predominância dos insaturados, vitaminas, carboidratos e pigmentos, que viabilizam o seu uso na complementação da dieta alimentar (ANUPAMA & RAVINDRA, 2000; JACOB-LOPES et al., 2006).

É fundamental que novas fontes de alimentos sejam encontradas para que as futuras gerações sejam alimentadas adequadamente. Uma fonte de alimento ideal deve ser completada nutricionalmente e requer um mínimo de solo, tempo e custo para produzir. Além de atender a esses critérios, a proteína unicelular pode ser produzida em uma variedade de materiais de resíduos (JAY, 1992).

No entanto, um dos principais fatores limitantes no uso de proteína unicelular, como alimento é o seu teor de ácido nucleico. A ingestão de uma dieta rica em ácido nucleico resulta na produção de ácido úrico por degradação do ácido nucleico, causando distúrbios de saúde, como a formação de gota ou de cálculos renais. Embora os índices altos de ácido nucleico tenham apresentado problemas no desenvolvimento precoce e no uso de proteína unicelular, estes compostos podem ser reduzidos a níveis inferiores a 2% por diversas técnicas (LOURENÇO, 2006).

2.6 PIGMENTOS

Todos os organismos fotossintéticos contêm pigmentos orgânicos para converter luz em energia (MASOJÍDEK et al., 2004). Neste sentido, a aparência colorida de microalgas é derivada de seus pigmentos, que absorvem a luz visível e iniciam reações de fotossíntese (HALL & RAO, 1999).

As microalgas são microrganismos reconhecidos como uma excelente fonte de pigmentos, os quais podem ser produzidos de formas renováveis, uma vez que produzem alguns pigmentos exclusivos de forma sustentável. Portanto, elas são uma fonte altamente confiável de pigmentos, que podem ser produzidos em grandes quantidades (METTING & PYNE,1986).

As três principais classes de pigmentos fotossintéticos que aparecem em algas são clorofilas, carotenóides e ficobilinas (HALL & RAO, 1999).

As clorofilas são substâncias lipossolúveis esverdeadas encontradas nos cloroplastos, as quais contém um anel de porfirina, capaz de captar a energia da luz para a realização de fotossíntese (MARQUEZ & SINNECKER, 2008). O papel principal nos sistemas de absorção de luz é desempenhado pela clorofila *a*, portanto, ela está presente em todas as espécies fotossintetizantes. Já as clorofilas *b, c* e *d* são pigmentos acessórios a fotossíntese, sendo que a maioria das algas possui alguma delas (LOURENÇO, 2006).

Os carotenoides são compostos lipofílicos, geralmente de cor amarela, laranja ou vermelha e são os pigmentos mais diversos encontrado na natureza, sendo considerados os mais importantes comercialmente (SASSO et al., 2012; VARELA et al., 2015). Da mesma forma que as clorofilas *b, c* e *d*, são pigmentos acessórios da fotossíntese, que também possuem função de proteger os fotossistemas quando há excesso de luz, funcionando como uma espécie de filtro. No grupo dos carotenoides destaca-se o β-caroteno, o qual é convertido pelo corpo humano em vitamina A e possui propriedades antioxidantes (MASOJÍDEK et al., 2004; DUFOSSÉ, 2005; SPOLAORE et al., 2006).

As ficobilinas estão presentes apenas em alguns tipos de algas e podem ser pigmentos azuis, sendo chamadas de ficocianinas, ou pigmentos vermelhos, sendo chamados de ficoeritrinas, estes pigmentos são capazes de aumentar o espectro de captação da luz pela fotossíntese e também atuam como reserva de nitrogênio (LOURENÇO, 2006).

2.7 CAROTENOIDES

Os carotenoides são pigmentos derivados de terpenos encontrados em cloroplastos e cromoplastos de plantas e organismos fotossintéticos, onde seu papel é apoiar a fotossíntese. São pigmentos lipossolúveis produzidos como metabólitos secundários em frutas, vegetais, algas, fungos e algumas bactérias (ZAGHDOUDI et al., 2015).

Com base em diferentes características químicas, os carotenoides podem ser classificados como xantofilas e carotenos. As Xantofilas são carotenoides com oxigênio presente nas moléculas, como a luteína, fucoxantina, astaxantina e zeaxantina. Os carotenos são carotenoides não oxigenados que contêm apenas carbono e hidrogênio. Exemplos de carotenos incluem β-caroteno, α-caroteno e licopeno (BRITTON, 2008; CUTTRISS et al., 2011; REDAELLI, 2012; VARELA et al., 2016).

Cada espécie de microalgas comumente apresenta entre 5 e 10 tipos de carotenoides de um universo de 600 carotenoides diferentes (DONATO et al., 2003; PLAZA et al., 2010).

São compostos bioativos responsáveis pela cor amarela, laranja ou vermelha dos alimentos; tal efeito decorre de sua estrutura básica que consiste em um esqueleto linear e simétrico com uma série de duplas ligações conjugadas, denominado cromóforo de absorção de luz (RODRIGUEZ-AMAYA, 2001). Vários estudos relataram que os principais carotenoides encontrados em microalgas são β-caroteno, zeaxantina, luteína, nostoxantina, oscilaxantinas, equinenona, mixoxantofila e cantaxantina (PRASANNA et al., 2010; WALTER & STRACK, 2011; RODRIGUES et al., 2015).

Em particular, os carotenoides das algas atraem a atenção como novos ingredientes alimentares funcionais, devido ao alto conteúdo encontrado, que pode atingir 0,2% dos carotenoides (TALERO et al., 2015). O aspecto biológico mais importante dos carotenoides para humanos é o papel da atividade da provitamina A e outras propriedades intrínsecas, como a ação antioxidante, anti-inflamatória e uma possível associação à prevenção de câncer (BECKER, 1994; GRAHAM & WILCOX, 2000; DUFOSSÉ et al., 2005; SPOLAORE et al., 2006; REDAELLI, 2012). Além disso, os carotenoides alimentares são conhecidos por reduzir o risco de doenças cardiovasculares, degeneração macular relacionada com a idade e câncer. O mecanismo que está associado a todos esses efeitos benéficos para a saúde são as propriedades antioxidantes dos carotenoides (MIYASHITA et al., 2014).

Uma vez que o organismo humano não é capaz de sintetizar carotenoides, e estes compostos apresentam propriedades que os tornam importantes na alimentação por serem benéficos à saúde, estes pigmentos vêm sendo uma das principais áreas de exploração biotecnológica de microalgas, com ampla gama de aplicações (MOGEDAS et al., 2009; OTA et al., 2009; PEREZ-GARCIA et al., 2011). Neste contexto, destaca-se o elevado potencial antioxidante atribuído aos carotenoides microalgais (FIEDOR & BURDA, 2014; CHUYEN & EUN, 2017; MANAYI et al., 2016). Esta propriedade se deve ao sistema de duplas ligações conjugadas presentes em sua estrutura (MERCADANTE, 2008), podendo ser influenciada pelo número de ligações duplas conjugadas, tipo de grupos terminais estruturais e oxigênio contendo substituintes (SAINI et al., 2015).

De acordo com *USDA (2013)*, do ponto de vista industrial e comercial, os carotenoides têm um mercado maduro e estável e suas aplicações incluem corantes industriais, para utilização em produtos alimentícios e ração para animais, cosméticos e suplementos (PULZ & GROSS, 2004; DEL CAMPO et al., 2007; YE et al., 2008).

2.8 BIOPRODUTOS DE ATIVIDADE BIOLÓGICA

Nas últimas décadas, as microalgas têm atraído grande interesse como um dos grupos mais promissores de organismos para isolar produtos naturais e ativos bioquímicos de alto valor agregado (PRASANNA et al., 2008; RODRÍGUEZ-MEIZOSO et al., 2008; HLAVOVA et al., 2015).

A exploração comercial em larga escala das microalgas teve início em 1950, motivada pelo elevado teor de proteínas da biomassa, para utilização como recurso alimentar alternativo (SPOLAORE et al., 2006; SUGANYA et al.,2016). Entretanto, o teor proteico já não é mais único argumento para promover a sua utilização, pelo fato das pesquisas estarem direcionadas à produção e isolamento de compostos bioativos encontrados nestes microrganismos (PRASANNA et al., 2010; COSTA et al., 2013). Algumas vantagens como o baixo custo das fontes de energia e nutrientes utilizados para a sua multiplicação, bem como as taxas de crescimento rápido e capacidade de acumular ou secretar metabólitos, fazem com que as microalgas tenham grande potencial de produção de biomoléculas (CHRISTENSON & SIMS, 2011).

Recentemente, alguns pesquisadores têm previsto enormes possibilidades das microalgas como fonte potencial de compostos bioativos, particularmente pelo grande interesse em seus pigmentos, na medida em que possuem importância terapêutica e uma vasta gama de aplicações, que lhe confere, consequentemente, um alto valor econômico (BOROWITZKA, 2013). Neste sentido, as microalgas são uma importante fonte produtora de pigmentos, como o β-caroteno, astaxantina, clorofila a e ficocianina, e vitaminas de interesse comercial, tais como a vitamina B12 e vitamina E, que poderão ser comercializados como constituintes de uma alimentação saudável, conhecida também por "*health food*", bem como em suplementos alimentares nas mais diversas formas (BAKER & GUNTER, 2004; PULZ & GROSS, 2004; KIM et al., 2007).

Aos pigmentos também são atribuídas propriedades físico-químicas que lhes permitem participar de uma série de reações e desempenhar papéis essenciais em várias atividades em nível celular e em sistemas alimentícios (ERIKSEN, 2008). Estas reações têm sido relacionadas à suas capacidades de modular reações de oxidação, através da desativação de espécies reativas de oxigênio (ERO's). ERO é um termo amplo que engloba radicais de oxigênio, como o radical peroxila ($ROO^\bullet$) e derivados de oxigênio não radicalares, tais como peróxido de hidrogênio (H_2O_2) e o oxigênio singlete ($^1O_2$) (HALLIWELL et al., 2007). Em

condições fisiológicas normais, as ERO's são essenciais e desempenham uma série de funções importantes, entretanto, em casos em que ocorre um desequilíbrio entre sua geração e consumo, conduzindo ao estresse oxidativo, elas podem se tornar extremamente deletérias e têm sido implicadas na fisiopatologia de doenças crônicas não degenerativas (VALKO et al., 2007). Os danos induzidos por ERO's podem afetar componentes presentes nos alimentos, nos quais alteram atributos sensoriais, nutricionais e toxicológicos (MIN & CHOE, 2002; WETTASINGHE & SHAHIDI, 2000). Diante do exposto, há grande demanda por antioxidantes tanto na dieta quanto para produtos alimentares, cosméticos, farmacêuticos e outros bens oxidáveis e observa-se a busca por fontes naturais de antioxidantes (DUFOSSÉ, 2006). Devido ao seu caráter natural, surgem os pigmentos microbianos como uma fonte antioxidante natural, que é independente da estação do ano e das condições geográficas, de rendimento controlável e de qualidade previsível (JOSHI et al., 2003). Desta forma, espera-se que os pigmentos de microalgas venham a superar os sintéticos bem como outras fontes naturais devido à sua sustentabilidade da produção e natureza renovável (DUFOSSÉ, 2005).

3. OBJETIVOS

3.1 OBJETIVO GERAL

Caracterizar os carotenoides e avaliar a atividade antioxidante da biomassa microalgal.

3.2 OBJETIVOS ESPECÍFICOS

- Identificar o perfil de carotenoides de três espécies de microalgas.
- Quantificar os carotenoides das três espécies de microalgais.
- Avaliar a atividade antioxidante dos extratos de carotenoides obtidos das biomassas microalgais.
- Identificar o perfil de carotenoides do extrato da microalga com maior potencial antioxidante em diferentes tempos de cultivo.
- Quantificar os carotenoides dos extratos da microalga com maior potencial antioxidante em diferentes tempos de cultivo.

4. CAPÍTULO: SINGLE-CELL PROTEIN AS A SOURCE OF BIOLOGICALLY ACTIVE INGREDIENTS FOR THE FORMULATION OF ANTI-OBESITY FOODS

Handbook of Food Bioengineering:

Volume 17: Alternative and replacement foods

Editors: Alexandre Grumezescu, Alina Maria Holban

Copyright: ©Academic Press 2018

Published: 2nd February 2018

Page Count: 472

SINGLE-CELL PROTEIN AS A SOURCE OF BIOLOGICALLY ACTIVE INGREDIENTS FOR THE FORMULATION OF ANTI-OBESITY FOODS

Luciana Dapieve Patias, Mariana Manzoni Maroneze, Stefania Fortes Siqueira, Cristiano Ragagnin de Menezes, Leila Queiroz Zepka, Eduardo Jacob-Lopes*

Department of Food Science and Technology, Federal University of Santa Maria (UFSM), 97105-900, Santa Maria, RS, Brazil

Abstract

According to the world health organization, obesity is a major public health problem all over the world. Several mechanisms are responsible for the development of obesity, but diets with adequate macronutrient composition seem to play an important role in controlling body weight. Currently, some authors have demonstrated the ability of proteins to affect food intake and appetite in humans. Thus, increased protein intake might promote weight loss, acting against the development of obesity. The scarcity of protein-rich food has forced mankind to search for alternative protein sources that can replace conventional ones. Microalgae-based processes is a field of food bioengineering and is considered to be the key to producing novel food ingredients with potential health benefits, mainly due to high protein levels found in biomass, besides the other components, such as fatty acids and pigments. In this sense, the purpose of this chapter is to elucidate the use of microalgae biomass as a food ingredient, emphasizing the ability of single-cell proteins as an anti-obesity factor, and evaluate the feasibility of their inclusion in foods for control of obesity.

Key words: obesity; protein; microalgae; technological properties

Introduction

The prevalence of obesity in humans is increasing in both developed and developing countries. Obesity is rapidly becoming a worldwide public health problem despite food shortages in many parts of the world (Heindel et al., 2015). According to estimates of the World Health Organization (WHO), in 2014, more than 1.9 billion adults were overweight, and more than 600 million of them were obese. The prevalence of obesity has doubled or even risen threefold in less than two decades and has caused approximately 2.8 million deaths per year (WHO, 2014).

Both overweight and obesity are associated with an increased risk of metabolic, cardiovascular and chronic inflammatory diseases, such as type 2 diabetes mellitus, dyslipidaemia, non-alcoholic fatty liver disease, hypertension, coronary heart disease, stroke

and osteoarthritis, as well as various forms of cancer (Moustafa and Froguel, 2013; Kusminski et al. 2016).

Obesity is primarily defined as the excess of fat mass of sufficient magnitude to produce adverse health consequences. It is diagnosed based on Body Mass Index (BMI). The BMI is defined as a person's weight in kilograms divided by the square of his height in meters (kg/m^2). WHO defined that a person whose BMI is greater than or equal to 25 is overweight and a BMI greater than or equal to 30 indicates obesity.

Losing weight can reverse the harmful health effects attributed to excess weight, and may improve or prevent obesity-related diseases. Current therapies for obesity are based on dieting and exercise, a select number of drugs for a fraction of patients, and stomach (bariatric) surgery for extremely obese individuals (Mohamed et al., 2014). Unfortunately, the above-mentioned strategies have only shown limited success, since lifestyle modification is difficult to persist and drugs and surgical procedures are frequently accompanied by many kinds of side-effect and complications (Hu et al., 2016).

As a consequence, nowadays, a huge interest has been aroused among food industry and consumers in products that can promote human health, especially on obesity control. Microalgal single-cell protein has been considered as part of a healthy diet due to their content of proteins, amino acids, bioactive peptides, fatty acids and pigments. Thus, this complex of high biological value compounds, when inserted in a food system, can induce body weight reduction and prevent diet-induced obesity (Kumar et al., 2015).

In this sense, the purpose of this chapter is to elucidate the microalgae biomass use as a food ingredient, emphasizing the ability of single-cell proteins as an anti-obesity factor, and evaluate the feasibility of their inclusion in foods for control of obesity, through technological advantages of the use of single-cell proteins in foods and food processing technologies manifested in terms of sensory, nutritional and performance properties.

Section 1 Weight control mechanism

Although obesity has been associated with serious health problems for a long time, only recently has it been regarded as a disease in the sense of being a specific target for medical therapy (Melnikova and Wages, 2006). Obesity treatments are focused on three types of therapy, which consist of dieting, pharmacotherapy and bariatric surgery. The first recommendation to the patients is the change in lifestyle (dieting and physical activity). If the patient is not able to achieve the target weight and health goal by lifestyle alone and meets the indications for drug therapy, then addition of adjunctive pharmacotherapy should be considered. As a third step, bariatric surgery can be considered for patients with more severe disease and who meet its indications (Kushner, 2014).

The main challenges in the treatment of obesity are weight loss and long-term weight maintenance, which is difficult to achieve since body-weigh regain appears to be the pitfall. Maintenance of body weight of individuals is done through energy balance, which involves equilibrium between calorie intake and energy utilization (physical activity, basal metabolism,

and adaptive thermogenesis). Energy balance in animals is governed by the First Law of Thermo dynamics, and is often expressed as a simple equation (Mohamed et al., 2014):

Energy Balance = Energy Intake - Energy Expenditure

The development of overweight and obesity is given by a positive energy balance and is a consequence of the easy and cheap availability of high-calorie foods, which is combined with sedentary lifestyle, as shown in Figure 1. Conversely, when energy expenditure exceeds energy intake, a state of negative energy balance ensues and the consequence is a loss of body mass (Mohamed 2014).

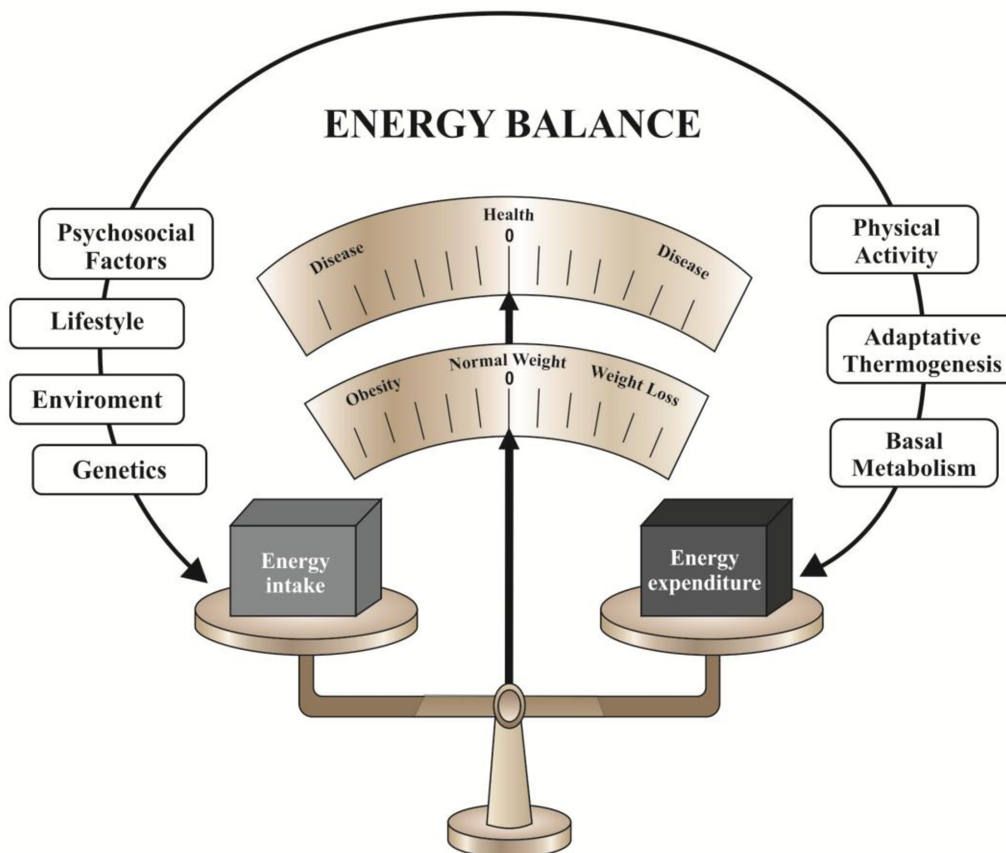


Figure 1. Fundamental principles of energy balance (Adapted from Mohamed et al., 2014).

Although there are many strategies for the treatment of obesity, this chapter will focus primarily on current diets for weight loss. Specifically, on higher-protein diets in body weight regulation.

1.1 Weight control diets

From the beginning of the twentieth century until the 1950s, a high-fat diet was recommended by the medical profession as a suitable diet for health maintenance and the treatment of diabetes. However, in the 1970s, a reduction in fat was recommended in the diet, as a means of lowering serum cholesterol and preventing heart disease (Stamler, 1982).

Then, low-fat diets emerged and they are probably one of the most commonly recommended types of diet. This occurs because, although controversy still exists over whether fat consumption is the major determinant of body fat mass, the generally accepted paradigm is that low-fat/high-carbohydrate diets promote weight loss. This type of diet can particularly promote weight loss if the consumed carbohydrate-rich foods are of plant origin, and have low glycemic index, but this consumption has also been associated with adverse effects because it can promote insulin resistance (D'Antona, 2014). Moreover, it is difficult to maintain for a long time, because it can be monotonous and offer less satiety than fat, when compared to the same amount of food in carbohydrates or proteins (MCmillam-Price & Brand-Miller, 2004).

Meal replacement diets also are well-known for high efficiency in weight loss. These diets are a form of low-calorie diet in which one or two full calorie daily meals are replaced by a low-calorie drink. Some recent studies evaluating the effect of meal-replacement for weight loss, and the results showed that in addition to weight loss, lipid and glucose levels, blood pressure and waist circumference also improved. However, these diet drinks do not contain a wide range of vitamins, minerals and antioxidants that are present in full meals (Clifton, 2008; Gelberg et al., 2015)

Recently, low-carbohydrate diets have been highlighted because of their advantages over conventional diets (rich in carbohydrates). These advantages include improvements to lipid profile elements and insulin sensitivity, as well as greater and more rapid weight loss (D'Antona, 2014). There are two popular forms of carbohydrate-restricted weight-loss diets. One replaces a moderate amount of carbohydrate with protein and is low in fat, while the other replaces the majority of the carbohydrate with both protein and fat (also known as the Atkins diet) (Clifton, 2008). The normal intake of protein is in the range of 12-18% of daily energy intake. In carbohydrate-restricted diet the protein intake may be in the range of 25-35% of daily energy intake. And this increase in protein content is responsible for the success of carbohydrate-restricted diets, because proteins not only increase and maintain satiety but also increase thermogenesis and basal energy expenditure.

1.2 Protein: a key macronutrient in weight loss and maintenance

Over the years, several theories have been put forward in order to demonstrate the isolated effect of dietary macronutrients on satiety (Westman et al., 2002; Veldhorst et al., 2008). It is well established that protein promotes greater satiety than either carbohydrates or fat, making people feel fuller and more satisfied for a longer period of time. This suggests that a modest increase in protein, at the expense of other macronutrients, may promote satiety and facilitate weight loss through reduced energy consumption (Paddon-Jones et al., 2008). The

mechanisms that may contribute to protein-induced satiety promote increases in energy expenditure, in concentrations of ‘satiety’ hormones, in concentrations of metabolites such as amino acids and altered gluconeogenesis.

The thermic effect of nutrients, also called diet-induced thermogenesis (DIT), is related to the stimulation of energy-requiring processes during the postprandial period. It is based on the amount of ATP required for the initial steps of metabolism and storage. The DIT is mostly indicated as a percentage increase in energy expenditure over the basic metabolic rate (Pesta and Samuel, 2014). DIT values are highest for protein (~15-30%), followed by CHOs (~5-10%) and fat (~0-3%). Thus, a high protein diet induces a greater thermic response in healthy subjects compared with a high fat diet (Veldhorst et al., 2008).

It has been hypothesized that protein-induced satiety is related to a relatively high increase in concentrations of anorexigenic hormones. Variations in concentrations of these hormones are directly recorded by the central nervous system and thereby may affect the control of food intake, but until now this lacks sufficient evidence (Veldhorst et al., 2008; Westerterp-Plantenga et al., 2012).

Metabolites, especially amino acids, contribute to the perception of postprandial satiety. In this sense, in 1956, Mellinkoff developed a theory called aminostatic hypothesis. This theory suggests that high concentrations of amino acids in blood or plasma, which cannot be conveyed to the synthesis of proteins, produce feelings of satiety while decreasing concentrations provide the sensation of hunger (Mellinkoff et al., 1956).

Finally, the gluconeogenesis mechanism has also been considered as a contributor to satiety, or better food intake regulation. The satiating effect of high protein feeding could be related to the improvement of glucose homeostasis through the modulation of hepatic gluconeogenesis and subsequent glucose metabolism (Westerterp-Plantenga et al., 2006).

Section 2 Single-cell proteins as an alternative source of protein

Earth has always been subject to rapid and drastic changes under the name of development. According to United Nations, the global population is expected to increase by 33% by the year 2050. This constitutes is a change from 7.3 billion people in 2010 to 9.7 billion in 2050. The world population will continue to grow exponentially over the next 50 years. Population growth and rapid urbanization exert direct influence over soil resources and food security. The UN Food and Agriculture Organization (FAO) estimates that farmers will have to produce 70% more food by 2050 to meet the needs of the world's expected 9-billion-strong population (United Nations, 2015).

In particular, protein supply poses a problem because essential amino acids cannot be replaced. The increasing world deficiency of protein is becoming a major problem for humankind. Since the early fifties, efforts have been made to explore new, alternate and unconventional proteins. For this reason, in 1966, new forms of protein were described by Carol L. Wilson at the Massachusetts Institute of Technology (MIT), e.g., single-cell proteins (SCP) (Suman et al., 2015). SCP refer to the dried cells of microorganisms such as yeasts, bacteria, fungi and microalgae, grown in large-scale culture systems for use as protein sources

in human food or animal feed. Not only proteins, but also free amino acids, lipids, carbohydrates, vitamins and minerals are often included in the single-cell protein term (Nalege et al., 2016).

It is fundamental that new food sources be found in order for future generations to be adequately fed. An ideal food source should be nutritionally complete and requires a minimum of land, time, and cost to produce. In addition to meeting these criteria, SCP can be produced on a variety of waste materials (Jay, 1992). Large-scale processes for SCP production show interesting features, including:

- Microorganisms have a very short generation time and can thus provide a rapid mass increase;
- High efficiency in substrate conversion;
- To a certain extent, their nutritional composition can be controlled by genetic manipulation;
- High protein content;
- A wide variety of methodologies, raw materials and microorganisms can be used to SCP production;
- Independence of seasonal factors;

However, a major limiting factor in the use of SCP as food is its nucleic acid content. Intake of a diet high in nucleic acid results in the production of uric acid by degradation of the nucleic acid, causing health disorders, such as gout or kidney stone formation. Although high nucleic acid contents presented problems in the early development and use of SCP, these compounds can be reduced to levels below 2% by techniques such as acid precipitation, acid or alkaline hydrolysis, or use of endogenous and bovine pancreatic RNAses.

2.1 Microorganisms used in SCP production

Microorganisms suitable for single cell protein production are divided into four main categories: bacteria, yeasts, fungi and algae. Some characteristics are desirable in microorganisms to be used for production of SCP, which will be listed below (Goldberg, 1985; Nalage et al., 2016):

- High growth rate and low generation time;
- Ability to grow on different substrates no requirement for expensive growth factors;
- Resistance to contamination and adverse conditions;
- Possibility of genetic modification;
- High nutritional content;
- High-quality protein, fat and carbohydrate content;
- Low content of nucleic acid;
- High digestibility;
- Absence of toxicity;
- Good sensorial and functional properties.

Cultivation conditions such as culture medium, temperature, salinity, light and pH not only affect the growth rate of the microorganisms but also influence the activity of cellular

metabolism and their composition. Under optimal conditions, the average contents of crude protein, fat, ash and nucleic acid in the main microorganisms that produce single cell proteins are given in Table 1.

Table 1. Average different compositions of the main groups of microorganisms (% dry weight).

Composition	Fungi	Algae	Yeast	Bacteria
Protein	30-45	40-70	45-55	50-65
Fat	2-8	7-30	2-6	1-3
Ash	9-14	8-10	5-10	3-7
Nucleic acid	7-10	3-8	6-12	8-12

(Adapted from Anupama and Ravindra, 2000)

Among the microorganisms used for the production of SCP, microalgae stand out because of their high protein (40-70%) and fat (7-30%) content, which still have high levels of essential amino acids and essential fatty acids, respectively. Microalgae are also highly digestive due to thin wall and low nucleic acid contents (7-10%). They can be harvested by simple and less expensive methods. In addition, they contain a high percentage of digestible proteins (60-75%), vitamins, amino acids, and other components with functional properties.

Section 3 What are microalgae?

Microalgae are photosynthetic microorganisms that are found in both marine and freshwater environments. They are considered to be simple organisms because they are not organized into organs as in higher plants. These organisms are a polyphyletic and highly diverse group of prokaryotic and eukaryotic organisms. The classification into divisions is based on various properties such as pigmentation, chemical nature of photosynthetic storage product, organization of photosynthetic membranes, and other morphological features. The most known microalgal classes are *Cyanophyceae*, *Chlorophyceae*, *Bacillariophyceae* and *Chrysophyceae* (Charcon-Lee and González-Mariño, 2010).

The first interest in microalgae occurred during the Second World War (1945), when these organisms were investigated as a potential source of a number of products such as antibiotics and a good source of protein (Jegathese and Farid, 2014). Ever since, many research groups have dedicated time and effort to present microalgae to the population as a very important and abundant source of protein, based on their quantity and quality (Charcon-Lee and González-Mariño, 2010).

The main commercial large-scale culture of microalgae started in the early 1960s in Japan with the culture of *Chlorella*, followed by *Spirulina* in the early 1970s in Lake

Texcoco, Mexico. In the 1980s, large-scale algae production facilities were established in Asia, India, the USA, Israel and Australia (Wijffels et al., 2013).

Microalgae have emerged as a good source of protein and kept as such, but nowadays these microorganisms have attracted wide interest as potential tools to produce different compounds, including specialty chemicals, pharmaceuticals, health food and biofuels (Hlavova et al., 2015).

3.1 Microalgal biomass production

The technology to produce microalgal biomass depends on the selected application and commercial value of the compounds that can be extracted. In fine chemical industries, closed systems are preferred for microalgal production to obtain the desired products, as costs of microalgae production are offset by the high value of the extracted compounds. However, for environmental and energy applications, production costs should be lower. Besides the selection of low-cost operating bioreactors, the integration of processes will enhance the economic viability of microalgal culture. This can be achieved by integrating biomass production with wastewater treatment (secondary and/or tertiary) and CO_2 capture.

Microalgae cultivations can be categorized based upon carbon supply. In photoautotrophic cultivation, inorganic forms of carbon (CO_2 or bicarbonates) are supplied to the cultures. Light energy is transformed into chemical energy through photosynthesis. Some strains have the ability to use organic compounds in heterotrophic metabolism. Still, there are species which can use both; organic and inorganic carbon sources are called mixotrophs.

3.1.1 Autotrophic production systems

Currently, photoautotrophic production of microalgal biomass is the most established method and used on a large scale. Autotrophic production systems are generally classified according to their design conditions as “open” or “closed” systems. “Open” Systems are outdoor facilities that include ponds, lagoons, deep channels, shallow circulating units and others, while “closed” systems are vessels or tubes with walls made of transparent materials located in outdoors facilities under sun light irradiation or indoor facilities under artificial irradiation (Razzak et al., 2013).

Open systems can be categorized into natural water systems (lakes, lagoons, and ponds) and artificial water systems (ponds, tanks and containers). Open-culture systems are normally less expensive to build and operate, more durable than large closed reactors and with a large production capacity when compared with closed systems (Mata et al., 2010). They also have lower energy input requirement, and regular maintenance and cleaning are easier and therefore they may have the potential to return large net energy production (Brennan and Owende, 2010).

However, open ponds are more susceptive to weather conditions, not allowing control of water temperature, evaporation and lighting. Also, they require an extensive land area and are very susceptible to contamination from other microalgae or bacteria. In respect to biomass

productivity, open pond systems are less efficient when compared with closed photobioreactors.

Closed systems, mainly known as photobioreactors (PBRs) are designed to overcome some of the major problems associated with the described open pond production systems. The main closed systems are tubular, flat plate, and column PBRs. The advantages of using PBRs in biomass production process are quite clear: they offer better control over culture conditions and growth parameters (pH, temperature, mixing, CO_2 and O_2), prevent evaporation, reduce CO_2 losses, allow to attain higher microalgae densities or cell concentrations, promote higher volumetric productivities, offer a safer and more protected environment, thus preventing contamination or minimizing invasion by competing microorganisms (Mata et al., 2010).

Despite their advantages, PBRs have several drawbacks that need to be considered and solved. They include: high cost, overheating, bio-fouling, oxygen accumulation, difficulty in scaling up, high cost of building, operating and of algal biomass cultivation, and cell damage by shear stress and deterioration of material used for the photo-stage (Mata et al., 2010).

3.1.2 Heterotrophic production systems

Heterotrophic growth in the dark, supported by an exogenous carbon source, is an important ability of some species of the photosynthetic organism. This metabolic route, where possible, overcomes major limitations of single-cell proteins from microalgae, that is, the dependency on light which significantly complicates the process (Francisco et al., 2014). Also, heterotrophic cultivations also are characterized for being inexpensive, simple for construction of facilities and easy to maintain on a full-scale, since it requires just a conventional fermenter as a bioreactor (Perez-Garcia et al., 2011)

Commercial fermenters come in a wide range of sizes: from 10 to 100,000 L. Photobioreactors and fermenters have many features in common: pH and temperature control, harvesting, mixing and degassing. Compared to photobioreactors, the significant differences of fermenters are their energy source, oxygen supply, and sterility, as well as some advantages such as high biomass yield, lack of a light requirement, and ease of monoculture control (Masojídek and Torzillo, 2014).

However, heterotrophic cultures present some limitations, such as limited number of microalgal species that can grow heterotrophically, contamination and competition with other microorganisms, inability to produce light-induced metabolites and high dependence of carbon substrates. Microalgae can assimilate a variety of organic carbon sources (such as glucose, acetate, glycerol, fructose, sucrose, lactose, galactose, and mannose) for growth. It is estimated that about 80% of total culture medium cost is attributed to an exogenous organic carbon source in heterotrophic cultivation. In this sense, some studies have thus focused on finding cheaper organic carbon sources, such as agro-industrial wastes and starch (Francisco et al., 2014; Francisco et al., 2015).

In addition to meeting the demand for organic carbon, the use of wastewater in these cultivations also contributes to agro-industrial waste management, making the single-cell protein production economically feasible (Maroneze et al., 2014).

3.1.3 Mixotrophic production systems

Mixotrophic cultivation is when microalgae undergo photosynthesis and use both organic compounds and inorganic carbon (CO_2) as a carbon source for growth. The mixotrophic metabolism means that biomass growth is not strictly dependent on photosynthesis, therefore light energy is not an absolutely limiting factor for growth as either light or organic carbon substrates can support the growth. These characteristics of mixotrophic cultures reduced photoinhibition and improved growth rates.

On the other hand, due to the presence of organic compounds in the medium, mixotrophic culture may be easily contaminated by heterotrophic microorganisms. Also, the cost of medium increases the cost of biomass production, as in heterotrophic cultivation. Another issue that should be taken into account is the design of PBR, to have a better control over culture conditions, increasing biomass productivities.

Section 4 Microalgae single-cell protein on obesity control

Currently, one of the principal research interests in food science is the extraction and identification of anti-obesity compounds of natural origin. The term “anti-obesity” used here refers to two meanings: the components that have been already proven to cause body weight loss and those that can possibly decrease fat accumulation by acting through the above-mentioned mechanisms (Hu et al., 2016). Single-cell proteins from microalgae have been appointed as a source with exploration potential for the production of those compounds. Based on their structure type, all anti-obesity compounds present in microalgae biomass were arranged in three categories: (1) proteins, (2) lipids and (3) pigments, which will be detailed below.

4.1 Proteins

As previously mentioned, moderate intake of protein diet plays a crucial role in body weight loss and weight maintenance. The proposed mechanisms for weight loss include increased satiety and thermogenesis, accretion of fat free mass and lowering food intake, resulting in decreased body weight (Manikkam et al., 2015). Microalgae have been identified as excellent reservoirs of proteins and derivatives having potent biological properties (Samarakoon and Jeon, 2012). Proteins are present in microalgae in diverse forms and locations such as cell wall components, in the form of enzymes and also bound to pigments and carbohydrates (Conde et al., 2013).

The nutritive value of microalgal SCP proteins is comparable, and in many cases superior, to that of most conventional protein feed supplements in term of gross protein content, unique amino acid quality and composition and nutritional acceptability. In terms of quantity, these phototrophic microorganisms have the ability to biosynthesize large quantities of proteins, which can amount to up to 70% of dry weight under optimized cultivation conditions, making microalgae a superior source of proteins when compared to eggs and soy, for example.

Proteins are made up of different amino acids and hence the nutritional quality of a protein is determined by the content, proportion and availability of its amino acids, the so-called amino acid profile. Plants are capable of synthesizing all amino acids, while humans and animals are limited to the biosynthesis of certain amino acids only (non-essential amino acids); the remaining amount must be present in the diet, hence their classification is essential. Microalgal SCPs are not only attractive for their high protein content, but also for their amino acid profile, which presents all essential amino acids (Becker, 2004). This profile is very similar across species and relatively unaffected by growth phase and cultivation conditions.

Morever, the protein fraction also presents bioactive peptides, which are protein hydrolysates that act as physiological modulators of metabolism during intestinal digestion (Samarakoon and Jeon, 2012; Manikkam et al., 2015; Hu et al., 2016). These peptides usually consist of 3-20 amino acids, and their activity depends on their amino acid composition and sequence. Based on their structural, compositional, and sequential properties, they may exhibit different kinds of bioactivities such as antioxidant, antihypertensive, immunomodulatory, anticancer, hepeto-protective, anticoagulant and anti-obesity.

The antiobesity effects of bioactive peptides are related to the inhibition effects of the angiotensin converting enzyme (ACE) on the renin-angiotensin system (RAS). This regulatory enzyme converts angiotensin-I (ANG I) into angiotensin-II (ANG II). ANG II is a physiologically important constituent that performs a big number of functions, e.g., growth of adipose tissue, which affects the endocrine and metabolic systems.

The hyperactivity of the renin-angiotensin system (RAS) plays an important role in the onset of obesity. Many studies have correlated the different components (qrenin, AGT, ANG II, ACE and ANG II receptors) of RAS with body fat and adipose tissue, which is markedly expanded in obesity. Moreover, it is known that ANG II may promote adipocyte hypertrophy by increasing the expression of fatty acid synthase (FAS) within fat cells (Jones et al., 1997).

In this respect, ACE inhibition has played a fundamental role in the therapeutical treatment of obesity. Recent studies have also demonstrated the role of ACE inhibition in adipogenesis inhibition, body weight control in addition to improvement of body composition, glucose intolerance and lipid metabolism (Jayasooriya et al., 2008, Mathai et al., 2008). Thus, several studies have demonstrated the ability of bioactive peptides or ACE inhibitors to perform these functions, both in *in vitro* and *in vivo* studies (Ohta et al., 1997; Li et al., 2007; Mathai et al., 2008; Lee et al., 2010).

4.2 Lipids

Over several decades, epidemiological data indicated an inverse correlation between prevalence of metabolic diseases and consumption of polyunsaturated fatty acids (PUFAs). Fish and fish oils are still considered as the most common sources of n-3 PUFAs. On the other hand, microalgae appear as an alternative source of these fatty acids (FAs) (Paniagua-Michel, 2015).

The average lipid content in microalgae varies between 1% and 40%, but can reach up to 85% of dry weight, depending on the growing conditions. Algal lipids are typically composed of glycerol, sugars, or bases esterified to fatty acids. The most important lipids are the essential polyunsaturated fatty acids such as α-linolenic acid, eicosapentaenoic acid (EPA), and docosahexaenoic acid (DHA) (Fig. 2) (Charcon-Lee and González-Mariño, 2010).

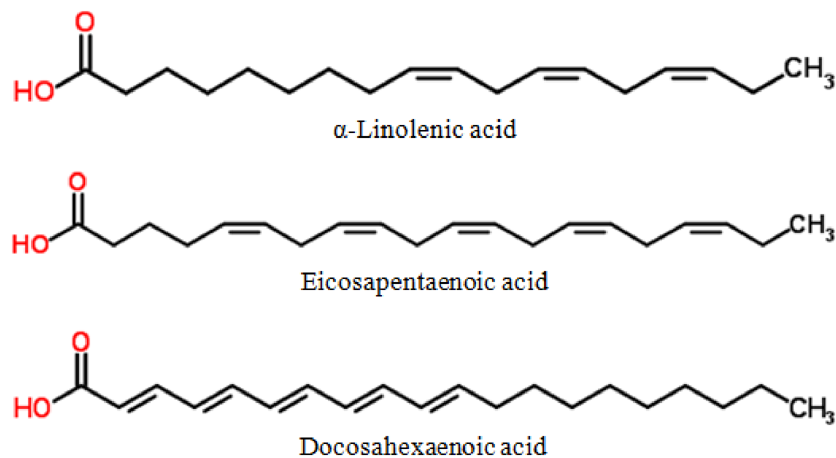


Figure 2. Chemical structure of typical microalgal biomass fatty acids.Available in http://www.chemspider.com/.

The higher level of n-3 PUFA in plasma is related to lower BMI, waist and hip circumference, suggesting that microalgal oil may contribute to a healthy weight status and prevention of adiposity. This hability of n-3 PUFAs is associated with their storage as triglycerides in mature adipocytes. An excess of triglyceride storage promotes hyperplasia and hypertrophy of adipocytes leading to obesity, and omega-3 PUFAs, especially EPA and DHA, can inhibit adipocyte hypertrophy and decrease the lipid content of adipose tissue (Yook et al., 2015).

Although the beneficial effect of microalgal oil on obesity is widely accepted, whether it could regulate appetite is still controversial. In fact, more researchers tend to support that the anti-obesity effect of fish oil is independent of feeding behavior (Hu et al., 2016).

4.3 Pigments: carotenoids

Carotenoids are terpene-derived pigments found in chloroplasts and chromoplasts of plants and photosynthetic organisms, where their role is to support photosynthesis. Based on different chemical characteristics, carotenoids can be classified as xanthophylls and carotenes.

Xanthophylls are carotenoids with oxygen present in the molecules, such as lutein (Fig. 3), fucoxanthin, astaxanthin, and zeaxanthin. Carotenes are unoxygenated carotenoids which contain only carbon and hydrogen. Examples of carotenes include β -carotene (Fig. 3), α -carotene, and lycopene (Cuttriss et al., 2011).

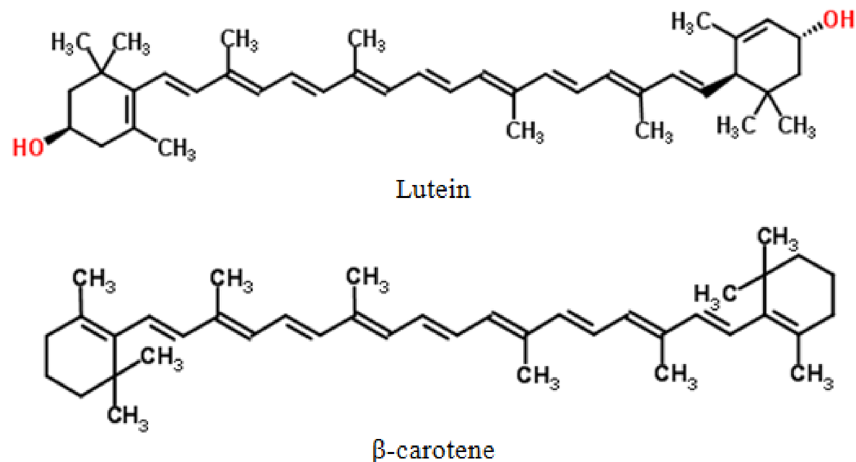


Figure 3. Chemical structure of a xanthophyll (lutein) and a carotene (β-carotene). Available in http://www.chemspider.com/.

In particular, carotenoids of algae are attracting attention as new functional food ingredients, due to the high content found, which can reach 0.2% of carotenoids (Talero et al., 2015). The most important biological aspect of carotenoids for humans is the role of provitamin A activity. In addition, dietary carotenoids are known to reduce the risk of cardiovascular diseases, age-related macular degeneration and cancer. The mechanism that is associated to all these beneficial health effects is the antioxidant properties of carotenoids (Miyashita et al., 2015). With regard to anti-obesity effect of carotenoids, yet there is little information in the literature, except for fucoxanthin and astaxanthin (Hu et al., 2016).

4.3.1 Fucoxanthin

Fucoxanthin (Fig. 4) is a carotenoid with a unique structure including an allenic bond and oxygenic functional groups, such as epoxy, hydroxyl, carbonyl and carboxyl groups in the polyene hydrocarbon chain (Pádua et al., 2015). It has no provitamin A activity, but shows strong antioxidant properties. In addition, fucoxanthin was recently reported to have anti-obesity and anti-diabetic effects. It has attracted much attention in the food industry and nutrition studies because of the unique mechanism of these effects (Maeda et al., 2013).

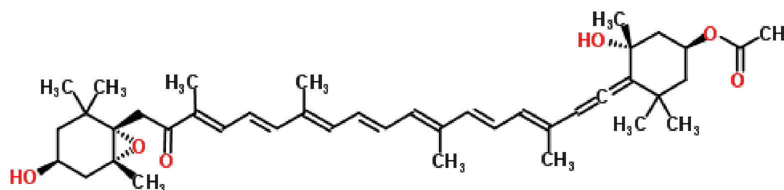


Figure 4. Chemical structure of fucoxanthin. Available in http://www.chemspider.com/.

The most popular effect of fucoxanthin from algae is reduced abdominal weight through thermogenesis mechanism (Maeda et al., 2013; Myiashita et al., 2014; Muradian et al., 2015; Hu et al., 2016). Thermogenesis is a major function of brown adipose tissue (BAT), found in hibernating animals, small rodents, and newborns that require active thermogenesis to protect them from cold exposure and maintain body temperature. BAT establishes non-shivering thermogenesis to dissipate excess energy as heat and increase energy expenditure as a result. Thus, BAT plays a significant role in energy balance control (Hu et al., 2016). A key regulator in this process is uncoupling proteins (UCP), which discharge the proton gradient generated in oxidative phosphorylation, resulting in energy dissipation via thermogenesis. On the other hand, the white adipose tissue (WAT) is the main site of energy storage in mammals, with the substrate being deposited as triacylglycerols at a high energy density, where an excess of WAT has a negative impact on the health.

Fucoxanthin controls energy expenditures in abdominal WAT, and reduces excess lipid in WAT. This effect of fucoxanthin is attributable to the induction of the uncoupling protein 1 (UCP1) in abdominal WAT, leading to fatty acid oxidation and heat production in WAT. UCP1 is normally expressed only in BAT, not in WAT. Thus, UCP1 can dissipate energy through oxidation of fatty acids and heat production. Furthermore, fucoxanthin improves insulin resistance and ameliorates blood glucose levels (Maeda et al., 2013; Myiashita et al., 2014).

4.3.2 Astaxanthin

Oxidative stress plays critical roles in the pathogenesis of various diseases, such as diabetes, cardiovascular disease, and atherogenic processes. Furthermore, many authors have described a strong relation between obesity and oxidative stress (Fernández-Sánchez et al., 2011). These authors point out that oxidative stress is particularly higher in the adipose tissue of obese individuals, and that this oxidative stress on adipocytes might deleteriously affect the secretion of adipokines, leading to low adiponectin concentration and high free fatty acid (FFA) concentration in the blood. Other reports of recent studies have described that several types of antioxidant supplementation in overweight and obese individuals can ameliorate abnormalities of adipose tissue and lipid metabolism (Kimura et al., 2014).

Astaxanthin (Fig. 5), a xanthophyll ketocarotenoid found in several marine animals and produced predominantly by blue-green algae *Haematococcus pluvialis,* acts as a strong antioxidant. It is more effective than other antioxidants such as β-carotene and α-tocopherol in preventing lipid peroxidation, and it has been of notable recent interest for pharmaceutical applications that include anti-ageing protection due to UV light photo oxidation and

inflammation (Haque et al., 2016). Astaxanthin is ubiquitous in nature, especially in the marine environment, and is probably best known for eliciting the pinkish-red hue to the flesh of salmonids, as well as shrimp, lobsters and crayfish (Kimura et al., 2014).

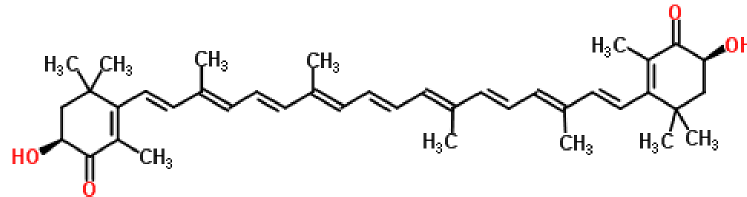


Figure 5. Chemical structure of astaxanthin. Available in http://www.chemspider.com/.

Ikeuchi et al. (2007) also confirmed the anti-obesity effect of astaxanthin in another obese animal model, where astaxanthin was found to inhibit the increases in body weight and weight of adipose tissue, and to reduce liver weight, liver triglycerides, plasma triglycerides, and total cholesterol.

Section 5 Nutritional properties of microalgae single cell proteins

Proteins are essential nutrients for animals and human organisms and as such they must be present in adequate amounts in food. Besides the quantitative aspect, the nutritional value should be taken into account. The nutritive value of protein will depend mainly of digestibility and the bioavailability of essential amino acids and their biological value.

5.1 Digestibility

Protein digestibility is an important factor in determining nutritional value and should therefore be understood as the part or portion of the protein that can be hydrolyzed by the digestive enzymes to amino acids and would thus be available biologically, provided there was no interference with the absorption of amino acids for animal or human organisms (Donadio et al., 2011; Sgarbieri, 1996).

Digestion and absorption should always be considered as inherent parts of protein quality. A protein can be predicted as having good quality on the basis of its amino acid score, but in practice it could only have poor quality because it is poorly digested and/or absorbed. Thus, when making recommendations for protein requirements, factors which might affect digestibility or absorption should be considered (FAO, 1981). The digestibility of a food is measured by the ratio between absorbed nitrogen and nitrogen ingested with the diet, expressed as a percentage (Paniagua-Michel, 1992).

Microalgae have complex carbohydrate cell walls. Microalgal cell wall polysaccharides are more diverse than terrestrial plants. These long-chain sugar molecules are difficult to break down and therefore also to digest. As a result, they have little nutritional

value. This means that they are low in food calories but they still curb hunger (Valdez, 2012; Batista et al., 2013).

The cell wall represents about 10% of the microalgal dry matter and consists of a variety of macromolecules, the amounts and chemical composition of which are group-, species-, and even strain-specific. In general, at least two major components, one fibrillary and one mucilagenous, were identified in the microalgal cell wall. Microfibrils form the most inert and resistant part of the cell wall, with the most common one of the skeletal components being cellulose. As already indicated, this cellulosic cell wall poses a serious problem in digesting/utilizing microalgal biomass, since it is not digestible for humans and other non-ruminants (Becker, 2004).

Effective treatments are therefore necessary to disrupt the cell wall, making the microalgal protein and other constituents accessible for digestive enzymes. With the exception of the cyanobacteria *Spirulina* sp. and *Aphanizomenon flos-aquae*, most of all other microalgae of commercial importance have that rigid indigestible cell wall, which mandates that the microalgal cell be ruptured. This can be achieved by either physical methods, as boiling, various types of high temperature drying, to a certain extent even sun drying (freeze drying per se will not break the cellulosic cell wall), or by chemical methods like autolysis or breaking the hydrogen bonds by using phenol, formic acid or urea (Gellenbeck, 2011)

The major problem encountered with the latter methods is the necessity of recovering the solvent and ensuring that the product is not toxic because of the residues of the chemicals used (Salazar et al., 1996). Analyses of the cell walls of cyanobacteria revealed the absence of cellulosic material and a close relationship with the structure of Gram negative bacteria. Therefore, the cell wall of *Spirulina* sp. does not represent a barrier to proteolytic enzymes as demonstrated by the fact that this microalga, in general, can be digested by monogastric vertebrates like humans without previous physical or chemical rupture of the cell wall (Shinohara et al., 1986; Tanaka et al., 1994).

In order to demonstrate the necessity of microalgal cell wall rupture, one should always keep in mind that proper processing of the biomass is the key process for almost all applications of the algal biomass (Becker, 2014).

5.2 Bioavailability of essential amino acids and biological value

The biological value measures protein quality by calculating the nitrogen used for tissue formation divided by the amount of nitrogen absorbed from food. This product is multiplied by 100 and expressed as a percentage of nitrogen utilized. The biological value provides a measurement of how efficient the body utilizes protein consumed in the diet. A food with a high value correlates to a high supply of essential amino acids. Animal sources typically possess a higher biological value than vegetable sources because vegetable sources lack one or more essential amino acids. There are, however, some inherent problems with this rating system. The biological value does not take into consideration several key factors that influence the digestion of protein and interaction with other foods before absorption. The biological value also measures a protein's maximal potential quality but not its estimate at requirement levels (Belay et al., 1993).

It is not sufficient that the protein has amino acids in adequate quantities and proportions to meet the requirements of various organisms that need this protein as a source of nitrogen and essential amino acids. Amino acids, particularly essential ones, have to be bioavailable. Amino acids are considered bioavailable when they are absorbed in their

metabolically active form and can perform their specific functions in various tissues and organs. Amino acid bioavailability is defined as the bioavailable fraction of the total amount ingested as an amino acid of the protein, which is absorbed in a metabolically active form and utilized by the body. The bioavailability of many amino acids is varied depending not only on the nature of the protein, but also on the processing which the protein and / or food is subjected to during storage and domestic and industrial preparation (Molina et al., 2003; Sgarbieri, 1996).

A cyanobacterium as a source of single-cell protein has certain advantages over the use of other microorganisms because of its rapid growth and the quantity and quality of protein. Among microalgae, the genus *Spirulina* contains about 60 to 70% of proteins, nucleic acids and amino acids recommended by the Food and Agriculture Organization. It also contains betacarotene and absorbable iron, and other minerals and high levels of vitamins, phenolic compounds, gammalinolenic acid and other essential fatty acids (Von Der Weid et al., 2000). The essential amino acids isoleucine, leucine, lysine, methionine, phenylalanine, threonine, tryptophan and valine were found in the biomass of *Spirulina*, and generally presented larger amounts of essential amino acids than the theoretical quantities recommended in dietary protein for children aged 2 to 5 years (FAO, 1991), the exception being lysine which accounted for 2.95% of the dry biomass of *Spirulina* as compared with the amount of 5.8%, recommended in the dietary protein of children by the Food and Agriculture Organization.

The use of alternative protein sources has drawn the attention of researchers into studying the action of microalgal biomass in the body of rats, which are constantly used in animal research, focusing on hormonal, psychological, immunological and nutritional assessments. Zepka et al. (2010) evaluated the nutritional parameters for daily intake of *Aphanothece*, using rats as the experimental model. The rats fed the test diet (*Aphanothece*) had a lower body weight gain, lower cholesterol and lower glycemic indexes than rats fed the control diet as shown in Table 2. These measures indicate that the use of *Aphanothece* biomass is a possible important source of single-cell proteins.

Table 2. Biometric evaluation, glycemic index and total cholesterol values.

Parameter	Dietary group	
	Casein	*Aphanothece*
Initial body weight (%)	76.8	76.5
Final body weight (%)	89.8	24.7
Total cholesterol (mg/dl)	105.0	83.6
Glycemic index (mg/dl)	154.0	115.0

(Adapted from Zepka et al., 2010)

Section 6 Criteria development of products from single cell proteins

The marine environment is highly diverse and offers a wide variety of applications with numerous potential areas for exploitation. Microalgae are one of the richest and most promising sources of renewable natural resource not yet utilized on a large scale, which can be applied to many aspects of food and beverage production. In the context of food,

microalgae are mostly used as food ingredients, and as sources of bioactive substances and high-value chemicals and pharmaceuticals.

These microorganisms offer interesting possibilities as new food sources for the development of new food products to meet changing eating habits of consumers, and these opportunities are essential to the food industry, given its need for continuous innovation. Moreover, the use of microalgae is likely to increase in view of the need for additional food sources as the world population continues to grow (Yuan, 2008; Bocanegra et al., 2009).

Microalgal constituents such as hydrocolloids (agar), carrageen and alginate also offer technological advantages for the industry when used as ingredients and gelling, thickening and emulsifying agents in food and beverage production. More recently, there has been growing interest in microalgal-based products as sources of bioactive ingredients that have many applications in processing healthier foods and developing functional foods (Bocanegra et al., 2009). A growing understanding of the relationship between diet and health is leading to new insights into the effect of food ingredients on physiological function and health. These possibilities generate scientific and public interest, inducing increased consumer demand for healthy, nutritious foods with additional health-promoting functions, such as functional foods (Jim et al., 2005).

Whatever the ultimate purpose of their use, both specific components extracted from microalgae and whole dehydrated and powdered ones have been used in food and beverage production. Microalgae are considered to be a good supplement and fortified food in diets for malnourished adults, as a single cell protein (Spolaore et al., 2006). There are various potential strategies for introducing qualitative and quantitative modifications in foods and beverages in order to achieve healthier products. In particular, strategies associated with product processing are especially promising. The basic idea is to be able to enhance the concentrations of compounds with beneficial physiological effects and/or reduce the concentrations of others with adverse health implications. Which explains their utility as natural ingredients in reformulation processes for healthy food and beverage production, it is the microalgae can be endowed with functional effects by the presence of various nutrients and bioactive compounds in microalgae, and by the technological opportunities that microalgae offer in product reformulation (Mendis and Kim, 2011).

Thus, the following sections consider the different aspects of the opportunities offered by the use of whole microalgae in the development of healthier foods and beverages. It gives a brief account of the presence of various different nutrients and the technological advantages of using them as natural ingredients in reformulation processes for healthy foods and beverages.

Section 7 Technological properties of the use of single cell proteins

The major stages involved in the development of food products aim to (i) identify the beneficial interactions between a food product, or a specific ingredient, and one or more functions of the organism; (ii) deal with technological aspects; (iii) demonstrate efficacy and the necessary intake level to achieve the desired effect, safety at efficacy levels and bioavailability; (iv) gain approval for health-enhancing marketing claims by showing sufficiency of scientific evidence; (v) communicate benefits to consumers; and (vi) conduct

in-market confirmation of efficacy and consumer acceptance (Siro et al., 2008; Venugopal, 2009). The development of a marine product usually involves a similar sequence of steps with contextual differences (Freitas et al., 2012).

The development of food requires new process technologies to be identified and applied to food manufacture, generating opportunities for new and different products. The viability of incorporating microalgae biomass in food systems is conditioned by the type and intensity of applied processing (e.g. thermal, mechanical), by the nature of the food matrix (e.g. emulsion, gel, viscosity) and by the interactions with other food components (e.g. proteins, polysaccharides, lipids, sugars, salts). In addition to coloring and nutritional purposes, introducing microalgae as ingredients in food systems, can also impart significant changes in their microstructure and rheological properties (Day et al., 2009). The rheological properties and the potential use of whole microalgae as ingredients in healthy food formulations are discussed in the following subsections.

7.1 Solubility

The solubility of a protein is the most important functional property since the properties of microalgal biomass are based on the protein fraction and this should be soluble in order to be applicable in food systems. Several functional properties, such as thickening, foaming, emulsification, and gelation, of proteins are affected by solubility. Solubility of a protein is fundamentally related to its hydrophilicity/hydrophobicity balance and it is also related to the pH, where it is minimal at the isoelectric point, making the environmental pH the most important factor when it comes to the degree of protein solubility. It is also influenced by temperature, ionic strength, amino acid composition, molecular weight, conformation, content of polar, nonpolar groups in amino acids, temperature, and processing conditions (Bolontrade et al., 2016; Zhuang et al., 2016). Proteins may be modified by product processing conditions, and heat treatment affects their structure and solubility. Iinsolubility is a major obstacle in the inclusion of protein in food products. High solubility may indicate the ideal feature for application in foods (Jacob-Lopes et al., 2006).

The determining factor of protein solubility is the pH of the medium. The degree of protein solubility in an aqueous medium is the result of electrostatic and hydrophobic interactions among protein molecules. Solubility is increased if electrostatic repulsion between the molecules is higher than hydrophobic interactions. Protein solubility is affected by a sensitive balance between repulsive and attractive intermolecular forces and proteins are soluble when electrostatic repulsion between proteins is greater than hydrophobic interactions (Ba et al., 2016).

In order to be soluble, proteins should be able to interact as much as possible with the solvent. At the isoelectric point (PI), proteins have a net zero charge, attractive forces predominate, and molecules tend to associate, resulting in insolubility. Above the pI, the net charge is negative and solubility is enhanced. Protein-water interactions increase at pH values higher or lower than the pI because proteins carry a positive or negative charge (Matak et al., 2015). The functionality of proteins can be studied more effectively if a systematic study is first made of protein solubility under various ionic conditions. The mechanism of the ionic strength effect on protein solubility is poorly understood and probably involves solvation,

electrostatic and salting in and salting out phenomena (Chen et al., 2016; Bertram et al., 2004).

The low solubility of the microalgal protein, especially in the basic pH region, may be an indication that the cell wall inhibits the solubility of the protein (Matak et al., 2015). Studies shown by Guil-Guerrero et al., (2004) in microalgae *Tricornutum* sp. demonstrated that after the cell wall rupture process was defatted, biomass and trials show an increase electrostatic repulsion between molecules larger than hydrophobic interactions and thus there was a 50% increase in solubility of biomass which can be used in the formulation of acid foods, such as milk analogue products and protein-rich carbonated beverages.

7.2 Emulsifying capacity

Emulsification is an important process in the manufacturing of many formulated foods. Food emulsions are classified as macroemulsions with droplet size of 0.2 to 50 μm. Emulsion represents a heterogeneous mixture of fat globules. Food emulsions can be of the oil in water (O/W) or water in oil (W/O) type. The difference between O/W and W/O emulsions is that an O/W emulsion commonly exhibits a creamy texture, while a W/O system has greasy textural properties (Chronakis, 2001).

Protein emulsifying activity is the ability of a protein to participate in emulsion formation and to stabilize the newly created emulsion. Emulsifying capacity is the ability of a protein solution or suspension to emulsify oil. Emulsifying properties are useful functional characteristics which play an important role in the development of new sources of plant protein products for use as foods. Proteins are the components that dominate in most food emulsions (Almada, 2008).

A significant number of foods are emulsions, dispersions, and foams, and in these systems, proteins, in combination with lipids and carbohydrates, are important stabilizers. The characteristics used to describe emulsifying properties of proteins are EC, ES, and emulsifying activity (EA). They are used to describe the emulsifying properties of proteins in food emulsion systems. EC is presented as the amount of oil (ml) that is emulsified under specific conditions by 1 g protein. The emulsifying capacity of an emulsifier depends on its ability to form the adsorption films around the globules, and to lower the interfacial tension at the oil-water interface. Emulsion stability is the capacity of emulsion droplets to remain dispersed without separation by creaming, coalescing, and flocculation. Emulsifying activity is presented as the maximal interfacial area per 1 g of protein of a stabilized emulsion (Sgarbieri, 1996; Sari et al., 2013).

In certain foods, a natural protein ingredient is an effective stabilizer. Proteins are effective surface-active agents because they are capable of lowering the interfacial tension between hydrophobic and hydrophilic components in foods. Proteins participate in the formation of oil-in-water and water-in-oil emulsions and stabilize the emulsions that are formed. A stabilizing effect of proteins in the emulsion system results from the formation of a protective barrier around fat droplets, preventing emulsion coalescence (Sari et al., 2013).

The emulsifying capacity of proteins depends on the shape, charge, and hydrophobicity of the protein molecules, neutrality of dipoles, and hydration of polar groups. Emulsion stability depends on the magnitude of these interactions. To produce stable emulsions, one should select protein material that is soluble, has the ability to adsorb rapidly at the interface, has well-distributed charged groups, and has the ability to form a strong cohesive film (Vanthoor-Koopmans et al., 2013).

The interfacial behavior of extracted lipids confirms that remaining traces of lipids in protein powder have only a minor influence on the surface activity of *Spirulina* protein. The surface active components are likely to be protein and/or protein-pigment complexes rather than individual protein molecules. Present knowledge of such physicochemical properties emphasizes the high potential of applicability of *Spirulina* microalgal protein in the food industry for foams and emulsions (Chronakis et al., 2000).

Evaluations have been made of the capacity of the biomass of the microalga *Chlorella vulgaris* as a fat mimetic and its ability as an emulsifier. Pea protein emulsions with an addition of *Chlorella vulgaris* (green, 60% protein, and orange–carotenogenic, 6% protein) were prepared at different protein and oil contents. The addition of *Chlorella vulgaris* proved to be beneficial in terms of enabling lesser oil contents in the emulsions without disturbing their structural and textural properties. Although the microalgal biomass has a high protein content, it cannot be used as the only emulsifier in these types of emulsion systems. Possible interactions between pea protein and microalgal biomass can also contribute to the reinforcement of the emulsion structure via the formation of physical entanglements. The effect was more significant for Chlorella vulgaris, which must be due to its higher protein content. As a consequence, the total oil content can be reduced in this case, yielding emulsions with the same rheological and sensory properties. For this reason, it was considered that the biomass acted as a fat mimetic with a mechanism that resembles that of xanthan gum (Raymundo et al., 2005).

In order to study the properties of the respective food emulsions, were also measured in terms of viscoelastic properties and steady state flow behavior and texture properties (Raymundo et al., 2005; Pignolet et al., 2013). A study was conducted on the effect of addition of oil on the viscoelastic properties of the 3% pea emulsions with 2% *Chlorella vulgaris*. These emulsions present mechanical spectra typical of protein-stabilized emulsions in which an elastic network develops owing to the occurrence of an extensive bridging flocculation process. It can be observed from the dynamic measurements that for a certain protein and microalgae concentration, a higher oil content induces a reinforcement of the emulsion structure, which is beneficial for the production of pâté from microalgal biomass (Raymundo et al., 2005)

7.3 Foaming and gelation properties

The proteins exhibit foaming properties and are important in the production of a variety of foods. The foam consists of a two-phase system consisting of air cells separated by a thin continuous liquid layer called the lamellar phase. Food foams are usually very complex

systems, including a mixture of gases, liquids, solids, and surfactants. The size distribution of air bubbles in foam influences the appearance and textural properties of foam products by means of a uniform distribution of small air bubbles, thus imparting body, smoothness, and lightness to the food. In food, body and smoothness in foams of is related to the formation of air bubbles that allow volatilization flavors with enhanced palatability of the foods (Drenckhan and Saint-Jalmes, 2015; Cantat et al., 2013; Dietrich et al., 2013).

The most widely used protein foaming agents are: egg white, gelatins, casein, other milk proteins, soy proteins, and gluten. Protein foaming agents should possess the following properties: they should stabilize foams rapidly and effectively at low concentrations and perform as an effective foaming agent over the pH range which exists in various foods (Stevenson, 2012; Testouri et al., 2010).

Our understanding of the functions of proteins in the formation of adsorbed protein films has been improved by studies in which radio-labelled proteins were used to obtain surface concentrations (Matsumiya and Murray, 2016). Foamability and whippability of foaming agents are used interchangeably in the literature. The term whippability is applied when foam is obtained by a high blending or whipping treatment. The term foamability is applied when foam is prepared by injecting air or gas through the protein solution. The foaming properties of proteins are influenced by the source of the protein, methods and thermal parameters of processing, including protein isolation, temperature, pH, protein concentration, mixing time, method of foaming. Among many factors influencing foaming capacity (FC) of proteins, type of foaming equipment and method of agitation are important. Speed of whipping is important to foam properties and consumer acceptance (Niaounakis, 2015; Rodríguez et al., 2015)

In order for a protein to be a good foaming agent, it should possess the following attributes (Damodaran, 1994; Von et al., 2014):

- It should be rapidly absorbed at the air-water interface during whipping or bubbling;
- It should undergo rapid conformational change and rearrangement at the air-water interface and rapidly reduce surface tension;
- It should be able to form a cohesive, viscoelastic film through intermolecular interactions.

The first two criteria are essential for better foamability, whereas the third criterion is important for stability. Graham and Phillips (1976) demonstrated that the single most important factor for foamability of a protein solution is the rate at which the protein can reduce the interfacial tension as new interfacial areas are continuously created during bubbling or whipping.

Gelation, water and fat absorption capacity, emulsification capacity, foaming capacity and stability of algal proteins are also comparable with those of terrestrial plants. Moreover, the great genetic diversity of microalgae and genetic engineering of microalgal proteins may be able to lead to a great variety of protein products with better yields than other protein feed supplements (Chronakis and Madsen, 2011).

Spirulina sp. powder has a very high foaming capacity, especially if the sample is defatted. Its high foaming capacity, which is twice as much as that of egg protein, appears to be a remarkable property of a spray dried, defatted powder of microalgae. Foaming capacity in the same sample without defatting was nearly 50% lower. It is not clear whether this is due

to a loose lipoprotein complex being formed in the presence of fat, and this may also have some bearing in the drastic reduction of foam properties of proteins. The spray-dried *Spirulina* sp. powder has a much higher emulsification activity and slower kinetics, resulting in higher emulsion stability as compared to a spray-dried defatted sample or to egg protein (Chronakis and Madsen, 2011; Niaonakis, 2015).

The major processed products from microalgae are hydrocolloids, including carrageenan, agars and alginates. These polysaccharides are extracted from microalgal biomass and used as gelling agents in a variety of foods and health-care products. Solutions of *Spirulina* sp. protein isolate form elastic gels during heating at90°C. Subsequent cooling at ambient temperatures causes a further pronounced increase in the elastic moduli and network elasticity. Microalgae biomass protein isolates have good gelling properties with fairly low minimum critical gelling concentrations (Chronakis and Madsen, 2011).

Studies have also been made of the molecular forces of thermal association and gelation of *Spirulina* sp. Protein hydrophobic interactions contribute substantially to the facilitation of molecular association during gelation and to the stabilization of the gel structure the microalgae protein. Hydrogen bonds reinforce the rigidity of the network of the protein on cooling and further stabilize the structure of *Spirulina* sp. protein gels but they are not the only ones to form a network structure. Intermolecular sulfhydryl and disulfide bonds have been found to play a minor role in the network strength of microalgae protein gels but affect the elasticity of the structures formed. Both time and temperature at isothermal heat-induced gelation at 40–80°C substantially affect network formation and the development of the elastic modulus of the microalgae protein gels. This is also attributed to the strong temperature dependence of hydrophobic interactions. It is likely that the aggregation, denaturation and gelation properties of *Spirulina* sp. microalgal protein isolate are controlled by protein-protein complexes rather than individual protein molecules (Chronakis, 2001).

7.4 Viscosity

Consumers' choice of several liquid and semi-solid products depends on the viscosity or consistency of the product. Viscosity or consistency of solutions is greatly influenced by solution type (Dickinson, 2015). Large molecular weight soluble polymers greatly increase viscosity even at low concentrations. This again depends on several molecular properties such as size, shape, flexibility, and hydration. Solutions of randomly coiled polymers display greater viscosity than do solutions of compact folded polymers with the same molecular weight. Thus, polysaccharides and gum, which are large, highly flexible and hydrophilic polymers, exhibit high viscosity even at low concentrations. These hydrocolloids are often used in food products as thickening agents (Lam and Nickerson, 2013).

Hydrocolloids are a diverse group of long-chain polymers that are readily dispersive, fully or partially soluble, and prone to swell in water. They change the physical properties of the solution to form gels, or enable thickening, emulsification, coating and stabilization (Schmitt and Ravaine, 2013). The presence of many hydroxyl groups conspicuously increases their affinity for binding water, thus rendering them as hydrophilic. In addition, hydrocolloids produce a dispersion, which is intermediate between a true solution and a suspension, and exhibit the properties of a colloid. Consequently, they are aptly termed as 'hydrocolloids' (Saha and Bhattacharya, 2010).

Food colloids can represent an important part of our everyday diet, with products such as sauces, dressings, yoghourt, mayonnaise and ice creams. Hydrocolloids have a splendid array of functional properties in these food colloid systems. And they function as thickeners, gelling agents, foaming agents, edible coatings, emulsifiers, stabilizers, etc. The principal reason for extensive use of hydrocolloids in the food industry is their ability to bind with water and modify the properties of food ingredients. The modification of rheological characteristics is helpful to modifying foods' sensory properties (Valdez, 2012). Hydrocolloids are employed as extremely significant food additives. Indeed, glance through the handbook of food additives and you will find a large quantity of hydrocolloids listed for food use (Smith and Hong-Shum, 2011).

Certain proteins, such as gelatin, myosin, etc., that have large axial ratios exhibit high viscosity even at low concentrations. The viscosity of protein solutions generally increases exponentially with protein concentration this is attributable to an increased interaction between the hydrated protein molecules. Also, the ability of the protein to absorb water and swell affects its viscosity.

The viscosity of *Spirulina* sp. protein isolate decreases when the temperature increases, as observed in most proteins. The decrease in the viscosity of *Spirulina* sp. protein at temperatures of 10 to 50°C, follows an Arrhenius-type of dependence. Above 60°C, the viscosity increase is closely related to the dissociation-denaturation process. Lower viscosities have been observed for protein solutions dissolved at pH 9 as a result of increased protein solubility. At a pH of approximately 5, closer to the isoelectric point of 3.5, viscosity was seen to be higher than at neutral pH. This is because solubility decreased, as the *Spirulina* sp. protein tends to form aggregates, which include the core that is not accessible for maximum hydration. The changes in viscosity at such conditions are mainly relative to changes (increase) in particle size and are obviously of practical importance to the stability and processing of the *Spirulina* sp. Protein dispersions can be used in formulating beverages, yogurts and soups dietetic (Chronakis, 2001).

7.5 Sensory properties

Functional foods supplemented with microalgae biomass are sensorily much more convenient and variable, thus combining health benefits with attractiveness to consumers (Pulz and Gross, 2004). From the sensory standpoint, the major obstacles are represented by the powder like consistency of the dried biomass, its dark green color and its slightly fishy smell, which limit the incorporation of the microalgal material into conventional foods. In addition to palatability, the attractiveness of a food product to eaters is an important contributor to the value of food. Typical sensorial characteristics of a product should be maintained in order to meet commercial success in the development of functional foods (Babuskin et al., 2014).

It is known from studies on microalgae that they have the characteristic of producing a wide array of volatile compounds that may influence the flavor or aroma of the biomass. These compound classes include unsaturated aldehydes, dimethyl sulfide, and organohalogens. Such volatiles may positively or negatively affect microalga-enriched food materials. There is also evidence that some microalgae have marine or seafood aromas. For instance, norisoprenoids derived from microalgal carotenoids impart a positive green flavor in natural sea salts. Carotenoid degradation products, together with long-chain aldehydes derived from microalgal unsaturated fatty acids, have a positive influence on flavor (Donadio et al., 2011; Zhang et al., 2009).

The full use of the volatile fraction of microalgal/cyanobacterial biomass may represent an improvement in the supply of a large volume of inputs to many different types of industry (Santos et al., 2016). These authors conducted a study using heterotrophic microalgae reactors supplemented with sucrose and fructose and detected odors mainly classified as fruity, spice and floral compounds.

Short dough cookies and biscuits are widely consumed food products, appreciated for their taste, versatility, convenience, conservation, texture and appearance. *Chlorella vulgaris* biomass has already been used experimentally in food products. The incorporation of this biomass can provide a coloring effect and other functional characteristics such as antioxidant activity, and provide nutritional supplements (e.g. fiber, fatty acids and oligoelements) (Gouveia et al., 2005; Raymundo et al., 2005).

Moreover, microalgal extracts are often used to improve the texture of food products such as carrageenans; linear sulphated polysaccharides extracted from edible red seaweed are often used in prepared foods such as yoghurt and ice cream. In the presence of potassium carrageenan ions bind dairy proteins into a firm aqueous gel, which both thickens then product, making it feel more substantial, and helps to prevent the components from separating. There are many other uses of carrageenan. A *Spirulina* sp. extract, containing carrageenan, can be added to bread dough to increase moisture content, decrease stickiness of the dough and produce acceptably firmer bread (Mamat et al., 2014).

Section 8 The use of microalgae as ingredients in healthy food formulations

A wide variety of microalgae have been used for different purposes in food and beverage formulations. When added to these products, microalgae offer interesting technological, nutritional and health benefits. (Jiménez-Colmenero et al., 2001; Arihara, 2006; Jiménez-Colmenero, 2007). They have been used to enhance nutritional value, provide human health benefits and impart desirable technological and sensory characteristics in response to different problems associated with reformulation processes (Jiménez-Colmenero et al. 2001, Arihara, 2006, Weiss et al., 2010).

Numerous combinations of microalgae with other health foods can be found in the market in the form of tablets, powders, capsules, pastilles and liquids; also, their extract are included in noodles, beverages and cereals, providing health-promoting effects (Spolaore et al., 2006; Belay, 1996). *Arthrospira* is used in human nutrition because of its high protein content and its excellent nutritive value (Soletto et al., 2005; Desmorieux and Decaen, 2005; Rangel-Yagui et al., 2004). In addition, this microalga has various possible health-promoting effects: the alleviation of hyperlipidemia, suppression of hypertension, protection against renal failure, growth promotion of intestinal *Lactobacillus*, and suppression of elevated serum glucose level (Yamaguchi, 1997; Liang et al., 2004). Their *Arthrospira*-based products (tablets and powder) are distributed in over 20 countries around the world. Many other companies sell a wide variety of nutraceuticals made from microalgae. Table 3 shows the different commercial presentation of food products from microalgae.

Table 3. Products and ingredients from microalgae commercially applied.

Commercial products	Characteristics	Company	References
Microalgal flour	*Chlorella* is used to replace dairy fats, vegetable oils and egg yolks in products, as ice cream to cookies, cakes, dressings, chocolate milk and pasta sauce.	Alga Via	http://algavia.com/how-we-innovate/
Bread and Cookies	*Chlorella* is inserted into the dough loaves for reduce oil content and replace the use of eggs, it is also used to create a healthier product, and maintain texture and structure bread.	Solazyme	http://solazymeindustrials.com/
Spaghetti	*Chlorella vulgaris* and *Spirulina* it is added to the mass of spaghettis with the intention completely remove butter, oil and egg yolks and maintain texture, mouthfeel and overall flavor.	Weihai Qingzheng	http://seaweed.foodmate.com/
Juice powder	Juice powder is enriched with *Aphanizomenonflos-aquae,* because microalgae contains every vitamin, mineral and amino acid that the body needs, thus becoming a healthier product at use of diets.	E_3Live® original	http://thepowerofjuice.com/blue-green-algae-e3live/
Dairy drinks	*Euglena* is used in the fermentation of yogurt by having excellent stability in low pH beverages means no grittiness from the protein. When fermented presents sensory characteristics acceptable by having similar flavor to the grape.	Euglena & Yogurt	https://www.euglena.jp/en/solution/
Extract Astaxanthin	*Haematococcus pluvialis* improved texture, emulsification, water binding and flavor delivery when added to food. Astaxanthin is principally consumed by the salmon feed industry.	Organic Herb Inc. (Hunan)	http://www.organic-herb.com/
Capsules	*Chlorella*,*Spirulina*, *Haematococcuspluvialis* and *Dunaliella*, works well for people who need to re-mineralize their body and boost their daily nutrient intake.	Ocean Drop	https://oceandrop.com.br
Docosahexaenoicacid (DHA)	Produced from microalgae *Crypthecodinium* and *Schizochytrium*, DHA has several applications including infant formulas, products for pregnant and nursing women, food and beverage products and dietary supplements.	Omega Tech (USA)	http://www.dhagold.com/
ß-Carotene	*Dunaliella* is rich in beta-carotene and is used as natural food colorants and as additive for animal feed.	Nature Beta Technologies	http://wondercare.co.in/nature/nature.html

Dairy products are nutrient-rich foods which contribute substantial amounts of many nutrients to the diet, including calcium, potassium, phosphorus, magnesium, zinc, protein, vitamins A, D and B12 and riboflavin. Dairy products, and especially cheese, have been reformulated to incorporate different types of microalgae to improve their nutritional quality. The combination of both kinds of foods helps to achieve healthy products enriched with various essential nutrients. Milk and dairy products are the main sources of calcium in nutrition; however, calcium in cheese is locked into casein. People lacking casein-degrading enzymes cannot reabsorb calcium from dairy products and can therefore develop some kind of hypocalcaemic status (Anderson and Sjöberg, 2001). Thus, the addition of microalgae rich in calcium could increase their concentration in dairy products.

In order to make a dairy product a source of iodine, Shrestha et al. (2011) used microalgae-enriched products as a source of iodine and as they were likely to be acceptable to consumers. The effect of adding the microalga *Chlorella* (0.5–1%) on the physical and sensory properties of processed cheese was investigated by Jeon (2006). Although the moisture, protein and fat contents and the degree of oiling off was similar in the *Chlorella* sp. and control cheeses, there were some differences in texture and color. However, the cheese with 0.5% *Chlorella* sp. was preferred (optimum sensory parameters) over the 1% *Chlorella* sp. and control cheeses. Also, Heo et al. (2006) investigated the effect of adding *Chlorella* powder (0–2.0%) on the growth of lactic acid bacteria, ripening velocity and sensory properties of Appenzeller cheese. Lactic acid bacteria counts were higher in the cheese containing added microalgae than in the control Appenzeller cheese. The optimum level of addition of *Chlorella* sp. powder that would produce cheese of acceptable quality was 0.5%.

Cereal-based products (pasta, bakery, etc.) are staples in many countries and are well accepted by many sectors of the population because, among other reasons, of their low cost, ease of preparation, versatility, sensory attributes, nutritional quality and long shelf-life. However, various strategies can be assayed in order to improve nutritional and health properties, including the incorporation of microalgae as ingredients.

Among the cereal-based products, pasta is considered to be very versatile in both nutritional and gastronomic terms. Nutritionists consider pasta to be highly digestible, providing significant quantities of complex carbohydrates, B-vitamins and iron, apart from containing low levels of sodium and total fat (Douglas and Matthews, 1982). Since it is low in protein and essential amino acids. Recent research studies have proposed to improve the nutritional properties of pasta by adding supplements from high-protein sources such as soy flours, soy isolates, whey proteins, etc. (Prabhasankar et al., 2007). In this context, edible microalgae have been used as optional ingredients in pasta products to further improve their nutritional properties and help to make microalgae popular amongst non-microalgae eaters. Prabhasankar et al. (2009) studied the effect of different levels (0–30%) of *Chorella vulgaris* on the sensory, cooking, nutritional and biofunctional quality of pasta. They reported that pasta containing up to 20% microalgae had acceptable sensory characteristics and better biofunctional properties. Pasta with added microalgae resulted in improved amino acid and fatty acid profiles, higher total phenolic contents and antioxidant activity and higher fucoxanthin and fucosterol contents.

Section 9 Regulation on novel foods and novel food ingredients

There is no universally accepted regulation governing functional foods, given the wide range of products without precise boundaries, and the varying views on what is considered sufficient scientific substantiation to draw conclusions about their functionality. A functional food is "a food that beneficially affects one or more target functions in the body beyond adequate nutritional effects in a way that is relevant to either an improved state of health and well-being and/or reduction of risk of disease. It is consumed as part of a normal food pattern. It is not a pill, a capsule or any form of dietary supplement" (Coppens et al., 2006).

Microalgae can be well suited for use as functional food because they contain constituents that are well known to have health benefits with few if any side effects, and they are uncommon in other foods. These include Omega-3 fatty acids and some pigments, such as β-carotene and astaxanthin (Siró et al. 2008).

Governments around the world have regulated functional food differently hence there is considerable overlap and variability about what constitutes a functional food product and what constitutes a nutraceutical one.

9.1 USA

Functional food is called dietary supplements under US regulation. They are easier to market if they consist of substances documented as Generally Recognized as Safe (GRAS), which is based on evidence that the substance has been part of the human diet over a long term with little or no adverse effect. However, this produces a dilemma for suppliers because when food products are marked as GRAS, they may be seen as ordinary by consumers rather than as having particular health functions (Burdock et al., 2006).

Any claim made on the package is regulated by the Food and Drug Administration (FDA) and must be supported by data; but if data are generated from clinical studies, the FDA is inclined to classify the product as a drug and the burden for proof of efficacy is extremely high and expensive. Likewise, any claim that a product can treat a disease makes the product a drug, not a food product. On the other hand, claims made in advertising (not on the package) are regulated by the Federal Trade Commission (FTC), not the FDA. The FDA has the power to pre-empt sale, whereas the FTC can only order the producer to cease and desist, and that can be challenged. A product that can avoid the FDA tests can thus have a substantial market lifetime before FTC can successfully intervene (Burdock et al., 2006).

Spirulina has a GRAS certificate through scientific procedures, for use as an ingredient in beverages and beverage bases, breakfast cereals, fresh fruits and fruit juices, frozen dairy desserts and mixes, grain products and pastas, milk products, plant protein products, processed fruits and fruit juices, processed vegetables and vegetable juices, snack foods, soft candy, and soups and soup mixes at a level of 0.5-3 grams *Spirulina* per serving (g/serving).

Under FDA regulations, claims are permitted provided they are expressed as nutrient content, health, qualified health or structure/function claims. The content of a specific nutrient can be listed, and compared with other foods. Health claims may suggest that the product might reduce the risk of a disease, and if the data are incomplete or inconclusive they must be qualified by saying so. The Significant Scientific Agreement (SSA) standard puts the burden of proof on the producer to show that the claims are supported by published studies and opinions from qualified professionals. Structure/function claims can propose an effect of the nutrient on specific body structures or functions, such as joints or eyes, if there is supporting

evidence, and the claim does not suggest that it can treat a disease. Such constraints on claims make it particularly difficult for producers to distinguish their product from competitors, except for the amount of a specific nutrient they contain (Roberfroid, 2002; Patel et al., 2008; Holdt and Kraan, 2011; Ahmad et al., 2011).

9.2 The European Union

According to the EU Regulation EC258/97 (EU, 1997), novel foods are food products and food ingredients that had not been used for human consumption to a signify cant degree within the European Community before 1997. The EU has a category of Foods for Particular Nutritional Use (PARNUTS) regulated between the categories of Food and Medicine (Coppens et al. 2006). Generally, the test is special preparation to meet the "particular nutritional requirements of certain categories of persons whose digestive processes or metabolism are disturbed; or of certain categories of persons who are in a special physiological condition and who are therefore able to obtain special benefit from controlled consumption of certain substances in foodstuffs." The supplier must notify the competent authority of their intent to market as PARNUTS.

The Food Safety regulation mentioned above states that food safety must be proven by a prolonged period of consumption. When this condition is not met - i.e. food products are new to the market without a history of safe use - these products are not authorized on the European market without having performed a safety assessment beforehand (Serafini et al., 2010).

The Novel Food Regulation applies to foods and food ingredients which present a new or modified primary molecular structure; which consist of micro-organisms, fungi or microalgae, which consist of or are isolated from plants and ingredients isolated from animals, whose nutritional value, metabolism or level of undesirable substances has been significantly changed by the production process. Important principles applied in this regulation are that novel foods and food ingredients must be safe for consumers (not being dangerous or nutritionally disadvantageous) and properly labelled so as not to mislead consumers (Enzing et al, 2014).

Within PARNUTS, the category Foods for special medical purposes requires medical supervision. EU member nations vary in how they interpret the regulations. Suppliers' claims for their product will determine whether it is classified as a Food, a Medicine or as a PARNUTS product (Verhagen et al., 2009).

9.3 Brazil

In Brazil, it is possible to declare some foods as having functional and/or health properties since ANVISA developed legislation that defines "Food Functional Properties and/or Health Claims". In this sense, the Functional Property Claim of a food product is defined as a claim "concerning the metabolic or physiological role that the nutrient or non-nutrient has in growth, development, maintenance and/or other normal functions of the human organism" (Brazil, 1999). This attribute must appear on the food label in a sentence such as

"Food fibers help bowels work well. Intake must be associated with a balanced diet and healthy habits" (Brazil, 2006).

Functional properties and/or health claims on a food label are tools that allow the consumer to know the possible benefits the food can offer. Generally speaking, information on the benefits inherent in foods and nutrition, when described on labels, helps consumers select a healthy diet (Hawkes, 2004).

It is reasonable that, for a product to be classified as food, it must comply with decree 986/69, which defines food as "any or mixed substances designed to offer the regular elements for the formation, maintenance and development of the human organism (Brazil, 1969). In 1999, ANVISA created a new category known as "new food products" (BRAZIL, 1999), defined as "food or substances not previously consumed in the country, or food with consumed substances previously that can be added or used in higher levels as compared to food used in regular diets" (Brazil, 1999).

ANVISA issued RDC resolution 2/2002 on January 7, 2002, which approved the Technical Rules for Bioactive and Isolated Probiotic Substances with Functional Properties or Health Claims. After this resolution, Brazilian legislation recognized two food categories with functional properties and/or health claims. The first includes food items with functional properties and/or health claims, and the second includes bioactive substances and isolated probiotics with functional properties and/or health claims.

An analysis of ANVISA's records shows that the same product can be classified into different food categories, i.e., it can be classified as a New Food or Ingredient and as a Bioactive Substance or Isolated Probiotic with Functional Properties and/or health claims. What makes this classification different, in a preliminary analysis, is that the isolated probiotic alone can ensure that the food product has potential for functional properties and/or health claims.

ANVISA authorizes the use of microalgae *Spirulina* and *Chlorella* as a food ingredient for the formulation of functional foods provided it meets the following criteria: recommended daily consumption of the product should not result in the ingestion above 1.6 3 grams per serving; it presents the specifications of the ingredient, including identification of microalgae species and their place of cultivation; it presents an analysis report, using recognized methodology, on the content of inorganic contaminants in ppm: mercury, lead, cadmium and arsenic.

Final considerations

Microalgae single-cell proteins, as well as microalgal products, have attracted a great deal of attention for their health benefits. Among these benefits, anti-obesity properties are worth of notice, since obesity with its comorbidities has become a major public worldwide health problem.

The main allegation of the use of microalgae in anti-obesity diets is their high protein content. This macronutrient plays an important role in body weight regulation diets, mainly because of their mechanism of protein-induced satiety. Moreover, microalgae single-cell

proteins also present other high-value anti-obesity compounds, such as peptides, essential fatty acids and pigments.

The major reasons for considering microalgae as a potential feedstock to produce foods or food ingredients with functional properties include (i) high content of digestible proteins, amino acids, essential fatty acids, pigments and other bioactive compounds; (ii) low nucleic acid contents; (iii) good sensorial and functional properties and (iv) the fact that they are robust microorganisms, hence they present resistance to contamination and adverse conditions and simple nutritional requirements.

The incorporation of microalgae into some foods and beverages that have high consumer acceptance is a good opportunity to popularize the health benefits of microalgae among consumers, even those unaccustomed to this type of food.

In recent years, many efforts have been made to incorporate microalgae into different foods and beverages. Several microalgae-based products with health claim can already be found on the market. They can be in the form of tablets, powders, capsules, pastilles and liquids. Also, their extract is included in noodles, beverages and cereals, providing health-promoting effects.

References

Ahmad, M.F., Ashraf, S.A., Ali Ahmad, F., Ansari, J.A., Siddiquee, M.R.A., 2011. Nutraceutical market and its regulation. Am. J. Food Technol. 6, 342-347.

Almada, A. L., 2008. Scope, importance and market opportunities of nutraceuticals and functional foods: a special emphasis on the future directions, in: Bagchi, D. (Ed.) Nutraceutical and Functional Food Regulations in the United States and Around the World. Food Science and Technology International Series. Academic Press., New York, pp. 3-9.

Anderson, J.J.B., Sjöberg, H.E., 2001. Dietary calcium and bone health in the elderly: uncertainties about recommendations. Nutr. Res. 21, 263-268.

Anupama, P., Ravindra., 2000. Value-added food: Single cell protein. Biotechnol. Adv. 18, 459-479.

ANVISA (Brazilian Sanitary Surveillance Agency), 2009. Lista dos Novos Ingredientes aprovados. Relatório em Alimentos com alegações de propriedades funcionais e ou de saúde, novos alimentos/ingredientes, substâncias bioativas e probióticos.

Arihara, K., 2006. Strategies for designing novel functional meat products. Meat. Sci. 74, 219-229.

Ba, F., Ursu, A.V., Loroche, C., Djelveh, G., 2016. *Haematococcus pluvialis* soluble proteins: Extraction, characterization, concentration/fractionation and emulsifying properties. Bioresource Technol. 200, 147-152.

Babuskin, S., Radhakrishnan, K., Babu, P.A.S., Sivarajan, M., Sukumar, M., 2014. Effect of photoperiod, light intensity and carbon sources on biomass and lipid productivities of *Isochrysis galbana*. Biotechnol. Lett. 36, 1653-1660.

Batista, A.P., Gouveia, L., Bandarra, N.M., Franco, J.M., Raymundo, A., 2013. Comparison of microalgal biomass profiles as novel functional ingredient for food products. Algal Res. 2, 164-173.

Becker, W., 2004. Microalgae in human and animal nutrition, in: Richmond, A. (Ed.), Handbook of Microalgal Culture: Biotechnology and Applied Phycology. Blackwell Publishing Ltd., London, pp. 312-351.

Belay, A., Kato, T., Ota, Y., 1996. *Spirulina* (*Arthrospira*): potential application as an animal feed supplement. J. Appl. Phycol. 8, 303-311.

Bocanegra, A., Bastida, S., Bened, I.J., Denas, S., Sanchez-Muniz, F., 2009. Characteristics and nutritional and cardiovascular-health properties of seaweeds. J. Med. Food. 12, 236-258.

Bolontrade, A.J., Scilling, A.A., Añón, M.C., 2016. Amaranth proteins foaming properties: Film rheology and foam stability-Part 2. Colloid. Surf. B. 141, 642-650.

Brazil., 1969. Decreto-Lei nº 986: Institui normas básicas sobre alimentos. Diário Oficial da União.

Brazil., 199. Lei n.9.782: O Congresso Nacional através do MS define o Sistema Nacional de Vigilância Sanitária e cria a Agência Nacional de Vigilância Sanitária. Diário Oficial da União.

Brazil., 2006. Manual de orientação para os conselheiros e agentes envolvidos na execução do Programa Nacional de Alimentação Escolar. Ministério da Educação. Fundo Nacional de Desenvolvimento da Educação.

Brennan, L., Owende, P., 2010. Biofuels from microalgae—A review of technologies for production, processing, and extractions of biofuels and co-products. Renew. Sust. Energ. Rev. 14,557-577.

Burdock, G.A., Carabin, I.G., Griffiths, J.G., 2006. The importance of GRAS to the functional food and nutraceutical industries. Toxicology. 221, 17-27.

Cantat, I., Cohen-Addad, S., Elias, F., Graner, F., Hohler, R., Pitois, O., 2013. Foams-structure and dynamics, ed. Oxford, London.

Chacón-Lee, T.L., Gonz´alez-Mariño, G.E., 2010. Microalgae for “healthy” foods-possibilities and challenges. Compr. Rev. Food Sci. F. 9, 655-675.

Chen, X., Zou, Y., Han, M., Pan, L., Xing, T., Xu, X., Zhou, G., 2016. Solubilisation of myosin in a solution of low ionic strength L-histidine: Significance of the imidazole ring. Food Chem. 196, 42-49.

Chronakis, I.S., 2001. Gelation of edible blue-green algae protein isolate (*Spirulina platensis strain Pacifica*): thermal transitions, rheological properties, and molecular forces involved. J. Agr. Food Chem. 49, 888-898.

Chronakis, I.S., Galatanu, A.N., Nylander, T., Lindman, B., 2000. The behaviour of protein preparations from blue-green algae (*Spirulina platensis strain Pacifica*) at the air/water interface. Colloid. Surface. A. 173, 181-192.

Chronakis, I.S., Madsen, M., 2011. Algal protein, in: Phillips, G.O., Williams, P.A., Handbook of Food Proteins (Eds.). Woodhead Publishing Inc., United Kingdom, pp. 353-394

Clifton, P.M., 2008. Dietary treatment for obesity. Nat. Clin. Pract. Gastroenterol. Hepatol. 5, 672-681.

Conde, E., Balboa, E.M., Parada, M., Falqué E., 2013. Algal proteins, peptides and amino acids, in: Dominguez, H. (Ed.), Functional Ingredients from Algae for Foods and Nutraceuticals. Woodhead Publishing, Cambridge, pp.135-169.

Coppens, P., Da silva, M. F., Pettman, S., 2006. European regulations on nutraceuticals, dietary supplements and functional foods: A framework based on safety. Toxicology. 221, 59-74.

Cuttriss, A. J., Cazzonelli, C. I., Wurtzel, E. T., & Pogson, B. J., 2011. Carotenoids, in: F. Rébeillé & R. Douce (Eds.), Biosynthesis of Vitamins in Plants. Part A, Vitamins A, B1, B2, B3, B5. Academic Press., London, pp. 1-36.

D'Antona, G., 2014. Essential Amino Acid Supplementation for the Prevention and Treatment of Obesity, in: Watson, R. (Ed.), Nutrition in the Prevention and Treatment of Abdominal Obesity. Academic Press., New York, pp. 447-469.

Damodaran, S., 1994. Structure-function relationship of food proteins, in: Hettiarachchy, N.S., Ziegler, G.R. (eds.), Protein Functionality in Food Systems. New York, pp 1-38.

Day, L., Seymour, R.B., Pitts, K.F., Konczak, I., Lundin, L., 2009. Incorporation of functional ingredients into foods. Trends. Food Sci. Technol. 20, 388-395.

Desmorieux, H., Decaen, N., 2005. Convective drying of *Spirulina* in thin layer. J. Food Eng. 66, 497-503.

Dickinson, E., 2015. Structuring of colloidal particles at interfaces and the relationship to food emulsion and foam stability. J. Colloid. Interf. Sci. 449, 38-45.

Dietrich, N., Mayoufi, N., Poncin, S., Li, H.Z., 2013. Experimental investigation of bubble and drop formation at submerged orifices. Chem. Pap. 67, 313-25.

Donadio, C., Bialecki, A., Valla, A., Dufosse, L., 2011. Carotenoid-derived aroma compounds detected and identified in brines and speciality sea salts (*fleur de sel*) produced in solar salterns from Saint-Armel (France). J. Food Compos. Anal. 24, 801-810.

Douglas, J.S., Matthews, R.H., 1982. Nutrient content of pasta products. Cereal Foods World. 27, 558-561.

Drenckhan, W., Saint-Jalmes, A., 2015. The science of foaming. Adv. Colloid. Interface Sci. 222, 228-259.

Enzing, C., Ploeg, M., Sijtsma, M.B.L., 2014. Microalgae-based products for the food and feed sector: an outlook for Europe, first ed. Joint Research Centre, Spain.

EU., 1997. Regulation (EC) No. 258/97: European Parliament and of the Council concerning novel foods and novel food ingredients. CELEX-EUR Official Journal L.

FAO (Food and Agriculture Organization), 1981. Protein digestibility and absorption: effects of fiber and the extent of individual variation. Report of a joint FAO/WHO Expert Consultation on Energy and Protein Requirements.

FAO (Food and Agriculture Organization), 1991. Protein Quality Evaluation. Report of a joint FAO/WHO Expert Consultation.

FDA (Food and Drug Administration), Generally Recognized as Safe (GRAS). Available in http://www.fda.gov/Food/IngredientsPackagingLabeling/GRAS/default.htm

Fernández-Sánchez, A., Madrigal-Santillán, E., Bautista, M., Esquivel-Soto, J., Morales-González, A., Esquivel-Chirino, C., Durante-Montiel, I., Sánchez-Rivera, G., Valadez-Vega, C., Morales-González, J.A., 2011. Inflammation, Oxidative stress and Obesity. Int. J. Mol. Sci. 12, 117-132.

Fleurence, J., 1999. Seaweed proteins: Biochemical, nutritional aspects and potential uses. Trends Food Sci. Technol. 10, 25-28.

Francisco, E.C., Franco, T.T., Wagner, R., Jacob-Lopes, E., 2014. Assessment of different carbohydrates as exogenous carbon source in cultivation of cyanobacteria. Bioprocess Biosyst. Eng. 37, 1497-1505.

Francisco, E.C., Franco, T.T., Zepka, L.Q., Jacob-Lopes, E., 2015. From waste-to-energy: the process integration and intensification for bulk oil and biodiesel production by microalgae. J. Env. Chem. Eng. 3, 482-487.

Freitas, A.C., Rodrigues, D., Rocha-Santos, T.A.P., Gomes, A.M.P., Duarte, A.C., 2012. Marine biotechnology advances towards applications in new functional foods. Biotechnol. Adv. 30, 1506-1515.

Gelberg, H.A., Kwan, C.L., Mena, S.J., Erickson, Z.D., Baker, M.R., Chamberlin, V., Nguyen, C., Rosen, J.A., Shah, C., Ames, D., 2015. Meal replacements as a weight loss tool in a population with severe mental illness. Eating. behaviors. 19, 61-64.

Gellenbeck, K.W., 2011. Utilization of algal materials for nutraceutical and cosmeceutical application-what do manufacturers need to know? J. Appl. Phycology. 24, 1-5.
Goldberg, I., 1985. Single cell protein, first ed. Springer-Verlag, Berlin.

Gouveia, L., Rema, P., 2005. Effect of microalgal biomass concentration and temperature on ornamental goldfish (*Carassius auratus)* skin pigmentation. Aquac. Nutrition. 11, 19-23.

Graham, N., Phillips, M.C., 1976. The conformation of proteins at the air-water interface and their role in stabilizing foams, in: Akers, R. J. (eds.), Foams. Academic Press, New York, pp. 237-255.

Guill-Guerrero, J.L., Navarro-Juárez, R., López-Martínez, J.C., Campra-Madrid, P., Rebolloso-Fuentes, M.M., 2004. Functional properties of the biomass of the three microalgal species. J. Food Eng. 65, 511-517.

Haque, F., Dutta, A., Thimmanagari, M., Chiang, W., 2016. Intensified green production of astaxanthin from Haematococcus *pluvialis*. Food Bioprod. Process. 99, 1-11.

Hawkes, C., 2004. Nutrition labels and health claims: the global regulatory environment. First ed. World Health Organization, Geneva.

Heindel, J.J., Newbold, R., Schug, T.T., 2015. Endocrine disruptors and obesity. Nat. Rev. Endocrinol. 11, 653-661.

Heo, J.Y., Shin, H.J., Oh, D.H. Cho, S.K., Yang, C.J., Kong, Y.K., Lee, S.S.L., Choi, K.S., Choi, S.H., Kim, S.C., Coi, H.Y., Bae, I., 2006. Quality properties of Appenzeller cheese added with *Chlorella*. Korean J. Food Sci. Anim. Resour. 26, 525-531.

Hlavova, M., Turoczy, Z., Bisova, K., 2015. Improving microalgae for biotechnology-From genetics to synthetic biology. Biotechnol. Adv. 33, 1194-1203.

Holdt, S.L., Kraan, S., 2011. Bioactive compounds in seaweeds: functional food application and legislation. J. Appl. Phycol. 23, 543-597.

Hu, X., Tao, N., Wang, X., Xiao, J. Wang, M., 2016. Marine-derived bioactive compounds with anti-obesity effect: A review. J. Func. Foods. 21, 372-387.

Ikeuchi, M., Koyama, T., Takahashi, J., Yazawa, K., 2007. Effects of astaxanthin in obese mice fed a high-fat diet. Biosci. Biotechnol. Biochem. 71, 893-899.

Jacob-Lopes, E., Zepka, L.Q., Queiroz., M.I., Netto, F.M., 2006. Caracterização da fração protéica da cianobactéria *Aphanothece Microscopica Nägeli* cultivada no efluente da parboilização do arroz. Ciênc. Tecnol. Aliment. 26, 482-488.

Jay, M.J., 1992. Modern Food Microbiology, sixth ed. Van Nostrand Reinhold, New York.

Jayasooriya, A.P., Mathai, M.L., Walker, L.L., Begg, D.P., Denton, K.A., Cameron-Smith, D., Egan, G.F., McKinley, M.J., Rodger, P.D., Sinclair, A.J., Wark, J.D., Weisinger, H.S., Jois, M., Weisinger, R.S., 2008. Mice lacking angiotensin-converting enzyme have increased energy expenditure, with reduced fat mass and improved glucose tolerance. Proc. Natl. Acad. Sci. 105, 6531-6536.

Jegathese, S.J.P., Farid, M., 2014. Microalgae as a renewable source of energy: A niche opportunity. J. Rew. Energ. 4, 1-10.

Jeon, H., 2006. Effect of *Chlorella* addition on the quality of processed cheese. J. Korean Soc. Food Sci. Nutr. 35, 373-377.

Jiménez-Colmenero, F., 2007. Healthier lipid formulation approaches in meat-based fat. Trends. Food Sci. Technol. 18, 567-578.

Jiménez-Colmenero, F., Carballo, J., Cofrades, S., 2001. Healthier meat products: Their role as functional foods. Meat Sci, 59, 5-13.

Jin, H.Z., Fu, X.J., Kim, E.H., Jung, S.T., Park, Y.K., 2005. Properties of rice extrudates with adding the powder and extracts of seaweeds. J. Henan. Univ. Technol. Nat. Sci. 26, 5-8.

Jones, B.H., Standridge, M.K., Moustaid, N., 1997. Angiotensin II increases lipogenesis in 3T3-L1 and human adipose cells. Endocrinology. 138, 1512-1519.

Kimura, M., Iida, M., Yamauchi, H., Suzuki, M., Shibasaki, T., Saito, Y., Saito, H., 2014. Astaxanthin supplementation effects on adipocyte size and lipid profile in OLETF rats with hyperphagia and visceral fat accumulation. J. Funct. Foods. 11, 114-120.

Kumar, S.A., Magnusson, M., Ward, L.C., Paul, N.A., Brown, L. A., 2015. Green algae mixture of *Scenedesmus* and *Schroederiella attenuates* obesity-linked metabolic syndrome in rats. Nutrients. *7*, 2771-2787.

Kushner, R.F., 2014. Weight Loss Strategies for Treatment of Obesity. Prog. Cardiovasc. Dis. 56, 465-472.

Kusminski, C.M., Bickel, P.E., Scherer, P.E., 2016. Targeting adipose tissue in the treatment of obesity-associated diabetes. Nat. Rev. Drug. Discov. 5, 1-22.

Lam, R.S.H., Nickerson, M.T., 2013. Food proteins: A review on their emulsifying properties using a structure-function approach. Food Chem. 141, 975-984.

Lee, S.H., Qian, Z.J., Kim, S.K., 2010. A novel angiotensin I am converting enzyme inhibitory peptide from tuna frame protein hydrolysate and its antihypertensive effect in spontaneously hypertensive rats. Food Chem. 118, 96-102.

Li, G.H., Qu, M.R., Wan, J.Z., You, J.M., 2007. Antihypertensive effect of rice protein hydrolysate with in vitro angiotensin I-converting enzyme inhibitory activity in spontaneously hypertensive rats. Asia Pac. J. Clin. Nutr. 16, 275-280.

Liang, S., Xueming, L., Chen, F., Chen, Z., 2004. Current microalgal health food R&D activities in China. Hydrobiologia. 512, 45-48.

Maeda, H., 2013. Anti-obesity and anti-diabetic activities of algae, in: Dominguez, H. (Ed.), Functional Ingredients from Algae for Foods and Nutraceuticals. Woodhead Publishing, Cambridge, pp.453-472.

Mamat, H., Matanjun, P., Ibrahim, S., Amin, S.F.M., Hamid, M.A., Rameli, A.S., 2014. The effect of seaweed composite flour on the textural properties of dough and bread. J. Appl. Phycol. 26, 1057-1062.

Manikkam, V., Vasiljevic, T., Donkor, O.N., Mathai, M.L., 2016. A review of potential marine-derived hypotensive and anti-obesity peptides. Crit. Rev. Food Sci. Nutr. 56, 92-112.

Maroneze, M.M., Barin, J.S., Menezes, C.R., Queiroz, M.I., Zepka, L.Q., Jacob-Lopes, E., 2014. Treatment of cattle-slaughterhouse wastewater and the reuse of sludge for biodiesel production by microalgal heterotrophic bioreactors. Scientia Agri. 71, 521-524.

Mata, T.M., Martins, A.A., Caetano, N.S., 2010. Microalgae for biodiesel production and other applications: A review. Renew. Sust. Energ. Rev. 14, 217-232.

Matak, K.E., Tahergorabi, R., Jaczynski, J., 2015. A review: Protein isolates recovered by isoelectric solubilization/precipitation processing from muscle food by-products as a component of nutraceutical foods. Food Res. Int. 77, 269-703.

Mathai, M.L., Naik, S., Sinclair, A.J., Weisinger, H.S., Weisinger, R.S., 2008. Selective reduction in body fat mass and plasma leptin induced by angiotensin-converting enzyme inhibition in rats. Int. J. Obes. 32, 1576-1584.

Matsumiya, K., Murray, B.S., 2016. Soybean protein isolate gel particles as foaming and emulsifying agents. Food hydrocolloid. 60, 206-215.

MCmillan-Price, J., Brand-Miller, J., 2004. Dietary approaches to overweight and obesity. Clin. dermatol. 22, 310-314.

Mellinkoff, S.M., Frankland, M., Boyle, D., Greipel, M., 1956. Relationship between serum amino acid concentration and fluctuations in appetite. J. Appl. Physiol. 8, 535-538.

Melnikova, I., Wages, D., 2006. Anti-obesity therapies. Nat. Rev. Drug Discov. 5, 369-370.
Mendis, E., Kim, S.K., 2011. Present and future prospects of seaweeds in developing functional foods. Adv. Food Nutr. Res. 64, 1-15.

Miyashita, K., and Hosokawa, M., 2014. Carotenoids as a Nutraceutical Therapy for Visceral Obesity, in: Watson, R. (Ed.), Nutrition in the Prevention and Treatment of abdominal Obesity. Academic Press, New York, pp.329-340.

Mohamed, G.A., Ibrahim, S.R.M., Elkhayat, E.S., El Dine, R.S., 2014. Natural anti-obesity agents. Bull. Fac. Pharm. Cairo Univ. 52, 269-284.

Molina, G.E., Belarbi, E.H., Acién-Fernandez. F.G., 2002. Recovery of microalgal biomass and metabolites: process option and economics. Biotechnol. Advan. 20, 491-515.

Moustafa, J.S.E.S., Froguel, P., 2013. From obesity genetics to the future of personalized obesity therapy. Nat. Rev. Endocrinol. 9, 402-413.

Muradian, K.H., Vaiserman, A., Min, K.J., Fraifeld, V.E., 2015. Fucoxanthin and lipid metabolism: A minireview. Nutr. Metab. Cardiovas. 25, 891-897.

Nalage, D.N., Khedkar, G.D., Kalyankar, A.D., Sarkate, A.P., Ghodke, S.R., Bedre, B.V., Khedkar, C.D., 2016. Single Cell Proteins, in; *Caballero, B., Finglas, P.M. Toldrá*, F. (Eds.), Encyclopedia of Food and Health. Academic Press., New York, pp.790-794.

Niaonakis, M., 2015. Biopolymers: Processing and Products, first ed. William Andrew, United Kingdom.

Ohta, T., Iwashita, A., Sasaki, S., Kawamura, Y., 1997. Antihypertensive action of the orally administered protease hydrolysates of chum salmon head and their angiotensin iconverting enzyme inhibitory peptides. Food Sci. Technol. Int. 3, 339-343.

Paddon-Jones, D., Westman, E., Mattes, R.D., Wolfe, R.R., Astrup, A., Westerterp Plantenga, M., 2008. Protein, weight management, and satiety. Am. J. Clin Nutr. 87, 1558-1561.

Pádua, D., Rocha, E., Gargiulo, D., Ramos, A.A., 2015. Bioactive compounds from brown seaweeds: Phloroglucinol, fucoxanthin and fucoidan as promising therapeutic agents against breast cancer. Phytochem. Letters. 14, 91-98.

Paniagua-Michel, J., 2015. Microalgal Nutraceuticals, in: Kim, S.K. (Ed.), Handbook of Marine Microalgae. Academic Press, New York, pp.255-267.

Paniagua-Michel, J., Dujardin, E., Sironval, C., 1992. Le Tecuitlatl, concentre de *Spirulines des Aztecs*. Bull Acad. Roy. Sci. Beg. 10, 253-263.

Patel, D., Dufour, Y., Domigan, N., 2008. Functional food and nutraceutical registration process in Japan and China: Similarities and differences. J. Pharm. Pharm. Sci. 11, 1-10.

Perez-Garcia, O., Escalante, F.M.E., De-Bashan. L.E., Bashan, Y., 2011. Heterotrophic cultures of microalgae: Metabolism and potential products. Water Res. 45, 11-36.

Pesta, D.H., Samuel, V.T., 2014. A high-protein diet for reducing body fat: mechanisms and possible caveats. Nutr. Metab. 11, 53-61.
Pignolet, O., Jubeau, S., Vaca-Garcia, C., Michaud, P., 2013. Highly valuable microalgae: biochemical and topological aspects. J. Ind. Microbiol. Biotechnol. 40, 781-796.

Prabhasankar, P., Ganesan, P., Bhaskar, N., Hirose, A., Nimishmol, S., Gowda, L.R., Hosokawa, M., Miyashita, K., 2009. Edible Japanese seaweed, wakame (*Undaria pinnatifida*) as an ingredient in pasta: Chemical, functional and structural evaluation. Food Chem. 115, 501-508.

Prabhasankar, P., Jyotsna, R., Indrani, D., Rao, G.V., 2007. Influence of whey protein concentrate, additives, their combination on the quality and microstructure of vermicelli made from Indian *T. durum* wheat variety. J. Food Eng. 80 ,1239-1245.

Pulz, O., Gross, W.O., 2004. Valuable products from biotechnology of microalgae, Appl. Microbiol. Biotechnol. 65, 635–648.

Rangel-Yagui, C.O., Godoy-Danesi, E.D., Carvalho, J.C.M., Sato, S., 2004. Chlorophyll production from *Spirulina platensis:* cultivation with urea addition by fed-batch process. Bioresour. Technol. 92, 133-141.

Raymundo, A., Gouveia, L., Batista, A.P., Empis, J., Sousa, I., 2005. Fat mimetic capacity of *Chlorella vulgaris* biomass in oil-in-water food emulsions stabilised by pea protein. Food Res. Int. 38, 961-965.

Razzak, S.A., Hossain, M.M., Lucky, R.A., Bassi, A.S., Lasa, H., 2013. Integrated CO_2 capture, wastewater treatment and biofuel production by microalgae culturing-A review. Renew. Sust. Energ. Rev. 27, 622-653.

Roberfroid, M.B., 2002. Global view on functional foods: European perspectives, Br. J. Nutr. 88, S133–S138.

Rodríguez, S.D., Staszewski, M.V., Pilosof, A.M.R., 2015. Green tea polyphenols-whey proteins nanoparticles: Bulk, interfacial and foaming behavior. Food Hydrocolloid. 50, 108-115.

Saha, D., Bhattacharya, S., 2010. Hydrocolloids as thickening and gelling agents in food: a critical review. J Food Sci. Technol. 47, 587-597.

Salazar, M., Chamorro, G.A., Salazar, S., Steele, C.E., 1996. Effect of *Spirulina* maxima consumption on reproduction and peri-and postnatal development in rats. Food Chem. Toxicol. 34, 353-59.

Samarakoon, K., Jeon, Y.J., 2012. Bio-functionalities of proteins derived from marine algae - A review. Food Res. Int. 48, 948-960.

Santos, A.B., Fernandes, A.S., Wagner, R., Jacob-Lopes, E., Zepka, L.Q., 2016. Biogeneration of volatile organic compounds produced by *Phormidium autumnale* in heterotrophic bioreactor. J. Appl. Phycol. 28, 1561-1570.

Sari, Y.W., Bruins, M.E., Sanders, J.P.M., 2013. Enzyme assisted protein extraction from rapeseed, soybean, and microalgae meals. Ind. Crops. Prod. 43, 78-83.

Schmitt, V., Ravaine, V., 2013. Surface compaction versus stretching in pickering emulsions stabilised by microgels. Curr. Opin. Colloid. Interface. Sci. 18, 532-564.

Serafini, M., Stanzione, A., Scarpa, B., Foddai, S., 2010. Novel Food: A New Reality in Food Ingredient. J. Clin. Gastroenterol. 44, S47–S48.

Sgarbieri, V.C., 1996. Proteins in food protein: properties, degradation, modification, first ed. Editor-Livraria Varela, São Paulo.

Shinohara, K., Okura, Y., Koyano, T., Murakami, H., Kim, E., Omura, H., 1986. Growth-promoting effects of an extract of a thermophilic blue-green alga, *Synechococcus elongatus var.* on human cell lines. Agric. Biol. Chem. 50, 2225-2230.

Shrestha, M., Woehlk, H., Tchetseubu Saha, G., 2011. Studies on drying and comminution of young brown seaweed for manufacture of algae-enriched dairy products. Die Milchwirschaft. 332-336.

Siró, I., Kapolna, E., Kapolna, B., Lugasi, A., 2008. Functional food. Product development, marketing and consumer acceptance – A review. Appetite. 51, 456–467.

Smith, J., Hong-Schum., 2011. Food Additives-Data Book, second ed. Wiley-Blackwell, New York.

Soletto, D., Binaghi, L., Lodi, A., Carvalho, J.C.M., Converti, A., 2005. Batch and fed-batch cultivations of *Spirulina platensis* using ammonium sulphate and urea as nitrogen sources. Aquaculture. 243, 217-224.

Spolaore, P., Joannis-Cassan, C., Duran, E., Isambert, A. 2006. Commercial applications of microalgae. J. Biosci. Bioeng. 101, 87-96.

Stamler, J., 1982. Diet and coronary heart disease. Biometrics. 38, 95-118.

Staszewski, V., Pizones, M., Ruiz-Henestrosa, V.M., Pilosof, A.M.R., 2014. Green tea polyphenols-β-lactoglobulin nanocomplexes: Interfacial behavior, emulsification and oxidation stability of fish oil. Food Hydrocolloid. 35, 505-511.

Stevenson P., 2012. Foam engineering: Fundamentals and Applications, first ed. John Wiley & Sons Ltd, Chichester.

Suman, G., Nupur, M., Anuradha, S., Pradeep. B., 2015. Single Cell Protein Production: A Review. Int. J. Curr. Microbiol. App. Sci. 4, 251-262.

Talero, E., García-Mauriño, S., Ávila-Román, J., Rodríguez-Luna, A., Alcaide, A., Motilva, V., 2015. Bioactive compounds isolated from microalgae in chronic inflammation and cancer. Mar Drugs. 30, 6152-6209.

Tanaka, K., Konishi, F., Himeno, K., Taniguchi, K., Nomoto, K., 1984. Augmentation of antitumor resistance by a strain of unicellular green algae, *Chlorella vulgaris*. Cancer Immunol. Immun. 17, 90-94.

Testouri, A., Honorez, C., Barillec, A., Langevin, D., Drenckhan, W., 2010. Highly structured foams from chitosan gels. Macromolecules. 43, 6166-6173.

United Nations., 2015. World Population Prospects: The 2015 Revision, Key Findings and Advance Tables. Department of Economic and Social Affairs, Population Division.

Valdez, P.J., Nelson, M.C., Wang, H.Y., 2012. Hydrothermal liquefaction of *Nannochloropsis* sp.: Systematic study of process variables and analysis of the product fractions. Biomass Bioenerg. 46, 317-331.

Vanthoor-Koopmans, M., Wijffels, R.H., Barbosa, M., Eppink, M.H.M., 2013. Biorefinery of microalgae for food and fuel. Bioresour. Technol. 135, 142-149.

Veldhorst, M., Smeets, M.A., Soenen, S., Hochstenbach-Waelen, A., Hursel, R., Diepvens, K., Lejeune, M., Luscombe-Marsh, N., Westerterp-Plantenga, M., 2008. Protein-induced satiety: Effects and mechanisms of different proteins. Physiol. Behav. 94, 300-307.

Venugopal, V., 2009. Functional foods: an overview, in: Venugopal, V. (Eds.), Marine Products for Healthcare: Functional and Bioactive Nutraceutical Compounds from the Ocean. CRC Press, New York, pp. 1-22.

Verhagen, H., Vos, E., Francl, S., Heinonen, M., Van Loveren, H., 2010. Status of nutrition and health claims in Europe. Arch. Biochem. Biophys. 501, 6-15.

Von Der Weid D., Dillon, J.C., Falquet, J., 2000. Malnutrition: a silent massacre, first ed. Antenna Technology, Genève.

Weiss, J., Gibis, M., Schuh, V., Salminen, H., 2010. Advances in ingredient and processing systems for meat and meat products. Meat. Sci. 86, 196-213.

Westerterp-Plantenga, M.S., Lemmens, S.G., Westerterp, K.R., 2012. Dietary protein-its role in satiety, energetics, weight loss and health. Br. J. Nutr. 108, 105-112.

Westerterp-Plantenga, M.S., Luscombe-Marsh, N., Lejeune, M.P.G.M., Diepvens, K., Nieuwenhuizen, A., Engelen, M.P.K.L., Deutz, N.P., Azzout-Marniche, D., Tome, D., Westerterp, K.R., 2006. Dietary protein, metabolism, and body-weight regulation: dose–response effects. Int. J. Obesity. 30, 16-23.

Westman, E.C., Yancy, W.S., Edman, J.S., Tomlin, K.F., Perkins, C.E., 2002. Effect of 6-month adherence to a very low carbohydrate diet program. Am. J. Med. 113, 3-6.
WHO (World Health Organization), 2014. Obesity and overweight. Fact Sheet. 311.

Wijffels, R.H., Kruse, O., Hellingwerf, K.J., 2013. Potential of industrial biotechnology with cyanobacteria and eukaryotic microalgae. Curr. Opin. Biotechnol. 24, 405-413.

Yamaguchi, K., 1997. Recent advances in microalgal bioscience in Japan, with special reference to utilization of biomass and metabolites: a review. J. Appl. Phycol. 8, 487-502.

Yook, J.S., Kim, K.A., Park, J.E., Lee, S.H., Cha, Y.S., 2015. Microalgal oil supplementation has an anti-obesity effect in C57BL/6J Mice fed a high fat diet. Prev. Nutr. Food Sci. 20, 230-237.

Yuan, Y.V., 2008. Marine Algal Constituents, in: Barrow, C., Shahidi, F. (Eds.), Marine Nutraceuticals and Functional Foods, CRC Press, Taylor & Francis Group, New York, pp. 259-296.

Zepka, L.Q., Jacob-Lopes, E., Goldbeck, R., Souza-Soares, L.A., Queiroz, M.I. 2010. Nutritional evoluation of single-cell protein produced by *Aphanothece microscopica Nägeli*. Bioresour. Technol. 101, 7107-7111.

Zhang, Z., Li, T., Wang, D., Zhang, L., Chen, G., 2009. Study on the volatile profile characteristics of oyster *Crassostrea gigas* during storage by a combination sampling method coupled with GC/MS. Food Chem. 115, 1150-1157.

Zhuang, X., Jiang, X., Han, M., Zhuang-Li, K., Zhao, L., Xing-Lian, X., Guang-Hong, Z., 2016. Influence of sugarcane dietary fier on water states and microstructure of myofibrillar protein gels. Food Hidrocolloid. 57, 253-261.

5. ARTIGO: CAROTENOID PROFILE OF THREE MICROALGAE / CYANOBACTERIA SPECIES WITH PEROXYL RADICAL SCAVENGER CAPACITY

Periódico: Food Research International

Recebido em: 18 de março de 2017.

Aceito em: 28 de junho 2017.

In Press: 2017

ARTICLE IN PRESS

Food Research International xxx (xxxx) xxx–xxx

Contents lists available at ScienceDirect

Food Research International

journal homepage: www.elsevier.com/locate/foodres

Carotenoid profile of three microalgae/cyanobacteria species with peroxyl radical scavenger capacity

Luciana D. Patias[a], Andrêssa S. Fernandes[a], Fabiane C. Petry[b], Adriana Z. Mercadante[b], Eduardo Jacob-Lopes[a], Leila Q. Zepka[a,*]

[a] Department of Food Technology and Science, Federal University of Santa Maria (UFSM), P.O. Box 5021, Santa Maria 97105-900, Brazil

[b] Department of Food Science, University of Campinas (UNICAMP), Monteiro Lobato, 70, Campinas 13083-862, Brazil

ARTICLE INFO

Keywords:
Algae
Blue-green algae
Carotenoids
Bioactive compounds
Antioxidant capacity
HPLC-PDA-MS/MS

ABSTRACT

Carotenoids from cyanobacteria *Aphanothece microscopica Nageli* and green microalgae *Chlorella vulgaris* and *Scenedesmus obliquus* were identified. The total carotenoid content, based on dry weight of biomass, of *A. microscopica Nägeli*, *C. vulgaris* and *S. obliquus* were 1398.88 μg/g, 1977.02 μg/g and 2650.70 μg/g, respectively. A total of 23 different carotenoids were separated in all the extracts, the major ones being all-*trans*-β-carotene (29.3%) and all-*trans*-lutein (28.1%) in *Scenedesmus*; all-*trans*-echinenone (22.8%) and all-*trans*-β-carotene (17.7%) in *Chlorella*; all-*trans*-echinenone (28.3%) and all-*trans*-β-carotene (26.2%) in *Aphanothece*. The carotenoid extracts were shown to be a potent scavenger of peroxyl radical, with values of 31.1 (*Chlorella*), 14.0 (*Scenedesmus*) and 7.3 (*Aphanothece*) times more potent than α-tocopherol.

1. Introduction

The carotenoids have high commercial values, especially for their high demand as bioactive compounds. Thus, antioxidant pigments are currently the most marketed products from microalgae, renovating the interest in increasing the research and development of these compounds in microalgae biomass (Poojary et al., 2016). The global market of carotenoids was US$ 1.2 billion in 2010 and is expected to increase to $ 1.4 billion by 2018. The highest market shares are β-carotene and astaxanthin, with an average price close to USD 2500/kg (Suganya, Varman, Masjuki, & Renganathan, 2016).

The total number of reported carotenoids that has been fully characterized is about 1167, this number includes the enormous variety of carotenoids in microalgae (Britton, Liaaen-Jensen, & Pfander, 2004; Yabuzaki, 2017). Microalgae produce carotenoids with structural characteristics very different from those commonly found in fruits and vegetables, in particular, acetylenic carotenoids, ketocarotenoids and glycosylate carotenoids (Poojary et al., 2016; Rodrigues, Menezes, Mercadante, Jacob-Lopes, & Zepka, 2015; Crupi et al., 2013; Haugan & Liaaen-Jensen, 1994; Hertzberg, Liaaen-Jensen, & Siegelman, 1971).

Diatoxanthin, crocoxanthin, echinenone, canthaxanthin and myxoxanthophyll are secondary bioactive carotenoids of microalgal origin, which may have diverse industrial applications (Grama et al., 2014). 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 (Solovchenko et al., 2013). Echinenone and canthaxanthin are considered to be stronger antioxidants than β-carotene; astaxanthin shows high colorant potential (Papp et al., 2013).

Currently, microalgal carotenoids are mainly produced from *Dunaliella salina* and *Haematococcus pluvialis*. Nevertheless, there are inherent shortcomings for these strains, such as slow growth, insufficient yield, and strict nutritional requirements (Guo et al., 2016). By contrast, the microalgae *Scenedesmus obliquus*, *Chlorella vulgaris* and *Aphanothece microscopica Nägeli* grow rapidly, have substantial carotenoid content, and perform robustly in bioreactors (Francisco, Neves, Jacob-Lopes, & Franco, 2010; Maroneze et al., 2016). Therefore, these microalgae can be considered as potential alternative producers of carotenoids.

Thus, the objective of our study is to identify the carotenoid composition, and determine the peroxyl radical scavenger capacity of the carotenoid extracts of three microalgae species. The results may draw attention to the importance of these species as alternative sources of bioactive compounds, with an elevated potential for industrial exploitation.

* Corresponding author.
E-mail addresses: jacoblopes@pq.cnpq.br (E. Jacob-Lopes), lqz@pq.cnpq.br (L.Q. Zepka).

http://dx.doi.org/10.1016/j.foodres.2017.06.069
Received 16 March 2017; Received in revised form 27 June 2017; Accepted 28 June 2017
0963-9969/ © 2017 Elsevier Ltd. All rights reserved.

Please cite this article as: Patias, L.D., Food Research International (2017), http://dx.doi.org/10.1016/j.foodres.2017.06.069

2. Materials and methods

2.1. Microorganisms and culture media

Axenic cultures of *Aphanothece microscopica Nägeli* (RSMan92), *Chlorella vulgaris* (CPCC90) and *Scenedesmus obliquus* (CPCC05) were used in the experiments. Stock cultures were propagated and maintained in synthetic BG11 medium (Braun-Grunow medium) (Rippka, Deruelles, Waterbury, Herdman, & Stanier, 1979). The incubation conditions were 30 °C, photon flux density of 15 μmol/m^2/s and a photoperiod of 12 h were used.

2.2. Microalgal biomass production

The biomass productions were made in phototrophic conditions. The cultivations were performed in a bubble column photobioreactor (Maroneze et al., 2016) operating under a batch regime, fed on 2.0 L of BG11 medium. The experimental conditions were as follows: initial cell concentration of 100 mg/L, isothermal reactor operating at a temperature of 25 °C, photon flux density of 150 μmol/m^2/s and continuous aeration of 1VVM (volume of air per volume of culture per minute) with the injection of air enriched with 15% carbon dioxide. The biomasses were separated from the culture medium by centrifugation. It was subsequently freeze dried for 24 h at − 50 °C above − 175 μm Hg, and then stored under refrigeration until the time of analysis. The cultivations were performed twice, and in duplicate.

2.3. Carotenoid extraction

The carotenoids were exhaustively extracted from the freeze-dried sample (0.2 ± 0.02 g) with ethyl acetate and methanol in a mortar with a pestle followed by centrifugation (Hitachi, Tokyo, Japan) for 7 min at 1500 × *g* (Mandelli, Miranda, Rodrigues, & Mercadante, 2012). The extraction procedure was repeated until the supernatant becomes colorless, which was reached approximately after 9 extractions with ethyl acetate and 5 with methanol. 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 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.

2.4. HPLC-PDA-MS/MS analysis

The carotenoids were analyzed by high performance liquid chromatography HPLC (Shimadzu, Kyoto, Japan) equipped with quaternary pumps (model LC-20AD), online degasser, and injection valve with a 20 μL loop (Rheodyne, Rohnert Park-CA, USA). The equipment was connected in series to a PDA detector (model SPD-M20A) and a mass spectrometer with an ion-trap analyzer and atmospheric pressure chemical ionization (APCI) source (model AmaZon speed ETD, Bruker Daltonics, Bremem, Germany). The carotenoid separation was performed on a C30 YMC column (5 μm, 250 × 4.6 mm) (Waters, Wilmington-DE, USA). HPLC-PDA-MS/MS parameters were set as previously described by De Rosso and Mercadante (2007). Prior to HPLC–PDA–MS/MS analysis, the carotenoid extract was solubilized in methanol (MeOH): methyl-terbutyl-ether (MTBE) (70:30) and filtered through Millipore membranes (0.22 μm). The mobile phase consisted in a mixture of MeOH and MTBE. A linear gradient was applied from 95:5 to 70:30 in 30 min, to 50:50 in 20 min. The flow rate was 0.9 mL/min. The identification was performed according to the following combined information: elution order on C30 HPLC column, co-chromatography with authentic standards, UV–visible spectrum (λ max, spectral fine structure, peak *cis* intensity), and mass spectra characteristics (protonated molecule ([M + H]$^+$) and MS/MS fragments), compared with data available in the literature (Britton, 1995; De Rosso & Mercadante, 2007; Rodrigues et al., 2015; Van Breemen, Dong, & Pajkovic, 2012; Zepka & Mercadante, 2009).

The carotenoids were quantified by HPLC-PDA, using external calibration curves for all-*trans*-violaxanthin, all-*trans*-zeaxanthin, all-*trans*-lutein, all-*trans*-β-carotene and all-*trans*-α-carotene of five concentration levels. All-*trans*-luteoxanthin and 9-*cis*-neoxanthin were quantified using the curve of all-*trans*-violaxanthin; the isomers zeaxanthin, 13-*cis*-antheraxanthin, all-*trans*-antheraxanthin and all-*trans*-diatoxanthin were quantified using the curve of all-*trans*-zeaxanthin; the isomers lutein and all-*trans*-crocoxanthin were quantified using the curve of all-*trans*-lutein; and the isomers and epoxides of the β-carotene, all-*trans*-canthaxanthin, all-*trans*-myxoxanthophyll, all-*trans*-β-cryptoxanthin, all-*trans*-echinenone, 9-*cis*-echinenone were quantified using the curve of all-trans-β-carotene. Total carotenoid content was calculated as the sum of the contents of each individual carotenoid separated on the C30 column.

2.5. Peroxyl radical scavenging assay for lipophilic extracts

The antioxidant capacity assay of the lipophilic extracts was carried out according to Rodrigues, Mariutti, Chisté, and Mercadante (2012). The dry carotenoid extracts were suspended in dichloromethane and pooled together to compose the stock solution. Aliquots of the stock solution were taken to prepare the working solutions in five different concentrations (42, 57, 109, 204 and 321 μL). After evaporation under N_2 flow, they were dissolved in DMSO/MTBE (10:1, *v/v*) and homogenized. The assays were carried out in a microplate reader (Synergy Mx Biotek, Winooski-VT, USA). The ROO· scavenging capacity was measured by monitoring the effect of the carotenoid extract or α-tocopherol standard on the fluorescence decay resulting from the ROO· induced oxidation of the C_{11}-BODIPY$^{581/591}$ probe. ROO· was generated by thermal decomposition of AIBN at 41 ± 0.5 °C. The ROO· scavenging capacity was calculated as the ratio of the slop of the curve representing the sample concentration against the net area under the curve, and the slope of the curve representing α-tocopherol concentration against the net area under the curve.

2.6. Statistical analysis

Descriptive statistics, analysis of variance (one-way ANOVA) and Tukey's test ($p < 0.05$) were applied to experimental data. The analyses were performed with the software Statistica 7.0 (StatSoft, Tulsa-OK, USA).

3. Results and discussion

A total of 23 different carotenoids were separated in the extracts of *Scenedesmus obliquus*, *Chlorella vulgaris*, and *Aphanothece microscopica Nägeli* (Fig. 1). The separated carotenoids were identified or tentatively identified based on the combined information obtained from chromatographic elution, co-chromatography with standards, UV/visible, and mass spectra characteristics (Table 1). Since a detailed description of carotenoid identification using the above information was already reported by Rodrigues et al. (2015), Rodrigues et al. (2014) and De Rosso and Mercadante (2007) only considerations regarding the carotenoids not identified in these previous reports were discussed below.

Diatoxanthin (peak 7) is an acetylated carotenoid mainly found in microalgae with low spectral fine structure (III/II = 9%). The molecular mass of diatoxanthin was confirmed by the protonated molecule ([M + H]$^+$) at *m/z* 567 and by consecutive losses of two hydroxyl groups, at *m/z* 549 [M + H − 18]$^+$ and 531 [M + H − 18 − 18]$^+$, verified in the MS/MS spectrum. In addition, a fragment at low abundance was observed at *m/z* 475, resulting from the loss of toluene [M

ARTICLE IN PRESS

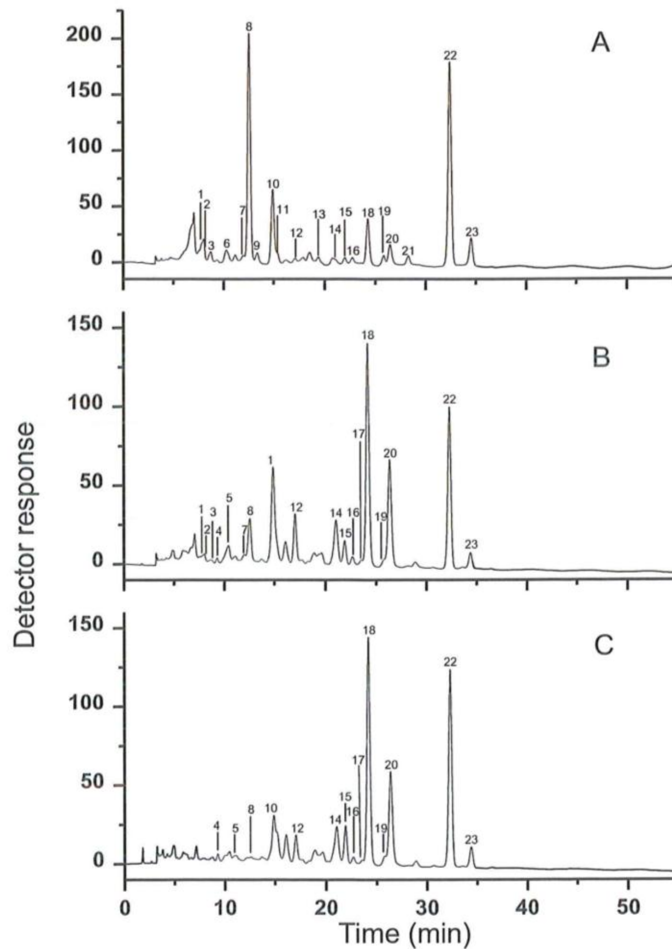


Fig. 1. Chromatogram, obtained by HPLC–DAD, of the carotenoid extract from *Scenedesmus obliquus* (A), *Chlorella vulgaris* (B), and *Aphanothece microscopica Nägeli* (C). See text for chromatographic conditions. Peak identification and characterization are given in Table 1. Chromatogram was processed at 451 nm.

+ H − 92]$^+$ (Crupi et al., 2013; Van Breemen et al., 2012). Another characteristic feature of the APCI-MS/MS was the fragment detected at *m/z* 393, resulting from the cleavage of the double bond allylic to the acetylenic carbon.

Peak 17 was identified as crocoxanthin, considering the lack of spectral fine structure and λ_{max} at 419, 444, 466 nm, [M + H]$^+$ at *m/z* 551. The MS/MS spectrum showed fragments at *m/z* 533 and 459, due to the loss of water and toluene, respectively. As expected, the fragment at *m/z* 393 was detected, as crocoxanthin is also an acetylated carotenoid.

The total carotenoid contents from biomasses were 2650.70 µg/g, 1977.02 µg/g, and 1398.88 µg/g, as dry weight, for *Scenedesmus obliquus*, *Chlorella vulgaris* and *Aphanothece microscopica Nägeli*, respectively.

Twenty carotenoids were identified in *Scenedesmus* (Fig. 1A). All-*trans*-β-carotene was the major (29.3%), followed by all-*trans*-lutein (28.1%), all-*trans*-zeaxanthin (9.9%) and all-*trans*-echinenone (6.2%), as shown in Table 2. The predominant *cis* isomers were 9-*cis*-β-carotene (4.6%), 9-*cis*-β-echinenone (4.0%), 13-*cis*-β-carotene (1.7%), 15-*cis*-zeaxanthin (1.4%), 9-*cis*-zeaxanthin (1.1%) and 9-*cis*-lutein (1.0%) (Table 2). The green algae represents an case of accumulating large amounts of β-carotene, and *cis*-β-carotene (Ben-Amotz & Avron, 1990; Hu, Lin, Lu, Chou, & Yang, 2008; Dewapriya & Kim, 2014). Content of the high bioavailability stereoisomer of β-carotene, the 9-*cis* stereoisomer, 13-*cis* and 15-*cis* stereoisomers of β-carotene, is highest in green algae among all the natural carotenoids sources. The 9-*cis* to all-*trans* ratio is proportional to the integral light intensity during cell's division cycle (Ben-Amotz, Lers, & Avron, 1988). About geometric isomers of lutein it has been reported that 9-*cis*-lutein is produced by *Scenedesmus protuberans* (Erdoğan, Çağır, Dalay, & Eroğlu, 2015).

In addition, 9-*cis*-neoxanthin (2.2%) was only identified in carotenoid extract from *Scenedesmus* biomass. In fact, allenic carotenoids are very limited in microalgae (Takaichi & Mirauro, 1998; Takaichi, 2011).

The composition of carotenoid in *Chlorella vulgaris* can be seen in Fig. 1B and Table 2. Among the 18 carotenoids identified in the *Chlorella* carotenoid extract, 15 compounds were common to *Scenedesmus* carotenoid profile. The results from Table 2 showed that the main differences between *Scenedesmus* and *Chlorella* extracts were the amounts of zeaxanthin derivates and keto carotenoids.

A total of 14 carotenoids were identified in the *Aphanothece microscopica Nägeli* extract (Fig. 1C and Table 2). It is interesting to note that

ARTICLE IN PRESS

Table 1
Chromatographic, UV–vis spectrum and mass characteristics, obtained by HPLC–PDA–MS/MS of microalgal carotenoids.

Peak[a]	Carotenoid	t_R (min)[b]	UV–Vis characteristics			Fragment ions (positive mode) (*m/z*)	
			λ_{max} (nm)[c]	III/II (%)[d]	A_B/II (%)[e]	$[M + H]^+$	MS/MS
1	all-*trans*-violaxanthin	7.6	414, 437, 466	56	0	601	583 $[M + H - 18]^+$, 565 $[M + H - 18 - 18]^+$, 509 $[M + H - 92]^+$, 221
2[g]	9-*cis*-neoxanthin	7.9–8	328, 412, 435, 464	75	22	601	583 $[M + H - 18]^+$, 565 $[M + H - 18 - 18]^+$, 547 $[M + H - 18 - 18 - 18]^+$, 509 $[M + H - 92]^+$
3[g]	all-*trans*-luteoxanthin	8.6–8.7	406, 421, 447	62	0	601	583 $[M + H - 18]^+$, 565 $[M + H - 18 - 18]^+$, 509 $[M + H - 92]^+$, 491 $[M + H - 92 - 18]^+$, 221
4[g]	13-*cis*-antheraxanthin	9.3	441/443	0	nd[f]	585	567 $[M + H - 18]^+$, 549 $[M + H - 18 - 18]^+$, 531 $[M + H - 18 - 18 - 18]^+$, 220
5[g]	15-*cis*-lutein	10.2	330, 416, 440, 465	14	26	569	551 $[M + H - 18]^+$, 533 $[M + H - 18 - 18]^+$
6[g]	all-*trans*-antheraxanthin	10.4	419, 445, 471	50	0	585	567 $[M + H - 18]^+$, 549 $[M + H - 18 - 18]^+$, 531 $[M + H - 18 - 18 - 18]^+$, 493 $[M + H - 92]^+$, 221
7[g]	all-*trans*-diatoxanthin	12.0	425, 449, 472	9	nd[f]	567	549 $[M + H - 18]^+$, 535, 531 $[M + H - 18 - 18]^+$, 475 $[M + H - 92]^+$, 393
8	all-*trans*-lutein	12.5–12.6	420, 444, 472	59	0	569	551 $[M + H - 18]^+$ (in − source), 533 $[M + H - 18 - 18]^+$, 495 $[M + H - 18 - 56]^+$
9[g]	15-*cis*-zeaxanthin	13.3	420, 449, 474	16	nd[f]	569	551 $[M + H - 18]^+$, 533 $[M + H - 18 - 18]^+$, 477 $[M + H - 92]^+$
10	all-*trans*-zeaxanthin	14.8–14.9	425, 450, 476	30	0	569	551 $[M + H - 18]^+$, 533 $[M + H - 18 - 18]^+$, 477 $[M + H - 92]^+$
11[g]	9-*cis*-lutein	15.2	331, 415, 441, 467	50	11	nd[f]	551 $[M + H - 18]^+$ (in source), 533 $[M + H - 18 - 18]^+$, 495 $[M + H - 18 - 56]^+$
12[g]	all-*trans*-canthaxanthin	17.0	470/472	0	0	565	547 $[M + H - 18]^+$, 509 $[M + H - 56]^+$, 459 $[M + H - 106]^+$, 363, 203
13[g]	9-*cis*-zeaxanthin	19.3	419, 446, 470	33	nd[f]	569	551 $[M + H - 18]^+$, 533 $[M + H - 18 - 18]^+$, 477 $[M + H - 92]^+$
14[g]	all-*trans*-myxoxanthophyll	20.9–21.1	449, 473, 505	48	0	nd[f]	nd[f]
15[g]	β-carotene-5,6-epoxide	21.9	419, 445, 473	64	0	553	535 $[M + H - 18]^+$, 461 $[M + H - 92]^+$, 205
16[g]	all-*trans*-β-cryptoxanthin	22.6–22.7	425, 450, 476	18	0	553	535 $[M + H - 18]^+$, 461 $[M + H - 92]^+$
17[g]	all-*trans*-crocoxanthin	23.6	419, 444, 466	66	0	551	533 $[M + H - 18]^+$, 459 $[M + H - 92]^+$, 393
18[g]	all-*trans*-echinenone	24.1–24.2	459/462	0	0	551	533 $[M + H - 18]^+$, 427, 203
19[g]	13-β-carotene	25.8–25.9	338, 420, 445, 470	14	48	537	444 $[M + H - 92]^+$, 347
20	9-*cis*-echinenone	26.3–26.4	457/454	0	nd[f]	551	533 $[M + H - 18]^+$, 471 $[M + H - 80]^+$, 427
21	all-*trans*-α-carotene	28.1	419, 445, 473	62	0	537	413, 321
22	all-*trans*-β-carotene	32.2–32.3	425, 451, 478	33	0	537	444 $[M + H - 92]^+$, 399, 355
23[g]	9-*cis*-β-carotene	34.3–34.4	421, 446, 472	30	nd[f]	537	444 $[M + H - 92]^+$

[a] Numbered according to the chromatograms shown in Figs. 1, 2 and 3.
[b] t_R: Retention time on the C_{30} column.
[c] Linear gradient Methanol:MTBE.
[d] Spectral fine structure: Ratio of the height of the longest wavelength absorption peak (III) and that of the middle absorption peak (II).
[e] Ratio of the *cis* peak (A_B) and the middle absorption peak (II).
[f] Not detected.
[g] Tentatively identified.

Aphanothece and *Chlorella* are species from different classes (Cyanophyceae and Chlorophyceae, respectively), and show 14 carotenoids in common. The major carotenoids in *Aphanothece* were all-*trans*-echinenone (28.3%), all-*trans*-β-carotene (26.2%), 9-*cis*-echinenone (14.3%), and all-*trans*-zeaxanthin (7.3%) (Table 2).

Taking into account the structures identified in this work, a biosynthetic pathway was proposed for biotechnological production of carotenoids from *Scenedesmus obliquus*, *Chlorella vulgaris* and *Aphanothece microscopica Nägeli* (Fig. 2). All carotenoids indentified in the extract from biomasses cultivated in this study are derived from all-*trans*-β-carotene or all-*trans*-α-carotene, exceptionally, myxoxanthophyll (Fig. 2). In the present study, echinenone (peak 18) and zeaxanthin (peak 10) were between the major carotenoids in the biomasses. According to the proposed scheme (Fig. 2), two different sub paths were observed from β-carotene in the three biomasses, the keto and xanthophyll pathway.

The keto pathway, lead to the production of echinenone (peak 18), canthaxanthin (peak 12), and 9-*cis*-echinenone (peak 20). The production of ketocarotenoids from β-carotene (peak 22) involves the introduction of keto moieties at the 4,4′ position of the β-rings. These conversions of β-carotene into echinenone (one keto group) and canthaxanthin (two keto groups) is carried out by the β-carotene ketolase (Armstrong, 1997; Grünewald, Hirschberg, & Hagen, 2001; Misawa, Kajiwara, Kondo, Yokoyama, & Satomi, 1995; Sun, Cunningham, & Gantt, 1998). With few exceptions, ketocarotenoids are not usually found in higher plants; however, microalgae can easily synthesize them (Albrecht, Takaichi, Steiger, Wang, & Sandmann, 2000; Fujisawa et al., 2008; Gharibzahedi, Razavi, & Mousavi, 2013).

In the xanthophyll pathway (Fig. 2), the introduction of a hydroxyl group at the 3 position of the β-carotene (peak 22) results in β-cryptoxanthin (peak 16) and of a second hydroxyl group (3′ position), in zeaxanthin (peak 10). The enzyme responsible for the hydroxylation of the β-ring is β-carotene hydroxylase (Armstrong, 1997; Grünewald et al., 2001).

The C-7,8 double bond of zeaxanthin can be oxidized to the triple bond to rise diatoxanthin (peak 7). Little is known about the enzymatic process for biosynthesis of peak 7, and not yet understood which enzyme catalyses the conversion of zeaxanthin into the acetylene diatoxanthin (Borowitzka, Beardall, & Raven, 2016).

All three biomasses have these class-specific acetylenic carotenoids (Table 2, Fig. 2), *Scenedesmus* and *Chlorella* contain diatoxanthin (peak 7) and crocoxanthin (peak 17) was detected in *Chlorella* and *Aphanothece*.

When formed, zeaxanthin is easily transformed to epoxy compounds (Fig. 2). The epoxy groups are introduced into 5,6 and 5′,6′ positions of zeaxanthin to produce violaxanthin (peak 1) through antheraxanthin (peak 6). The enzyme that catalyses this reaction in the biosynthesis of violaxanthin is the zeaxanthin epoxidase (Jin, Feth, & Melis, 2003; Niyogi, Björkman, & Grossman, 1997).

In the carotenoid extract from green microalgae, three epoxide-

Table 2
Quantitative characterization of carotenoids in microalgal extracts (μg/g dry weight).

Peak	Carotenoids	*Scenedesmus*	*Chlorella*	*Aphanothece*
1	all-*trans*-violaxanthin	31.67^{a} ± 1.2	13.42^{b} ± 0.2	nd
2	9-*cis*-neoxanthin	60.66^{a} ± 2.3	20.53^{b} ± 0.3	nd
3	all-*trans*-luteoxanthin	54.56^{a} ± 2.0	17.39^{b} ± 0.3	nd
4	13-*cis*-antheraxanthin	nd	18.57^{a} ± 0.3	13.85^{b} ± 0.8
5	15-*cis*-lutein	nd	72.19^{a} ± 1.3	22.99^{b} ± 1.3
6	all-*trans*-antheraxanthin	38.19 ± 1.4	nd	nd
7	all-*trans*-diatoxanthin	21.13^{a} ± 0.8	21.66^{a} ± 0.3	nd
8	all-*trans*-lutein	746.99^{a} ± 8.7	111.26^{b} ± 2.0	10.30^{c} ± 0.6
9	15-*cis*-zeaxanthin	38.45 ± 1.4	nd	nd
10	all-*trans*-zeaxanthin	262.58^{a} ± 5.1	271.01^{b} ± 4.9	103.14^{c} ± 6.1
11	9-*cis*-lutein	28.85 ± 1.1	nd	nd
12	all-*trans*-canthaxanthin	10.13^{a} ± 0.3	104.01^{b} ± 1.8	51.57^{c} ± 3.0
13	9-*cis*-zeaxanthin	30.68 ± 1.1	nd	nd
14	all-*trans*-myxoxanthophyll	17.48^{a} ± 0.6	129.89^{b} ± 2.3	92.40^{c} ± 5.5
15	5,6-epoxide-β-carotene	20.57^{a} ± 0.7	49.23^{b} ± 0.8	62.07^{c} ± 3.7
16	all-*trans*-β-cryptoxanthin	23.76^{a} ± 0.9	15.05^{b} ± 0.2	10.09^{c} ± 0.6
17	all-*trans*-crocoxanthin	nd	8.09^{a} ± 0.1	5.17^{b} ± 0.3
18	all-*trans*-echinenone	166.80^{a} ± 6.4	452.63^{b} ± 3.2	396.44^{c} ± 3.7
19	13-*cis*-β-carotene	46.96^{a} ± 1.8	17.17^{b} ± 0.3	17.20^{b} ± 1.0
20	9-*cis*-echinenone	106.51^{a} ± 2.0	263.20^{b} ± 1.7	200.10^{c} ± 1.9
21	all-*trans*-α-carotene	42.03 ± 1.6	nd	nd
22	all-*trans*-β-carotene	778.18^{a} ± 9.9	352.29^{b} ± 2.3	367.82^{c} ± 2.0
23	9-*cis*-β-carotene	124.42^{a} ± 4.7	40.43^{b} ± 0.7	45.74^{c} ± 2.7
	Total carotenoids	2650.70^{a}	1977.02^{b}	1398.88^{c}

Values are average and standard deviation of triplicates.
nd: not detected.
Different letters in the same line differ significantly by Tukey test (α = 0.05).

derivatives zeaxanthin (all-*trans*-violaxanthin, 9-*cis*-neoxanthin, all-*trans*-luteoxanthin) and one acetylenic-derivative zeaxanthin (diatoxanthin) were formed. However, these compounds were not identified in cyanobacteria *Aphanothece*. These facts indicated that the formation of epoxide compounds, epoxy-furanoid rearrangement, and acetylenic-derivative from zeaxanthin were the main difference between carotenoid profile from green microalgae and cyanobacteria in the present study.

Table 3
ROO· scavenging capacity of carotenoids from microalgal extracts.

Microalgae	Value α-tocopherol relative
Chlorella vulgaris	31.1^{a} ± 0.4
Scenedesmus obliquus	14.0^{b} ± 0.9
Aphanothece microscopica Nägeli	7.3^{c} ± 0.2

Values are average and standard deviation of triplicates.
Different letters in the same column differ significantly by Tukey test (α = 0.05).

Zeaxanthin was also the precursor of 15-*cis*-zeaxanthin (peak 9) and 9-*cis*-zeaxanthin (peak 13). The antheraxanthin was not detected in carotenoid extracts from *Chlorella* and *Aphanothece* but its 13-*cis*-isomer was identified (peak 4).

All-*trans*-neoxanthin was not detected in the different biomasses evaluated in this study; however, as expected the geometric isomer 9-*cis*-neoxanthin (peak 2), was identified in the class Chlorophyceae (*Scenedesmus* and *Chlorella*), and not found in the class Cyanophyta (*Aphanothece*). This occurs because carotenoid composition between prokaryotic and eukaryotic microalgae is significantly different, consequently, during symbiosis of cyanobacteria to eukaryotic cells, carotenoids might be considerably restructured (Ahmed et al., 2014; Takaichi, 2011; Takaichi & Mirauro, 1998).

All-*trans*-lutein (peak 8), 15-*cis*-lutein (peak 5), 9-*cis*-lutein (peak 11), and crocoxanthin (peak 17) are derivatives of α-carotene (peak 21). Lutein was the second principal compound in the carotenoid profile, but crocoxanthin and 15-*cis*-lutein were not detected in *Scenedesmus*. Probably, in *Scenedesmus*, the synthesis stops at the intermediate compound lutein. However, crocoxanthin was identified in *Chlorella* and *Aphanothece* extract, suggesting a route to produce acetylenic carotenoids (Fig. 2). Crocoxanthin is found mainly in microalgae

Fig. 2. Chemistry mechanism for biotechnology production of carotenoids from *Scenedesmus obliquus*, *Chlorella vulgaris*, and *Aphanothece microscopica Nägeli*. Description of the illustration: 1 Cyclization; 2 Ketolation; 3 *Cis*-isomerization; 4 Hydroxylation; 5 Acetylation; 6 Epoxidation; 7 Epoxide-furanoxide rearrangement; 8 Glycosylation; * Intermediate compound not identified (All-*trans*-neoxanthin) between the peak one and peak two.

ARTICLE IN PRESS

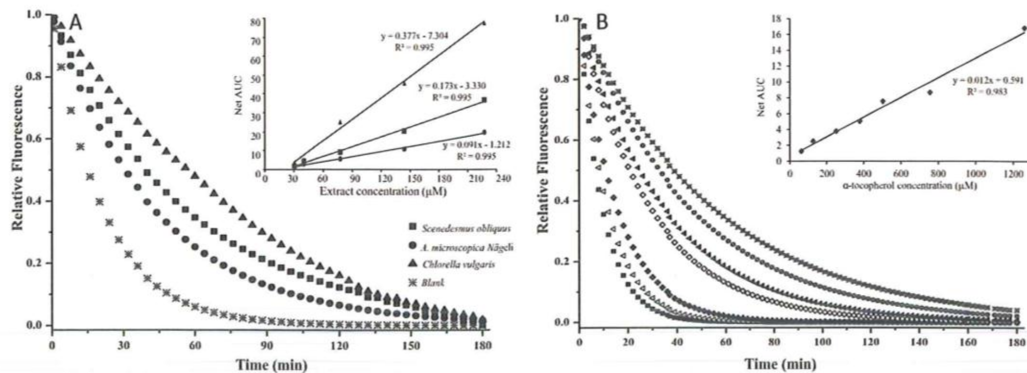


Fig. 3. (A) Fluorescence decay of C11-BODIPY581/591 induced by peroxyl radicals in the presence of concentrations at 70 μM of extract microalgae. Inset: linear relationship between the standards concentrations and net AUC values from the fluorescence decay curves of C11-BODIPY581/591 oxidation. (B) Fluorescence decay of C11-BODIPY581/591 induced by peroxyl radicals in the presence of different concentrations of α-tocopherol. Inset: linear relationship between α-tocopherol concentrations (62; 126; 252; 504; 756; 1260 and blank in 0 μM) and net AUC values from the fluorescence decay curves of C11-BODIPY581/591 oxidation.

(Bjerkeng, Storebakken, & Liaaen-Jensen, 1990).

Considering the substantial carotenoid content in *Scenedemus*, *Chlorella*, and *Aphanotece* biomasses and the carotenoid profile with structural characteristics very different from those commonly found in fruits and vegetables, such as a greater number of conjugated double bonds, of keto groups, and of acetylenic bond, the peroxyl radical scavenger capacity of the carotenoids extracts was exploited.

The antioxidant capacity of the pigments extracts are shown in the Table 3 and Fig. 3. The value of the ROO˙ scavenging capacity found for the carotenoid extract from *Chlorella* (31.1) was twice higher to that found for *Scenedesmus* (14.0) extract and four times higher than that of *Aphanothece* (7.3) extract. The *Scenedesmus obliquus* carotenoid extract showed high contents of carotenoids; however the major contents of ketocarotenoids was present in *Chlorella* extract (Table 2). Indeed, a positive effect on the antioxidant capacity was related due to the addition of keto groups at the terminal rings when these groups integrate the chromophore (Rodrigues et al., 2012). Carbonyl groups have free electron pairs, which might contribute to the electron delocalization across the polyene chain (Di Mascio, Kaiser, & Sies, 1989).

4. Conclusion

The *C. vulgaris*, *S. obliquus* and *A. microscopica Nägeli* presented the ability to produce a meaningful content of carotenoids under photoautotrophic conditions, indicating the potential as a renewable source of these pigments. Additionally, these extracts proved to be potent scavenger of peroxyl radical. The higher antioxidant activity in the microalgae *C. vulgaris* was favored by its carotenoid profile, which contains a great number of conjugated double bonds. Moreover, as far as we are concerned, this is the first report on the carotenoid profile and antioxidant capacity of *S. obliquus* and *A. microscopica Nägeli.*

Acknowledgements

The authors are grateful to the National Academic Cooperation Program PROCAD/CAPES and National Counsel of Technological and Scientific Development (552440/2011-6 and 405640/2013-7) (CNPq) for the financial support.

References

Ahmed, F., Fanning, K., Netzel, M., Turner, W., Li, Y., & Schenk, P. M. (2014). Profiling of carotenoids and antioxidant capacity of microalgae from subtropical coastal and brackish waters. *Food Chemistry, 165*, 300–306.

Albrecht, M., Takaichi, S., Steiger, S., Wang, Z. Y., & Sandmann, G. (2000). Novel hydroxycarotenoids with improved antioxidative properties produced by gene combination in *Escherichia coli*. *Nature Biotechnology, 18*(8), 843–846.

Armstrong, G. A. (1997). Genetics of eubacterial carotenoid biosynthesis: A colorful tale. *Brazilian Journal of Microbiology, 51*, 629–659.

Ben-Amotz, A., & Avron, M. (1990). The biotechnology of cultivating the halotolerant alga *Dunaliella*. *Trends in Biotechnology, 8*, 121–126.

Ben-Amotz, A., Lers, A., & Avron, M. (1988). Stereoisomers of β-carotene and phytoene in the alga *Dunaliella bardawil*. *Plant Physiology, 86*(4), 1286–1291.

Bjerkeng, B., Storebakken, T., & Liaaen-Jensen, S. (1990). Response to carotenoids by rainbow trout in the sea: Resorption and metabolism of dietary astaxanthin and canthaxanthin. *Aquaculture, 91*(1), 153–162.

Borowitzka, M. A., Beardall, J., & Raven, J. A. (2016). *The physiology of microalgae. Vol. 6*. Springer.

Britton, G. (1995). UV/visible spectroscopy. In G. Britton, S. Liaaen-Jensen, & H. Pfander (Eds.), *Carotenoids: Spectroscopy* (pp. 13–62). Basel: Birkhäuser Verlag.

Britton, G., Liaaen-Jensen, S., & Pfander, H. (2004). *Carotenoids: handbook*. Basel: Birkhäuser Verlag.

Crupi, P., Toci, A. T., Mangini, S., Wrubl, F., Rodolfi, L., Tredici, M. R., & Antonacci, D. (2013). Determination of fucoxanthin isomers in microalgae (*Isochrysis* sp.) by high performance liquid chromatography coupled with diode array detector multistage mass spectrometry coupled with positive electrospray ionization. *Rapid Communications in Mass Spectrometry, 27*(9), 1027–1035.

De Rosso, V. V., & Mercadante, A. Z. (2007). Identification and quantification of carotenoids, by HPLC-PDA-MS/MS, from Amazonian fruits. *Journal of Agricultural and Food Chemistry, 55*, 5062–5072.

Dewapriya, P., & Kim, S. K. (2014). Marine microorganisms: An emerging avenue in modern nutraceuticals and functional foods. *Food Research International, 56*, 115–125.

Di Mascio, P., Kaiser, S., & Sies, H. (1989). Lycopene as the most efficient biological carotenoid singlet oxygen quencher. *Archives of Biochemistry and Biophysics, 274*(2), 532–538.

Erdoğan, A., Çağır, A., Dalay, M. C., & Eroğlu, A. E. (2015). Composition of carotenoids in *Scenedesmus protuberans*: Application of chromatographic and spectroscopic methods. *Food Analytical Methods, 8*(8), 1970–1978.

Francisco, E. C., Neves, D. B., Jacob-Lopes, E., & Franco, T. T. (2010). Microalgae as feedstock for biodiesel production: Carbon dioxide sequestration, lipid production and biofuel quality. *Journal of Chemical Technology and Biotechnology, 85*(3), 395–403.

Fujisawa, M., Watanabe, M., Choi, S. K., Teramoto, M., Ohyama, K., & Misawa, N. (2008). Enrichment of carotenoids in flaxseed (*Linum usitatissimum*) by metabolic engineering with introduction of bacterial phytoene synthase gene crtB. *Journal of Bioscience and Bioengineering, 105*(6), 636–641.

Gharibzahedi, S. M. T., Razavi, S. H., & Mousavi, S. M. (2013). Ultrasound-assisted formation of the canthaxanthin emulsions stabilized by arabic and xanthan gums. *Carbohydrate Polymers, 96*(1), 21–30.

Grama, B. S., Chader, S., Khelifi, D., Stenuit, B., Jeffryes, C., & Agathos, S. N. (2014). Characterization of fatty acid and carotenoid production in an *Acutodesmus* microalga isolated from the Algerian Sahara. *Biomass and Bioenergy, 69*, 265–275.

Grünewald, K., Hirschberg, J., & Hagen, C. (2001). Ketocarotenoid biosynthesis outside of plastids in the unicellular green alga *Haematococcus pluvialis*. *Journal of Biological Chemistry, 276*, 6023–6029.

Guo, B., Liu, B., Yang, B., Sun, P., Lu, X., Liu, J., & Chen, F. (2016). Screening of diatom strains and characterization of *Cyclotella cryptica* as a potential fucoxanthin producer. *Marine Drugs, 14*(7), 125.

Haugan, J. A., & Liaaen-Jensen, S. (1994). Algal carotenoids 54. Carotenoids of brown algae (*Phaeophyceae*). *Biochemical Systematics and Ecology, 22*(1), 31–41.

Hertzberg, S., Liaaen-Jensen, S. T., & Siegelman, H. W. (1971). The carotenoids of blue-

green algae. *Phytochemistry, 10*(12), 3121–3127.

Hu, C. C., Lin, J. T., Lu, F. J., Chou, F. P., & Yang, D. J. (2008). Determination of carotenoids in *Dunaliella salina* cultivated in Taiwan and antioxidant capacity of the algal carotenoid extract. *Food Chemistry, 109*(2), 439–446.

Jin, E. S., Feth, B., & Melis, A. (2003). A mutant of the green alga *Dunaliella salina* constitutively accumulates zeaxanthin under all growth conditions. *Biotechnology and Bioengineering, 81*, 115–124.

Mandelli, F., Miranda, V. S., Rodrigues, E., & Mercadante, A. Z. (2012). Identification of carotenoids with high antioxidant capacity produced by extremophile microorganisms. *World Journal of Microbiology and Biotechnology, 28*, 1781–1790.

Maroneze, M. M., Siqueira, S. F., Vendruscolo, R. G., Wagner, R., De Menezes, C. R., Zepka, L. Q., & Jacob-Lopes, E. (2016). The role of photoperiods on photobioreactors: A potential strategy to reduce costs. *Bioresource Technology, 219*, 493–499.

Misawa, N., Kajiwara, S., Kondo, K., Yokoyama, A., & Satomi, Y. (1995). Canthaxanthin biosynthesis by the conversion of methylene to keto groups in a hydrocarbon b-carotene by a single gene. *Biochemical and Biophysical Research Communications, 209*, 867–876.

Niyogi, K. K., Björkman, O., & Grossman, A. R. (1997). *Chlamydomonas* xanthophyll cycle mutants identified by video imaging of chlorophyll fluorescence quenching. *Plant Cell, 9*, 1369–1380.

Papp, T., Csernetics, Á., Nagy, G., Bencsik, O., Iturriaga, E. A., Eslava, A. P., & Vágvölgyi, C. (2013). Canthaxanthin production with modified *Mucor circinelloides* strains. *Applied Microbiology and Biotechnology, 97*(11), 4937–4950.

Poojary, M. M., Barba, F. J., Aliakbarian, B., Donsì, F., Pataro, G., Dias, D. A., & Juliano, P. (2016). Innovative alternative technologies to extract carotenoids from microalgae and seaweeds. *Marine Drugs, 14*(11), 214.

Rippka, R., Deruelles, J., Waterbury, J. B., Herdman, M., & Stanier, R. Y. (1979). Generic assignments, strain histories and properties of pure cultures of cyanobacteria. *Journal of General Microbiology, 111*, 1–61.

Rodrigues, D. B., Flores, E. M. M., Barin, J. S., Mercadante, A. Z., Jacob-Lopes, E., & Zepka, L. Q. (2014). From waste to natural pigments: Productions of microalgal carotenoids in agroindustrial wastewater. *Food Research International, 65*, 144–148.

Rodrigues, D. B., Menezes, C. R., Mercadante, A., Jacob-Lopes, E., & Zepka, L. Q. (2015). Bioactive pigments from microalgae *Phormidium autumnale*. *Food Research International, 77*, 273–279.

Rodrigues, E., Mariutti, L. R., Chisté, R. C., & Mercadante, A. Z. (2012). Development of a novel micro-assay for evaluation of peroxyl radical scavenger capacity: Application to carotenoids and structure-activity relationship. *Food Chemistry, 135*, 2103–2111.

Solovchenko, A., Solovchenko, O., Khozin-Goldberg, I., Didi-Cohen, S., Pal, D., Cohen, Z., & Boussiba, S. (2013). Probing the effects of high-light stress on pigment and lipid metabolism in nitrogen-starving microalgae by measuring chlorophyll fluorescence transients: Studies with a Δ5 desaturase mutant of Parietochloris incisa (*Chlorophyta, Trebouxiophyceae*). *Algal Research, 2*(3), 175–182.

Suganya, T., Varman, M., Masjuki, H. H., & Renganathan, S. (2016). Macroalgae and microalgae as a potential source for commercial applications along with biofuels production: A biorefinery approach. *Renewable and Sustainable Energy Reviews, 55*, 909–941.

Sun, Z., Cunningham, F. X., Jr., & Gantt, E. (1998). *Differential expression of two isopentenyl pyrophosphate isomerases and enhanced carotenoid accumulation in a unicellular chlorophyte95. Differential expression of two isopentenyl pyrophosphate isomerases and enhanced carotenoid accumulation in a unicellular chlorophyte* (pp. 11482–11488).

Takaichi, S. (2011). Carotenoids in algae: Distributions, biosyntheses and functions. *Marine Drugs, 9*(6), 1101–1118.

Takaichi, S., & Mirauro, M. (1998). Distribution and geometric isomerism of neoxanthin in oxygenic phototrophs: 9′-cis, a sole molecular form. *Plant and Cell Physiology, 39*(9), 968–977.

Van Breemen, R. B., Dong, L., & Pajkovic, N. D. (2012). Atmospheric pressure chemical ionization tandem mass spectrometry of carotenoids. *International Journal of Mass Spectrometry, 312*, 163–172.

Yabuzaki, J.. Carotenoids database. (2017). http://carotenoiddb.jp/ (Accessed in May 26, 2017).

Zepka, L. Q., & Mercadante, A. Z. (2009). Degradation compounds of carotenoids formed during heating of a simulated cashew apple juice. *Food Chemistry, 117*, 28–34.

6. MANUSCRITO: CAROTENOID PROFILE OF MICROALGAE *Chlorella vulgaris* AT DIFFERENT HARVESTING TIMES

Em fase de revisão

CAROTENOID PROFILE OF MICROALGAE *Chlorella vulgaris* AT DIFFERENT HARVESTING TIMES

Luciana Dapieve Patias[1], Eduardo Jacob-Lopes[1]
[1]*Department of Food Science and Technology, Federal University of Santa Maria (UFSM), 97105-900, Santa Maria, RS, Brazil*

ABSTRACT

Microalgae are a group of microorganisms largely studied due to their biotechnological importance as valuable sources, has been successfully developed in the mass-production of carotenoids. The objective of this study was to characterize the carotenoids profile in extracts of microalgae *Chlorella vulgaris* (CPCC 90) at different harvesting times. Carotenoids from *Chlorella vulgaris* colleted in different times 0, 72 and 168 hs were identified. The total carotenoids content, based on dry weight of biomass, of *Chlorella vulgaris* were 1977.02 µg/g, 1426.70 µg/g and 1990.65 µg/g, *at* 0, 72 and 168h, respectively. The carotenoid profile in *Chlorella vulgaris* time 0h, were 18 identified carotenoids: the major one was the all-*trans*-echinenone (22,9 %), followed by the all-*trans*-β-carotene (17,8 %) and the all-*trans*-zeaxanthin (13,7 %). At 72 h, 12 carotenoids were identified: the major one was the all-*trans*-lutein (27,7 %), followed by the all-*trans*-β-carotene (19,9 %) and the all-*trans*-zeaxanthin (13,5 %). However, only four carotenoids were identified at 168 h: the major one was the all-*trans*-lutein (80,9 %), followed by the all-*trans*-β-carotene (12,9 %) and the all-*trans*-zeaxanthin (4,0 %). Among all carotenoids, the lutein was the one with greater concentration in different harvesting times. The present study was able to present that the harvesting time can be important to define which phase accumulates specific compounds of carotenoids extract, allowing one to predict it. It is possible to improve the performance and optimization of the operational conditions, since the microalgae *Chlorella* should be considered a potential feedstock for the diversity of natural carotenoid production, regarding the industrial interests.

Keywords: microalgae-based process; pigment; chlorophyceae; HPLC-PDA-MS/MS.

1. INTRODUCTION

Microalgae have received increasing attention because of their potential as a natural source of carotenoids and other beneficial bioproducts (Gong & Bassi, 2016). Especially the strains belong to Chlorophyceae family, as *Chlorella vulgaris* is one of the largely studied microalgae for their biotechnological importance as valuable sources, has been successfully developed in mass-producing carotenoids (Pignolet et al., 2013; Safi et al., 2014; Kyriakopoulou et al., 2015).

The microalgae *Chlorella vulgaris* can be considered as potential alternative in the carotenoids production because they grow rapidly, possess a high carotenoids content and are robust in bioreactors (Maroneze et al., 2016). However, the microalgae carotenoid production has not been economical enough yet to compete with traditional synthetic chemical methods and other technologies, such as extraction of plant-based sources (Gong & Bassi, 2016).

High commercial values, especially for their high demand as bioactive compounds, renewing interest in increasing the research and development of these compounds in microalgae biomass (Morowvat & Ghasemia, 2016). The global market for carotenoids was $ 1.5 billion, with β-carotene, lutein and astaxanthin occupying more than 60 percent market share, and the global market hit $ 1.8 billion in 2019 (Business Communications Company, 2015).

Stress conditions are often applied to high carotenoids in microalgae, but different carotenoids have varied responses to stress conditions. This represents a growth strategy most used to increase the production of carotenoids. Investigation of the parameters involved in this process can help to achieve high productivity of carotenoids. Limited biomass production under stress can be counteracted by a multi-stage growth strategy application (Hodgson et al., 2016).

To significantly improve carotenoid production, future research should be focused on cultivation optimization strategies. The study of growth physiology and the effect of time on the synthesis of carotenoids of these microorganisms is of particular interest (Faraloni & Torzillo, 2017). Thus, the objective of this study was to characterize the carotenoids profile in extracts of microalgae *Chlorella vulgaris* (CPCC 90) at different harvesting times.

2. MATERIALS AND METHODS

2.1. Microorganisms and culture media

Axenic culture of *Chlorella vulgaris (*CPCC90) was used in the experiments. Stock culture was propagated and maintained in synthetic BG11 medium (Braun-Grunow medium) (Rippka et al., 1979). Maintenance conditions used were 25°C and constant light intensity 15μmol/m^2/s.

2.2. Microalgal biomass production

The cultivations were performed in a bubble column photobioreactor (Maroneze et al., 2016) operating under a batch regime, fed on 2.0 L of BG11 medium. The experimental conditions were as follows: initial cell concentration of 100 mg/L, isothermal reactor operating at a temperature of 25°C, photon flux density of 150 μmol/m^2/s and continuous aeration of 1VVM (volume of air per volume of culture per minute) with the injection of air enriched with 15% of carbon dioxide. The cultures were carried out under phototrophic conditions, one 72 hours and the other of 168 hours. The cultivations were performed twice, and in duplicate.

2.3. Obtained microalgae biomasses

The biomass of the bioreactor was obtained by centrifugation, the supernatant was discarded and the remaining biomass frozen in a freezer at -18°C for 24 hours. After were lyophilized for 24 hours under vacuum conditions: from 0.200 to 0.300 and μHg temperature of -37°C condenser, and then stored under refrigeration until the time of analysis.

2.4. Carotenoid extraction

The carotenoids were exhaustively extracted from the freeze-dried sample (0.2 ± 0.02 g) with ethyl acetate and methanol in a mortar with a pestle followed by centrifugation (Hitachi, Tokyo, Japan) for 7 min at 1500 x g (Mandelli et al; 2012). The extraction procedure was repeated until the supernatant becomes colorless, which was reached approximately after 9 extractions with ethyl acetate and 5 with methanol. 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 (16h) with 10% (w/v) methanolic KOH at room temperature. The alkali was removed by washing with distilled water, and each extract was once again concentrated in rotary evaporator, flushed with N_2 and kept at -37˚C in the dark until chromatographic analysis. All extractions were performed in triplicate.

2.5. HPLC-PDA-MS/MS carotenoids analysis

The carotenoids were analyzed by high performance liquid chromatography HPLC (Shimadzu, Kyoto, Japan) equipped with quaternary pumps (model LC-20AD), online degasser, and injection valve with a 20µL loop (Rheodyne, Rohnedert Park, CA, USA). The equipment was connected in series to a PDA detector (model SPD-M20A) and a mass spectrometer with an ion-trap analyzer and atmospheric pressure chemical ionization (APCI) source (model Amazon speed ETD, Bruker Daltonics, Bremem, Germany). The carotenoid separation was performed on a C_{30} YMC column (5µm, 250 x 4.6 mm) (Waters, Wilmington, DE, USA). HPLC-PDA-MS/MS parameters were set as previously described by De Rosso & Mercadante (2007). Prior to HPLC-PDA-MS/MS analysis, the carotenoid extract was solubilized in methanol (MeOH): methyl-terbutyl-ether (MTBE) (70:30) and filterd through Millipore membranes (0.22 µm). The mobile phase consisted in a mixture of MeOH and MTBE. A linear gradient was applied from 95:5 to 70:30 in 30 min, to 50:50 in 20 min. The flow rate was 0.9 mL/min^{-1}. The identification was performed according to the following combined information: elution order on C_{30} HPLC column, co-chromatography with authentic standards, UV-Visible spectrum (λmax, spectral fine structure, peak *cis* intensity), and mass spectra characteristics (protonated molecule ($[M + H]^+$) and MS/MS fragments), compared with data available in the literature (Britton, 1995; De Rosso & Mercadante, 2007; Zepka & Mercadante, 2009; Van Breemen et al., 2012; Rodrigues et al., 2015).

The carotenoids were also quantified by HPLC-PDA, using external calibration curves for all-*trans*-violaxanthin, all-*trans*-zeaxanthin, all-*trans*-lutein, all-*trans*-β-carotene and all-*trans*-α-carotene of five concentration levels. All-*trans*-luteoxanthin and 9-c*is*-neoxanthin were quantified using the curve of all-*trans*-violaxanthin; the isomers zeaxanthin, 13-c*is*-antheraxanthin, all-*trans*-antheraxanthin and all- *trans*-diatoxanthin were quantified using the curve of all-*trans*-zeaxanthin; the isomers lutein and all-*trans*- crocoxanthin were quantified

using the curve of all- *trans*-lutein; and the isomers and epoxides of the β-carotene, all-*trans*-canthaxanthin, all-*trans*-myxoxanthophyll, all-*trans*-β-cryptoxanthin, all-*trans*-echinenone, 9-cis-echinenone were quantified using the curve of all-*trans*-β-carotene. Total carotenoid content was calculated as the sum of the contents of each individual carotenoid separated on the C30 column.

2.6. Statistical analysis

Descriptive statistics, analysis of variance (one-way ANOVA) and tukey's test ($p < 0.05$) were applied to experimental data. The analyses were performed with the software Statistica 7.0 (STATSOFT, Tulsa, OK, USA).

3. RESULTS AND DISCUSSION

3.1. Identification and quantification of carotenoids

The chromatograms obtained from the HPLC (Figure 1A, B and C) show the carotenoids found in biomasses *Chlorella vulgaris* at different times. A total of 18 different carotenoids were separated in the extracts. The separated carotenoids were identified or tentatively identified based on the combined information obtained from chromatographic elution, co-chromatography with standards, UV/visible, and mass spectra characteristics (Table 1). Since a detailed description of carotenoid identification using the above information was already reported by De Rosso & Mercadante (2007), Rodrigues et al. (2014), Rodrigues et al. (2015) and Patias et al. (2017). Only considerations regarding the carotenoids amount will be discussed, since the identifications have already been made by the cited authors.

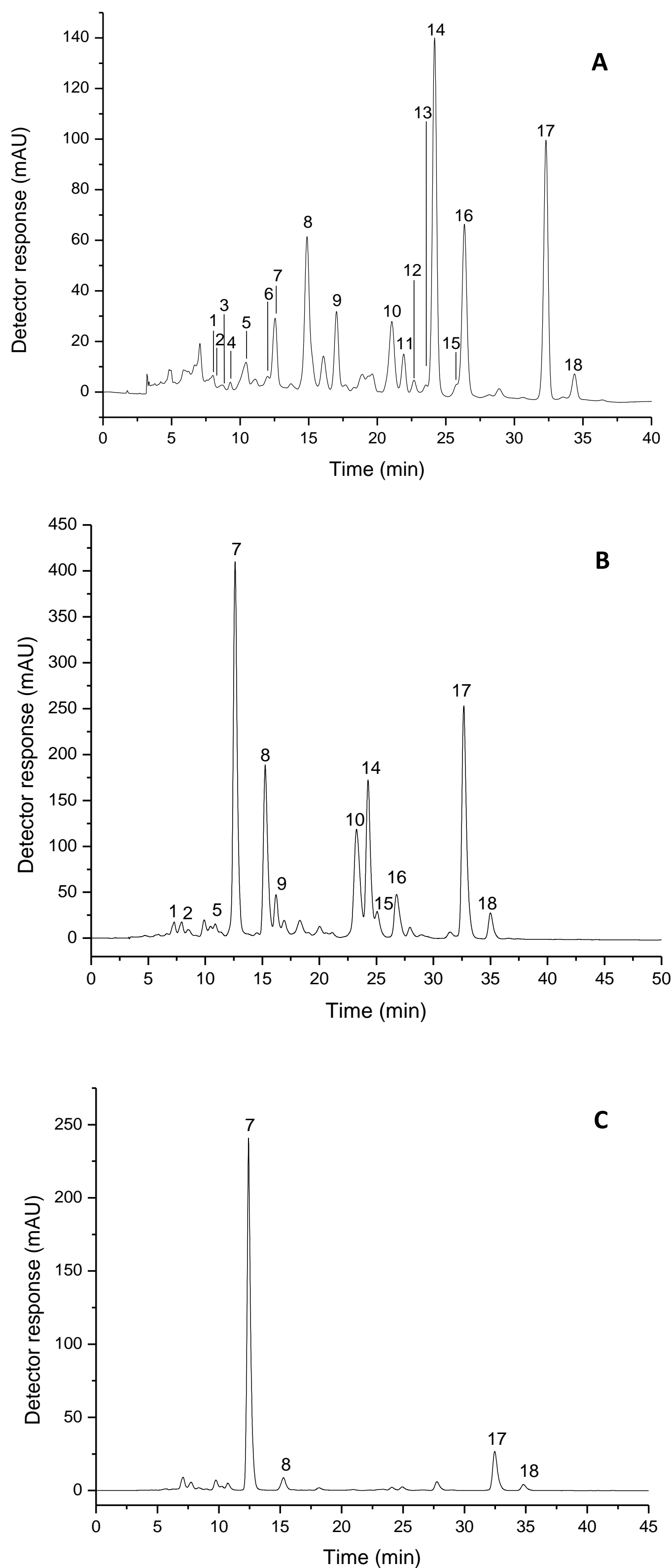


Fig. 1. Chromatogram, obtained by HPLC–DAD, of the carotenoid extract from *Chlorella vulgaris* – 0 h (A), 72 h (B), and 168 h (C). See text for chromatographic conditions. Peak identification and characterization are given in Table 1. Chromatogram was processed at 451 nm.

Table 1 Chromatographic, UV–vis spectrum and mass characteristics, obtained by HPLC–PDA–MS/MS of *Chlorella vulgaris* carotenoids.

Peak[a]	Carotenoid	t_R (min)[b]	UV-Vis characteristics			Fragment ions (positive mode) (*m/z*)	
			λ_{max} (nm)[c]	III/II (%)[d]	A_B/II (%)[e]	$[M+H]^+$	MS/MS
1	all-*trans*-violaxanthin	7.3	415, 438, 468	56	0	601	583 $[M+H-18]^+$, 565 $[M+H-18-18]^+$, 509 $[M+H–92]^+$, 221
2[g]	9-*cis*-neoxanthin	7.9	330, 412, 435, 463	75	22	601	583 $[M+H-18]^+$, 565 $[M+H-18-18]^+$, 547 $[M+H-18-18-18]^+$, 509 $[M+H–92]^+$
3[g]	all-*trans*-luteoxanthin	8.6-8.7	406, 421, 447	62	0	601	583 $[M+H-18]^+$, 565 $[M+H-18-18]^+$, 509 $[M+H-92]^+$, 491 $[M+H–92-18]^+$ 221
4[g]	13- *cis*-antheraxanthin	9.3	441/443	0	nd[f]	585	567$[M+H-18]^+$, 549 $[M+H-18-18]^+$, 531 $[M+H-18-18-18]^+$, 220
5[g]	15-*cis*-lutein	10.8	330, 437, 454, 464	14	26	569	551 $[M+H-18]^+$, 533 $[M+H-18-18]^+$
6[g]	all-*trans*-diatoxanthin	12	425,449,472	9	nd[f]	567	549 $[M+H-18]^+$, 535,531 $[M+H-18-18]^+$, 475 $[M+H-92]^+$,393
7	all-*trans*-lutein	12.4-12.6	420, 443, 471	59	0	569	551 $[M+H-18]^+$ (in-source), 533 $[M+H-18]^+$, 495 $[M+H-18-56]^+$
8	all-*trans*-zeaxanthin	15.2-15.3	425, 449, 474	30	0	569	551 $[M+H-18]^+$, 533 $[M+H-18-18]^+$, 477 $[M+H-92]^+$
9[g]	all-*trans*-canthaxanthin	16.0	469	0	0	565	547 $[M+H-18]^+$, 509 $[M+H-56]^+$, 459 $[M+H-106]^+$, 363, 203
10[g]	all-*trans*-myxoxanthophyll	21.2	449, 473, 504	48	0	nd[f]	nd[f]
11[g]	β-carotene-5,6-epoxide	21.9	419, 445, 473	64	0	553	535 $[M+H-18]^+$, 461$[M+H-92]^+$, 205
12[g]	all-*trans*- β- cryptoxanthin	22.6-22.7	425,450,476	18	0	553	535 $[M+H-18]^+$, 461$[M+H-92]^+$
13[g]	all-*trans*- crocoxanthin	23.6	419,444,466	66	0	551	533 $[M+H-18]^+$, 459$[M+H-92]^+$,393
14[g]	all-*trans*-echinenone	24.2	469	0	0	551	533 $[M+H-18]^+$, 427, 203
15[g]	13-β-carotene	25	338, 418, 444, 467	14	48	537	444 $[M+H-92]^+$, 347
16	9-*cis*-echinenone	26.7	466	0	nd[f]	551	533 $[M+H-18]^+$, 471 $[M+H-80]^+$, 427
17	all-*trans*-β-carotene	32.5-32.7	450, 467,476	33	0	537	444 $[M+H-92]^+$, 399, 355
18[g]	9-*cis*-β-carotene	34.8-34.9	420, 445, 471	30	nd[f]	537	444 $[M+H-92]^+$

[a]Numbered according to the chromatograms shown in Figure 1. [b]t_R: Retention time on the C_{30} column. [c]Linear gradient Methanol: MTBE. [d]Spectral fine structure: Ratio of the height of the longest wavelength absorption peak (III) and that of the middle absorption peak (II). [e]Ratio of the *cis* peak (A_B) and the middle absorption peak (II). [f]Not detected. [g]Tentatively identified.

The total carotenoids contents from biomasses were 1977.02 µg/g, 1426.70 µg/g and 1990.65 µg/g, as dry weight, for *Chlorella vulgaris* at 0, 72 and 168h, respectively.

The carotenoid profile in *Chlorella vulgaris* at 0h can be seen in (Figure 1A), totalizing eighteen identified carotenoids. All-*trans*-echinenone (22,9 %), was the major, followed by all-*trans*-β-carotene (17,8 %) and all-*trans*-zeaxanthin (13,7 %), as shown in Table 2. In the *Chlorella vulgaris* at 72 h, twelve carotenoids were identified (Figure 1B), all-*trans*-lutein (27,7 %) was the major, followed by all-*trans*-β-carotene (19,9 %) and all-*trans*-zeaxanthin (13,5 %), as shown in Table 2. However, only four carotenoids were identified in the *Chlorella vulgaris* at 168 h (Figure 1C and Table 2), all-*trans*-lutein (80,9 %) was the major, followed by all-*trans*-β-carotene (12,9 %) and all-*trans*-zeaxanthin (4,0 %).

Among the eighteen carotenoids identified in *Chlorella vulgaris* extract at 0h, twelve compounds were common in time 72h; and only four compounds were found in *Chlorella vulgaris* at 168h. It is interesting to highlight that all the identified carotenoids in the biomass extract grown in this study at 0h have had a higher number of compounds. However, at time 72h, the number of compounds was reduced from 18 to 12 compounds, and some compounds increased their quantity. In the 168h only 4 compounds were identified and it was possible to verify that all-*trans*-lutein has had its highest concentration comparing to the three harvesting times.

According to Begum et al. (2015), the pigment content depends on the microalgae species and culture conditions. Some factors must be taken into account regarding the microalgal pigments production that can be used for different applications. Temperature, salinity, irradiation, wavelength, photoperiods, pH, nutrient limitation, nitrogen supplements, pesticides and heavy metals affect the production of microalgal pigments.

Microalgae can synthesize some unique types of carotenoids, and the *Chlorella vulgaris* in harvesting different times, has showed the presence of characteristic ketocarotenoids, acetylenic carotenoids and glycosylated carotenoids, with structural characteristics very different from those commonly found in fruits and vegetables (Rodrigues et al, 2015; Poojary et al., 2016). Besides few exceptions, ketocarotenoids are not usually found in higher plants; however, microalgae can easily synthesize them (Fujisawa et al., 2008; Gharibzahedi et al., 2013). This information should be important in order to improve a more sustainable way natural carotenoids production.

It is interesting to emphasize that throughout the cultures it was possible to verify that in the highest evaluated time there was a large accumulation of all-*trans*-lutein in the extract.

It indicates the microalgae potential as source of this pigment, verifying that the harvesting time has influence on the biosynthesis of carotenoids, affecting the content and production of specific carotenoids. Lutein is one of the main photosynthetic pigments in the xanthophyllic family, and contains a large conjugated carbon system attached with hydroxyl or carbonyl groups (Ho et al., 2014).

The lutein is a carotenoid that has received special attention from the health, pharmaceutical, cosmetic and food industries, because it is widely used for the ophthalmological conditions and cancer treatment, and it has also been applied as a natural dye in cellular pigmentation and in the food industry (Fernandez-Sevilla et al., 2010; Xu et al., 2013; Yaakob, 2014; Ho, 2015).

The most important factors that affect lutein content in microalgae are temperature, light, pH, the availability and source of nitrogen, salinity (or ionic strength) and the presence of oxiding substances (or redox potential); however, specific growth rate also plays a crucial role in the biosynthesis of lutein (Wei et al., 2008; Guedes et al., 2011; Campenni et al., 2013).

The high lutein accumulation observed at 168h can be explained by the effect of increased exposure time to light, since irradiation is one of the factors that affect the bioaccumulation of this pigment (Macías-Sanchez et al., 2008; Guedes et al., 2011).

Currently, one of the challenges that most severely hampers the industrialization of the microalgae process of producing lutein is to efficiently conduct a dynamic optimization process within a control scheme that can greatly improve the profitability process (Del Rio-Chanona et al., 2016). In this sense, the microalgae C*hlorella* demonstrated efficiency in the production of this carotenoid.

Table 2 Quantitative characterization of carotenoids in microalgal extracts of *Chlorella vulgaris* (µg/g dry weight).

Peak	Carotenoids	0 h	72 h	168 h
1	all-*trans*-violaxanthin	13.42^{b} ± 0.2	11.09^{h} ± 0.7	nd
2	9-*cis*-neoxanthin	20.53^{b} ± 0.3	12.34^{h} ± 0.7	nd
3	all-*trans*-luteoxanthin	17.39^{b} ± 0.3	nd	nd
4	13- *cis*-antheraxanthin	18.57^{a} ± 0.3	nd	nd
5	15-*cis*-lutein	72.19^{a} ± 1.3	11.94^{h} ± 0.7	nd
6	all-*trans*-diatoxanthin	21.66^{a} ± 0.3	nd	nd
7	all-*trans*-lutein	111.26^{b} ± 2.0	395.28^{a} ± 1.7	1611.57^{a} ± 2.5
8	all-*trans*-zeaxanthin	271.01^{b} ± 4.9	192.26^{c} ± 3.0	80.46^{c} ± 0.1
9	all-*trans*-canthaxanthin	104.01^{b} ± 1.8	39.00^{f} ± 2.4	nd
10	all-*trans*-myxoxanthophyll	129.89^{b} ± 2.3	171.90^{d} ± 3.1	nd
11	5,5-epoxide- β-carotene	49.23^{b} ± 0.8	nd	nd
12	all-*trans*- β-cryptoxanthin	15.05^{b} ± 0.2	nd	nd
13	all-*trans*-crocoxanthin	8.09^{a} ± 0.1	nd	nd
14	all-*trans*-echinenone	452.63^{b} ± 3.2	188.36^{c} ± 1.2	nd
15	13-*cis*-β-carotene	17.17^{b} ± 0.3	30.27^{g} ± 1.8	nd
16	9-*cis*-echinenone	263.20^{b} ± 1.7	57.84^{e} ± 3.5	nd
17	all-*trans*-β-carotene	352.29^{b} ± 2.3	284.78^{b} ± 1.8	257,67^{b} ± 0.4
18	9-*cis*-β-carotene	40.43^{b} ± 0.7	31.64^{fg} ± 1.9	40.95^{d} ± 0.1
	Total carotenoids	1977.02^{b}	1426.70^{c}	1990.65^{a}

Values are average and standard deviation of triplicates. nd: not detected. Different letters in the same line differ significantly by the Tukey test (p > 0.05).

4. CONCLUSION

The present study was able to demonstrate that the harvesting time can be important to define which phase accumulates specific compounds of carotenoids extract, allowing one to predict it. It is possible to improve the performance and optimization of the operational conditions, since the microalgae *Chlorella* should be considered as a potential feedstock for the production of several diversity of natural carotenoids of industrial interest.

REFERENCES

BEGUM, H. et al. Availability and utilization of pigments from microalgae. **Critical reviews in food science and nutrition**, 56, 2209-2222, 2016.

BRITTON, G. UV/visible spectroscopy. In BRITTON, G.; LIAAEN-JENSEN, S.; PFANDER, H. (Eds). **Carotenoids: Spectroscopy** (p.13-62). Badel: Birkhäuser Verlag, 1995.

BUSINESS COMMUNICATIONS COMPANY, 2015.The Global Market for carotenoids.

CAMPENNI, Luca et al. Carotenoid and lipid production by the autotrophic microalga Chlorella protothecoides under nutritional, salinity, and luminosity stress conditions. **Applied microbiology and biotechnology**, 97, 1383-1393, 2013.

DE ROSSO, V.V., MERCADANTE, A.Z. Identification and quantification of carotenoids, by HPLC-PDA-MS/MS, from Amazonian fruits. **Journal of agricultural and food chemistry,** 55, 5062-5072, 2007.

DEL RIO-CHANONA, E. A. et al. An efficient model construction strategy to simulate microalgal lutein photo-production dynamic process. **Biotechnology and bioengineering**, in press, 2017.

FARALONI, C.; TORZILLO, G. Synthesis of Antioxidant Carotenoids in Microalgae in Response to Physiological Stress. In: **Carotenoids**. InTech, 2017.

FERNÁNDEZ-SEVILLA, J.M.; FERNÁNDEZ, F.G.A; GRIMA, E. Molina. Biotechnological production of lutein and its applications. **Applied microbiology and biotechnology**, 86, 27-40, 2010.

FUJISAWA, M. et al. Enrichment of carotenoids in flaxseed (*Linum usitatissimum*) by metabolic engineering with introduction of bacterial phytoene synthase gene crtB. **Journal of bioscience and bioengineering**, 105, 636-641, 2008.

GHARIBZAHEDI, S.M.T.; RAZAVI, S.H; MOUSAVI, S.M. Ultrasound-assisted formation of the canthaxanthin emulsions stabilized by arabic and xanthan gums. **Carbohydrate polymers**, 96, 21-30, 2013.

GONG, M.; BASSI, A. Carotenoids from microalgae: A review of recent developments. **Biotechnology advances**, 34, 1396-1412, 2016.

GUEDES, A.C; AMARO, H.M.; MALCATA, F.X. Microalgae as sources of carotenoids. **Marine drugs**, 9, 625-644, 2011.

HO, Shih-Hsin et al. Perspectives on engineering strategies for improving biofuel production from microalgae - a critical review. **Biotechnology advances**, 32, 1448-1459, 2014.

HO, SHIH-HSIN. et al. Effects of nitrogen source availability and bioreactor operating strategies on lutein production with Scenedesmus obliquus FSP-3. **Bioresource technology**, 184, 131-138, 2015.

HODGSON, P., BARROW, C. ADHOLEYA, A. A review on the assessment of stress conditions for simultaneous production of microalgal lipids and carotenoids. **Frontiers in microbiology**, 7, 1-19, 2016.

KYRIAKOPOULOU, K.; PAPADAKI, S.; KROKIDA, M. Life cycle analysis of β-carotene extraction techniques. **Journal of food engineering**, 167, 51-58, 2015.

MANDELLI, F. et al. Identification of carotenoids with high antioxidant capacity produced by extremophile microorganisms. **World journal of microbiology and biotechnology,** 28, 1781-1790, 2012.

MACÍAS-SÁNCHEZ, M.D. et al. Extraction of carotenoids and chlorophyll from microalgae with supercritical carbon dioxide and ethanol as cosolvent. **Journal of separation science**, 31, 8, 1352-1362, 2008.

MARONEZE, M.M. et al. The role of photoperiods on photobioreactors: A potential strategy to reduce costs. **Bioresource technology,** 219, 493-499, 2016.

MOROWVAT, M.; GHASEMI, Y. Culture medium optimization for enhanced β-carotene and biomass production by Dunaliella salina in mixotrophic culture. **Biocatalysis and Agricultural Biotechnology**, v. 7, p. 217-223, 2016.

PATIAS, L.D. et al. Carotenoid profile of three microalgae/cyanobacteria species with peroxyl radical scavenger capacity. **Food research international**, in press, 2017.

PIGNOLET, O. et al. Highly valuable microalgae: biochemical and topological aspects. **Journal of industrial microbiology and biotechnology,** 40, 781-796, 2013.

POOJARY, M.M. et al. Innovative alternative technologies to extract carotenoids from microalgae and seaweeds. **Marine drugs**, 14, p. 214, 2016.

RIPPKA, R. et al. Generic assignments, strain histories and properties of pure cultures of cyanobacteria. **Journal of general microbiology,** 111, 1-61, 1979.

RODRIGUES, D.B. et al. Production of carotenoids from microalgae cultivated using agroindustrial wastes. **Food research international**, 65, 144-148, 2014.

RODRIGUES, D.B. et al. Bioactive pigments from microalgae *Phormidium autumnale*. **Food research international,** 77, 273-279, 2015.

SAFI, C. et al. Extraction of lipids and pigments of Chlorella vulgaris by supercritical carbon dioxide: influence of bead milling on extraction performance. **Journal of applied phycology**, 26, 1711-1718, 2014.

VAN BREEMEN, R.B.; DONG, L.; PAJKOVIC, N.D. Atmospheric Pressure Chemical Ionization Tandem Mass Spectrometry of Carotenoids. **International journal of mass spectrometry,** 312, 163-172, 2012.

XU, Y.; PURTON, S.; BAGANZ, F. Chitosan flocculation to aid the harvesting of the microalga Chlorella sorokiniana. **Bioresource technology**, 129, 296-301, 2013.

WEI, Dong et al. Enhanced production of lutein in heterotrophic Chlorella protothecoides by oxidative stress. **Science in China Series C: Life Sciences**, 51, 1088-1093, 2008.

YAAKOB, Z. et al. An overview: biomolecules from microalgae for animal feed and aquaculture. **Journal of biological research**, 21, p. 6, 2014.

ZEPKA, L.Q.; MERCADANTE, A.Z. Degradation compounds of carotenoids formed during heating of a simulated cashew apple juice. **Food chemistry,** 117, 28-34, 2009.

7. CONCLUSÃO

O presente estudo demonstrou a importância das três espécies estudadas: *Chlorella vulgaris*, *Scenedesmus obliquus* e *Aphanotece microscopica Nägeli* como fontes alternativas de compostos bioativos. Estas espécies apresentaram capacidade de produzir um conteúdo significativo de carotenoides sob condições fotoautotróficas, indicando potencial como fonte renovável desses pigmentos.

Quanto à capacidade antioxidante, os extratos provaram ser um potente eliminador de radical peroxil. Sendo que, a maior atividade antioxidante foi demonstrada pela espécie *Chlorella vulgaris*, a qual foi favorecida pelo seu perfil de carotenoides, que contém estruturas com um grande número de duplas ligações conjugadas.

O estudo também mostrou que o tempo de cultivo pode ser importante para definir em qual fase podemos acumular certos carotenoides de interesse, e prever o desempenho e otimização de condições operacionais.

Os resultados encontrados neste trabalho são promissores do ponto de vista de que as microalgas devem ser consideradas como uma matéria-prima potencial para a produção de uma grande diversidade de carotenóides naturais.

REFERÊNCIAS

ANUPAMA, P.; RAVINDRA, L. Value-added food: Single cell protein. **Biotechnology advances**, v. 18, n. 6, p. 459-479, 2000.

AL HATTAB, M.; GHALY, A. Production of Biodiesel from Marine and Freshwater Microalgae: A Review. **Algae,** v. 14, p. 18, 2015.

BAKER, R.; GUNTER, C. The role of carotenoids in consumer choice and the likely benefits from their inclusion into products for human consumption. **Trends in Food Science & Technology,** n. 15, p. 484-488, 2004.

BECK, C. et al. The diversity of cyanobacterial metabolism: genome analysis of multiple phototrophic microorganisms. **BMC Genomics**, v. 13, n. 56, p. 1-17, 2012.

BECKER, E.W. **Microalgae - Biotechnology and Microbiology**. Cambridge: Cambridge University Press, 1994.

BEER, L.L. et al. Engineering algae for biohydrogen and biofuel production. **Current Opinion in Biotechnology**, v. 20, p. 264-271, 2009.

BOROWITZKA, M.A.; MOHEIMANI, N.R. Sustainable biofuels from algae. **Mitigation and Adaptation Strategies for Global Change**, v. 18, n. 1, p. 13-25, 2013.

BRITTON, G.L.; PFANDER, H. **Carotenoids: Handbook**. Badel, Birkhäuser Verlag. 2004.

BRITTON, G.; LIAAEN-JENSEN, S.; PFANDER, H. **Carotenoids: Natural Functions**. Vol. 4. 2008.

BRUTEMARK, A.; GRANÉLI, E. Role of mixotrophy and light for growth and survival of the toxic haptophyte *Prymnesium parvum*. **Harmful Algae**, v. 10, n. 4, p. 388-394, 2011.

BLUNT, J.; MUNRO, M.; UPJOHN, M. The role of databases in marine natural products research. In: **Handbook of marine natural products**. Springer Netherlands, p. 389-421. 2012.

CARDOSO, A.S; VIEIRA, G.E.G; MARQUES, A.K. Uso de microalgas para obtenção de biocombustíveis. **Revista Brasileira Biociências**, Porto Alegre, v. 9, p. 542 - 549, 2011.

CHAE, S.R.; HWANG, E.J.; SHIN, H.S. Single cell protein production of *Euglena gracilis* and carbon dioxide fixation in an innovative photo-bioreactor. **Bioresource technology**, v. 97, n. 2, p.322-329, 2006.

CHEN, C.Y. et al. Cultivation, photobioreactor design and harvesting of microalgae for biodiesel production: A critical review. **Bioresource technology**, v. 102, n. 1, p. 71-81, 2011.

CHOJNACKA, K.; MARQUEZ-ROCHA, F. Kinetic and stoichiometric relationships of the energy and carbon metabolism in the culture of microalgae. **Biotechnology**, v. 3, n. 1, p. 21-34, 2004.

CHUYEN, H.V.; EUN, J. Marine carotenoids: bioactivities and potential benefits to human health. **Critical reviews in food science and nutrition**, v. 57, n. 12, p. 2600-2610, 2017.

COSTA, M. Exploring bioactive properties of marine cyanobacteria isolated from the portuguese coast: High potential as a source of anticancer compounds. **Marine Drugs**, v. 12, n. 1, p. 98-114, 2013.

CHACÓN-LEE, T.L., GONZALEZ-MARIÑO, G.E. Microalgae for "healthy" foods-possibilities and challenges. **Comprehensive Reviews in Food Science**. v. 9, p. 655-675, 2010.

CHRISTENSON, L.; SIMS, R. Production and harvesting of microalgae for wastewater treatment, biofuels, and bioproducts. **Biotechnology advances**, v. 29, n. 6, p. 686-702, 2011.

CUTTRISS, A.J. et al. Carotenoids, in: RÉBEILLÉ, F.; DOUCE, R. (Eds.), Biosynthesis of Vitamins in Plants. Part A, Vitamins A, B1, B2, B3, B5. **Academic Press.,** London, pp. 1-36, 2011.

DEL CAMPO, J.A.; GARCÍA-GONZÁLEZ, M.; GUERRERO, M.G. Outdoor cultivation of microalgae for carotenoid production: current state and perspectives. **Applied Microbiology and Biotechnology**, v. 74, n. 6, p. 1163-1174, Apr. 2007.

DONATO, M.; VILELA, M.H; BANDARRA, N.M. Fatty acids, sterols, α- tocopherol and total carotenoids composition of *Diacronema vlkianum*. **Journal of Food Lipids**, v. 10, n. 4, p. 267-276, 2003.

DONG, Q.L.; ZHAO, X.M. In situ carbon dioxide fixation in the process of natural astaxanthin production by a mixed culture of *Haematococcus pluvialis* and *Phaffia rhodozyma*. **Catalysis Today**, v. 98, n. 4, p. 537-544, 2004.

DUFOSSÉ, L. et al. Microorganisms and microalgae as sources of pigments for food use: a scientific oddity or an industrial reality? **Trends in Food Science and Technology**, v. 16, n. 9, p. 389-406, Sept. 2005.

DUFOSSÉ, L. Microbial production of food grade pigments. **Food Technology and Biotechnology**, v. 44, n. 3, p. 313-321, 2006.

ERIKSEN, N.T. Production of phycocyanin - a pigment with applications in biology, biotechnology, foods and medicine. **Applied Microbiology and Biotechnology**, v. 80, n. 1, p. 1-14, Aug. 2008.

FRANCISCO, E.C. et al. Microalgae as feedstock for biodiesel production: carbon dioxide sequestration, lipid production and biofuel quality. **Journal of Chemical Technology and Biotechnology**, v. 85, n. 3, p. 395-403, 2010.

FRANCISCO, E.C. et al. Assessment of different carbohydrates as exogenous carbon source in cultivation. **Bioprocess and Biosystems Engineering** v. 37, p. 1497-1505, 2014.

FRANCISCO, E. et al. From waste-to-energy: the process integration and intensification for bulk oil and biodiesel production by microalgae. J. **Environmental Chemical Engineering,** v. 3, p. 482-487, 2015.

FERREIRA, S.P.; SOUZA-SOARES, L.; COSTA, J.A.V. Microalgas: uma fonte alternativa na obtenção de ácidos gordos essenciais. **Revista de Ciências Agrárias**, v. 36, n. 3, p. 275-287, 2013.

FIEDOR, J.; BURDA, K. Potential role of carotenoids as antioxidants in human health and disease. **Nutrients**, v. 6, n. 2, p. 466-488, 2014.

GAFFNEY, M., O'ROURKE, R., MURPHY, R. Manipulation of fatty acid and antioxidant profiles of the microalgae Schizochytrium sp. through flax seed oil supplementation**. Algal Research**, v. 6, p.195-200, 2014.

GIMPEL, J.A.; HENRÍQUEZ, V.; MAYFIELD, S.P. In metabolic engineering of eukaryotic microalgae: potential and challenges come with great diversity. **Frontiers in microbiology**, v. 6, 2015.

GOLDBERG, I. **Single cell protein**. First ed. Springer-Verlag, Berlin. 1985.

GONG, M.; BASSI, A. Carotenoids from microalgae: a review of recent developments. **Biotechnology advances**, v. 34, n. 8, p. 1396-1412, 2016.

GOUVEIA, L. et al. Microalgae-source of natural bioactive molecules as functional ingredients. **Food Science and Technology Bulletin - Functional Foods**. v. 7, p.21-37, 2010.

GRAHAM, L.E.; WILCOX, L.W. **Algae**. Prentice Hall: Upper Saddle River, N.J. 640 p., 2000.

GROBBELAAR, J.U. In: RICHMOND, A. (Ed.), **Handbook of Microalgal Culture**: **Biotechnology and Applied Phycology**. Blackwell Publishing Ltd., Oxford, 97-115, 2004.

GUIL-GUERRERO, J.L. et al. Functional properties of the biomass of three microalgal species. **Journal of food engineering**, v.65, n.4, p.511-517, 2004.

GUO, B. et al. Screening of diatom strains and characterization of *Cyclotella cryptica* as a potential fucoxanthin producer. **Marine drugs**, v. 14, n. 7, p. 125, 2016.

HALL. D.O., RAO, K.K. Photosynthetic apparatus, 6 th ed. *In*: **Photosynthesis, The Press Syndicate of The University of Cambridge**, p.33-57, 1999.

HALLIWELL, B., GUTTERIDGE, J. **Radicals in biology and medicine**. 4th ed., New York: Oxford University Press Inc, 2007.

HLAVOVA, M.; TUROCZY, Z.; BISOVA, K. Improving microalgae for biotechnology - From genetics to synthetic biology. **Biotechnology Advances**, v. 33, p. 1194-1203, 2015.

HILDEBRAND, M. et al. Metabolic and cellular organization in evolutionarily diverse microalgae as related to biofuels production. **Current opinion in chemical biology**, v. 17, n. 3, p. 506-514, 2013.

IBAÑEZ & CIFUENTES, A. Benefits of using algae as natural sources of functional ingredients. **Journal of the Science of Food and Agriculture**, v. 93, n. 4, p. 703-709, 2013.

JACOB-LOPES, E. et al. Protein characterisation of the Aphanothece Microscopica Nägeli cyanobacterium cultivated in parboiled rice effluent. **Food Science and Technology**, v. 26, n. 2, p. 482-488, 2006.

JACOB-LOPES, E. et al. Characteristics of thin-layer drying of the cyanobacterium Aphanothece microscopica Nägeli. **Chemical Engineering and Processing: Process Intensification**, v. 46, n. 1, p. 63-69, 2007.

JAY, J.M.; LOESSNER, M.J.; GOLDEN, D.A. **Modern food microbiology**. Springer Science & Business Media, 1992.

JEONG, H.J. et al. Growth, feeding and ecological roles of the mixotrophic and heterotrophic dinoflagellates in marine planktonic food webs. **Ocean science journal**, v. 45, n. 2, p. 65-91, 2016.

JOSHI, V.K. et al. Microbial pigments. **Indian Journal of Biotechnology**, v. 2, n. 3, p. 362-369, July 2003.

KHAN, S.A. et al. Prospects of biodiesel production from microalgae in India. **Renewable and Sustainable Energy Reviews**, v. 13, n. 9, p. 2361-2372, 2009.

KIM, M.K. et al. Enhanced production of *Scenedesmus* ssp. (green microalgae) using a new medium containing fermented swine wastewater. **Bioresource Technology,** n. 98, p. 2220-2228, 2007.

KUMAR, R.R. et al. Enzymatic and non-enzymatic antioxidant potentials of *Chlorella vulgaris* grown in effluent of a confectionery industry. **Journal of Food Science and Technology**, v. 51, n. 2, p. 322-328, Feb. 2014.

KOSINKOVA, J. et al. Measuring the regional availability of biomass for biofuels and the potential for microalgae. **Renewable and Sustainable Energy Reviews,** v. 49, p. 1271-1285, 2015.

LEE, R.E (Ed). **Phycology**. 4 th ed. Cambridge University Press. Colorado State University, USA, 2008.

LEITE et al. **Algal biofuels: Challenges and opportunities.** Bioresource Technology, v. 145, p.134–141, 2013.

LÓPEZ-GONZÁLEZ, D. et al. Energetic, economic and environmental assessment of the pyrolysis and combustion of microalgae and their oils. **Renewable and Sustainable Energy Reviews,** v.51, p. 1752-1770, 2015.

LOURENÇO, S.O. **Cultivo de microalgas marinhas – princípios e aplicações.** Rima: São Carlos, 2006.

MAADANE, A. et al. Antioxidant activity of some Moroccan marine microalgae: Pufa profiles, carotenoids and phenolic content. **Journal of biotechnology**, v. 215, p. 13-19, 2015.

MADIGAN, M.T.; MARTINKO, J.M.; PARKER, J. **Microbiologia de Brok**. 10 ed. São Paulo; Pratice Hall Inc., 608p. 2004.

MANAYI, A. et al. Lutein and cataract: from bench to bedside. **Critical reviews in biotechnology**, v. 36, n. 5, p. 829-839, 2016.

MARKOU, G.; GEORGAKAKIS, D. Cultivation of filamentous cyanobacteria (blue-green algae) in agroindustry wastes and wastewaters: a review. **Applied Energy**, v. 88, n. 10, p. 3389-3401, Oct. 2011.

MARKOU, G.; NERANTZIS, E. Microalgae for high-value compounds and biofuels production: a review with focus on cultivation under stress conditions. **Biotechnology advances**, v. 31, n. 8, p. 1532-1542, 2013.

MARQUEZ, U.M.L.; SINNECKER, P. Chlorophylls in foods: Sources and stability. **Food colorants chemical and functional properties**. CRC Press, Baca Raton, 2008.

MASOJÍDEK, J.; TORZILLO, G.; KOBLÍŽEK, M. Photosynthesis in microalgae. In: RICHMONT, A. (Ed.) **Handbook of microalgal culture**: biotechnology and applied phycology. Oxford: Blackwell Publishing Ltd. p. 20-39, 2004.

MARONEZE, M.M. et al. Treatment of cattle-slaughterhouse wastewater and the reuse of sludge for biodiesel production by microalgal heterotrophic bioreactors. **Scientia Agricola,** v. 71, p. 521-524, 2014.

MARONEZE, M. M. et al. The role of photoperiods on photobioreactors–A potential strategy to reduce costs. **Bioresource technology**, v. 219, p. 493-499, 2016.

MATA, T.M., MARTINS, A.A., CAETANO, N.S. Microalgae for biodiesel production and other applications: A review. **Renewable & Sustainable Energy Reviews,** v. 14, p. 217-232, 2010.

MERCADANTE, A. Z. Carotenoids in foods: sources and stability during processing and storage. In: SOCACIU, C. (Ed.). **Food colorants**: chemical and functional properties. Boca Raton: CRC Press, p. 213-235, 2008.

METTING, B.; PYNE, J.W. Biologically active compounds from microalgae. **Enzyme and Microbial Technology**, v.8, n.7, p.386-394, 1986.

MIAO, X.; WU, Q. Biodiesel production from heterotrophic microalgal oil. **Bioresource Technology**, v. 97, n. 6, p. 841-846, 2006.

MIYASHITA, K.; HOSOKAWA, M. Carotenoids as a Nutraceutical Therapy for Visceral Obesity, in: Watson, R. (Ed.), Nutrition in the Prevention and Treatment of abdominal Obesity. **Academic Press**, New York, p. 329-340, 2014.

MIN, D.B.; CHOE, E.O. Effects of singlet oxygen oxidation on the flavor of foods and stability of vitamins. **Food Science and Biotechnology**, v. 11, n. 5, p. 582-586, 2002.

MOGEDAS, B. et al. β-Carotene production enhancement by UV-A radiation in *Dunaliella bardawil* cultivated in laboratory reactors. **Journal of bioscience and bioengineering**, v. 108, n. 1, p. 47-51, 2009.

NALAGE, D.N. et al. Single Cell Proteins, in: Caballero, B., Finglas, P.M. Toldrá, F. (Eds.), **Encyclopedia of Food and Health.** Academic Press., New York, p. 790-794, 2016.

NORTON, T.A.; MELKONIAN, M.; ANDERSEN, R.A. Algal biodiversity. **Phycologia**, v. 35, n. 4, p. 308-326, 1996.

OLAIZOLA, M. Commercial development of microalgal biotechnology: from the test tube to the marketplace. **Biomolecular Engineering**, v. 20, p. 459-466, 2003.

OLVERA-NOVOA, M.A. et al. Effect of the use of the microalga Spirulina maxima as fish meal replacement in diets for tilapia, *Oreochromis mossambicus* (Peters), fry. **Aquaculture Research**, v. 29, n. 10, p. 709-715, 1999.

PEREIRA, C.M.P. et al. Biodiesel renovável derivado de microalgas: avanços e perspectivas tecnológicas. **Química Nova**, v. 35, n. 10, 2012.

PEREZ-GARCIA, O. et al. Heterotrophic cultures of microalgae: metabolism and potential products. **Water Research**, v. 45, n. 1, p. 11-36, 2011.

PLAZA, M., CIFUENTES, A., IBANEZ, E. In the search of new functional food ingredients from algae. **Trends in Food Science & Technology,** v. 9, n. 1, p. 31-39, 2008.

PLAZA, M. et al. Facts about the formation of new antioxidants in natural samples after subcritical water extraction. **Food Research International**, v. 43, n. 10, p. 2341-2348, 2010.

PRASANNA, R.; JAISWAL, P.; KAUSHIK, B.D. Cyanobacteria as potential options for environmental sustainability - promises and challenges. **Indian Journal of Microbiology**, v. 48, n. 1, p. 89-94, 2008.

PRASANNA, R. et al. Rediscovering cyanobacteria as valuable sources of bioactive compounds (Review). **Applied Biochemistry and Microbiology**, v. 46, n. 2, p. 119-134, 2010.

PRAVEENKUMAR, R. et al. Breaking dormancy: an energy-efficient means of recovering astaxanthin from microalgae. **Green Chemistry**, v. 17, n. 2, p. 1226-1234, 2015.

PULZ, O.; GROSS, W. Valuable products from biotechnology of microalgae. **Applied microbiology and biotechnology**, v. 65, n. 6, p. 635-648, 2004.

POOJARY, M.M. et al. Innovative alternative technologies to extract carotenoids from microalgae and seaweeds. **Marine drugs**, v. 14, n. 11, p. 214, 2016.

QUEIROZ, M.I. et al. Fish processing wastewater as a platform of the microalgal biorefineries. **Biosystems Engineering,** v. 115, p. 195-202, 2013.

RAVINDRA, P. et al. Value-added food: Single cell protein. **Biotechnology advances**, v. 18, n. 6, p. 459-479, 2000.

RAZZAK, S.A. et al. Integrated CO_2 capture, wastewater treatment and biofuel production by microalgae culturing-A review. **Renewable & Sustainable Energy Reviews.** v. 27, p. 622-653, 2013.

REDAELLI, C. **Estudo da produção de lipídeos e carotenoides por Chlorella minutissima em fotobiorreator.** Dissertação (Mestrado em Engenharia Química). Universidade Federal do Rio Grande do Sul, Porto Alegre, 2012.

RICHIMOND, A. **Handbook of microalgal culture:** Biotechnology and applied phycology. Oxford: Blackwell Science, 566 p., 2004.

RODRIGUEZ-AMAYA, D.B. **A guide to carotenoid analysis in foods**. Washington: ILSI Press. 64 p. 2001.

RODRIGUEZ-GARCIA, I.; GUIL-GUERRERO, J.L. Evaluation of the antioxidant activity of three microalgal species for use as dietary supplements and in the preservation of foods. **Food Chemistry**, v. 108, n. 3, p. 1023-1026, June 2008.

RODRIGUES, D. B. et al. Bioactive pigments from microalgae *Phormidium autumnale*. **Food Research International,** v. 77, p. 273-279, 2015.

RODRÍGUEZ-MEIZOSO, I. et al. Pressurized fluid extraction of bioactive compounds from Phormidium species. **Journal of Agricultural and Food Chemistry**, v. 56, p. 3517-3523, 2008.

SAINI, R.K.; NILE, S.H.; PARK, S.W. Carotenoids from Fruits and Vegetables: Chemistry, Analysis, Occurrence, Bioavailability and Biological Activities. **Food Research International**, v. 76, p. 735-750, 2015.

SASSO, S. et al. Microalgae in the postgenomic era: a blooming reservoir for new natural products. **FEMS microbiology reviews**, v. 36, n. 4, p. 761-785, 2012.

SPOLAORE, P. et al. Commercial applications of microalgae. **Journal of bioscience and bioengineering**, v. 101, n. 2, p. 87-96, 2006.

STENGEL, D.B.; CONNAN, S.; POPPER, Z.A. Algal chemo diversity and bioactivity: sources of natural variability and implications for commercial application. **Biotechnology advances**, v. 29, n. 5, p. 483-501, 2011.

SUGANYA, T. et al. Macroalgae and microalgae as a potential source for commercial applications along with biofuels production: a biorefinery approach. **Renewable and Sustainable Energy Reviews**, v. 55, p. 909-941, 2016.

SUMAN, G. et al. Single cell protein production: a review. **International Journal of Current Microbiology and Applied Sciences**, v. 4, p. 251-262, 2015.

TALERO, E. et al. Bioactive compounds isolated from microalgae in chronic inflammation and cancer. **Mar Drugs**. 30, 6152-6209, 2015.

THAJUDDIN, N.; SUBRAMANIAN, G. Cyanobacterial biodiversity and potential applications in biotechnology. **Current Science**, v. 89, n. 1, p. 47, 2005.

TIRICHINE, L.; BOWLER, C. Decoding algal genomes: tracing back the history of photosynthetic life on Earth. **The Plant Journal**, v. 66, n. 1, p. 45-57, 2011.

VALKO, M. et al. Free radicals and antioxidants in normal physiological functions and human disease. **The International Journal of Biochemistry and Cell Biology**, v. 39, n. 1, p. 44-84, Jan. 2007.

VARELA, J.C. et al. Production of carotenoids by microalgae: achievements and challenges. **Photosynthesis research**, v. 125, n. 3, p. 423-436, 2015.

VARELA, J.C. et al. Erratum to: Production of carotenoids by microalgae: achievements and challenges. **Photosynthesis research**, v. 127, n. 2, p. 285-286, 2016.

WALTER, M.H.; STRACK, D. Carotenoids and their cleavage products: biosynthesis and functions. **Natural Product Reports**, v. 28, n. 4, p. 663-692, Feb. 2011.

WATERBURY, J. The cyanobacteria–isolation, purification and identification the prokaryotes. *In*: DWORKIN, M. et al. (Eds): **The Prokaryotes**. 3 ed., v. 4, p. 1053-1073. New York: Springer, 2006.

WEN, Z.Y.; CHEN, F. Heterotrophic production of eicosatetraenoic acid by microalgae. **Biotechnology Advances**, v. 21, n. 4, p. 273-294, 2003.

WETTASINGHE, M.; SHAHIDI, F. Scavenging of reactive-oxygen species and DPPH free radicals by extracts of borage and evening primrose meals. **Food Chemistry**, v. 70, n. 1, p. 17-26, 2000.

WIJFFELS, R.H.; KRUSE, O.; HELLINGWERF, K.J. Potential of industrial biotechnology with cyanobacteria and eukaryotic microalgae. **Current Opinion in Biotechnology**, v. 24, n. 3, p. 405-413, 2013.

YE, Z.; JIANG, J.; WU, G. Biosynthesis and regulation of carotenoids in Dunaliella: progresses and prospects. **Biotechnology Advances**, v. 26, n. 4, p. 352-360, 2008.

YEN, HONG-WEI; BRUNE, D.E. Anaerobic co-digestion of algal sludge and waste paper to produce methane. **Bioresource Technology**, v. 98, n. 1, p. 130-134, 2007.

XU, H.; MIAO, X.; WU, Q. High quality biodiesel production from a microalga *Chlorella protothecoides* by heterotrophic growth in fermenters. **Journal of Biotechnology**, v. 126, n. 4, p. 499-507, 2006.

ZAGHDOUDI, K. et al. Accelerated solvent extraction of carotenoids from: Tunisian Kaki (*Diospyros kaki* L.), peach (*Prunus persica* L.) and apricot (*Prunus armeniaca* L.). **Food Chemistry**, v. 184, p. 131-139, 2015.