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

BIORREFINARIA DE MICROALGAS: PRODUÇÃO DE QUÍMICOS DE ALTO VALOR A PARTIR DE EFLUENTES AGROINDUSTRIAIS

DISSERTAÇÃO DE MESTRADO

Daniele Bobrowski Rodrigues

Santa Maria, RS, Brasil
2014

BIORREFINARIA DE MICROALGAS: PRODUÇÃO DE QUÍMICOS DE ALTO VALOR A PARTIR DE EFLUENTES AGROINDUSTRIAIS

Daniele Bobrowski Rodrigues

Dissertação apresentada ao curso de Mestrado do Programa de Pós-Graduação em Ciência e Tecnologia dos Alimentos, Área de Concentração em Qualidade dos Alimentos, da Universidade Federal de Santa Maria (UFSM, RS), como requisito parcial para obtenção do grau de **Mestre em Ciência e Tecnologia dos Alimentos.**

Orientador: Profª. Drª. Leila Queiroz Zepka

Santa Maria, RS, Brasil
2014

FICHA CATALOGRÁFICA

Universidade Federal de Santa Maria
Centro de Ciências Rurais
Programa de Pós-Graduação em Ciência e Tecnologia dos Alimentos

A Comissão Examinadora, abaixo assinada,
aprova a Dissertação de Mestrado

BIORREFINARIA DE MICROALGAS: PRODUÇÃO DE QUÍMICOS DE ALTO VALOR A PARTIR DE EFLUENTES AGROINDUSTRIAIS

elaborada por
Daniele Bobrowski Rodrigues

como requisito para obtenção do grau de
Mestre em Ciência e Tecnologia dos Alimentos

COMISSÃO EXAMINADORA

Prof[a]. Dr[a]. Leila Queiroz Zepka (UFSM)
(Presidente/Orientadora)

Prof[a]. Dr[a]. Adriana Zerlotti Mercadante (UNICAMP)

Prof[a]. Dr[a]. Veridiana Vera de Rosso (UNIFESP)

Prof. Dr. Eduardo Jacob Lopes (UFSM)
(Membro suplente)

Santa Maria, 18 de fevereiro de 2014.

AGRADECIMENTOS

A Deus que, incomparável na sua infinita bondade, me guiou e me deu força quando em mim já não havia.

A *Dicaiada* que, mais que uma família, é uma base sólida, uma plataforma que nos impulsiona para voos mais altos. O amor incondicional de vocês me dá segurança.

Ao Leonardo, pela paciência, auxílio, cuidado, generosidade e amor sincero em todos os momentos. Contigo tudo fica mais fácil e melhor.

A minha orientadora, Profª. Leila, por ampliar meus horizontes ao longo desses dois coloridos anos que dividimos. Pela confiança depositada em meu trabalho.

Ao meu co-orientador, Prof. Eduardo, pela orientação precisa.

A Profª. Drª. Adriana Zerlotti Mercadante pelas oportunidades de realização de análises em seu laboratório e de dar continuidade aos estudos na área que tanto gosto.

Ao Prof. Dr. Eliseu Rodrigues pela grande contribuição no trabalho, pelo auxilio e conselhos valiosos.

As minhas queridas filhas Jéssica Righi, Grazielle Castagna, Karine Schio, Mariany Deprá e Bruna Nunes pela ajuda nos experimentos e amizade.

A Andriéli Borges e Érika Francisco por serem minhas companhias no laboratório e me ajudarem.

A Sarai Kachuk pela ajuda nos últimos e cruciais momentos.

A Gabriela, Luís, Tatiele, Karem, Alberto, Mariane Bittencourt, Raquel Vendrusculo, Daniele Ferreira e aos demais colegas de Lab 111 e do NTA que compartilharam comigo esta caminhada, pelos conhecimentos trocados, disponibilidade para ajudar e amizade valiosa. Em especial à nossos anjos Matheus e Carol (*in memorian*), que nos deixaram imagem de dedicação, de alegria e de amor pelo que se faz, e que agora brilham lá no céu.

As colegas de mestrado por dividirem comigo as alegrias e desesperos deste dois anos. Por cada palavra, cada conversa séria e cada sorriso. Especialmente as colegas Luana Maurer e Bruna Tischer, pela parceria de sempre!

Aos grandes amigos do NIDAL, que sempre me auxiliaram tanto. Since 2007.

A Profª. Tatiana Emanuelli, pelo suporte sempre que necessário.

A Drª. Lilian Mariutti, pela ajuda e incentivo.

Aos colegas e amigos do laboratório de Química dos Alimentos (FEA/UNICAMP) que me receberam tão bem e me ajudaram muito.

A professora Águeda Castagna e toda a equipe LABAC pela possibilidade de utilização do leitor de microplacas.

Ao professor Érico Flores e equipe CEPETRO pela disposição dos equipamentos para a extração assistida por ultrassom.

A Capes pelo apoio financeiro através da concessão da bolsa de Mestrado.

Aos Professores membros da banca pela presença e correções sempre oportunas e bem colocadas.

A Universidade Federal de Santa Maria pela formação proporcionada.

A todos que contribuíram de forma direta ou indireta para realização deste trabalho.

Muito obrigada!

RESUMO

Dissertação de Mestrado
Programa de Pós-Graduação em Ciência e Tecnologia dos Alimentos
Universidade Federal de Santa Maria

BIORREFINARIA DE MICROALGAS: PRODUÇÃO DE QUÍMICOS DE ALTO VALOR A PARTIR DE EFLUENTES AGROINDUSTRIAIS

AUTORA: DANIELE BOBROWSKI RODRIGUES
ORIENTADORA: LEILA QUEIROZ ZEPKA
Data e Local da Defesa: Santa Maria, 18 de fevereiro de 2014.

Cianobactérias são seres de estrutura procarionte que reúnem interessantes características para a utilização biotecnológica em sistemas de biorrefinaria. A *Phormidium* sp. é uma cianobactéria que possui uma versatilidade metabólica que a permite crescer em condições fotossintéticas e heterotróficas, partir de resíduos industriais, gerando uma biomassa quimicamente atrativa. Em face disto, o trabalho teve por objetivos avaliar a produção de químicos de alto valor por *Phormidium* sp. a partir de resíduos agroindustriais. O foco foi direcionado a (i) identificação e quantificação de carotenoides da biomassa obtida heterotroficamente a partir de efluente agroindustrial (ii) a identificação e quantificação de carotenoides da biomassa cultivada fotoautotroficamente e a avaliação da capacidade antioxidante do extrato (iii) quantificação das ficobiliproteínas da biomassa cultivada fotoautotroficamente a avaliação da capacidade antioxidante do extrato e (iv) quantificação das clorofilas da biomassa cultivada fotoautotroficamente a avaliação da capacidade antioxidante do extrato. Os resultados indicaram que a biomassa microalgal obtida do cultivo heterotrófico apresentou um teor de carotenoides de 183,03 ± 0,9 $\mu g.g^{-1}$ enquanto que na cultivada em condições fotossintéticas o teor foi de 714,3 ± 0,9 $\mu g.g^{-1}$. Os carotenoides majoritários em ambos os extratos foram all-*trans*-β-caroteno, all-*trans*-luteína e all-*trans*-zeaxantina. O extrato de carotenoides foi capaz de desativar o radical peroxila 28 vezes mais eficientemente que o padrão de α-tocoferol. O conteúdo de ficocianina encontrado foi 20,05 ± 1.1 $g.100g^{-1}$ biomassa e após o fracionamento apresentou uma pureza de 4,8 e atividade antioxidante de 237,4 ± 0.7 $\mu mol\ trolox.g^{-1}$ biomassa. Em termos de clorofila, foram encontradas concentrações de 3400 $\mu g.g^{-1}$ e capacidade de desativar o radical peroxila relativa ao α-tocoferol de 84,9. Os resultados encontrados demonstram que a biomassa microalgal de *Phormidium* sp. possui potencial para exploração biotecnológica como uma fonte renovável e de baixo custo de compostos bioativos de alto valor agregado.

Palavras-chave: Capacidade antioxidante. Carotenoides. Cianobactéria. Clorofila. Ficocianina.

ABSTRACT

Master Dissertation
Post-Graduate Program in Food Science and Technology
Federal University of Santa Maria

MICROALGAL BIOREFINERY: PRODUCTION OF HIGH ADDED VALUE CHEMICALS FROM AGROINDUSTRIAL WASTE

AUTHOR: DANIELE BOBROWSKI RODRIGUES
ADVISOR: LEILA QUEIROZ ZEPKA
Place and Date: Santa Maria, February 18, 2014.

Cyanobacteria are prokaryote microorganisms that gather interesting features for the biotechnological use in biorefinery systems. *Phormidium* sp. is a cyanobacterium that has metabolic versatility, grown in both photosynthetic and heterotrophic conditions, from industrial residues, generating a chemically attractive biomass. On face of it, the work aimed to evaluate the production of high added value chemicals by *Phormidium* sp. from agro-industrial residues. It focuses on (i) identification and quantification of carotenoids from heterotrophically grown biomass in agro-industrial wastewater (ii) identification and quantification of carotenoids from photoautotrophically grown biomass and evaluation of the antioxidant capacity of the extract (iii) quantification of phycobiliproteins from photoautotrophically grown biomass and evaluation of the antioxidant capacity of the extract (iv) quantification of chlorophyll from photoautotrophically grown biomass and the evaluation of the antioxidant capacity of the extract. The results showed that the microalgal biomass from heterotrophic growth had 183.03 ± 0.9 $\mu g.g^{-1}$ of total carotenoids while in the biomass from photosynthetic growth this value was 714.3 ± 0.9 $\mu g.g^{-1}$. The major carotenoids in both extracts were all-*trans*-β-carotene, all-*trans*-lutein and all-*trans*-zeaxanthin. The carotenoid extract was able to scavenge the peroxyl radical 28 times more than the α-tocopherol standard. The content of phycocyanin found was 20.05 ± 1.1 $g.100g^{-1}$ and after fractionation achieved a purity ratio of 4.8 as well as antioxidant capacity of 237.4 ± 0.7 μmol trolox equivalents.g^{-1}. In terms of chlorophyll, the biomass showed the content of 3400 $\mu g.g^{-1}$ and the capacity to scavenge the peroxyl radical 84.9 in relation to α-tocopherol standard. The results show that *Phormidium* sp. microalgal biomass has potential for biotechnological exploitation as a renewable and low cost source of bioactive compounds of high added value.

Keywords: Antioxidant capacity. Carotenoids. Cyanobacteria. Chlorophyll. Phycocyanin.

LISTA DE ILUSTRAÇÕES

CAPÍTULO 1

CAPÍTULO 2

CAPÍTULO 3

LISTA DE TABELAS

CAPÍTULO 2

CAPÍTULO 3

SUMÁRIO

INTRODUÇÃO

O crescente interesse em produtos naturais com possíveis atividades bioativas direciona o desenvolvimento de tecnologias que empregam microrganismos, dentre eles as microalgas, que são capazes de sintetizar compostos específicos. A biomassa microalgal é considerada uma fonte potencial de compostos bioativos como carotenoides, ficobiliproteínas, clorofilas, esteróis e ácidos graxos poli-insaturados. Estudos vêm sendo realizados no sentido da utilização deste tipo de biomassa como matéria-prima em sistemas de biorrefinarias, que embora viáveis biotecnologicamente, têm no alto custo de produção da biomassa a maior barreira para sua comercialização.

Para a produção de biomassa microalgal, fotobiorreatores são amplamente utilizados e tem sido considerados uma possibilidade viável para biofixação de dióxido de carbono. Em culturas de grande escala, entretanto, a absorção de luz é atenuada pelo sombreamento mútuo das células, afetando seriamente a produtividade e a qualidade dos produtos da biomassa das algas (MARKOU; GEORGAKAKIS, 2011), além do custo elevado da energia elétrica (IP; CHEN, 2005). Uma alternativa para estes inconvenientes tecnológicos do processo seria o cultivo heterotrófico, a partir do qual o fornecimento energético para os microrganismos decorre da assimilação de uma fonte de carbono exógena, como por exemplo, mono e polissacarídeos (FRANCISCO et al., 2014). O emprego de substratos desta natureza traria um alto custo à produção (DUCAT et al., 2011) e novas fontes de carbono de menor custo são requeridas. Resíduos agroindustriais com alta carga orgânica surgem como uma possibilidade real de utilização como substrato nestes sistemas e desta forma, a associação entre o tratamento de águas residuárias e a geração de bioprodutos a partir da biomassa microalgal, estabelecendo um sistema de biorrefinaria, pode representar um significativo avanço na direção de reduzir os custos de produção. Gupta et al. (2013) salientam a importância de fontes de carbono de baixo custo para a produção em modo heterotrófico.

Em nível mundial, pesquisas e programas de demonstração estão sendo realizados no sentido de desenvolver as tecnologias necessárias para a expansão da utilização de fontes de energia de microalgas e geração industrial de produtos químicos (SHEEHAN, 2009; WIJFFELS et al., 2013). Da mesma forma que há a necessidade de pesquisas visando o desenvolvimento e o aperfeiçoamento dos sistemas de produção, a fim de torná-los viáveis economicamente, também se faz necessária a identificação e quantificação dos bioprodutos

que podem ser obtidos das microalgas, bem como suas possíveis atividades bioativas, para que este sistema se estabeleça.

Cianobactérias são reconhecidamente uma fonte rica de pigmentos como clorofilas, carotenoides e ficobiliproteínas (DUFOSSÉ et al., 2005). A estes compostos têm sido relacionados efeitos benéficos à saúde, que são atribuídos principalmente às suas propriedades antioxidantes (KRINSKY; JOHNSON, 2005; KU et al., 2013; NAGARAJ et al., 2012; ROMAY et al., 1998). Os danos induzidos por espécies reativas de oxigênio (ERO's) podem afetar moléculas biológicas, culminando com a perda de sua estrutura e consequentemente, sua função. Espécies radicalares afetam, ainda, componentes presentes nos alimentos, nos quais alteram atributos sensoriais, nutricionais e toxicológicos, e outros bens oxidáveis, como cosméticos, farmacêuticos e plásticos. Compostos antioxidantes capazes de diminuir ou evitar estas reações são amplamente requeridos e observa-se um aumento nas investigações no campo de antioxidantes naturais (DUFOSSÉ, 2006).

A utilização de sistemas baseados em fontes renováveis pode ser uma alternativa para a produção de compostos de interesse, a nível de processo industrial. Entretanto, para a consolidação do conceito de biorrefinaria microalgal, primeiramente são necessárias investigações acerca dos componentes de interesse que podem ser obtidos da biomassa gerada a partir de resíduos. Isso inclui a avaliação da produção destes químicos de alto valor agregado, bem como a estimativa de sua produção industrial. Em adição, maiores estudos são necessários para estabelecer a potencialidade da biomassa de *Phormidium* sp. como uma fonte natural de antioxidantes.

Baseado no fato de que a determinação de compostos é fundamental para estabelecer o potencial de utilização de qualquer insumo, o presente trabalho fundamenta-se em um estudo exploratório acerca da potencialidade de produção de pigmentos naturais por *Phormidium* sp. sob o escopo de uma biorrefinaria.

OBJETIVOS

Objetivo geral

O objetivo geral deste estudo foi a avaliação da produção de químicos de alto valor por *Phormidium* sp. a partir de resíduos agroindustriais.

Objetivos específicos

a) Identificação e quantificação de carotenoides da biomassa obtida heterotroficamente a partir de efluente agroindustrial;

b) Identificação e quantificação de carotenoides da biomassa cultivada fotoautotroficamente e a avaliação da capacidade antioxidante do extrato;

c) Quantificação das ficobiliproteínas da biomassa cultivada fotoautotroficamente a avaliação da capacidade antioxidante do extrato;

d) Quantificação das clorofilas da biomassa cultivada fotoautotroficamente a avaliação da capacidade antioxidante do extrato.

CAPÍTULO 1

REVISÃO BIBLIOGRÁFICA

1 Microalgas

Microalgas constituem um grupo amplo e heterogêneo de microrganismos eucariontes e procariontes, estes últimos contemplados nas divisões Cyanophyta e Prochlorophyta (MUTANDA et al., 2011). A divisão Cyanophyta reúne as cianobactérias, microrganismos Gram-negativos que exibem ampla diversidade fisiológica, metabólica e morfológica, incluindo seres de formas unicelulares, filamentosas ou agrupados em colônias (BECK et al., 2012). Cianobactérias podem ser encontradas em diversos habitats e condições ambientais, como ambiente marinho, em água doce, solo, em regiões polares e desertos (ROTHSCHILD; MANCINELLI, 2001; WHITTON; POTTS 2000) e de acordo com Rodolfi et al. (2009), devido à seleção evolutiva e mudanças nas condições ambientais, há uma grande variedade de espécies encontradas em ambientes extremos. Estima-se que existam cerca de 6280 representantes deste grupo (NABOUT et al., 2013).

O potencial comercial e biotecnológico das microalgas representa um recurso inexplorado, uma vez que das possíveis espécies existentes, relativamente poucas foram estudadas em detalhe, do ponto de vista bioquímico e fisiológico (WATERBURY, 2006). Esta grande biodiversidade, que resulta na variabilidade da composição química e das características metabólicas, aliada em determinados casos ao melhoramento genético e desenvolvimento de novas tecnologias de cultivo, faz destes microrganismos um importante campo de pesquisa básica e aplicada, e vem permitindo o uso das microalgas em diversas aplicações (IBAÑEZ; CIFUENTES, 2013). Exemplos desta aplicações são o emprego da biomassa microalgal para a obtenção de alimentos (DRAAISMA et al., 2013), produção de energia (WIJFFELS; BARBOSA, 2010) e para a obtenção de compostos bioativos (COSTA et al., 2013), que possuem alto valor no mercado mundial, entre outras substâncias de interesse (JACOBSEN; FRIGAARD, 2014; MILEDGE, 2011).

As cianobactérias são assim denominadas pela ausência de organização celular e estruturas definidas, fato que as assemelha às bactérias. Apesar de sua típica organização celular procariótica, assim como os eucariontes possuem um elaborado sistema de membranas internas responsáveis tanto pelo o transporte de elétrons da cadeia respiratória quanto pela fotossíntese (SINGH et al., 2011). Embora as microalgas sejam principalmente fotoautotróficas, um número considerável destes microrganismos possui um metabolismo versátil, sendo capaz de crescer também heterotroficamente, a partir da assimilação de

substratos orgânicos para a manutenção de suas estruturas na ausência de luminosidade (FRANCISCO et al., 2014).

1.1 Metabolismo das microalgas

Cianobactérias são seres robustos que podem utilizar até três vias metabólicas para obtenção de energia, a respiração, a fotossíntese e a fixação de nitrogênio (QUEIROZ et al., 2013). O modo mais comum de cultivo destes microrganismos é o crescimento autotrófico, através da utilização de CO_2 atmosférico, nutrientes e luz. Segundo Beck et al. (2012), estes simples requerimentos potencializam o interesse na exploração tecnológica destes organismos.

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 de água para reduzir o CO_2, a fim de formar moléculas orgânicas. O processo da 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. As clorofilas e carotenoides são componentes lipofílicos associados em complexos proteína-clorofila. Estes pigmentos absorvem a região da luz vermelha (650-700 nm) e a região da luz azul (400-500 nm), respectivamente. As ficobilinas são hidrofílicas e absorvem a luz na região do vermelho-alaranjado (600-650 nm). Elas 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 (MARKOU; GEORGAKAKIS, 2011; MASOJÍDEK et al., 2004). 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 (BEER et al., 2009). Na fase seguinte, os produtos das reações com luz

são subsequentemente consumidos pela redução de CO_2 a carboidratos (MASOJÍDEK et al., 2004). 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 necessárias para o crescimento celular (KAUNY; SÉTIF, 2014). Já os substratos para a hidrogenase, hidrogênio e elétron, são supridos tanto via cadeia transportadora de elétrons fotossintéticos como via fermentação do carboidrato armazenado. Em microalgas, bem como em plantas superiores, as duas reações acima citadas podem operar independentemente, dependendo da qualidade e quantidade de iluminação e da concentração de substratos (MARKOU; GEORGAKAKIS, 2011; MULLINEAUX, 2014).

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 decompostos 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 antioxidantes (KUMAR et al., 2014).

Outra via metabólica importante é a respiração. Em um sentido amplo, todos os organismos, incluindo microalgas, utilizam as mesmas vias metabólicas para realizar a respiração. O metabolismo das microalgas se assemelha, com apenas pequenas diferenças ao das plantas superiores. O cultivo heterotrófico no escuro é uma importante habilidade de algumas espécies de cianobactérias fotossintéticas e vem demonstrando grande potencial para o cultivo de microalgas em geral (IP; CHEN, 2005). 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). Em grande parte dos casos, o cultivo de heterotrófico gera menos custos de produção, pode ser realizado em instalações mais simples, além da maior facilidade para a manutenção em larga escala (FRANCISCO et al., 2014). A respiração no escuro, além de fornecer energia para a manutenção das vias anabólicas, fornece carbono como bloco construtor para a biossíntese de compostos orgânicos de interesse, e assim, pode gerar maiores rendimentos, além de permitir uma ampliação das possibilidades em relação aos sistemas autotróficos (SMITH, 1982).

As microalgas possuem substancial capacidade de bioconversão de material orgânico e nutrientes presentes em águas residuárias, de forma relativamente rápida, de baixo custo e ecologicamente segura, com benefícios adicionais de recuperação e reciclo, além da geração de co-produtos na forma de biomassa. Neste sentido, sua utilização em efluentes agroindustriais ao explorar seu metabolismo heterotrófico seria uma alternativa em relação às formas convencionais de tratamento (KUMAR et al., 2014; QUEIROZ et al., 2007; QUEIROZ et al., 2013).

2 Sistemas de biorrefinarias industriais: geração de compostos de alto valor agregado

Recursos renováveis têm ganhado atenção como insumo para a indústria e tem estimulado o desenvolvimento de novas formas integradas de produção (BOUAID et al., 2010). Denomina-se biorrefinaria a esta nova abordagem de obtenção de matérias-primas e produtos renováveis em um processo integrado, de forma sustentável (CHERUBINI, 2010; OCTAVE; THOMAS, 2009). Desta forma, as biorrefinarias são sistemas que combinam as tecnologias necessárias entre a concepção, exploração das matérias primas biológicas (biomassa) e a produção de insumos intermediários e produtos finais, isto é conseguido usando uma combinação sinérgica entre conversões biológicas e químicas (KAMM; KAMM, 2004). Uma vez purificados, os novos produtos e subprodutos obtidos podem ser comercializados ou destinados à alimentar indústrias convencionais.

Dada a sua natureza renovável, a conversão de biomassa de microalgas em uma gama de bioprodutos de alto valor agregado estabelece um sistema de biorrefinaria microalgal. Microalgas têm sido consideradas como potenciais biocatalisadores em reações de conversão, e assim geram bioprodutos de natureza intracelular, além de metabólitos extracelulares, passíveis de utilização como insumos intermediários ou produtos finais de uma série de consumíveis (SPOLAORE et al., 2006). Embora o conceito de biorrefinaria seja bem estruturado, a maior barreira para uma aplicação em grande escala é a ausência de tecnologias de processamento de baixo custo.

Dentro deste contexto, as microalgas, particularmente as cianobactérias vêm sendo utilizadas na conversão de resíduos industriais, utilizando os diferentes constituintes dos efluentes para o seu crescimento, o que leva, consequentemente, a uma produção massiva de

biomassa. A biomassa resultante destas conversões é uma fonte renovável potencialmente rica em produtos de valor agregado. Tanto a biomassa microalgal quanto os demais produtos do seu metabolismo apresentam elevado potencial de reutilização em diversos segmentos industriais. Esta co-geração de insumos é atrativa, uma vez que os custos de produção podem ser reduzidos com créditos para tratamento de águas residuais, bem como com a redução da emissão de gases (FENG et al., 2011; WU et al., 2012), limitando o impacto ambiental das produções. Levando isso em consideração, o uso de subprodutos industriais como matérias-primas em sistemas de biorrefinaria se apresenta como uma alternativa para a produção de compostos bioativos, tanto do ponto de vista ambiental como econômico (BRENNAN; OWENDE, 2010; EKMAN; BÖRJESSON, 2011; OHARA, 2003).

De acordo com Jones e Mayfield (2012), a concretização da exploração de processos microalgais dependerá da criação de sistemas com aproveitamento global, completamente otimizado e eficiente, que use todos os componentes da biomassa microalgal, o que somente poderá ser obtido sob o escopo de uma biorrefinaria. Ao produzir múltiplos produtos, uma biorrefinaria pode tirar vantagem das diferenças de componentes da biomassa e intermediários, e maximizar o valor derivado da matéria-prima de biomassa de acordo com a situação do mercado e disponibilidade da biomassa (LUO et al., 2011; SUBHADRA; GRINSON-GEORGE, 2011).

3 Bioprodutos com atividade biológica

As cianobactérias são consideradas fábricas celulares naturais capazes de sintetizar uma série de compostos úteis (GERSHWIN; BELAY, 2008). A exploração comercial em larga escala das microalgas data de 1950, motivada pelo elevado teor de proteínas da biomassa, para utilização como recurso alimentar alternativo (SPOLAORE et al., 2006). Atualmente, o conteúdo protéico já não é o argumento único para promover a sua utilização, com pesquisas direcionadas à produção e isolamento de compostos bioativos encontrados nestes microrganismos (COSTA et al., 2013; PRASANNA et al., 2010). Nos últimos anos, microalgas foram identificadas como um dos grupos mais promissores de organismos para isolar produtos naturais e ativos bioquímicos de alto valor agregado (PRASANNA et al., 2008). Elas podem sintetizar, metabolizar, acumular e secretar uma grande diversidade de

compostos orgânicos de alto valor agregado com potencial de aplicação em indústrias como farmacêutica, alimentícia e cosmética (YAMAGUCHI, 1996). O elevado grau de diversidade das bioatividades é devido ao seu amplo espectro de metabólicos primários e secundários (SINGH et al., 2011), muitos com estruturas não encontradas em outras matrizes.

Dentre os bioprodutos de microalgas com possíveis propriedades bioativas, há grande interesse por pigmentos naturalmente presentes, na medida em que possuem importância terapêutica e uma vasta gama de aplicações, que lhe confere, consequentemente, um alto valor econômico (BOROWITZKA, 2013; RASTOGI; SINHA, 2009). Carotenoides, ficocianinas e clorofilas possuem 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. À estes pigmentos têm-se atribuídas propriedades anti-inflamatórias, imunossupressoras e hepatoprotetoras (ERIKSEN, 2008).

Estas ações 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; GUTTERIDGE, 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 patofisiologia de doenças crônico 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). Pigmentos microbianos surgem como uma fonte, devido ao seu caráter natural, produção independente da estação do ano e das condições geográficas e rendimento controlável e de qualidade previsível (JOSHI et al., 2003). Segundo Dufoseé et al. (2005) 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. Brennan e Owende (2010) listam uma série de vantagens ambientais e econômicas que os bioprodutos de microalgas exibem em relação às outras fontes vegetais, como necessidade menor de água do que as culturas terrestres, reduzindo

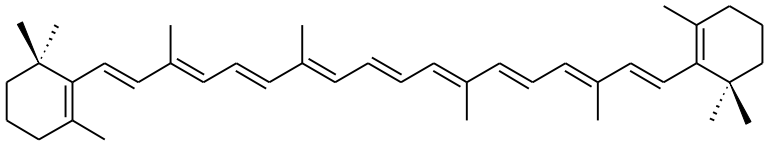
assim a carga sobre as fontes de água doce, e possibilidade de crescimento em água salobra e em terras não aráveis, sem comprometer a produção de alimentos, além da produção de biomassa gerar parte da biofixação de CO_2 atmosférico. Ainda, os nutrientes necessários para o cultivo de microalgas podem ser obtidos a partir de águas residuárias, por conseguinte, existe o potencial duplo de tratamento de águas residuais com simultânea produção de biomassa verde, que pode ser utilizada como fertilizante, por exemplo, e também pode produzir co-produtos de alto valor agregado.

Cianobactérias reúnem a combinação única da eficiente capacidade fotossintética e simplicidade das exigências nutricionais típicas de plantas com atributos biotecnológicos próprios de células microbianas, como altas taxas de crescimento, capacidade para produzir grandes quantidades de produtos desejados sob condições controladas, e adaptabilidade a ambientes distintos (DUCAT et al., 2011). Todos estes atributos, aliados a possibilidade de utilização de substratos orgânicos provenientes de resíduos, podem determinar vantagens tecnológicas e comerciais na produção destes compostos e tornaria possível sua produção a nível industrial.

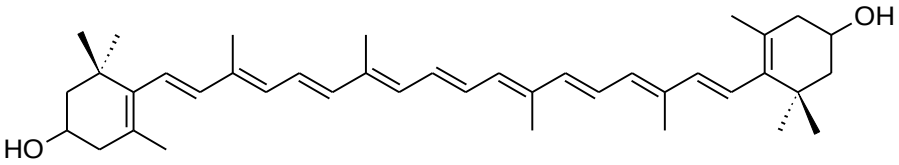
3.1 Carotenoides

Os carotenoides são geralmente compostos tetraterpênicos construídos por 8 unidades isoprenoides. A estrutura básica consiste em um esqueleto linear e simétrico com uma série de ligações duplas conjugadas (c.d.b.), que gera um sistema de ressonância de elétrons π e corresponde ao cromóforo. Os elétrons se deslocam ao longo de toda a cadeia de poliênica e absorvem luz na região visível do espectro eletromagnético, com forte absorção na região de 400 a 500 nm (MERCADANTE, 2008). Além da cor, essa característica estrutural é responsável por suas funções e atividades, como reatividade química, forma molecular, e atividade como desativadora de espécies reativas (BRITTON, 1995).

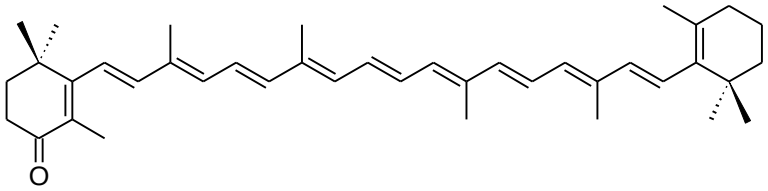
Segundo Hertzberg e Liaaen-Jensen (1966) e Powls e Britton (1976) são característicos de cianobactérias β-caroteno, zeaxantina, equinenona, mixoxantofila, e 4,4'-dioxocarotenoide como cantaxantina, mostrados na Figura 1.



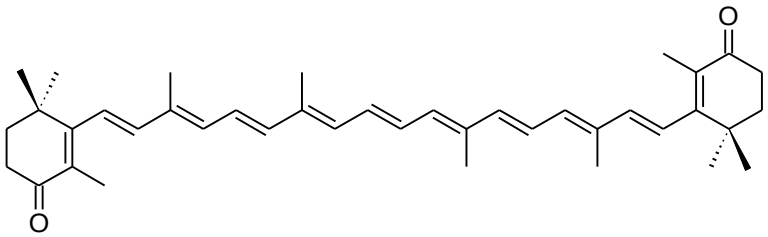
All-*trans*-β-caroteno



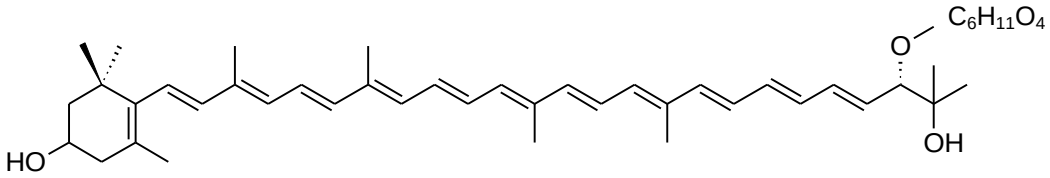
All-*trans*-zeaxantina



All-*trans*-equinenona



All-*trans*-cantaxantina



All-*trans*-mixoxantofila

Figura 1 - Estrutura dos principais carotenoides encontrados em cianobactérias.

Em cianobactérias, assim como nos demais organismos fotossintéticos, carotenoides são pigmentos acessórios. Absorvem a luz em uma região do espectro solar que clorofilas têm fraca absorção e protegem os organismos de danos fotooxidativos causados por oxigênio singlete e estados excitados de moléculas fotossensibilizadoras, e dos radicais peroxil

formados durante a produção de oxigênio (DOMONKOS et al., 2013). Todos os carotenoides envolvidos diretamente na fotossíntese são chamados carotenoides primários. Carotenoides secundários estão presentes nas células principalmente após a exposição a estímulos ambientais específicos (via carotenogênese), como exposição à luz de alta intensidade, privação de nutrientes, mudanças de temperatura e estresse oxidativo (LEYA et al., 2009; SKJÅNES et al., 2013). Portanto, as condições de produção da espécie podem ser estabelecidas de tal forma que a biossíntese de carotenoides desejados seja favorecida.

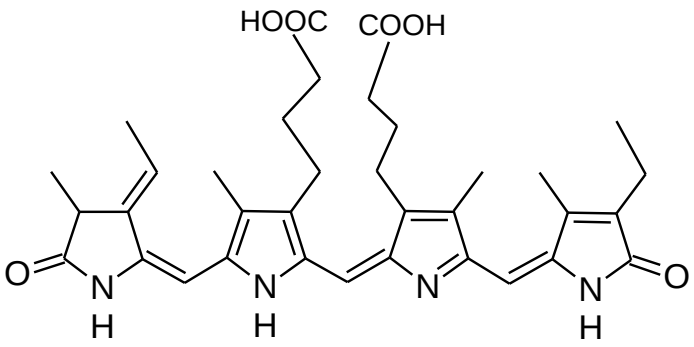
Estes compostos têm propriedades que os tornam importantes tanto em alimentos e na saúde humana, e de acordo com Perez-Garcia et al. (2011), são uma das principais áreas de exploração biotecnológica de microalgas, com uma ampla gama de aplicações. Do ponto de vista industrial e comercial, os carotenoides têm um mercado maduro e estável (USDA, 2013) e aplicações incluem corantes industriais, para aplicação em produtos alimentícios e ração para animais, cosméticos e suplementos (DEL CAMPO et al., 2007; PULZ; GROSS, 2004). As principais fontes industriais de carotenoides são a síntese química e a extração a partir de plantas e microalgas, com destaque para β-caroteno produzido por *Dunaliella* e astaxantina produzida por *Haematococcus*.

Os benefícios à saúde dos carotenoides são atribuídos, pelo menos em parte, à sua atividade como antioxidantes (KRINSKY; JOHNSON, 2005). Três mecanismos foram propostos para a remoção de radicais por carotenoides: a transferência de elétrons entre o radical e o carotenoide, a captação do hidrogênio alílico e adição do radical ao sistema de ligações duplas conjugadas (EL-AGAMEY et al., 2004).

3.2 Ficocianinas

A ficocianina (C-PC) é um pigmento azul com propriedades fluorescentes que se encontra apenas em cianobactérias, criptofíceas e rodofíceas (ERIKSEN, 2008), e é o membro mais estudado da classe das ficobiliproteínas. Ficobiliproteínas são pigmentos hidrossolúveis formados por grupos prostéticos tetrapirróis lineares (bilinas) covalentemente ligadas à proteínas específicas por uma ligação tioéter através de resíduos de cisteína (BERMEJO et al., 1997). As bilinas correspondem ao cromóforo da molécula. Os espectros de absorção de luz e emissão de fluorescência são determinados pela extensão das ligações duplas conjugadas nos

cromóforos, e com base em suas características espectrais, as ficobiliproteínas de cianobactérias são comumente classificados em ficoeritrina (C-PE) com cromóforo ficoeritrobilina, e ficocianina (C-PC) e aloficocianina (C-APC), com cromóforo ficocianobilina (Figura 2). O cromóforo ficoeritrobilina contém 6 ligações duplas conjugadas e absorve em menores comprimentos de onda que ficocianobilina com 8 ligações duplas conjugadas (WOLK, 1973). Assim, C-PE tem pico de absorção máximo entre 540 e 570 nm, C-APC entre 650 nm a 655 nm e C-PC entre 610 nm a 620nm (HSIEH et al., 2013).



Ficocianobilina

Figura 2 - Estrutura da ficocianobilina encontrada em cianobactérias.

Fonte: Adaptado de Eriksen (2008).

Não só a cor e as propriedades fluorescentes, mas também seu potencial terapêutico e antioxidante estão relacionados ao seu cromóforo covalentemente ligado a cadeia tetrapirrólica aberta. Ficocianobilina pode ser enzimaticamente reduzida a ficocianorubina a qual é estruturalmente semelhante a bilirrubina, um antioxidante natural do plasma que protege lipídeos da oxidação (SØRENSEN et al., 2013). Um crescente número de investigações revelam propriedades farmacológicas como antioxidante, anti-inflamatória, neuroprotetora e hepatoprotetora (BENEDETTI et al., 2004; BHANDARI; SHARMA, 2011; BHAT; MADYASTHA, 2000; ROMAY et al. 2003). A interação de C-PC com radicais peroxila indicam que o principal alvo destes radicais é a parte bilina (LISSI et al., 2000).

Este grupo possui uma vasta gama de aplicações industriais, como corante alimentício, em cosméticos e como marcadores de fluorescência altamente sensíveis utilizados no

diagnóstico clínico. Segundo Spolaore et al. (2006) há 17 anos atrás o mercado global das ficobiliproteínas já era estimado em US$ 50 milhões com preços variando de US$ 3 a US$ 25/mg. As principais fontes naturais de ficobiliproteínas são a cianobactéria S*pirulina* para C-PC (azul) e a rodofícea *Porphyridium* para C-PE (vermelho).

3.3 Clorofilas

Quimicamente, a clorofila não é uma molécula isolada, mas compreende uma família de substâncias semelhantes entre si, designadas clorofila *a*, *b*, *c*, *d*, *e* e *f*. São moléculas complexas, pertencentes à classe das porfirinas, formadas por 4 anéis pirrólicos planares em arranjo simétrico e um quinto anel isocíclico, localizado ao lado do anel pirrólico. Os anéis estão ligados entre si por pontes metilênicas e a molécula contém um átomo de magnésio no seu interior, coordenado aos anéis por 4 átomos de nitrogênio. No quarto anel pirrólico, o ácido propiônico ali existente é esterificado por um álcool acíclico de cadeia longa, o fitol, conferindo à clorofila um caráter hidrofóbico (GROSS, 1991). A clorofila *a* (Figura 3), o mais abundante e importante componente dessa família, corresponde a aproximadamente 75% dos pigmentos verdes encontrados na natureza. A clorofila *b* difere desta por possuir um grupamento aldeído (CHO) em C-7, em vez do grupo metila.

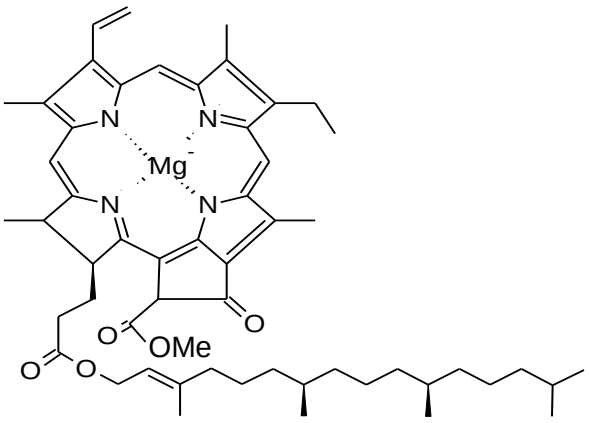


Figura 3 - Estrutura da clorofila *a*.

Estas moléculas são atualmente empregadas como corante natural em indústrias farmacêuticas e de alimentos, além de estarem vinculadas a propriedades nutracêuticas em atividades funcionais de promoção à saúde, como ação anti-inflamatória, auxiliar de cicatrização e controle de cristais de oxalato de cálcio (FERRUZI; BLAKESLEE, 2007). Adicionalmente, alguns estudos demonstram a redução no risco do câncer em dietas associadas ao consumo de clorofila *a* (BALDER et al., 2006). Os efeitos protetores do clorofilas dependem de sua capacidade de modular a ativação dos sistemas de desintoxicação de xenobióticos endógenos, bem como por suas propriedades antioxidantes e sequestradora de mutágenos (FERRUZZI; BLAKESLEE, 2007). Observa-se contudo, que são raros os estudos biológicos, o que tem sido relacionado principalmente à dificuldade em purificar esses pigmentos e à instabilidade química dos mesmos.

4 Referências

BALDER, H.F.; VOGEL, J.; JANSEN, M. C.; WEIJENBERG, M. P.; VAN DEN BRANDT, P. A.; WESTENBRINK, S.; VAN DER MEER, R.; GOLDBOHM, R. A. Heme and chlorophyll intake and risk of colorectal cancer in the Netherlands cohort study. **Cancer Epidemiology, Biomarkers and Prevention**, v. 15, n. 4, p. 717-725, Apr. 2006.

BECK, C.; KNOOP, H.; AXMANN, IM.; STEUER, R. The diversity of cyanobacterial metabolism: genome analysis of multiple phototrophic microorganisms. **BMC Genomics**, v. 13, n. 56, p. 1-17, Feb. 2012.

BEER, L. L.; BOYD, E. S.; PETERS, J. W.; POSEWITZ, M. Engineering algae for biohydrogen and biofuel production. **Current Opinion in Biotechnology**, v. 20, n. 3, p. 264-271, June 2009.

BENEDETTI, S.; BENVENUTI, F.; PAGLIARANI, S.; FRANCOGLI, S.; SCOGLIO, S.; CANESTRARI, F. Antioxidant properties of a novel phycocyanin extract from the blue-green alga *Aphanizomenon flos-aquae*. **Life Sciences**, v. 75, n. 19, p. 2353–2362, Sep. 2004.

BERMEJO, R.; TALAVERA, E. M.; ALVAREZ-PEZ, J. M.; ORTE, J. C. Chromatographic purification of biliproteins from *Spirulina platensis* high-performance liquid chromatographic separation of their α and β subunits. **Journal of Chromatography A**, v. 778, n. 1-2, p. 441-450, Aug. 1997.

BHANDARI; R. P.; SHARMA, P. K Photosynthetic and biochemical characterization of pigments and UV-absorbing compounds in *Phormidium tenue* due to UV-B radiation. **Journal of Applied Phycology**, v. 23, n. 2, p. 283-292, Apr. 2011.

BHAT, V.B.; MADYASTHA, K. M. C-phycocyanin: a potent peroxyl radical scavenger in vivo and in vitro. **Biochemical and Biophysical Research Communications**, v. 275, n. 1, p. 20-25, Aug. 2000.

BOROWITZKA, M. A. High-value products from microalgae—their development and commercialisation. **Journal of Applied Phycology**, v. 25, n. 3, p. 743-756, June 2013.

BOUAID, A.; MARTÍNEZ, M.; ARACIL, J. Biorefinery approach for coconut oil valorisation: A statistical study. **Bioresource Technology**, v. 101, n. 11, p. 4006-4012, June 2010.

BRENNAN, L.; OWENDE, P. Biofuels from microalgae — A review of technologies for production, processing, and extractions of biofuels and co-products. **Renewable and Sustainable Energy Reviews**, v. 14, n. 2, p. 557-577, Feb. 2010.

BRITTON, G. Structure and properties of carotenoids in relation to function. **The FASEB Journal**, v. 9, n. 15, p. 1551–1558, Dec. 1995.

CHERUBINI, F. The biorefinery concept: using biomass instead of oil for producing energy and chemicals. **Energy Conversion and Management**, v. 51, n. 7, p. 1412-1421, July 2010.

COSTA, M.; GARCIA, M.; COSTA-RODRIGUES J.; COSTA M. S.; RIBEIRO, M. J.; FERNANDES, M. H.; BARROS, P.; BARREIRO, A.; VASCONCELOS, V.; MARTINS, R. 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, Dec. 2013.

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.

DOMONKOS, I.; KIS, M.; GOMBOS, Z.; UGHY, B. Carotenoids, versatile components of oxygenic photosynthesis. **Progress in Lipid Research**, v. 52, n. 4, p. 539-561, Oct. 2013.

DRAAISMA, R. B.; WIJFFELS, R. H.; SLEGERS, P. M.; BRENTNER, L. B.; ROY, A., BARBOSA, M. J. Food commodities from microalgae. **Current Opinion in Biotechnology**, v. 24, n. 2, p. 169-177, Apr. 2013.

DUCAT, D. C.; WAY, J. C.; SILVER, P. A. Engineering cyanobacteria to generate high-value products. **Trends in Biotechnology**, v. 29, n. 2, p. 95-103, Feb. 2011.

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

DUFOSSÉ, L.; GALAUP, P.; YARON, A.; ARAD, S. M.; BLANC, P.; MURTHY, K. N. C.; RAVISHANKAR, G. A. 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.

EKMAN, A.; BÖRJESSON, P. Environmental assessment of propionic acid produced in an agricultural biomass-based biorefinery system. **Journal of Cleaner Production**, v. 19, n. 11, p. 1257–1265, July 2011.

EL-AGAMEY, A.; LOWE, G. M; MCGARVEY, D. J; MORTENSEN. A.; PHILLIP, D. M.; TRUSCOTT, T. G.; Young, A. J. Carotenoid radical chemistry and antioxidant/pro-oxidant properties. **Archives of Biochemistry and Biophysics**, v. 430, n.1, p. 37-48, Oct. 2004.

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.

FENG, Y.; LI, C.; ZHANG, D. Lipid production of *Chlorella vulgaris* cultured in artificial wastewater medium. **Bioresource Technology**, v. 102, n. 1, p. 101–105, Jan. 2011.

FERRUZZI, M. G.; BLAKESLEE, J. Digestion, absorption, and cancer preventative activity of dietary chlorophyll derivatives. **Nutrition Research**, v. 27, n. 1, p. 1-12, Jan. 2007.

FRANCISCO, E. C.; FRANCO, T. T.; WAGNER, R.; JACOB-LOPES, E. Assessment of different carbohydrates as exogenous carbon source in cultivation of cyanobacteria. **Bioprocess and Biosystems Engineering**, Jan. 2014. [Epub ahead of print]

GERSHWIN, M. E.; BELAY, A. (Eds) ***Spirulina*** **in human nutrition and health**. Boca Raton: CRC Press, Boca Raton, 2008.

GROSS, J. **Pigments in vegetables**: chlorophylls and carotenoids. New York: Van Nostrand Reinhold, 1991. 351 p.

GUPTA, V.; RATHA, S. K.; SOOD, A.; CHAUDHARY, V.; PRASANNA, R. New insights into the biodiversity and applications of cyanobacteria (blue-green algae)—Prospects and challenges. **Algal Research**, v. 2, n. 2, p. 79-97, Mar. 2013.

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

HERTZBERG, S., LIAAEN-JENSEN, S. SIEGELMAN, H. W. The carotenoids of blue-green algae. **Phytochemistry**, v. 10, n. 12, p. 3121-3127, Dec. 1971.

HERTZBERG, S.; LIAAEN-JENSEN, S. The carotenoids of blue-green algae—I. : The carotenoids of *Oscillatoria rubescens* and an *Athrospira* sp. **Phytochemistry**, v. 5, n. 4, p. 557-563, July 1966.

HSIEH, P.; PEDERSEN, J. Z.; ALBERTANO, P. Generation of reactive oxygen species upon red light exposure of cyanobacteria from *Roman hypogea*. **International Biodeterioration and Biodegradation**, v. 84, p. 258-265, Oct. 2013.

IBAÑEZ, E., 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, Mar 2013.

IP, P.; CHEN, F. Production of astaxanthin by the green microalga *Chlorella zofingiensis* in the dark. **Process Biochemistry**, v. 40, n. 2, p. 733-738, Feb. 2005.

JACOBSEN, J. H.; FRIGAARD, N. U. Engineering of photosynthetic mannitol biosynthesis from CO2 in a cyanobacterium. **Metabolic Engineering**, v. 21, p. 60-70, Jan. 2014.

JONES, C.S.; MAYFIELD, S. P. Algae biofuels: versatility for the future of bioenergy. **Current Opinion in Biotechnology**, v. 23, n. 3, p. 346-351, June 2012.

JOSHI, V. K.; ATTRI, D.; BALA, A.; BHUSHAN, S. Microbial pigments. **Indian Journal of Biotechnology**, v. 2, n. 3, p. 362-369, July 2003.

KAMM, B.; KAMM, M. Principles of biorefineries. **Applied Microbiology and Biotechnology**, v. 64, n. 2, p. 137-145, Apr. 2004.

KAUNY, J.; SÉTIF, P. NADPH fluorescence in the cyanobacterium *Synechocystis* sp. PCC 6803: A versatile probe for *in vivo* measurements of rates, yields and pools. **Biochimica et Biophysica Acta**, Jan. 2014. [Epub ahead of print]

KRINSKY, N. I.; JOHNSON E. J. Carotenoid actions and their relation to health and disease. **Molecular Aspects of Medicine**, v. 26, n. 6, p. 459-516, Dec. 2005

KU, C. S.; YANG, Y.; PARK, Y.; LEE. J. Health benefits of blue-green algae: prevention of cardiovascular disease and nonalcoholic fatty liver disease. **Journal of Medicinal Food**, v. 16, n. 2, p. 103-111, Feb. 2013.

KUMAR, R. R.; RAO, P. H.; SUBRAMANIAN, V. V.; SIVASUBRAMANIAN, V. 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.

LANFER-MARQUEZ, U. M.; SINNECKER, P. Analysis of Chlorophylls. In: SOCACIU, C. (Ed.). **Food colorants**: chemical and functional properties. Boca Raton: CRC Press, 2008. p. 429-446.

LEYA, T.; RAHN, A.; LÜTZ, C.; REMIAS, D. Response of arctic snow and permafrost algae to high light and nitrogen stress by changes in pigment composition and applied aspects for biotechnology. **FEMS Microbiology Ecology**, v. 67, n. 3, p. 432-43, Mar. 2009.

LISSI, E. A.; PIZARRO, M.; ASPEE, A.; ROMAY, C. Kinetics of phycocyanine bilin groups destruction by peroxyl radicals. **Free Radical Biology and Medicine**, v. 28, n. 7, p. 1051-1055, Apr. 2000.

LUO, G.; TALEBNIA, F.; KARAKASHEV, D.; XIE, L.; ZHOU, Q.; ANGELIDAKI, I. Enhanced bioenergy recovery from rapeseed plant in a biorefinery concept. **Bioresource Technology**, v. 102, n. 2, p. 1433-1439, Jan. 2011.

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

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., 2004. p. 20-39.

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, 2008. p. 213-235.

MILLEDGE, J. L. Commercial application of microalgae other than as biofuels: a brief review. **Reviews in Environmental Science and Biotechnology**, v. 10, n. 1, p. 31-41, Mar. 2011.

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.

MULLINEAUX, C. W. Electron transport and light-harvesting switches in cyanobacteria. **Frontiers in Plant Science**, v. 5, n. 7, Jan. 2014. [Published online]

MUTANDA, T.; RAMESH, D.; KARTHIKEYAN, S.; KUMARI, S.; ANANDRAJ, A.; BUX F. Bioprospecting for hyper-lipid producing microalgal strains for sustainable biofuel production. **Bioresource Technology**, v. 102, n. 1, p. 57-70, July 2011.

NABOUT, J. C.; ROCHA, B. S.; CARNEIRO, F. M., SANT'ANNA, C. L. How many species of Cyanobacteria are there? Using a discovery curve to predict the species number. **Biodiversity and Conservation**, v. 22, n. 12, p. 2907-2918, Nov. 2013.

NAGARAJ, S.; ARULMURUGAN, P.; RAJARAM, M. G.; KARUPPASAMY, K.; JAYAPPRIYAN, K. R.; SUNDARARAJ, R.; VIJAYANAND, N.; RENGASAMY, R. Hepatoprotective and antioxidative effects of C-phycocyanin from *Arthrospira maxima* SAG 25780 in CCl_4-induced hepatic damage rats. **Biomedicine and Preventive Nutrition**, v. 2, n. 2, p. 81-85, Apr./June 2012.

OCTAVE, S.; THOMAS, D. Biorefinery: Toward an industrial metabolism. **Biochimie**, v. 91, n. 6, p. 659-664, June 2009.

OHARA, H. Biorefinery. **Applied Microbiology and Biotechnology**, v. 62, n. 5-6, p. 474–477, Oct. 2003.

PEREZ-GARCIA, O.; ESCALANTE, F. M. E.; DE-BASHANA, L. E.; BASHAN, Y. Heterotrophic cultures of microalgae: metabolism and potential products. **Water Research**, v. 45, n. 1, p. 11-36, Jan. 2011.

POWLS, R.; BRITTON, G. A carotenoprotein, containing violaxanthin, isolated from *Scenedesmus obliquus* D_3. **Biochimica et Biophysica Acta**, v. 453, n. 1, p. 270-276, Nov. 1976.

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, Mar. 2008.

PRASANNA, R.; SOOD, A.; JAISWAL, P.; NAYAK, S.; GUPTA, V.; CHAUDHARY, V.; JOSHI, M.; NATARAJAN, C. Rediscovering cyanobacteria as valuable sources of bioactive compounds (Review). **Applied Biochemistry and Microbiology**, v. 46, n. 2, p. 119-134, Mar. 2010.

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

QUEIROZ, M. I.; HORNES, M. O.; MANETTI, A. G. S.; ZEPKA, L. Q.; JACOB-LOPES, E. Fish processing wastewater as a platform of the microalgal biorefineries. **Biosystems Engineering**, v. 115, n. 2, p. 195-202, June 2013.

QUEIROZ, M. I.; JACOB-LOPES, E.; ZEPKA, L. Q.; BASTOS, R.; GOLDBECK, R. The kinetics of the removal of nitrogen and organic matter from parboiled rice effluent by cyanobacteria in a stirred batch reactor. **Bioresource Technology**, v. 98, n. 11, p. 2163-2169, Aug. 2007.

RASTOGI, R. P.; SINHA, R. P. Biotechnological and industrial significance of cyanobacterial secondary metabolites. **Biotechnology Advances**, v. 27, n. 4, p. 521-539, July/Aug. 2009.

RODOLFI, L.; CHINI ZITTELLI, G.; BASSI, N.; PADOVANI, G.; BIONDI, N.; BONINI, G.; TREDICI, M. R. Microalgae for oil: strain selection, induction of lipid synthesis and outdoor mass cultivation in a low-cost photobioreactor. **Biotechnology and Bioengineering**, v. 102, n. 1, p. 100–112, Jan. 2009.

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.

ROMAY, C.; ARMESTO, J.; REMIREZ, D.; GONZÁLEZ, R.; LEDON, N.; GARCÍA, I. Antioxidant and anti-inflammatory properties of C-phycocyanin from blue-green algae. **Inflammation Research**, v. 47, n. 1, p. 36-41, Jan. 1998.

ROMAY, C.; GONZALEZ, R.; LEDON, N.; REMIREZ, D.; RIMBAU, V. C-phycocyanin: a biliprotein with antioxidant, anti-inflammatory and neuroprotective effects. **Current Protein and Peptide Science**, v. 4, n. 3, p. 207-216, June 2003.

ROTHSCHILD, L. J.; MANCINELLI, R. L. Life in extreme environments. **Nature**, v. 409, n. 6823, p. 1092-1101, Feb. 2001.

SHEEHAN, J. Engineering direct conversion of CO_2 to biofuel. **Nature Biotechnology**, v. 27, n. 12, p. 1128-1129, Dec. 2009.

SINGH, R. K.; TIWARI, S. P.; RAI, A. K.; MOHAPATRA, T. M. Cyanobacteria: an emerging source for drug discovery. **The Journal of Antibiotics**, v. 64, n. 6, p. 401-12, June 2011.

SKJÅNES, K.; REBOURS, C.; LINDBLAD, P. Potential for green microalgae to produce hydrogen, pharmaceuticals and other high value products in a combined process. **Critical Reviews in Biotechnology**, v. 33, n. 2, p. 172-215, June 2013.

SMITH, A. J. Modes of cyanobacterial carbon metabolism. In: CARR, N. G.; WHITTON, B. A. **The Biology of Cyanobacteria**. Berkeley: University of California Press, 1982. p. 47-86.

SØRENSEN, L.; HANTKE, A.; ERIKSEN, N. T. Purification of the photosynthetic pigment C-phycocyanin from heterotrophic *Galdieria sulphuraria*. **Journal of the Science of Food and Agriculture**, v. 93, n. 12, p. 2933-2938, Sep. 2013.

SPOLAORE, P.; JOANNIS-CASSAN, C.; DURAN, E.; ISAMBERT, A. Commercial applications of microalgae. **Journal of Bioscience and Bioengineering**, v. 101, n. 2, p. 87-96, Feb. 2006.

SUBHADRA, B.; GRINSON-GEORGE. Algal biorefinery-based industry: an approach to address fuel and food insecurity for a carbon-smart world. **Journal of the Science of Food and Agriculture**, v. 91, n. 1, p. 2-13, Jan. 2011.

USDA/ARS/NCAUR TECHNOLOGIES FOR TRANSFER. Natural carotenoids: what is this technology? Disponível em: <http://www.ars.usda.gov/SP2UserFiles/ Place/36200000/NatCarotenoid_flyer.pdf>. Acesso em: 27 dez. 2013.

VALKO, M.; LEIBFRITZ, D.; MONCOL, J.; CRONIN, M. T. D.; MAZUR, M.; TELSER, J. 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.

WATERBURY, J. The cyanobacteria–isolation, purification and identification the prokaryotes. In: DWORKIN, M.; FALKOW, S.; ROSENBERG, E.; SCHLEIFER, K.–H.; STACKEBRANDT, E. (Eds): **The Prokaryotes**. 3 rd. ed., vol. 4, New York: Springer, 2006. p. 1053–1073.

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, July 2000.

WHITTON, B. A.; POTTS, M. Introduction to the cyanobacteria. In: WHITTON, B. A.; POTTS, M. (Eds.). **The ecology of cyanobacteria**. Dordrecht: Kluwer Academic Publishers, 2000. p. 1-11.

WIJFFELS, R. H.; BARBOSA, M. J. An outlook on microalgal biofuels. **Science**, v. 329, n. 5993, p. 769-799, Aug. 2010.

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, June 2013.

WOLK, C. P. Physiology and cytological chemistry blue-green algae. **Bacteriological Reviews**, v 37, n. 1, p. 32-101, Mar. 1973.

WU, L. F.; CHEN, P. C.; HUANG, A. P.; LEE, C. M. The feasibility of biodiesel production by microalgae using industrial wastewater. **Bioresource Technology**, v. 113, special issue, p. 14–18, June 2012.

YAMAGUCHI, K. Recent advances in microalgal bioscience in Japan, with special reference to utilization of biomass and metabolites: a review. **Journal of Applied Phycology**, v. 8, n. 6, p. 487-502, Nov. 1996.

CAPÍTULO 2

FROM WASTE TO NATURAL PIGMENTS: PRODUCTION OF MICROALGAL CAROTENOIDS IN AGROINDUSTRIAL WASTEWATER

Submetido à revista Food Research International[1]

[1]O manuscrito foi formatado conforme as normas exigidas pela Revista.

From waste to natural pigments: production of microalgal carotenoids in agroindustrial wastewater

DANIELE B. RODRIGUES[a], ÉRICO M.M. FLORES[b], GRAZIELLE C.C. WEIS[a], JULIANO S. BARIN[a], ADRIANA Z. MERCADANTE[c], EDUARDO JACOB-LOPES[a], LEILA Q. ZEPKA[a]

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

[c]Department of Food Science, University of Campinas (UNICAMP), P.O. Box 6121, Campinas 13083-862, Brazil

*****Corresponding author: e-mail: lqz@pq.cnpq.br; Fax: 55-55-32208353; Phone: 55-55-32208254

Highlights

- Characterization of the carotenoid profile of *Phormidium* sp. is reported.
- *Phormidium* sp. grows heterotrophically using wastewater as substrate and effectively convert its components into biomass.
- The microalgal biomass shows potential to produce high amounts of β-carotene, zeaxanthin and lutein thought biorefinery systems.
- The carotenoid industrial production is estimated for industries with different capacities.
- This process can be considerate a potential biotechnological pathway to carotenoids production.

Abstract

The carotenoid profile from microalgae *Phormidium* sp. was assessed by high performance liquid chromatography coupled with a photodiode array and mass spectrometry detectors. A total of twenty carotenoids were separated in biomass from bioprocess for conversion of agroindustrial wastewater, and showed all-*trans*-β-carotene (70.22 μg.g^{-1}), all-*trans*-zeaxanthin (26.25 μg.g^{-1}), all-*trans*-lutein (21.92 μg.g^{-1}), all-*trans*-echinenone (19.87 μg.g^{-1}) and *cis*-echinenone (15.70 μg.g^{-1}) as the major ones. The single-cell carotenoids production was estimated to be 107,902.5 kg.year^{-1} in industrial scale. Based on these results, we observed the potential of *Phormidium* sp. to the production of microalgal carotenoid from agroindustrial wastewater.

Keywords*:* carotenoid; microalgae; industrial productivity; HPLC-DAD-MS/MS; *Phormidium* sp.

1. Introduction

Bioactive molecules such as carotenoids are produced in large numbers by many commercial and academic groups around the world, however the producing process of these structures is fraught with difficulty (Jez & Noel, 2000; Orchad et al., 2011).

Microalgae systems for chemicals production are an emergent area, representing a great promise for industrial application. Several processes have demonstrated their production capabilities mainly as pigments and additives for food and feed industries, but also for cosmetics industries. *Phormidium* is a genus of filamentous, unbranched cyanobacteria, with filaments of about 3-4 μm in diameter. Several species are known to live in extreme environments such as thermal springs, desert soils and polluted sites, and for this reason they have a wide potential for use in bioprocesses, because of their robustness and simple nutritional requirements (Guiry & Guiry, 2013).

Microalgae are recognized as an excellent source of carotenoids (Dufossé et al., 2005). The evolutionary and phylogenetic diversity also means a great diversity in the chemical composition of these organisms, with the ability to produce carotenoids with singular structural characteristics from those commonly found in conventional matrix, such as the length of the conjugated polyene chain of the carotenoid, an extension of the conjugated double bonds, and presence of hydroxyl groups (Albrecht et al., 2000; Mandelli et al., 2012). Studies involving the identification of carotenoid profile in microalgae can be traced to the classical works of Hertzberg and Liaaen-Jensen (1966) and Powls and Britton (1976), who reported β-carotene, echinenone, myxoxanthophyll, violaxanthin and 4,4'-dioxocarotenoid such as canthaxanthin in microalgae.

Biomass microalgae have long been thought to be a rich source of renewable bioproducts, but an understanding the major limitations for the economically

competitive is crucial. This consideration has been investigated, and different groups reported that the main hurdles to overcome are related to light provision, economical culture media, carbon sources, and cell harvesting (Jacob-Lopes & Franco, 2013). The cost of these parameters is a limiting factor in the commercialization of microalgae biomass products; however, microalgae have a mixotrophic tendencies and can combine both phototrophic and heterotrophic in different way. The wide metabolic capabilities of these microorganisms make possible the use of organic sources without commercial value, such as industrial effluents can be used to biomass growth in the dark used by a carbon source replacing the traditional support of light energy (Perez-Garcia et al., 2011). These knowledge makes them attractive for bioprospecting and potential exploitation as commercial sources of a wide range of natural pigments, mainly when their feedstock comes from biorefineries systems (Ohara, 2003; Dufossé et al., 2005, Octave & Thomas, 2009; Brennan & Owende, 2010; Queiroz et al., 2013; Borowitzka, 2013; Jacob-Lopes & Franco, 2013).

The biorefinery approach consists in sustainable processing of biomass into a wide range of valuable bioproducts and energy in an integrated process (IEA, 2009). The use of the heterotrophic harvest microalgal cultivate in industrial wastewater for carotenoid production may offer an inexpensive alternative to conventional forms to produced pigments.

A key issue on the viable production of the natural pigments is the general absence of low-cost processing technology. The agro-industries are an important world economic activity. In general, these processes involves significant water consumption, on average equal to 10 m^3 $tone^{-1}$ of product processed, resulting in a significant volume of wastewater. These wastewaters contains considerable quantities of carbon, nitrogen, phosphorus, metals, and solids being source of the raw material and suitable for

supporting microalgal growth. The agro-industrial residues are potentially available on a large scale and can generate a biomass, rich in natural pigments, that is cost competitive (Jacob-Lopes & Franco, 2010; Jacob-Lopes & Franco, 2013).

Thus, the objectives of this study was to identify, by HPLC–DAD coupled to mass spectrometry (MS/MS), the carotenoid profile of *Phormidium* sp. microalgal biomass obtained from heterotrophic bioprocess, and in parallel discuss the production potential of microalgal carotenoids in wastewater.

2. Material and Methods

2.1. Chemicals

Standards of all-*trans*-violaxanthin, all-*trans*-lutein, all-*trans*-zeaxanthin, all-*trans*-α-carotene, all-*trans*-β-carotene, were donated by DSM Nutritional Products (BASEL, Switzerland), with purities ranging from 95.0% to 99.9%, as determinated by HPLC-PDA. Methanol (MeOH), methyl tert-butyl ether (MTBE), acetone, petroleum ether and diethyl ether were obtained from Sigma Aldrich (St. Louis-MO, USA).

2.2 Microorganisms and culture media

Axenic cultures of *Phormidium* sp. were originally isolated from the Cuatro Cienegas desert (26º59'N, 102º03'W - Mexico). Stock cultures were propagated and maintained in solidified agar-agar (20 g/L) containing synthetic BG11 medium (Rippka et al., 1979). The incubation conditions used were 25 ºC, a photon flux density of 15 $\mu molm^{-2}\ s^{-1}$ and a photoperiod of 12/12 hours light/dark.

2.3 Microalgal biomass production

The biomass production was carried out under heterotrophic conditions, using the slaughterhouse wastewater as culture medium. The cultivations were performed in a bubble column bioreactor (Jacob-Lopes & Franco, 2013) operating under a batch regime, fed on 2.0 L of wastewater. The experimental conditions were as follows: initial concentration of inoculum of 100 mg/L, temperature of 26 ºC, pH adjusted to 7.6, carbon/nitrogen ratio of 30 (adjusted when necessary with glucose), aeration of 1 volume of air per volume of wastewater per minute, absence of light and a residence time of 168 h. The biomass was separated from the wastewater by centrifugation. It was subsequently freeze-dried for 24 h at -50 °C above -175 μm Hg. Two independent cultivations were performed in duplicate. Therefore, experimental data refer to the mean value of four repetitions.

2.4. Biomass concentration

Cell biomass was determined gravimetrically, filtering a known volume of culture through a 0.45 μm membrane filter (Millex FG®, Billerica-MA, USA), drying at 60 ºC for 24 h, followed by weighting.

2.5 Carotenoid extraction

The carotenoids were extracted by ultrasound-assisted extraction (UAE) using an ultrasonic processor (Sonics, Anaheim-CA, USA) with a 13 mm diameter probe. Dried samples were placed in a jacketed vessel through which water was circulated at

20 °C to avoid existence of hot spots. The extractions were carried out with cold acetone for 20 min and the amplitude applied for extraction was set to 50% (61 μm approximately). Samples were processed at a constant frequency of 20 kHz. The ultrasound probe was submerged to a depth of 25 mm in the sample. The sample suspension was centrifuged for 10 min at 3000 rpm. The extraction procedure was repeated until the supernatant became colorless. The carotenoids were transferred to petroleum ether/diethyl ether mixture [1:1 (v/v)], and saponified overnight with 10% (w/v) methanolic KOH at room temperature. The alkali was removed by washing the extract with distilled water and the extract concentrated in a rotary evaporator (T < 30 °C). In order to determine the total carotenoid concentration, the extract was dissolved in petroleum ether and the absorbance monitored spectrophotometrically at 451nm. Then, each extract was once again concentrated in a rotary evaporator (T < 30 °C), flushed with N_2 and kept at -18 °C in the dark until chromatographic analysis. All extractions were performed in triplicate and each triplicate was injected thrice into the HPLC. To avoid carotenoid degradation during analyses, the manipulation of the samples and extracts was conducted in absence of light.

2.6 HPLC-DAD-MS/MS analysis

Prior to HPLC-DAD-MS/MS analysis, the carotenoid extract was solubilized in MeOH:MTBE (70:30) and filtered through Millipore membranes (0.22 μm). 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 DAD detector (model SPD-M20A) and a

mass spectrometer with an ion-trap analyser and atmospheric pressure chemical ionization (APCI) source (model Esquire 4000, Bruker Daltonics, Bremem, Germany). The UV–visible spectra were obtained between 250 and 600 nm, and the chromatograms were processed at 451 nm. The MS parameters were set according to De Rosso and Mercadante (2007). Carotenoid separation was carried out on a C30 YMC column (5 μm, 250 x 4.6 mm id.) (Waters, Wilmington-DE, USA), with a mobile phase in linear gradient of MeOH and MTBE mixture from 95:5 to 70:30 in 30 min, to 50:50 in 20 min and maintaining this proportion for 15 min. The flow rate was 0.9 $mL.min^{-1}$ and the column temperature set to 29 °C.

The identification was performed according to the following combined information: elution order on C30 HPLC column, co-chromatography with standards, UV-visible spectrum characteristics [maximum absorption wavelength (λ max), spectral fine structure (% III/II) and peak *cis* intensity (% AB/AII)] and mass spectra characteristics (protonated molecules and their MS/MS fragments). This date were compared to authentic standards and data available in the literature (Britton, Liaaen-Jensen & Pfander, 2004; De Rosso & Mercadante, 2007; Zepka & Mercadante, 2009; Van Breemen, Dong, & Pajkovic 2012).

The carotenoids were quantified using five-point analytical curves of all-*trans*-violaxanthin (0.7-13.6 μg/mL), all-*trans*-lutein (1.0-59.5 μg/mL), all-*trans*-zeaxanthin (1.3-59.7 μg/mL), all-*trans*-α-carotene (1.0-49.2 μg/mL) and all-*trans*-β-carotene (1.1-30.2 μg/mL). All other xanthophylls and carotenes contents were estimated using the curve of all-*trans*-lutein and all-*trans*-β-carotene, respectively. The *cis*-isomers were estimated using the curve of the corresponding all-*trans*-carotenoid. Total carotenoid content was calculated as the sum of the contents of each individual carotenoid separated on the C30 column.

2.7 Statistical analysis

The data were analyzed using descriptive statistics, analysis of variance (ANOVA) and Tukey test through software Statistica 7.0 (StatSoft, Tulsa-OK,USA).

3. Results and discussion

3.1. Identification of carotenoids

The HPLC chromatogram (Figure 1) demonstrates the presence of twenty carotenoids in the extract of the *Phormidium* sp. biomass, which were identified or tentatively identified on the basis of the combined information obtained from chromatographic elution on a C30 column, co-chromatography with standards and characteristics of UV−Vis and mass spectra (Table 1). Since a detailed description of carotenoid identification using the above information was already reported by De Rosso and Mercadante (2007) and Van Breemen, Dong, and Pajkovic (2012) only some considerations of the most important aspects of this identification are discussed in present work.

Peak 11 (Figure 1, Table 1) was identified as all-*trans*-canthaxanthin, considering the lack of spectral fine structure due their two carbonyl groups to the chromophore, λ max at 472 nm and protonated molecule at *m/z* 565 (molecular weight 564 Da) along with loss of water indicating the presence of oxygen in the structure .

All-*trans*-myxoxanthophyll (peak 14), a major carotenoid glycoside distributed in cyanobacteria, was tentatively identified based on its UV-Vis characteristics (the

longest λ max at 505 nm consistent with a chromophore of 12 c.d.b., of which only one is in the ring, III/II ratio 50) and protonated molecule at *m/z* 731.

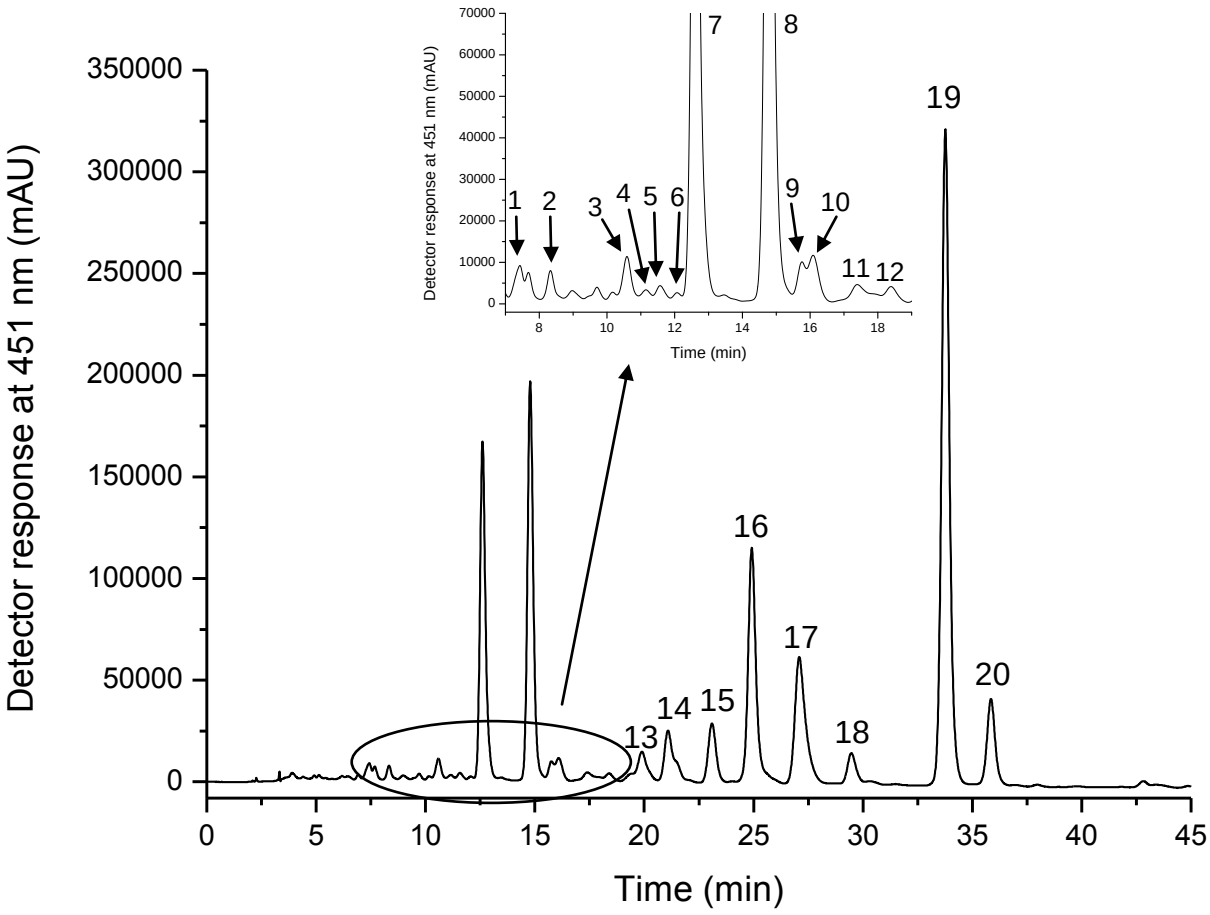


Figure 1 Chromatogram, obtained by HPLC-DAD, of the carotenoid extract from Phormidium sp. biomass. See text for chromatographic conditions. Peak identification and characterization are given in Table 1. Chromatogram was processed at 451 nm.

Table 1 Chromatographic, UV-Vis spectrum and mass characteristics obtained by HPLC-DAD-MS/MS of *Phormidium* sp. carotenoids.

Peak[a]	Carotenoid	t_R (min)[b]	UV-Vis characteristics			Fragment ions (positive mode) *(m/z)*		Carotenoid content ($\mu g.g^{-1}$)[f]
			$\lambda_{máx}$ (nm)[c]	III/II (%)[d]	AB/II (%)[e]	$[M+H]^+$	MS/MS	
1	All-*trans*-neoxanthin	7.7	415, 440, 468	77	0	601	583 $[M+H-18]^+$, 565, 509 $[M+H-92]^+$, 491$[M+H-18-92]^+$, 221	0.36 ± 0.1
2	9-*cis*-neoxanthin	8.3	327, 415, 437, 466	72	25	601	583 $[M+H-18]^+$, 565, 509 $[M+H-92]^+$, 491 $[M+H-18-92]^+$, 221	0.99 ± 0.2
3	9-*cis*-violaxanthin	10.6	330, 419, 437, 469	70	7	601	583 $[M+H-18]^+$, 565 $[M+H-18-18]^+$	1.40 ± 0.1
4	13-*cis*-lutein	11.1	334, 415, 437, 466	nc[g]	50	569	551 $[M+H-18]^+$, 533, 495, 477 $[M+H-92]^+$, 459 $[M+H-106]^+$	0.61 ± 0.5
5	13'-*cis*-lutein	11.6	330, 413, 439, 464	35	47	569	551 $[M+H-18]^+$, 533, 495, 477 $[M+H-92]^+$, 459 $[M+H-106]^+$	0.76 ± 0.5
6	13-*cis*-zeaxanthin	12.1	337, 420, 445, 471	Nc	45	569	551 $[M+H-18]^+$, 533, 495, 477 $[M+H-92]^+$, 459 $[M+H-106]^+$	0.06 ± 0.1
7	All-*trans*-lutein	12.6	420, 444, 472	62	0	569	551 $[M+H-18]^+$, 533 $[M+H-18-18]^+$, 495, 477 $[M+H-92]^+$, 459 $[M+H-106]^+$	21.92 ± 0.6
8	All-*trans*-zeaxanthin	14.8	425, 450, 477	30	0	569	551 $[M+H-18]^+$, 533 $[M+H-18-18]^+$, 495, 477 $[M+H-92]^+$, 459	26.25 ± 0.6
9	9-*cis*-lutein	15.8	330 , 417, 440, 467	50	6	569	551 $[M+H-18]^+$	0.79 ± 0.3
10	9-*cis*-zeaxanthin	16.1	337, 420, 445, 472	30	nc	569	551 $[M+H-18]^+$, 533 $[M+H-18-18]^+$	0.46 ± 0.2
11	All-*trans*-canthaxanthin	17.4	472	Nc	0	565	547 $[M+H-18]^+$	0.93 ± 0.2

12	Not identified 1	18.4	339, 420, 445, 468	36	21	567	535, 444	0.86 ± 0.3
13	Not identified 2	19.9	345, 421, 445, 471	40	25	569	551 $[M+H-18]^+$, 533 $[M+H-18-18]^+$, 495, 477 $[M+H-92]^+$, 459	2.65 ± 0.8
14	All-*trans*-myxoxantophyll	21.1	449, 474, 505	55	0	731	550, 476	3.15 ± 0.3
15	All-*trans*-zeinoxanthin	23.1	420, 446, 473	49	0	553	535 $[M+H-18]^+$, 461 $[M+H-92]^+$, 361	5.74 ± 0.5
16	All-*trans*-echinenone	24.9	463	Nc	0	551	533 $[M+H-18]^+$, 495, 459 $[M+H-92]^+$, 203	19.87 ± 0.9
17	*Cis*-echinenone	27.1	344, 452	Nc	19	551	533 $[M+H-18]^+$, 495, 459 $[M+H-92]^+$, 203	15.70 ± 0.1
18	All-*trans*-α-carotene	29.1	420, 445, 473	63	0	537	444 $[M–92]^+$	2.30 ± 0.5
19	All-*trans*-β-carotene	33.8	425, 451, 478	30	0	537	457 $[M+H-80]^+$, 444 $[M–92]^+$, 399 $[M-137]^+$, 177	70.22 ± 0.4
20	9-*cis*-β-carotene	35.8	340, 420, 447, 473	27	7	537	457 $[M+H-80]^+$, 444 $[M-92]^+$, 399 $[M-137]^+$, 177	8.00 ± 0.1
	Total carotenoids							183.03 ± 0.9

[a] Numbered according to the chromatogram 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 (AB) and the middle absorption peak (II).
[f] n=3 (dry weight).
[g] nc: not calculated.

Peak 17 was identified as an echinenone *cis*-isomer and demonstrated the same MS features as peak 16 (all-*trans*-echinenone). Considering the UV-Vis spectra characteristics, the lack of spectral fine structure was also related to the presence of conjugated carbonyl group and *cis*-configuration was assigned considering the hypsochromic shift and presence of *cis* peak when compared to that of the all-*trans*. As expected mass spectra of both isomers obtained in the positive ion mode showed the protonated molecule at *m/z* 551 and fragment ions in the MS/MS at *m/z* 533 $[M+H-18]^+$ and *m/z* 459 $[M+H-92]^+$, corresponding to the loss of one water molecule and toluene from the polyene chain, respectively.

In the analyzed circumstances, the chromatogram did not show the presence of all-*trans*-violaxanthin but its *cis*-isomer was detected. This can be attributed probably due the low stability demonstrated by this xanthophyll that can be isomerized during the extraction procedure. The isomer was identified as 9-*cis*-violaxanthin taking into account UV-Vis characteristics and elution order on C30 column compared with literature.

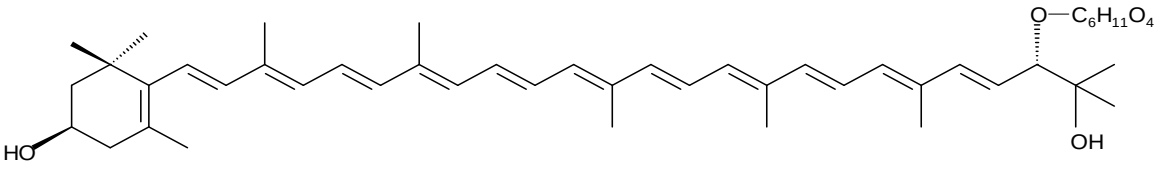
In the mass spectra of lutein, all isomers (*cis* and *trans*) demonstrate the characteristic higher intensity of fragment at *m/z* 551 $[M+H-18]^+$ than that of the protonated molecule at *m/z* 569 (molecular weight 568 Da), due the presence of the hydroxyl group in allylic position to the double bond in ε-ring (De Rosso and Mercadante, 2007). In contrast, the fragment at *m/z* 569 $[M+H]^+$ was more intense than the fragment at *m/z* 551 $[M+H-18]^+$ in all zeaxanthin isomers, which indicates that the hydroxyl group is not allylic to the double bond. Since mass spectra characteristics of isomers are similar, this tool is not able to differentiate geometrical isomers. Lutein isomers that elute before than the all-*trans* in a C30 column, and showed hypsochromic shifts and *cis* peak intensity of 7 and 5 nm and 50 and 47, respectively, were tentatively

identified as 13-*cis*-lutein (peak 4) and 13'-*cis*-lutein (peak 5). The lutein molecule is not symmetrical and possesses pairs of geometrical isomers 13 and 13' (Zechmeister, 1944). The distinction between the pair of stereoisomers 13 and 13' cannot be accomplished only by the UV-Vis and MS spectra. Thus, the assignment of the isomerization position of these carotenoids was tentatively identified. Zeaxanthin *cis*-isomer that elute before its relative all-*trans*-isomer was identified as 13-*cis* isomer, and the one that eluted after their respective all-*trans* in a C30 column, showed smoothest *cis* peak intensity than that the other isomer the slighter hypsochromic shift of its absorption maxima (445 nm), was assigned as 9-*cis*-zeaxanthin. The distinction among these isomers was possible, considering the elution order in a C30 column, as well as the intensity of the additional absorption band (*cis* peak), which is greater as the isomerization is nearer the center of the molecule (Zechmeister, 1944).

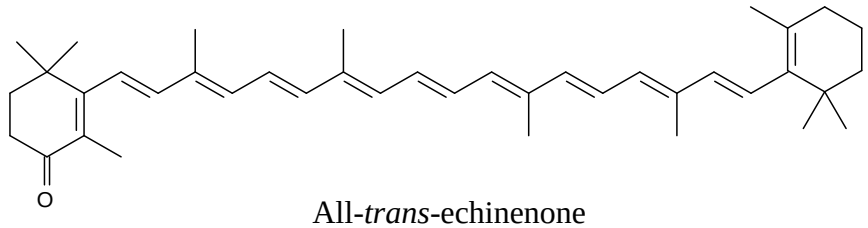
The peak 18 was identified as all-*trans*-α-carotene through chromatographic behavior on reversed phase, co-elution with authentic standard, and mass spectrum. The mass spectra showed the protonated molecule at *m*/*z* 537 and fragment ion in the MS/MS spectra at *m*/*z* 444, corresponding to the loss of the toluene. Lutein is one the oxygenated forms of α-carotene and was positively identified as the third major carotenoid in *Phormidium* extracts, besides the identification of the monohydroxy α-carotene derivative all-*trans*-zeinoxanthin.

Considering the quantitative profile, all-*trans*-β-carotene (70.22 $\mu g.g^{-1}$, peak 19) was dominant in the carotenoid extract of *Phormidium* sp. followed by all-*trans*-zeaxanthin (26.25 $\mu g.g^{-1}$, peak 8,) and all-*trans*-lutein (21.92 $\mu g.g^{-1}$, peak 7) which represented all together 65% (w/w) of the total carotenoid content. Moreover, cyanobacteria can synthesize some unique types of carotenoids, and the *Phormidium* extract showed the presence of characteristic ketocarotenoids and glycosylated

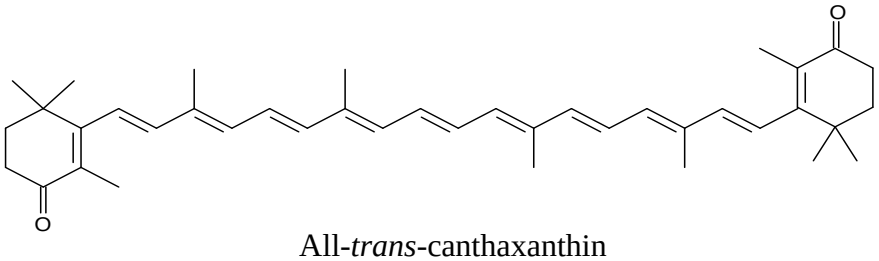
carotenoids in cyanobacteria such as all-*trans*-canthaxanthin (0.93 μg.g^{-1}, peak 11), all-*trans*-myxoxanthophyll (3.15 μg.g^{-1}, peak 14) and all-*trans*-echinenone (19.87 μg.g^{-1}, peak 16). The chemical structure of these characteristic carotenoids in cyanobacteria is shown in Figure 2.



All-*trans*-myxoxantophyll



All-*trans*-echinenone



All-*trans*-canthaxanthin

Figure 2 Structures of some carotenoids produced by the *Phormidium* sp.

The total carotenoid content was 183.03 μg.g^{-1} dry biomass and represents a substantial value when compared with the carotenoid content from other sources such as fruits and vegetables (Rodriguez-Amaya et al., 2008).

Pigment productivity is the main criterion for selection of production systems. Since carotenoids are intracellular products, overall carotenoids productivity is the product of carotenoids content multiplied by biomass productivity. In this sense, the microalgal biomass productivity in the cultivations on wastewater was 630 g/m^3.d, resulting in a total carotenoid production of 0.12g/m^3.d.

3.2 Sensitivity analyses

The biomass can be considered the first bio-product of microalgal biorefinery and the productivity of heterotrophic growth *Phormidium* sp. in different industrial capacities is shown in Table 2. Industrially, depending of the size of process plant it's possible to produce 5,690, 56,902 and 569,016 $ton_{biomass}$/year in a small (100m^3/d), medium (1,000m^3/d) and large industry (10,000m^3/d), resulting in a total carotenoids production of up to 107,902.5 kg/year. Individually, this represent a production (Table 3) of the majoritarian carotenoids: all-trans-β-carotene (397.1 to 39,717.3 kg/year), all-trans-zeaxanthin (150.6 to 15,061.8 kg/year), all-*trans*-lutein (140.8 to 14,083.1 kg/year), all-*trans*-echinenone (127.3 to 12,728.9 kg/year), *cis*-echinenone (99.9 to 9,991.9 kg/year), 9-*cis*-β-carotene (49.4 to 4,944.7 kg/year) and all-*trans*-zeinoxanthin (34.5 to 3,453.9 kg/year).

Table 2 Mass balance for microalgal biomass production in different industrial capacities

Industrial Capacity (m^3/d)	Production (ton/year)
100	5,690
1,000	56,902
10,000	569,016

Table 3 Mass balance for microalgal biomass production in different industrial capacities

Carotenoid	Industrial Capacity		
(kg.$year^{-1}$)	100 (m^3.d^{-1})	1000 (m^3.d^{-1})	10000 (m^3.d^{-1})
Total carotenoids	1,079.0	10,790.3	107,902.5
All-*trans*-β-carotene	397.1	3,971.7	39,717.3
All-*trans*-zeaxanthin	150.6	1,506.2	15,061.8
All-*trans*-lutein	140.8	1,408.3	14,083.1
All-*trans*-echinenone	127.3	1,272.9	12,728.9
Cis-echinenone	99.9	999.2	9,991.9

9-*cis*-β-carotene	49.4	494.5	4,944.7
All-*trans*-zeinoxanthin	34.5	345.4	3,453.9

These substantial productions potentially promote the commercial exploitation, the *Phormidium* biomass cultivated in agroindustrial wastewater could be considered a promising emerging platform of waste-pigment-utilization.

4. Conclusion

The detailed carotenoid composition of *Phormidium* sp. extracted by UAE was determined by HPLC-DAD-MS/MS for the first time and allowed the separation of 20 and identification of 18 carotenoids. In this study, the carotenoid production by *Phormidium* sp. was supported by the conversion of agro-industrial wastewater components into biomass heterotrophically. Using the established conditions it was possible to achieve the wastewater treatment with simultaneously production of biomass and their bioproducts. Sensitivity analysis demonstrated that substantial production of β-carotene, lutein, zeaxanthin, and echinenone, and that the total carotenoid production could reach values of 107,902.5 kg/year^{-1} for large industries. Therefore, the co-production of this high added value bioproduct can contribute to an overall increase of the economic feasibility of the whole integrated system following the biorefineries strategy. *Phormidium* sp. microalgal biomass is shown as an alternative, renewable and inexpensive source to obtain carotenoids with potential to be explored for a wide range of applications and a feedstock that can be best applied in industrial purposes.

Acknowledgements

The authors are grateful to the National Academic Cooperation Program PROCAD/CAPES for the financial support.

References

Albrecht, M., Takaichi, S., Steiger, S., Wang, Z., & Sandmann, G. (2009). Degradation compounds of carotenoids formed during heating of a simulated cashew apple juice. *Nature*, *18*, 843-846.

Borowitzka, M. A. (2013). High-value products from microalgae-their development and commercialization. *Journal of Applied Phycology*, *25*, 743-756.

Brennan, L., & Owende, P. (2010). Biofuels from microalgae - a review of technologies for production, processing, and extractions of biofuels and co-products. *Renewable and Sustainable Energy Reviews*, *14*, 557-577.

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

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.

Dufossé, L., Galaup, P., Yaron, A., Arad, S. M., Blanc, P., Murthy, K. N. C., & Ravishankar, G. A. (2005). Microorganisms and microalgae as sources of pigments for food use: a scientific oddity or an industrial reality? *Trends in Food Science & Technology*, *16*, 389-406.

Hertzberg, S., & Liaaen-Jensen, S. (1966). The carotenoids of blue green algae-I. The carotenoids of *Oscillatoria Rubescens* and an *Athrospira* sp. *Phytochemistry, 5*, 557-563.

IEA Bioenergy (2009). *Biorefineries: co-production of food, feed, chemicals, materials, fuels, power and heat from biomass*. Cape Town: IEA Bioenergy.

Jacob-Lopes, E., & Franco, T. T. (2010). Microalgae-based systems for carbon dioxide sequestration and industrial biorefineries. In M. N. B. Momba (Ed.), *Biomass* (pp. 135-146). Rijeka: Sciyo.

Jacob-Lopes, E., & Franco, T. T. (2013). From oil refinery to microalgal biorefinery. *Jornal of CO_2 Utilization, 2,* 1-7.

Jez, J. M., & Noel, J. P. (2000). A kaleidoscope of carotenoids. *Nature, 18,* 825-826.

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 & Biotechnology, 28,* 1781-1790.

Octave, S., & Thomas, D. (2009). Biorefinery: toward an industrial metabolism. *Biochimie, 91,* 659-664.

Ohara, H. (2003). Biorefinery. *Applied Microbiology and Biotechnology, 62*, 474-477.

Orchard, S., Al-Lazikani, B., Bryant, S., Clark, D., Calder, E., Dix, I., Engkvist, O., Forster, M., Gaulton, A., Gilson, M., Glen, R., Grigorov, M., Hammond-Kosack, K., Harland, L., Hopkins, A., Larminie, C., Lynch, N., Mann, R., Murray-Rust, P., Lo Piparo, E., Steinbeck, C. Henning D., Overington, J., & Thornton J. (2011). Minimum information about a bioactive entity (MIABE). *Nature Reviews Drug Discovery, 10,* 661-669.

Perez-Garcia, O., Escalante, F. M. E., de-Bashan, L. E., & Bashan, Y. (2011). Heterotrophic cultures of microalgae: metabolism and potential products. *Water Research, 45,* 11-36.

Powls, R., & Britton, G. (1976). A carotenoid containing violaxanthin, isolated from *Scenedesmus Obliquos* D_3. *Biochimica et Biophysica Acta, 453*, 270-276.

Queiroz, M. I., Hornes, M. O., Manetti, A. G. S., Zepka, L. Q., & Jacob-Lopes, E. (2013). Fish processing wastewater as a platform of the microalgal biorefineries. *Biosystems Engineering, 115*, 195-202.

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.

Rodriguez-Amaya, D. B., Kimura, M., Godoy, H. T., & Amaya-Farfan, J. (2008). Updated Brazilian database on food carotenoids: factors affecting carotenoid composition. *Journal of Food Composition and Analysis, 21*, 445-463.

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.

Zechmeister, L. (1944). Cis-trans isomerization and stereochemistry of carotenoids and diphenyl-polyenes. *Chemical Reviews, 34*, 267-344.

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.

Web references

Guiry, M. D., & Guiry, G. M. (2013) AlgaeBase: World-wide electronic publication, National University of Ireland, Galway. Retrieved 14 October 2013, from http://www.algaebase.org.

CAPÍTULO 3

SCAVENGING CAPACITY OF PIGMENTS FROM MICROALGAE AGAINST PEROXYL RADICALS

Em fase de revisão para ser submetido à revista Phytochemistry[1]

[1]O manuscrito foi formatado conforme as normas exigidas pela Revista.

Scavenging capacity of pigments from microalgae against peroxyl radicals

RODRIGUES D.B.[1], RODRIGUES, E.[2], MERCADANTE, A.Z.[3] JACOB-LOPES E.[1], ZEPKA L.Q.[1]

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

[2] Food Science and Technology Institute, Federal University of Rio Grande do Sul (UFRGS), Porto Alegre, 91501-970, Brazil.

[3]Department of Food Science, University of Campinas (UNICAMP), P.O. Box 6121, Campinas 13083-862, Brazil

*****Corresponding author: e-mail: lqz@pq.cnpq.br; Fax: 55-55-32208353; Phone: 55-55-32208254

Abstract

The profile of the three classes of pigments (carotenoids, chlorophylls and phycobiliproteins) and the peroxyl radical scavenger capacity of extracts from cyanobacteria *Phormidium* sp. was determined. Twenty-four carotenoids, three phycobiliproteins and two chlorophylls were identified. The major carotenoids of biomass were all-*trans*-β-carotene (225.44 $\mu g.g^{-1}$), all-*trans*-lutein (117.56 $\mu g.g^{-1}$) and all-*trans*-zeaxanthin (88.46 $\mu g.g^{-1}$). Furthermore, characteristic carotenoids in cyanobacteria such as echinenone (79.07 $\mu g.g^{-1}$), canthaxanthin (1.89 $\mu g.g^{-1}$) and myxoxanthophyll (18.83 $\mu g.g^{-1}$) were found. The chlorophyll *a* (2.700 $\mu g.g^{-1}$) and C-phycocyanin (2.05×10^5 $\mu g.g^{-1}$) were predominant pigments in the other fractions of color compounds from biomass. The carotenoid and chlorophyll extracts were shown to be potent scavengers of peroxyl radical, being almost 28 and 85 times more potent than α-tocopherol respectively, and for phycobiliproteins, the antioxidant capacity was 274 $\mu mol\ trolox.g^{-1}$ (dry wt).

Keywords: microalgae, carotenoid, chlorophyll, phycocyanin, peroxyl radical

Introduction

Cyanobacteria is a morphological diverse and widespread class of photosynthetic prokaryotes microorganisms (Hashtroudi et al., 2013). They have a structural complexity unusual for prokaryotes and gather the plant-like photosynthetic ability with the microbial capacity to produce desired compounds under controlled conditions and with high growth rates (Gupta et al., 2013). This determines that by means of solar energy these microorganisms can convert CO_2 and nutrients into biomass and produce valuable organic compounds, in a sustainable way (Jacob-Lopes and Franco, 2013).

Among these compounds, natural pigments comprise one of the most interesting components produced in cyanobacteria-based systems (Queiroz et al., 2013). Cyanobacterial pigments enable a highly efficient energy harvesting and trapping in photosynthesis and share the property of absorb light thanks to their characteristic conjugated systems of double bonds (Britton, 2009). Despite that the cyanobacteria mainly possess chlorophylls, important accessory pigments such as phycobiliproteins and carotenoids are also present in thylakoids membranes to relief in protect and enhance the light harvesting (Sloth et al., 2006; Yen et al., 2013).

In the last decades there has been an increase in knowledge of cyanobacteria, concerning to their biological significance, especially as a reservoir of new metabolites with therapeutic and bioregulatory potential (Singh et al., 2011). Cyanobacterial pigments display unique characteristics and have large industrial and biotechnological significance (Borowitzka, 2013). Carotenoids and phycocyanins have physicochemical properties that allow them to participate in a series of reactions and play essential role in trigger a series of activities in cell level and food systems. They have also been associated with a wide range of beneficial effects reviewed by Fiedor and Burda (2014) and Eriksen (2008). These actions is

in part related to their capacity to modulate oxidation reactions, through the scavenging of reactive oxygen species (ROS), and strong antioxidant activities *in vitro* and *in vivo* in animal models have been reported (Bhat and Madyastha, 2000; Heydarizadeh et al., 2013). Faced the worldwide demand for antioxidants to be applied in food, cosmetics and other oxidizable goods, the search for natural alternative sources increases yearly and microalgal biotechnology have gained a prominent place as a pathway for the sustainable production of these compounds (Guedes et al., 2011). In this sense, the aim of this study was to investigate the profile of carotenoids, chlorophylls and phycobiliproteins and the peroxyl radical scavenger capacity from *Phormidium* sp.

Results and Discussion

The pigment constituents of *Phormidium* sp. biomass consisted of three classes of compounds, carotenoids, chlorophylls and phycobiliproteins (Table 1).

The Figure 1 shows the HPLC chromatogram profile of the carotenoid extract from *Phormidium* sp. biomass, and the amounts of the twenty four carotenoids re presented (Table 1). The all zeaxanthin *cis*-isomers (peak 7 and 11) the fragment at *m/z* 569 [M + H]$^{+}$ was more intense than the fragment at *m/z* 551 [M + H – 18] $^{+}$, which indicates that the hydroxyl group is not allylic to the double bond. The tentative identification of zeaxanthin-*cis*-isomers was performed based on spectral features and chromatographic elution on a C_{30} column. Zeaxanthin *cis*-isomer that elute before its relative all-*trans*-isomer was identified as 13-*cis* isomer, and that one who elute after their respective all-*trans* in a C_{30} column, showed smoothest *cis* peak intensity that the other isomer and the slight hypsochromic shift of its absorption maxima (445 nm), be assigned as 9-*cis*-zeaxanthin.

Peak 12 was identified as all-*trans*-canthaxanthin, considering the lack of spectral fine structure due their two carbonyl groups in the conjugated double bonds (c.d.b.) system, λ max at 472 nm associated with this long chromophore (13 c.d.b.) and a protonated molecule at *m/z* 565.

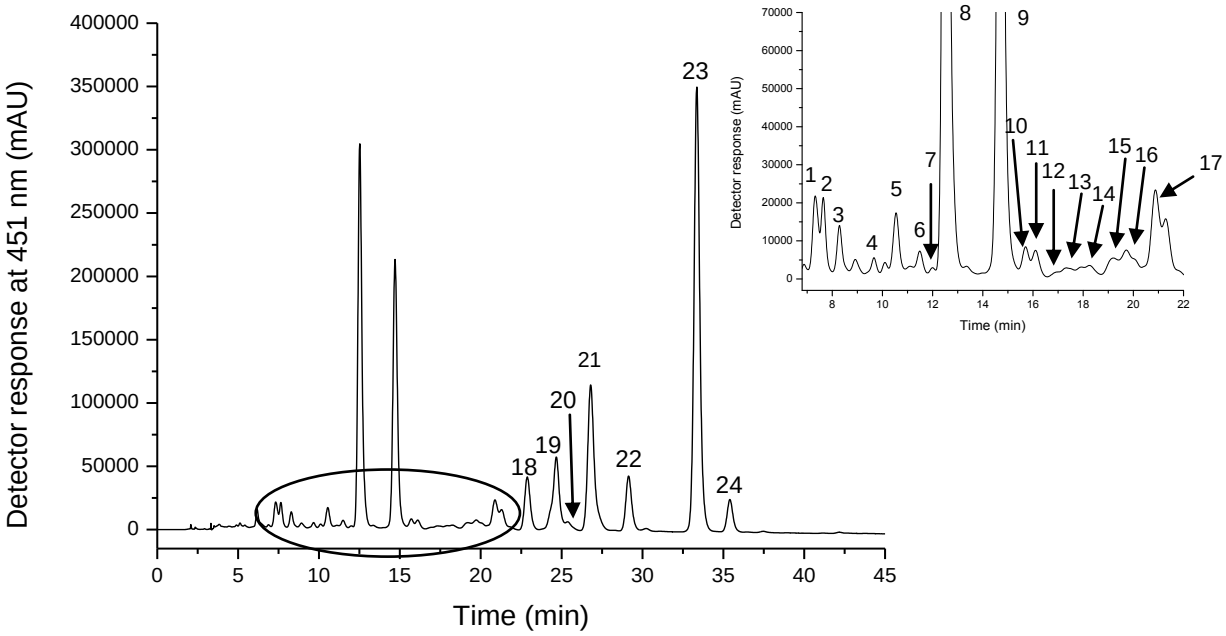


Figure 1. Chromatogram, obtained by HPLC-DAD, of the carotenoid extract from *Phormidium* sp. biomass. See text for chromatographic conditions. Peak identification and characterization is given in Table 1. Chromatogram was processed at 451 nm.

All-*trans*-myxoxanthophyll (peak 17), a major carotenoid glycoside distributed in cyanobacteria and exclusive them (Takaichi and Mochimaru, 2007), was tentatively identified based on its UV-Vis characteristics (the longest λmax at 505 nm consistent with a chromophore of 12 c.d.b., of which only one is in the ring, III/II ratio 50) and mass spectroscopic data (protonated molecule at *m/z* 731).

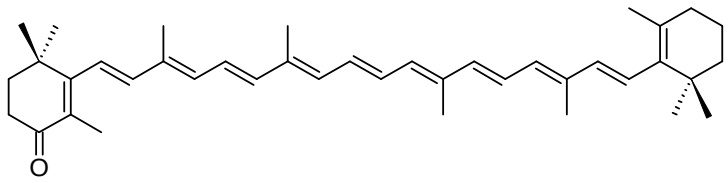
Table 1 Characterization of pigments in *Phormidium* sp. microalgal biomass.

Peak[a]	Pigment	t_R[b] (min)	UV-Vis characteristics			Fragment ions (positive mode) *(m/z)*		Pigment content ($\mu g.g^{-1}$ dw)[f]
			$\lambda_{máx}$[c] (nm)	III/II[d] (%)	AB/II[e] (%)	$[M+H]^+$	MS/MS	
				Carotenoids*				
1	13-*cis*-neoxanthin	7.3	326, 413, 437, 465	70	35	601	583 $[M+H-18]^+$, 565 $[M+H-18-18]^+$, 509 $[M+H-92]^+$, 491$[M+H-18-92]^+$, 221	5.33 ± 0.3
2	All-*trans*-neoxanthin	7.6	415, 439, 468	78	0	601	583 $[M+H-18]^+$, 565 $[M+H-18-18]^+$, 509 $[M+H-92]^+$, 491$[M+H-18-92]^+$, 221	3.51 ± 0.3
3	9-*cis*-neoxanthin	8.3	327, 414, 435, 464	75	29	601	583 $[M+H-18]^+$, 565 $[M+H-18-18]^+$, 509 $[M+H-92]^+$, 491 $[M+H-18-92]^+$, 221	5.19 ± 0.3
4	ni[g]	10.1	421, 444, 471	30	0	545	421, 395	1.18 ± 0.2
5	9-*cis*-violaxanthin	10.5	329, 419, 440, 465	70	9	601	583 $[M+H-18]^+$, 565 $[M+H-18-18]^+$	6.58 ± 0.7
6	13-*cis*-lutein	11.5	330, 416, 437, 464	35	46	569	551 $[M+H-18]^+$, 533 $[M+H-18-18]^+$, 495 $[M+H-18-56]^+$, 477 $[M+H-92]^+$, 459 $[M+H-106]^+$	3.16 ± 0.4
7	13-*cis*-zeaxanthin	12.0	334, 421, 440, 471	nc[h]	40	569	551 $[M+H-18]^+$, 533, 495, 477 $[M+H-92]^+$, 459 $[M+H-106]^+$	0.17 ± 0.1
8	All-*trans*-lutein	12.5	420, 444, 472	59	0	569	551 $[M+H-18]^+$, 533 $[M+H-18-18]^+$, 495 $[M+H-18-56]^+$, 477 $[M+H-92]^+$, 459 $[M+H-106]^+$	128.56 ± 0.5
9	All-*trans*-zeaxanthin	14.7	425, 450, 477	30	0	569	551 $[M+H-18]^+$, 533 $[M+H-18-18]^+$, 495 $[M+H-18-56]^+$, 477 $[M+H-92]^+$, 459 $[M+H-106]^+$	96.74 ± 0.4
10	9-*cis*-lutein	15.7	330, 419, 441, 468	50	5	569	551 $[M+H-18]^+$, 533 $[M+H-18-18]^+$, 495 $[M+H-18-56]^+$, 477 $[M+H-92]^+$	3.08 ± 0.3
11	9-*cis*-zeaxanthin	16.1	338, 420, 445 472	20	nc	569	551 $[M+H-18]^+$, 533 $[M+H-18-18]^+$, 495 $[M+H-18-56]^+$, 477 $[M+H-92]^+$, 459 $[M+H-106]^+$	1.10± 0.1
12	All-*trans*-canthaxanthin	17.2	472	nc	0	565	547 $[M+H-18]^+$	1.89 ± 0.2
13	Cis-carotenoid	17.9	330, 416, 444, 468	20	26	555	537	1.69 ± 0.3
14	*Cis*-carotenoid	18.3	339, 420, 442,	36	21	567	535, 444	1.96 ± 0.7

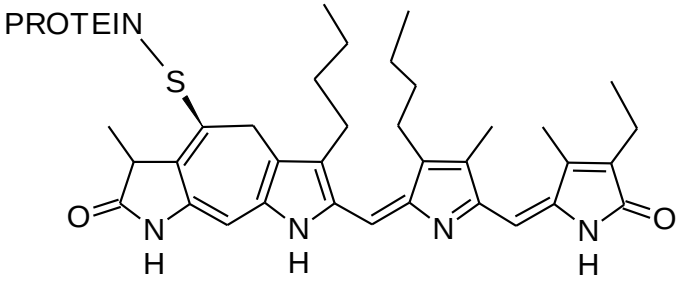
			465					
15	ni	19.2	420, 442, 468	80	0	567	551,535	2.56 ± 0.1
16	*Cis*-carotenoid	19.7	345, 421, 446, 471	30	25	569	551 $[M+H-18]^+$, 533 $[M+H-18-18]^+$, 495, 477 $[M+H-92]^+$, 459	3.50 ± 0.6
17	All-*trans*-myxoxantophyll	20.9	449, 474, 505	50	0	nd	550, 476	18.83 ± 0.5
18	All-*trans*-zeinoxanthin	22.9	420, 448, 473	48	0	553	535 $[M+H-18]^+$, 461 $[M+H-92]^+$, 361	25.81 ± 0.9
19	All-*trans*-echinenone	24.7	462	nc	0	551	533 $[M+H-18]^+$, 495, 459 $[M+H-92]^+$, 203	36.09 ± 0.7
20	15-*cis*-β-carotene	25.4	337, 420, 449, 471	5	50	537	457 $[M+H-80]^+$, 444 $[M–92]^+$, 399 $[M-137]^+$, 177	1.80 ± 0.3
21	*Cis*-echinenone	26.8	344, 452	Nc	18	551	533 $[M+H-18]^+$, 495, 459 $[M+H-92]^+$, 203	79.07 ± 0.9
22	All-*trans*-α-carotene	28.7	420, 444, 472	63	0	537	444 $[M–92]^+$	27.27 ± 0.8
23	All-*trans*-β-carotene	33.4	425, 451, 478	20	0	537	457 $[M+H-80]^+$, 444 $[M–92]^+$, 399 $[M-137]^+$, 177	246.54 ± 0.5
24	9-*cis*-β-carotene	35.4	340, 420, 447, 473	33	7	537	457 $[M+H-80]^+$, 444 $[M-92]^+$, 399 $[M-137]^+$, 177	12.73 ± 0.3
				Chlorophylls**				
1	Chlorophyll a	18.0	429, 660	na [k]	na		nd	2,700 ± 0.01
2	Chlorophyll b	7.2	460, 647	Na	na		nd	700 ± 0.01
				Phycobiliproteins***				
	C-PC	na	620	Na	na		na	2.05×10^5 ± 1.1
	C-APC	na	650	Na	na		na	100 ± 0.2
	C-PE	na	540	Na	na		na	9.2×10^3 ± 0.4
				Total pigments				
	Total carotenoids							714.31 ± 0.9
	Total chlorophylls							3,400 ± 0.1
	Total phycobiliproteins							2.14×10^5 ± 0.5

[a] Numbered according to the chromatogram 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 (AB) and the middle absorption peak (II). [f] n=3 (dry weight). [g] ni: not identified. [h] nc: not calculated. [i] nd: not detected. [j] not determined. [k] na: not applied. *Date referent to HPLC-DAD-MS/MS chromatogram showed in Figure 1. ** Date referent to HPLC-DAD chromatogram showed in Figure 2. *** Results from spectrophotometric analysis.

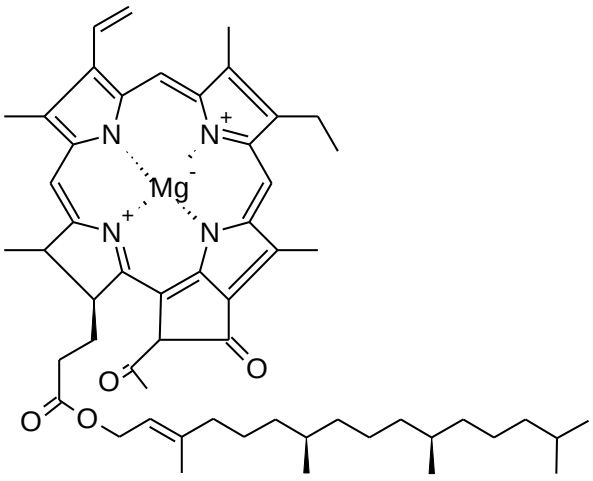
Peak 21 was identified as an echinenone *cis*-isomer and demonstrated the same MS features as peak 19 (all-*trans*-echinenone, Figure 2). Echinenone and echinenone *cis*-isomer are ketocarotenoids, which have one carbonyl group attached to the conjugated double bond system, extending the molecule chromophore to 12. Considering the UV-Vis spectra characteristics exhibit lack of spectral fine structure related to the presence of carbonyl group and hypsochromic shift and *cis* peak when compared to that of the all-*trans*. The mass spectra of the isomer obtained in the positive ion mode showed the protonated molecule at *m/z* 551 and fragment ions in the MS/MS at *m/z* 533 [M + H − 18] $^{+}$ and *m/z* 459 [M + H – 92] $^{+}$, corresponding to the loss of one water molecule and toluene group from the polyene chain, respectively.



All-*trans*-echinenone



C-phycocyanin



Chlorophyll a

Figure 2. Some characteristics structures of pigments present in *Phormidium* sp. microalgal biomass.

The total carotenoid content of *Phormidium* sp. grown under phototrophic conditions was 714.3 ± 0.9 $\mu g.g^{-1}$ (dry wt). The major carotenoids were all-trans-β-carotene (34.5%), all-*trans*-lutein (18.0%) and all-*trans*-zeaxanthin (13.5%), totally 66.0% (471.4 $\mu g.g^{-1}$). In addition to these carotenoids widely distributed in nature, interesting atypical structures were found in the carotenoid profile of *Phormidium* sp., such as all-*trans*-canthaxanthin (0.26%), all-*trans*-myxoxanthophyll (2.64%), all-*trans*-echinenone (11.07%), and echinenone *cis*-isomer, this value reaches 19.0% of total (135.7 $\mu g.g^{-1}$).

The complementary ca. 15% (107.2 $\mu g.g^{-1}$) was constituted by traces of other all-*trans* carotenoids (e.g., neoxanthin, zeinoxanthin, α-carotene) and mono-*cis* isomers (mainly 9-, 13-) of neoxanthin, violaxanthin, zeaxantin, lutein and β-carotene .

Additionally, the chromatogram of chlorophyll extract from *Phormidium* biomass is shown in Figure 3. Chlorophyll *b* (peak 1) showed UV-visible spectra (460 and 647nm) similar to those presented by Marquez and Sinnecker (2008) and Kamffer et al. (2010). The peak 2 was identified as chlorophyll *a*. The UV-visible spectrum, λ_{max} at 425 and 660 nm, was also similar to the data from the literature (Marquez and Sinnecker, 2008; Kamffer et al., 2010). In addition, the identity of both chlorophylls was confirmed by coelution with the standards. Chlorophyll *a* and chlorophyll *b* are distinguishable by their typical spectral properties. Since chlorophyll molecule contains a closed circuit of ten conjugated double bounds to absorb light spectrophotometrically (UV-Vis) are satisfactory to identify and estimate amounts of chlorophyll *a* and chlorophyll *b*. Spectrophotometric determinations reported in literature is that chlorophylls strongly absorb at 500 to 700 nm in the visible region and show a large typical band around 400 nm (Marquez and Sinnecker, 2008). The chlorophyll content indicate that the level of chlorophyll *a* was higher than that of chlorophyll *b* (2700 $\mu g.g^{-1}$ and 700 $\mu g.g^{-1}$, respectively).

Finally, in terms of phycobiliproteins, the results showed that *Phormidium* sp. contains 2.05×10^5 μg.g^{-1} (95.8%) of C-PC, 100.0 μg.g^{-1} (0.01%) of C-APC, and 9.2×10^3 μg.g^{-1} (4.19%) of C-PE. The purity of C-PC in the extract is commonly reported by a relative number that describes the ratio between absorbances from phycocyanobilin at 620 nm and all proteins in the extract at 280 nm (Bhaskar et al., 2005). The A_{620}/A_{280} ratio was 4.8 and according to Boussiba and Richmond (1979) C-PC extracts with purity numbers greater than 4.0 are described as analytical grade.

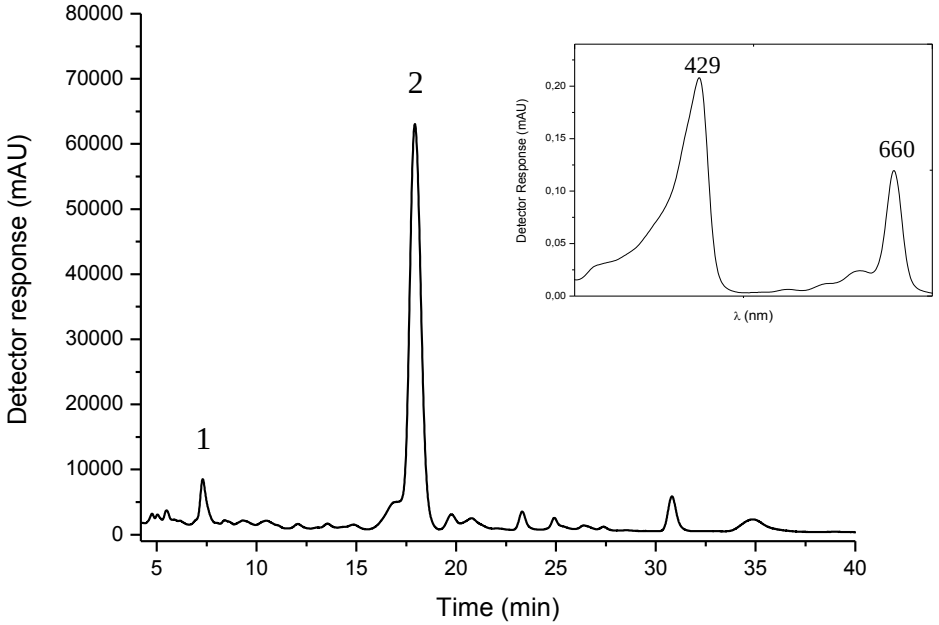


Figure 3. Chromatogram, obtained by HPLC-DAD, of the chlorophyll extract from *Phormidium* sp. biomass. Inset: Chlorophyll *a* spectrum. See text for chromatographic conditions. Peak identification and characterization is given in Table 1. Chromatogram was processed at 436 nm.

In terms of antioxidant capacity of the pigments extracts, the Table 2 shows the scavenger capacity against peroxyl radicals by hydrophilic and lipophilic extracts of *Phormidium* sp.

The carotenoid extract from *Phormidium sp.* was able to scavenge $ROO^{\bullet}$ and the net AUC values were linearly dependent to total carotenoid concentration ($r^2 > 0.99$) (data not shown). The carotenoid extract of *Phormidium* sp. was 28 (28.1 ± 0.2) times more potent scavenger of peroxyl radicals than α-tocopherol. The high antioxidant capacity of the *Phormidium* sp. carotenoid extract suggests the possible involvement of differentiated structures or other lipophilic compounds produced by cyanobacteria in the scavenging capacity. According to the structure activity relationship proposed by Rodrigues et al. (2012), the main features of carotenoid structure that allowed an increase of the capacity to scavenge $ROO^{\bullet}$ this method is chromophore length. Some carotenoids of the *Phormidium* sp. displayed longer conjugated double bond systems in its chemical structure, namely myxoxanthophyll, echinenone and canthaxanthin, with 12, 12 and 13 conjugated double bonds, respectively. Once in contact with radicals, the increase of the carotenoid c.d.b. system with the maximum overlap of the molecular orbitals promotes the formation of more resonance-stabilized products (Albrecht et al., 2000; Miller et al., 1996). Furthermore, a positive effect on the antioxidant capacity can be possible 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 et al., 1989), thus increasing the ability to stabilize the radical in question. The possible mechanism that may be involved in the scavenging of peroxyl radicals by carotenoids comprises radical addition to the polyene chain creating carotenyl adduct radicals, electron transfer with formation of carotenoid radical cations and abstraction of allylic hydrogen with formation of neutral radicals (El-Agamey et al., 2004; Skibsted, 2012). A combination of multiple variables, such as the polarity of the medium, the carotenoid structure and nature of the radical determines the preferred mode of antioxidant action (Mortensen and Skibsted, 2000).

Table 2 Scavenger capacity against peroxyl radicals by hydrophilic and lipophilic extracts of *Phormidium* sp. biomass.

Sample	$ROO^{\bullet}$	
	Hydrophilic [a]	Lipophilic [b]
Carotenoid Extract	nd [c]	28.1 ± 0.2
Chlorophyll Extract	nd	84.9 ± 0.7
C-PC Extract	237.4 ± 0.7	nd

[a] Micromoles of trolox equivalent per gram of *Phormidium* sp. biomass.
[b] α-tocopherol relative.
[c] not determined.

Another class pigments assessed by the peroxyl radicals scavenger capacity for lipophilic extracts was the chlorophyll. The choice of this assay is a function of lipophilic chlorophyll profile found in extract (Figure 3 and Table 1). The chlorophyll was able of scavenging peroxyl radical, being almost 85 times more potent than α-tocoferol (Table 2). Despite of the chlorophylls being the most abundant pigments in nature, their antioxidant capacity is not well studied. The scarcity of information could be attributed mainly to the difficulty to obtain chlorophylls and their derivatives in a purified form.

Finally, the fluorescence decay resulting from $ROO^{\bullet}$-induced oxidation of fluorescein probe in the absence of antioxidants (blank assay) and in the presence of phycobiliprotein extract from *Phormidium* sp. are showed in Figure 4a. In Figure 4b it is possible to visualize the typical analytical curve of trolox standard in the ORAC assay. The *in vitro* scavenging capacity against peroxyl radicals of phycobiliprotein extract from *Phormidium* sp. was 237.4 ± 0.7 μmol trolox equivalents.g^{-1} (Table 2). Bhat and Madyastha (2000) and Lissi et al. (200) related that their results indicate that the chromophore and not the apoprotein is mainly involved in the radical scavenging properties of C-PC. This corroborated with results from Piñero-Estrada et al. (2001). The chromophore fraction (bilin) is structurally close to the bile pigments bilirubin and biliverdin known important biological antioxidants. Stocker et al.,

(1987) described that bilirubin scavenges $ROO^{\bullet}$ radicals by donating a hydrogen atom attached to the C-10 bond of the tetrapyrrole molecule to form a carbon-centered radical with resonance stabilization through the whole bilirubin molecule. The scavenging capacity of C-PC against $ROO^{\bullet}$ was pointed out to be the responsible for hepatoprotective effects (Bhat and Madyastha, 2000). Their structure also have been described as effective singlet oxygen quenchers (Di Mascio et al., 1989) and alkoxyl, hydroxyl and peroxyl radicals scavenger, besides to react with peroxynitrite ($ONOO^{-}$) and hypochlorous acid (HOCl) (Romay et al., 2003). Despite the bilin chromophores are considered to be the main targets of free radicals, evidence exists that apo-biliproteins also exhibit anti-oxidant activities (Guan et al., 2009; Pleonsil at al., 2013).

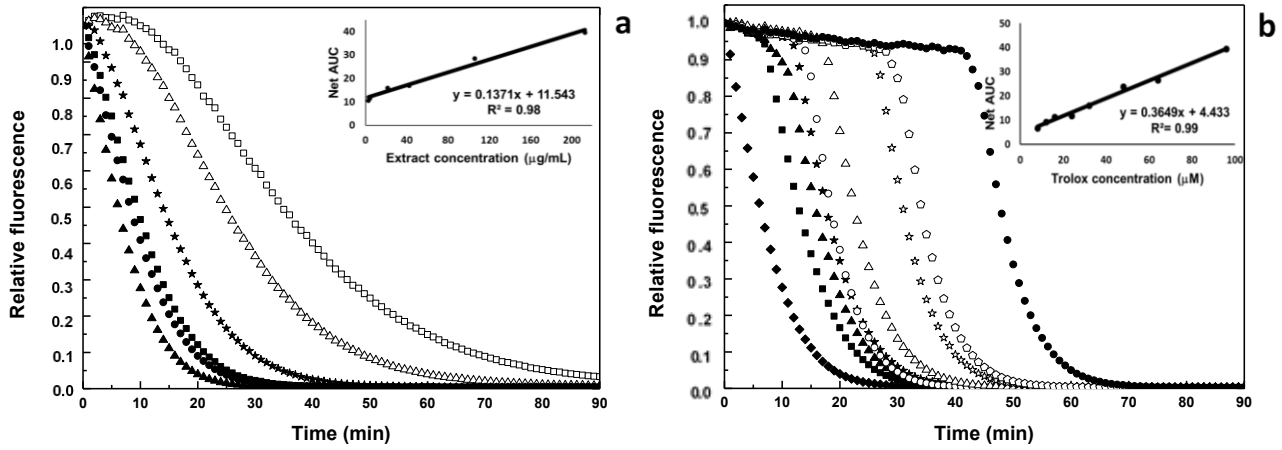


Figure 4. Fluorescence decay of fluorescein induced by peroxyl radicals in the presence of different concentrations of **(a)** phycobiliprotein hydrophilic extracts from *Phormidium* sp. and **(b)** trolox standard. Legend (a): blank (filled triangle), 2.3 μg/mL (filled circle), 4.3 μg/mL (filled square), 21.3 μg/mL (filled star), 106.0 μg/mL (open triangle) and 213.1 μg/mL (open square). Legend (b) blank (filled diamond), 8 μM (filled square), 12 μM (filled triangle), 16 μM (filled star), 24 μM (open circle) and 32 μM (open triangle), 48 μM (open star), 64 μM (open pentagon) and 96 μM (filled circle). Inset: linear relationship between (a) hydrophilic extract and (b) trolox standard concentrations and net AUC values from the fluorescence decay curves of fluorescein oxidation.

Based on these results, *Phormidium* sp. biomass could be considered a promising emerging platform to explore bioactive compounds. The biotechnology route overcome several drawbacks of the conventional systems, with high potential of sustainable production.

Conclusion

Phormidium sp. microalgal biomass presented the ability to produce a meaningful content of carotenoids, chlorophylls and phycocyanins under photoautotrophic conditions, indicating the potential of *Phormidium* sp. biomass as a renewable source of these pigments. Considering the different solubilities of the pigments synthetized by *Phormidium*, appropriated methods to assess the scavenging capacity of both lipophilic and hydrophilic peroxyl radicals was used. Thus, this study provides an actual contribution of the carotenoids, chlorophylls and phycocyanins on the antioxidant capacity in cyanobacteria against a biological relevant radical. Considering that many other bioactive compounds are present in microalgal cells, one of the main advantages of the use of microalgae biomass as a carrier of carotenoids, chlorophylls and phycocyanins is their positive impact on human health.

Experimental

Chemicals

Synthetic standards of all-*trans*-lutein, all-*trans*-zeaxanthin, all-*trans*-β-carotene, all-*trans*-α-carotene, and all-*trans*-zeinoxanthin, Trolox (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid), fluorescein, AAPH (α,α′-azodiisobutyramidine dihydrochloride), azobisisobutyronitrile (AIBN), dimethyl sulfoxide (DMSO), methyl tert-butyl ether (MTBE), methanol and dichloromethane were purchased from Sigma-Aldrich (St. Louis-MO, USA).

The fluorescent probe 4,4-difluoro-5-(4-phenyl-1,3-butadienyl)-4-bora-3a,4a-diaza-s-indacene-3-undecanoic acid (C_{11}-BODIPY$^{581/591}$, MW = 504.43 g.mol^{-1}) was purchased from Invitrogen (Carlsbad-CA, USA).

Microorganisms and culture media

Axenic cultures of *Phormidium* sp. were originally isolated from the Cuatro Cienegas desert (26º59'N, 102º03'W-Mexico). Stock cultures were propagated and maintained in solidified agar-agar (20 g/L) containing synthetic BG11 medium (Rippka et al., 1979). The incubation conditions used were 25 ºC, a photon flux density of 15 µmolm^{-2} s^{-1} and a photoperiod of 12/12 hours light/dark.

Microalgal biomass production

The biomass production was carried out in bubble column photobioreactor (Jacob-Lopes et al., 2010) operating in intermittent regime, fed with 2.0 L of BG11 medium (Ripka et al. 1979). The experimental conditions were as follows: initial concentration of inoculum of 100 mg/L, temperature of 25ºC, aeration of 1 volume of air per volume of medium per minute, with the injection of air enriched with 3.0% (v/v) of carbon dioxide, a photon flux density of 150 µmolm^{-2} s^{-1}, a light cycle of 24:0 (day:night) and a residence time of 168 h. The biomass was separated from the medium by centrifugation. It was subsequently freeze-dried for 24 h at −50 °C above -175 µmHg. The cultivations were performed twice, and in duplicate. Therefore, experimental data refer to the mean value of four repetitions.

Analytical methods

Carotenoid extraction

The carotenoids were exhaustively extracted from the freeze-dried sample (0.1 ± 0.02 g) with ethyl acetate and methanol in a mortar with a pestle followed by centrifugation (Hitachi, Tokyo, Japan) for 7 min at 3500 rpm (Mandelli et al., 2012). The extraction procedure was repeated until the supernatant become 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), resuspended 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.

HPLC-DAD-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 DAD detector (model SPD-M20A) and a mass spectrometer with an ion-trap analyzer and atmospheric pressure chemical ionization (APCI) source (model Esquire 4000, Bruker Daltonics, Bremem, Germany). The carotenoid separation was performed on a C_{30} YMC column (5 μm, 250 × 4.6 mm) (Waters, Wilmington-DE, USA). HPLC-DAD-MS/MS parameters were set as previously described by De Rosso and Mercadante (2007). The mobile phase consisted in a mixture of methanol 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 (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 et al., 2004; De Rosso & Mercadante, 2007; van Breemen et al., 2012). The carotenoids were also quantified by HPLC-PDA, using five-point analytical curves of all-*trans*-violaxanthin (0.7-13.6 μg/mL), all-*trans*-zeaxanthin (1.3-59.7 μg/mL), all-*trans*-lutein (1.0-59.5 μg/mL), and all-*trans*-β-carotene (1.1-30.2 μg/mL). All other xanthophyll and carotene contents were estimated using the curve of all-*trans*-lutein and all-*trans*-β-carotene, respectively. The *cis*-isomers were estimated using the curve of the corresponding all-*trans*-carotenoid. Total carotenoid content was calculated as the sum of the contents of each individual carotenoid separated on the C_{30} column.

Chlorophyll extraction

Chlorophyll was exhaustively extracted from the freeze-dried sample (0.2 ± 0.02 g) with acetone in a mortar with a pestle followed by centrifugation (Hitachi, Tokyo, Japan) for 10 min at 20000 rpm. The extraction procedure was repeated until the supernatant become colorless. The extract was partitioned in a mixture of petroleum ether/diethyl ether [1:1 (v/v)], and acetone was removed by washing with distilled water. The extract was concentrated in a rotary evaporator (T < 30 °C), flushed with N_2 and kept at -18 °C in the dark. The determination of the total chlorophyll concentration in the extract was determined spectrophotometrically (Molecular Devices Corp, Los Angeles-CA, USA) and calculated according Porra et al., 1989. In order to separate carotenoids from the chlorophyll, the samples was submitted to preparatory open column chromatography. Separation of the extract was carried out on a 25 ×300 mm glass column packed to a height of about 150 mm with MgO:Hyflosupercel (1:1) activated for 4 h at 110 °C. The carotenoids were eluted with a

gradient of petroleum ether with increasing concentrations of acetone and chlorophyll fraction was obtained in ethanol. The separation can be followed visually. The solvent was evaporated under N_2 flow, ressuspended in acetone and once quantified spectrophotometrically. This value calculated was subsequently used as a parameter to calculate the concentration of samples for peroxyl radical scavenging assay. The extract was partitioned in petroleum ether/diethyl ether [1:1 (v/v)], concentrated in a rotary evaporator (T < 30 °C), flushed with N_2 and kept at -18 °C in the dark until peroxyl radical scavenging assay and chromatographic analysis.

HPLC-DAD chlorophyll analysis

The chlorophyll was 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 DAD detector (model SPD-M20A). The carotenoid separation was performed on a C_{18} NST column (5 μm, 150 × 4.4mm) (Nano Separation Technologies, Sao Paulo, Brazil). HPLC-DAD parameters were set as previously described by Sartory (1985). The mobile phase consisted in binary solvent system. Solvent A consisted of 97% methanol and solvent B 97% acetone. Solvent delivery was programmed at 100% A for 15 min, then a linear gradient to 77% A:23% B at 20 min, then isocratical until 60 min. 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, compared with data available in the literature (Kamffer et al., 2010; Marquez and Sinnecker, 2008).

Peroxyl radical scavenging assay for lipophilic extracts

The antioxidant capacity assay was carried out according to Rodrigues et al. (2012) for lipophilic extracts. 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 concentrations (18.1, 21.7, 47.0, 86.8 and 137.4 μM for carotenoids and 15.8, 25.4, 50.8, 95.3 and 127.1 μM for chlorophylls). After evaporation under N_2 flow, they were redissolved in DMSO/MTBE (10:1, v/v) and homogenized. The assays were performed in a microplate reader (Synergy Mx Biotek, Winooski-VT, USA). The $ROO^{\bullet}$ scavenging capacity was measured by monitoring the effect of the carotenoid extract or α-tocopherol standard on the fluorescence decay resulting from the $ROO^{\bullet}$-induced oxidation of the C_{11}-$BODIPY^{581/591}$ probe. $ROO^{\bullet}$ was generated by thermal decomposition of AIBN at 41 ± 0.5 °C. Reaction mixtures in the wells contained the following reagents at the indicated final concentrations (final volume of 200 μL): 0.18 μM C_{11}-$BODIPY^{581/591}$ in DMSO, 175 mM AIBN in DMSO/MTBE (10:1, v/v) and the carotenoid extract (2.0, 2.4, 5.9, 9.6 and 15.3 μM) or chlorophyll extract (1.7, 2.8, 5.6, 10.6 and 14.1 μM) both dissolved in DMSO. The fluorescence measurements were expressed as relative fluorescence, using the fluorescence signal measured after 1 min of incubation as the initial reference, and measuring each 2 min until 181 min. The $ROO^{\bullet}$ scavenging capacity was calculated and expressed as a relative value that denotes how many times the extract is more efficient than α-tocopherol. α-tocopherol (252 and 504 μM) was used as positive control (Net AUC 4.60 ± 0.21 and 8.24 ± 0.48 respectively). The results correspond to two independent experiments performed in triplicate.

Phycobiliprotein extraction and ammonium sulfate fractionation

Freeze-dried biomass (1.0 ± 0.2 g) was extracted with 50 mM sodium phosphate buffer pH 6.8 in a mortar with a pestle followed by filtration. The filtrate containing phycocyanin was kept. Subsequently, to ensure complete recovery of the pigment, the biomass residue was submitted to repeated freezing and thawing cycles in the same buffer in the dark followed by centrifugation for 10 min at 20000 rpm at 4 °C (Hitachi, Tokyo, Japan), until the supernatant become colorless. The supernatant was pooled with the filtrate, constituting the extract of phycocyanin, and stored at -4 °C. The crude phycocyanin obtained was precipitated with ammonium sulfate 20% (m/v). The resulting solution was kept for 24 h and centrifuged at 20000 rpm for 10 min to allow the separation of remaining cell debris and proteins from the crude extract. The supernatant was subjected to 70% ammonium sulfate saturation, kept for 36 h and centrifuged again under the same conditions. The supernatant was discarded and the blue precipitate was resuspended in 20 mL of 50 mM sodium phosphate buffer (pH 6.8). The total purification procedure was carried out in the dark at 4 °C. The phycocyanin extract recovery (%) was calculated according Soni et al. (2006) and the purity of the pigment was assessed by calculating the ratio against the absorbance of total protein (280 nm), phycoerythrin (540 nm) and allophycocyanin (650 nm). The amount of phycobiliproteins was calculated according Bennett and Bogorad (1973). The UV-Vis absorption of C-PC and fluorescence emission and excitation spectra were obtained on a SpectraMax M5 (Molecular Devices Corp, Los Angeles-CA, USA). Data are the means of triplicate measurements from three independent experiments.

Peroxyl radical scavenging assay for hydrophilic extracts

The antioxidant capacity assay was carried out according the ORAC method developed by Ou et al. (2001) for hydrophilic samples. The $ROO^{\bullet}$ scavenging capacity was

measured by monitoring the effect of the phycocyanin extracts on the fluorescence decay resulting from $ROO^{\bullet}$-induced oxidation of fluorescein probe. $ROO^{\bullet}$ was generated by thermal decomposition of AAPH at 37 °C. Briefly, 25 μL of phycocyanobilin extract in phosphate buffer were placed in a 96-well microplate containing 150 μL of fluorescein (81 nM in 75 mM phosphate buffer, pH 7.4). The mixture was preincubated for 10 min at 37 °C followed by addition of 25 μL of AAPH (19 mM). The fluorescence signal was registered every min until 90 min in a SpectraMax M5 (Molecular Devices Corp, Los Angeles-CA USA) or until or until reach 0.5% of the initial fluorescence signal. The fluorescence was monitored for the emission wavelength at 528 ± 20 nm with excitation at 485 ± 20 nm. The relative fluorescence versus time was recorded and the area under curve (AUC) of the sample and of the blank were calculated. The results were expressed in μmol Trolox equivalent.g^{-1} of dry wt.

Statistical analysis

The data were analyzed using descriptive statistics through software Statistica 7.0 (StatSoft, Tulsa-OK,USA).

Acknowledgements

The authors are grateful to the National Academic Cooperation Program PROCAD/CAPES for the financial support.

References

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*. Nat. Biotechnol., 18, 843–846.

Bennett, A., Bogorad L., 1973. Complementary chromatic adaptation in a filamentous blue-green alga. J. Cell. Biol., 58, 419–435.

Bhaskar, S.U., Gopalaswamy, G., Raghu, R., 2005. A simple method for efficient extraction and purification of C-phycocyanin from *Spirulina platensis* Geitler. Indian J. Exp. Biol., 43, 277–279.

Bhat, V.B., Madyastha, K.M., 2000. C-phycocyanin: a potent peroxyl radical scavenger in vivo and in vitro. Biochem. Biophys. Res. Commun., 275, 20–25.

Boussiba, S., Richmond, A.E., 1979. Isolation and characterization of phycocyanins from the blue-green alga *Spirulina platensis*. Arch. Microbiol., 120, 155–159.

Borowitzka, M.A., 2013. High-value products from microalgae—their development and commercialisation. J. Appl. Physiol., 25, 743-756.

Britton, G., 2009. The biochemistry of natural pigments, first ed. Cambridge University Press, Cambridge.

Britton, G., Liaaen-Jensen, S., Pfander, H. (Eds.), 2004. Carotenoids: handbook. Birkhäuser Verlag, Badel.

de Rosso, V.V., Mercadante, A.Z., 2007. Identification and quantification of carotenoids, by HPLC-PDA-MS/MS, from Amazonian fruits. J. Agric. Food Chem., 55, 5062–5072.

Di Mascio, P., Kaiser, S., Sies, H., 1989. Lycopene as the most efficient biological carotenoid singlet oxygen quencher. Arch. Biochem. Biophys., 274, 532–538.

El-Agamey, A., Lowe, G.M, McGarvey, D.J, Mortensen. A., Phillip, D.M., Truscott, T.G., Young, A.J., 2004. Carotenoid radical chemistry and antioxidant/pro-oxidant properties. Arch. Biochem. Biophys, 430, 37–48.

Eriksen, N.T., 2008. Production of phycocyanin--a pigment with applications in biology, biotechnology, foods and medicine. Appl. Microbiol. Biotechnol., 80, 1–14.

Fiedor, J., Burda, K., 2014. Potential role of carotenoids as antioxidants in human health and disease. Nutrients, 6, 466–488.

Guan, X.Y., Zhang, W.J., Zhang, X.W., Li, Y.X., Wang, J.F., Lin, H.Z., Tang, X.X., Qin, S., 2009. A potent anti-oxidant property: fluorescent recombinant alpha-phycocyanin of Spirulina. J. Appl. Microbiol., 106, 1093–1100.

Guedes, A.C., Amaro, H.M., Malcata, F.X., 2011. Microalgae as sources of carotenoids. Mar. Drugs., 9, 625–644.

Gupta, V., Ratha, S.K., Sood, A., Chaudhary, V., Prasanna, R., 2013. New insights into the biodiversity and applications of cyanobacteria (blue-green algae)—Prospects and challenges. Algal Research, 2, 79–97.

Hashtroudi, M.S., Shariatmadari, Z., Riahi, H., Ghassempour, A., 2013. Analysis of *Anabaena vaginicola* and *Nostoc calcicola* from Northern Iran, as rich sources of major carotenoids. Food Chem., 136, 1148–1153.

Heydarizadeh, P., Poirier, I., Loizeau, D., Ulmann, L., Mimouni, V., Schoefs, B., Bertrand, M., 2013. Plastids of marine phytoplankton produce bioactive pigments and lipids. Mar. Drugs., 11, 3425–3471.

Jacob-Lopes, E., Franco, T.T., 2013. From oil refinery to microalgal biorefinery. Journal of CO_2 Utilization, 2, 1–7.

Jacob-Lopes, E., Scoparo, C.H., Queiroz, M.I., Franco, T.T., 2010. Biotransformations of carbon dioxide in photobioreactors. Energy Convers. Manage., 51, 894–900.

Kamffer, Z., Bindon, K.A., Oberholster, A., 2010. Optimization of a method for the extraction and quantification of carotenoids and chlorophylls during ripening in grape berries (*Vitis vinifera* cv. Merlot). J. Agric. Food. Chem., 58, 6578–6586.

Lissi, E.A., Pizarro, M., Aspee, A., Romay, C., 2000. Kinetics of phycocyanine bilin groups destruction by peroxyl radicals. Free Radical Biol. Med., 28, 1051–1055.

Mandelli, F., Miranda, V.S., Rodrigues, E., Mercadante, A.Z., 2012. Identification of carotenoids with high antioxidant capacity produced by extremophile microorganisms. World J. Microb. Biot., 28, 1781–1790.

Miller, N.J., Sampson, J., Candeias, L.P., Bramley, P.M., Rice-Evans, C.A., 1996. Antioxidant activities of carotenes and xanthophylls. FEBS Lett., 384, 240–242.

Mortensen, A., Skibsted L.H., 2000. Kinetics and mechanism of the primary steps of degradation of carotenoids by acid in homogeneous solution. J. Agric. Food. Chem., 48, 279−286.

Ou, B., Hampsch-Woodill, M., Prior, R.L., 2001. Development and validation of an improved oxygen radical absorbance capacity assay using fluorescein as the fluorescent probe. J. Agric. Food. Chem., 49, 4619−4626.

Piñero-Estrada, J.E., Bermejo-Bescós, P., Villar del Fresno, A.M., 2001. Antioxidant activity of different fractions of *Spirulina platensis* protean extract. Farmaco., 56, 497–500.

Pleonsil, P., Soogarun, S., Suwanwong, Y., 2013. Anti-oxidant activity of holo- and apo-C-phycocyanin and their protective effects on human erythrocytes. Int. J. Biol. Macromol., 60, 393–398.

Porra, R.J., Thompson, W.A., Kriedemann, P.E., 1989. Determination of accurate extinction coefficients and simultaneous equations for assaying chlorophylls *a* and *b* extracted with four different solvents: verification of the concentration of chlorophyll standards by atomic absorption spectroscopy. Biochim. Biophys. Acta, 975, 384–394.

Rippka, R., Deruelles, J., Waterbury, J.B., Herdman, M., Stanier, R.Y., 1979. Generic assignments, strain histories and properties of pure cultures of cyanobacteria. J. Gen. Microbiol., 111, 1–61.

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 Chem., 135, 2103–2111.

Singh, R.K., Tiwari, S.P., Rai, A.K., Mohapatra, T.M., 2011. Cyanobacteria: an emerging source for drug discovery. J. Antibiot., 64, 401–12.

Skibsted, L.H., 2012. Carotenoids in antioxidant networks. Colorants or radical scavengers. J. Agric. Food. Chem., 60, 2409–2417.

Sloth, J.K., Wiebe, M.G., Eriksen, N.T., 2006. Accumulation of phycocyanin in heterotrophic and mixotrophic cultures of the acidophilic red alga *Galdieria sulphurariai*. Enzyme Microb. Technol., 38, 168–175.

Soni, B., Kalavadia, B., Trivedi, U., Madamwar, D., 2006. Extraction, purification and characterization of phycocyanin from *Oscillatoria quadripunctulata*—Isolated from the rocky shores of Bet-Dwarka, Gujarat, India. Process Biochem., 41, 2017–2023.

Stocker, R., Yamamoto, Y., McDonagh, A.F., Glazer, A.N., Ames, B.N., 1987. Bilirubin is an antioxidant of possible physiological importance. Science, 235, 1043–1046.

Takaichi, S., Mochimaru, M., 2007. Carotenoids and carotenogenesis in cyanobacteria: unique ketocarotenoids and carotenoid glycosides. Cell. Mol. Life Sci., 64, 2607-2619.

van Breemen, R.B., Dong, L., Pajkovic, N.D., 2012. Atmospheric pressure chemical ionization tandem mass spectrometry of carotenoids. Int. J. Mass Spectrom., 312, 163–172.

Yen, H.W., Hu, I.C., Chen, C.Y. Ho, S.H., Lee. D.J., Chang, J.S., 2013. Microalgae-based biorefinery – from biofuels to natural products. Bioresour. Technol., 135, 166–174.

CONCLUSÃO GERAL

A produção de biomassa de *Phormidium* sp. a partir das rotas heterotrófica e fotossintética indicou a potencialidade de exploração de compostos bioativos a partir destas plataformas de produção.

A biomassa produzida heterotroficamente a partir de efluentes líquidos agroindustriais contêm 20 diferentes carotenóides, sendo o all-*trans*- β-caroteno (70,22 $\mu g.g^{-1}$), a all-*trans*-zeaxantina (26,25 $\mu g.g^{-1}$) e a all-*trans*-luteína (21,92 $\mu g.g^{-1}$) os mais relevantes quantitativamente. Adicionalmente, carotenoides característicos de microalgas como all-*trans*-equinenona (19,87 $\mu g.g^{-1}$), *cis*-equinenona (15,70 $\mu g.g^{-1}$), all-*trans*-mixoxantofila (3,15 $\mu g.g^{-1}$) e all-*trans*-cantaxantina (0,93 $\mu g.g^{-1}$) foram evidenciados.

As análises de sensibilidade demonstraram que substanciais produções destes pigmentos naturais podem ser obtidas por processos de biorrefinaria microalgal, com produções anuais de até 107.902,5 kg de carotenoides totais em paralelo a representativas produções individuais dos carotenoides majoritários da biomassa.

Quanto considerada a biomassa produzida fotossinteticamente, a partir do dióxido de carbono, foram evidenciados a ocorrência de três classes de pigmentos naturais: as ficobiliproteínas ($2{,}14\times10^{5}$ $\mu g.g^{-1}$), as clorofilas (3400 $\mu g.g^{-1}$) e os carotenoides (714,3 $\mu g.g^{-1}$).

Os extratos destes pigmentos demonstraram elevado potencial para desativação de radicais peroxila. Os extratos lipofílicos de carotenoides e clorofilas mostraram capacidade de desativar o radical peroxila 28 e 85 vezes maior que o padrão α-tocoferol, respectivamente. Adicionalmente, o extrato hidrofílico de ficocianina apresentou uma atividade antioxidante de 237 μM trolox /g de biomassa (base seca).

Globalmente, a biomassa da cianobactéria *Phormidium* sp. apresentou potencial de exploração como plataforma de biorrefino de moléculas de elevado valor agregado, aplicáveis extensivamente como intermediários e/ou produtos finais de inúmeros consumíveis.