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**ESTUDO DO POTENCIAL BIOATIVO DA FRAÇÃO CLOROFILADA
DA BIOMASSA DE *PHORMIDIUM AUTUMNALE***

DISSERTAÇÃO DE MESTRADO

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**Santa Maria, RS, Brasil
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**ESTUDO DO POTENCIAL BIOATIVO DA FRAÇÃO CLOROFILADA
DA BIOMASSA DE *PHORMIDIUM AUTUMNALE***

Gabriela Predebon Nogara

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

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BIOMASSA DE *PHORMIDIUM AUTUMNALE***

elaborada por
Gabriela Predebon Nogara

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

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Resumo

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

ESTUDO DO POTENCIAL BIOATIVO DA FRAÇÃO CLOROFILADA DA BIOMASSA DE *PHORMIDIUM AUTUMNALE*

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Microalgas são fontes reconhecidas de metabólitos com alto valor agregado, em grande parte pela atividade biológica destes compostos. Diversas espécies de cianobactérias produzem metabólitos secundários com caráter bioativo em função de sua estrutura molecular. Atualmente estuda-se muito a respeito de pigmentos pelo seu valor biológico, dando-se ênfase em sua bioatividade, porém a clorofila ainda é pouco elucidada. Apesar de seu importante papel biológico, as pesquisas sobre clorofila e seu potencial antioxidante e pró-oxidante ainda são escassas. A escassez de informações pode ser atribuída à dificuldade de se obter clorofilas e seus derivados em uma forma purificada e também à sua instabilidade, o que desencadeia um processo de degradação. Muitas incertezas ainda existem no campo científico sobre a clorofila natural, seus metabólitos, impacto sobre a saúde, devido seu alto conteúdo em alguns vegetais, ocorrência generalizada na natureza, e sua degradação durante o processamento de alimentos e digestão humana. A produção de compostos clorofilados pelas microalgas, em função de seu metabolismo diversificado, torna a biomassa microalgal uma fonte alternativa, com estruturas químicas diferenciadas quando comparada às matrizes alimentícias convencionais, sendo que seu sistema complexo de ligações conjugadas em anéis pirrólicos possibilita um caráter antioxidante, ou pró-oxidativo, atuando no sequestro de radicais livres ou desencadeando sistemas reacionais de degradação molecular. Neste sentido, o estudo destes pigmentos clorofilados visando um maior enfoque em suas estruturas químicas poderá auxiliar na elucidação do real potencial bioativo destas substâncias. Diante das diversas estruturas clorofiladas presentes no extrato de *Phormidium autumnale*, com diferentes polaridades, é fundamental escolher o método adequado para a avaliação do potencial antioxidante, além de investigar o mecanismo antioxidante desenvolvido por cada estrutura da molécula. Um total de

onze compostos clorofilados foram separados na biomassa de *Phormidium autumnale* no cultivo fotossintético. Entre os compostos majoritários foram encontrados clorofila a (1498.40µg / g), e feofitina b (613.23µg / g), onde os pigmentos totais clorofilados totalizaram 2720.19µg / g no extrato de biomassa. O potencial de eliminação de radicais livres foi analisado por dois métodos diferentes no que diz respeito à sua polaridade, sendo que para o método lipofílico o extrato de *Phormidium autumnale* mostrou-se 200 vezes mais potente do que o α -tocoferol na eliminação de radicais peroxil, e para o método hidrofílico o extrato de *Phormidium autumnale* apresentou atividade antioxidante de 219,82 mµ Trolox equivalente / g de extrato. Finalmente, observamos que a biomassa de *Phormidium autumnale* apresenta um potencial considerável na produção de compostos clorofilados em condições fototróficas, provando ser uma fonte alternativa para matrizes convencionais de pigmentos de clorofila, bem como se mostrou eficiente na eliminação de radicais livres, evidenciando seu potencial bioativo.

Palavras-Chave: clorofila, cultivo fototrófico, microalgas, compostos bioativos

Abstract

Master Dissertation
Post-Graduate Program in Food Science and Technology
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POTENTIAL STUDY OF FRACTION BIOACTIVE CHLOROPHYLLATED OF BIOMASS *PHORMIDIUM AUTUMNALE*

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Place and Date: Santa Maria, March 07, 2016.

Microalgae are recognized sources of metabolites with high added value, largely by biological activity of these compounds. Several species of cyanobacteria produce secondary metabolites with bioactive character due to its molecular structure. Currently studying a lot about pigments for its biological value, giving emphasis on its bioactivity, but chlorophyll is still poorly understood. Despite its important biological role, research on chlorophyll and its potential antioxidant and pro-oxidant are still scarce. The lack of information can be attributed to the difficulty of obtaining chlorophyll and its derivatives in a purified form and also to their instability, which triggers a process of degradation. Many uncertainties still exist in the scientific field of the natural chlorophyll, their metabolites, impact on health, due to its high content in some vegetables, widespread occurrence in nature, and its degradation during food processing and human digestion. The production of compounds of chlorophyll by microalgae, due to their diverse metabolism, makes biomass microalgal an alternative source, with different chemical structures as compared to conventional food matrix, and its complex system of conjugated bonds on pyrrole rings enables an antioxidant character, or pro-oxidative, acting in scavenging free radicals or reactive triggering systems of molecular degradation. In this sense, the study of these chlorophyll pigments designed to further focus on their chemical structure may help to elucidate the real potential bioactive these substances. Given the various chlorophyllous structures present in *Phormidium autumnale* extract with different polarities, it is crucial to choose the appropriate method for evaluating the antioxidant potential, and to investigate the antioxidant mechanism developed by each structure of the molecule. A total of eleven chlorophyll compounds were separated on biomass *Phormidium autumnale* the photosynthetic culture. Among the major compounds found chlorophyll a (1498.40µg / g) and pheophytin b (613.23µg / g), where the total

chlorophyll pigments totaled 2720.19 μ g / g in the biomass extract. The potential for scavenging free radicals was examined by two different methods with regard to their polarity, being that for the lipophilic method of the *Phormidium* extract *autumnale* proved to be 200 times more potent than α -tocopherol at the disposal of peroxy radicals , and the method the hydrophilic *Phormidium autumnale* extract showed antioxidant activity of 219.82 μ equivalent Trolox / g extract. Finally, we note that the biomass *Phormidium autumnale* has considerable potential in the production of chlorophyll compounds in phototrophic conditions, proving to be an alternative source to conventional arrays of chlorophyll pigments and proved efficient in eliminating free radicals, showing their potential bioactive .

Keywords: chlorophyll, autotrophic cultivation, microalgae, bioactive compounds

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INTRODUÇÃO

O cultivo de microalgas, devido seu metabolismo diversificado, torna-se atraente para a bioprospecção e exploração de fontes comerciais de alto valor, como os pigmentos naturais oriundos de seu metabolismo (BOROWITZKA, 2013; BRENNAN & OWENDE, 2010; DUFOSSE et al., 2005; JACOB-LOPES & FRANCO, 2013; OCTAVE & THOMAS, 2009; OHARA, 2003; QUEIROZ, HORNES, MANETTI, ZEPKA, & JACOB-LOPES, 2013; RODRIGUES et al., 2014). Estas moléculas provenientes do sistema metabólico das microalgas são substâncias químicas diferenciadas das demais moléculas encontradas na biomassa microalgal, que participam do processo fotossintético absorvendo energia na forma de luz e apresentam estrutura complexa pirrólica, com ligações duplas conjugadas. (SPOLAORE et al., 2006). As clorofilas, além de exercer papel fundamental no metabolismo de microalgas, representam uma grande promessa para aplicação industrial em vários setores, principalmente na área de bioativos e química fina, atuando em reações de sequestro de radicais livres (GUIRY & GUIRY, 2014).

O papel vital da molécula de clorofila está baseado na sua estrutura, que atua na absorção de energia na forma de luz, liberando-a na forma de elétrons, em diversas reações químicas que envolvam a troca de elétrons, atuando como antioxidante ou pró-oxidante. Além disso, estes compostos fotoquímicos têm efeitos importantes para a saúde humana, tais como antimutagênico e potente capacidade antioxidante para o sequestro de radicais livres e para evitar a oxidação de lipídeos (SIMONICH et al., 2007).

Além dos componentes da cadeia fotossintética, como a clorofila *a* e *b*, além da feofitina a um número de derivados de clorofila são formados em tecidos verdes como consequência do metabolismo natural ou durante o processamento. Entre os derivados de clorofila que mantêm a cadeia de fitol, existem quatro grupos principais de derivados de feofitina (feofitina *a*, feofitina *b*, hidroxifeofitina *a* e hidroxifeofitina *b*) que são formados quando o átomo de magnésio central do anel tetrapirrólico é facilmente substituído por dois hidrogênio-átomos como uma consequência do catabolismo da clorofila. O átomo de magnésio e a cauda de fitol também presentes

nesta estrutura, contribuem para a estabilidade energética da molécula de clorofila, sendo relevantes em processos de troca de elétrons e para a polaridade e miscibilidade dos compostos clorofilados em sistemas reacionais. Os compostos derivados de clorofila apresentam polaridades diferentes devido à sua estrutura molecular composta principalmente pela cauda de fitol e o íon Mg^{++} , além da estrutura do complexo pirrólico com ligações duplas conjugadas, influenciando também no caráter de absorção e emissão de energia, atuando diretamente no mecanismo de doação de elétrons, ou desencadeando reações oxidativas por fatores externos de energia. Essas peculiaridades das moléculas clorofiladas presentes na biomassa microalgal interferem na escolha do método antioxidante a ser usado, sendo fundamental priorizar métodos que contemplem o caráter hidrofílico ou lipofílico da estrutura analisada, para avaliar de forma eficiente o potencial antioxidante destas moléculas (LANFER-MARQUEZ, BARROS, SINNECKER, 2005). Estas atribuições químicas conferem a clorofila um caráter considerável de atividade biológica, sendo o enfoque do trabalho.

OBJETIVOS

Objetivo Geral

O objetivo geral do trabalho foi estudar a fração clorofilada da biomassa de *Phormidium autumnale*, através de suas estruturas moleculares, correlacionando sua estrutura química com seu potencial bioativo.

Objetivos Específicos

- Produzir biomassa de *Phormidium autumnale* em reator fototrófico;
- Identificar e quantificar a fração clorofilada à partir da biomassa de *Phormidium autumnale*;
- Determinar a atividade antioxidante na fração hidrofílica e lipofílica do extrato clorofilado da biomassa de *Phormidium autumnale*;
- Determinar a atividade antioxidante em padrões de clorofila, com caráter polar;
- Determinar a atividade antioxidante em padrões de clorofila, com caráter apolar;

CAPÍTULO 1

REVISÃO BIBLIOGRÁFICA

1. REVISÃO BIBLIOGRÁFICA

1.1 Microalgas

Microalgas são consideradas micro-organismos microscópicos fotossintéticos, podendo se proliferar em diversos habitats aquáticos, que incluem água doce e ambiente marinho, tolerando uma ampla faixa de temperatura e pH. Sob a denominação, as microalgas classificam-se como organismos com dois tipos de estrutura celular: estrutura procariótica, com representantes nas Divisões Cyanophyta (cianobactérias) e Prochlorophyta; estrutura celular eucariótica, com representantes nas divisões Chlorophyta, Euglenophyta, Rhodophyta, Haptophyta (Prymnesiophyta), Heterokontophyta (Bacillariophyceae, Chrysophyceae, Xantophyceae etc.), Cryptophyta e Dinophyta, segundo HOEK et al. (1995). 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 transporte de elétrons da cadeia respiratória quanto pela fotossíntese (SINGH et al., 2011). Cerca de 2.000 espécies de cianobactérias são conhecidas e podem ser divididos em 150 gêneros, a maioria sendo fototróficos obrigatórios (ANAGNOSTIDIS & KOMÁREK, 1985).

Além disso, as cianobactérias apresentam habilidade de armazenamento de nutrientes e uma alta produção de inúmeros metabólitos (PULZ & GROSS, 2004; PULZ et al., 2000; ANAGNOSTIDIS & KOMÁRIK, 1985). Esses microrganismos representam uma das fontes mais promissoras para novos bioprodutos, como proteínas, lipídeos, incluindo metabólitos secundários como pigmentos e alguns fármacos. (HU et al., 2013; GUEDES et al., 2011; HARUN et al., 2010; PULZ & GROSS, 2004). Um grande número de microalgas produzem pigmentos, como clorofila e carotenoides com estrutura química diferente das fontes convencionais (RODRIGUES et al., 2014).

O gênero *Phormidium* é uma cianobactéria filamentosa não ramificada e com conteúdo celular geralmente azul esverdeado, raramente marrom. Pode ser encontrada em solos, rochas úmidas, lama, plantas aquáticas, córregos, algumas em ambientes litorâneos. Outras espécies são encontradas em ambientes extremos como nascentes termais e solos de desertos (GUIRY & GUIRY, 2013; THOMAZEU et al., 2010). Por esta razão, apresentam grande potencial biocatalisador em bioprocessos, devido sua robustez e simples exigências nutricionais (AL-THUKAIR et al., 2007; GUIRIY & GUIRY, 2013). O gênero *Phormidium* apresenta a capacidade de adaptação a diferentes condições de luz, onde muitas vezes é um fator limitante das condições de habitats naturais, onde há uma variação dessa luminosidade. Compostos nitrogenados como nitrato podem ser consideradas as fontes preferidas de nitrogênio para a sua multiplicação celular.

O interesse no cultivo de microalgas é baseado na variedade de possibilidades para a sua aplicação, tais como na alimentação humana, produção de energia química e a extração de pigmentos, entre outras substâncias de valor industrial. Outro fator responsável pelo interesse nestes microrganismos é alta taxa de crescimento, determinando vantagens tecnológicas e comerciais quando comparado a técnicas convencionais de produção desses nutrientes microalgais (THAJUDDIN & SUBRAMANIAN, 2005). Estes microrganismos podem apresentar substâncias celulares (ácidos graxos, hidratos de carbono, proteínas e pigmentos), com composições variáveis e concentrações, o que reflete a natureza do organismo, a influência das condições de cultivo e do estado fisiológico de a cultura (TOKUSOGLU & ÜNAL, 2003).

O uso de microalgas para tratamento de águas residuais foi sugerido anos atrás (CALDWELL, 1946; OSWALD & GOTAAS, 1957). Estes microrganismos oferecem uma alternativa às formas convencionais dos tratamentos de águas residuais, devido à sua grande eficiência na remoção de poluentes, bem como a possibilidade de valorização dos resíduos por biotransformação em bioprodutos de valor agregado (QUEIROZ et al., 2007). Algumas espécies de microalgas atuam com o propósito de associar a produção de nutrientes a partir da bioconversão de compostos presentes no efluente, e assim empregar a biomassa gerada como complemento da dieta (JACOB-LOPES, 2006)

1.2 Metabolismo de microalgas

A produção de microalgas em larga escala tem sido estudado por décadas (BECKER, 1994; LEE, 2001), tendo em conta a grande variedade de produtos metabólicos práticos e potenciais, tais como lipídeos, enzimas, biomassa, polímeros, toxinas e pigmentos. Em condições de crescimento natural, as microalgas absorvem luz solar e assimilam o dióxido de carbono como fonte de carbono do ar e nutrientes dos habitats aquáticos. Este tipo de cultivo é denominado crescimento autotrófico, devido à capacidade fotossintética das microalgas (BRENNAN & OWENDE, 2010; PEREZ-GARCIA et al., 2011).

A fotossíntese é um processo complexo, através do qual a luz absorvida e carbono inorgânico são convertidos em matéria orgânica, contribuindo para todos os compostos orgânicos, cujo carbono é o principal elemento presente na biomassa de microalgas (incluindo cianobactérias), no valor de até 65% do peso seco (GROBBELAAR, 2004). O CO₂ é incorporado pela microalga através da reação com um açúcar de cinco carbonos conhecido como ribulose difosfato (Ciclo de Calvin), formando um composto instável de seis carbonos, que logo se quebra em duas moléculas de três carbonos (3-fosfoglicerato, conhecidas como PGA). O ciclo prossegue até que no final, é produzida uma molécula de glicose e é regenerada à molécula de ribulose difosfato (FAY, 1983).

Neste processo fotossintético, as microalgas produzem oxigênio, o que significa que utilizam a energia luminosa para extrair prótons e elétrons da água para reduzir o CO₂, a fim de formar moléculas orgânicas. A introdução de luz natural ou artificial permite o crescimento maciço de populações de microalgas (MANDALAM & PALSSON, 1998; YANG et al., 2000; SUH & LEE, 2003).

Durante a fotossíntese, as microalgas utilizam energia solar juntamente com vários nutrientes essenciais (C, N, P, S, K, Fe) para sintetizar seus compostos da biomassa e multiplicar suas células. Tais organismos precisam de quantidades específicas dos elementos essenciais para produção de biomassa, sendo que a deficiência de um dos elementos irá causar a redução do crescimento (SPAARGAREN, 1996).

Uma alternativa viável para culturas fototróficas, mas restrita a poucas espécies de microalgas, é o uso de sua capacidade de crescimento heterotrófico na ausência de luz. Neste caso, é necessária a utilização de compostos orgânicos para o crescimento da biomassa (DROOP, 1974). Além disso, algumas variedades de microalgas utilizam o meio de crescimento mixotrófico, combinando o processo fotossintético e heterotrófico. (BRENNAN & OWENDE, 2010; PEREZ-GARCIA et al., 2011) Neste caso, a fotossíntese é realizada como fonte primária de energia. No entanto, ambos os compostos orgânicos e CO₂ são essenciais. No cultivo anfitrófico os organismos são capazes de viver autotrófico ou heterotroficamente, dependendo da concentração de compostos orgânicos e de intensidade da luz disponível (MATA et al., 2010; CHOJNACKA & MARQUEZ-ROCHA, 2004).

1.3 Produtos de microalgas

O grande interesse pelo cultivo de microalgas é a capacidade de produção de metabólitos com alto valor industrial. Há dois recursos gerais a partir de microalgas que podem ser utilizados: a produção de biocombustíveis e coprodutos (BRENNAN & OWENDE, 2010; CHEN et al., 2007; SONG et al., 2008) . Além disso, podem ser utilizadas para o tratamento de águas residuais por remoção de amônia, nitrato e fosfato, por utilização desses contaminantes da água como nutrientes (WANG et al., 2008). Os biocombustíveis gerados a partir de microalgas incluem lipídeos, H₂, isoprenóides, hidratos de carbono, álcoois (diretamente ou por conversão de biomassa) e metano (a partir de digestão anaeróbia). Lipídios, principalmente na forma de triacilgliceróis (TAG), estão distribuídos na membrana celular e organelas intracelulares de microalgas, e partes destes contêm hidratos de carbono semelhantes ao petróleo.

Além de lipídeos, outros compostos com aplicações importantes em diversos setores industriais também podem ser extraídos da biomassa microalgal. Tais compostos são descritos como proteínas, açúcares, pigmentos (coprodutos), que podem ser utilizados para o setor de química fina, devido seu caráter bioativo (LI et al., 2008a, b.; RAJA et al., 2008).

Dentre as substâncias bioativas identificadas a partir de microalgas marinhas, os pigmentos receberam atenção especial devido sua atividade antioxidante, anticancerígena, anti-inflamatória, anti-obesidade e atividades neuroprotetoras (GUEDES et al., 2011; PANGESTUTI & KIM, 2011).

As três classes básicas de pigmentos encontrados nas microalgas marinhas são clorofilas, carotenoides e ficobiliproteínas. As clorofilas são substâncias lipossolúveis esverdeadas encontradas nos cloroplastos, as quais contém um anel de porfirina, capaz de captar a energia da luz para a realização de fotossíntese. A clorofila que está amplamente presente em microalgas contribui com seu potencial bioativo, podendo impedir processos oxidativos, devido à presença de um longo cromóforo de ligações duplas conjugadas, levando a estados de energia mais altos ou excitações das moléculas (MARQUEZ & SINNECKER, 2008).

2. Compostos bioativos

Uma das principais áreas de pesquisa em Ciência dos Alimentos é a tecnologia de obtenção e caracterização de novas fontes naturais com atividade biológica (RAMÍREZ et al., 2006; RODRÍGUEZ-MEIZOSO et al., 2008; YULIARI et al., 2008). Estas substâncias são preferidas pelos consumidores por terem uma origem natural, sendo comumente extraído a partir de plantas e algas, etc. (CHAROENSIDDHI & ANPRUNG, 2008; GOUVEIA et al., 2008; SHUI & LEONG, 2006). As algas são uma fonte natural de grande número de compostos bioativos provenientes de seu metabolismo secundário, que podem atuar como antioxidantes, antibacterianos, antivirais, etc. (PLAZA, CIFUENTES & IBÁÑEZ, 2008; PLAZA, HERRERO, CIFUENTES, & IBÁÑEZ, 2009).

O potencial bioativo destas microalgas deve-se a possibilidade de crescimento em diferentes condições ambientais, levando ao enriquecimento de alguns destes compostos. Esse caráter biológico está relacionado com a biodiversidade de compostos os quais as microalgas são capazes de sintetizar, de acordo com as condições do meio (RODRÍGUEZ-MEIZOSO et al., 2008). Estes mecanismos de defesa utilizados pelas microalgas podem resultar num elevado

nível de diversidade estrutural e química de compostos, provenientes de diferentes vias metabólicas (RODRIGUEZ-MEIOSO et al., 2010).

Alguns estudos mostram que as cianobactérias são fontes ricas de metabólitos secundários, representando um inexplorado recurso biológico para uma gama de diversidade desses metabólitos que podem ser polifenóis, ácidos graxos e pigmentos como carotenoides e a clorofila (CARDELLINA & MOORE, 2010).

A molécula de clorofila é conhecida pelo seu potencial bioativo, devido à sua atividade pró-oxidante e antioxidante. Segundo algumas pesquisas, o caráter pró-oxidante é explicado pelo fato deste pigmento promover e catalisar a foto-oxidação quando exposto à luz, devido às características fotossensibilizantes do anel porfirina dessa molécula que facilita a formação de oxigênio singlete, podendo desencadear o processo de autoxidação. No entanto, demais estudos demonstram um caráter antioxidante da molécula de clorofila quando esta não é exposta à luz, atuando como sequestradora de radicais livres ou radicais peroxila, inibindo o processo autoxidativo (LANFER- MARQUEZ, 2003).

Na busca de obtenção e caracterização destes compostos bioativos, a utilização de processos de extração considerados sustentáveis ambientalmente, vem sendo bastante elucidados. Nesse aspecto, a extração por ultrassom mostrou-se viável, diminuindo o tempo de extração e aumentando o rendimento (MA et al., 2008).

3. A Clorofila

Quimicamente, a clorofila não é uma molécula isolada, mas compreende uma família de substâncias semelhantes entre si, designadas de clorofila *a*, *b*, *c* e *d*. Estruturalmente são moléculas complexas, pertencentes à classe das porfirinas, formadas por 4 anéis pirrólicos e um quinto anel isocíclico, localizado ao lado do terceiro 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. No quarto anel pirrólico, o ácido propiônico ali existente é esterificado por um álcool acíclico de cadeia longa, geralmente o fitol, conferindo à clorofila um caráter hidrofóbico (GROSS, 1991; RÜDIGER & SCHOCH, 1988). A clorofila *b* difere da

clorofila *a* por uma pequena variação na substituição no anel pirrólico II. As clorofilas *c* e *d* são encontradas em algas (SCHWARTZ, LORENZO, 1990).

Grande variedade de pigmentos fotossintéticos é encontrada em algas as quais são dotadas de clorofila *a*, pigmento mais importante para a fotossíntese e que apresenta papel central no arranjo dos fotossistemas para a captação de energia luminosa. A maioria das algas dispõe de uma segunda clorofila, que pode ser a clorofila *b*, *c*, ou *d*, as quais apresentam diferenças estruturais na molécula. A maioria dos organismos fotossintéticos são capazes de utilizar essencialmente a região do espectro solar na faixa visível, estendendo-se desde 400 nm a 700 nm (BLANKENSHIP, 2002).

As clorofilas *b*, *c* e *d* (figura 1 e 2) são consideradas pigmentos acessórios da fotossíntese, pois o papel principal nos sistemas de absorção de luz é desempenhado pela clorofila *a*, cabendo às demais clorofilas aumentar o total de luz absorvido pelo organismo. A clorofila *d* pode ser encontrada em cianobactérias que vivem em condições de pouca luz. Além destas, fala-se também em clorofila do tipo *f*, que seria uma adaptação de organismos fotossintéticos para utilizar qualquer tipo de luz disponível (CALLAWAY, 2010). A Clorofila *f* foi isolada a partir de uma cianobactéria descaracterizada, que contém principalmente clorofila *a*. A evidência disponível sugere, assim, que não é clorofila *f* o principal fotopigmento (apenas cerca de 10-15% do total de clorofilas), mas funciona como um pigmento acessório de clorofila. O mesmo não é evidenciado na cianobactéria *Acaryochloris marina*, onde a clorofila *f* é predominante e responsável pela maioria das funções em reações fotossintéticas (CHEN, 2011).

A clorofila *a* é a mais difundida como fotopigmento na natureza, sendo que a clorofila *d* pode substituir na totalidade ou quase todas as funções da clorofila *a* em fotossíntese aeróbica. Este fotopigmento foi relatado pela primeira vez a partir de uma alga vermelha. No entanto, estudos demonstram alguns organismos que contém mais de 95% de clorofila *d* como seu importante fotopigmento, com apenas pequenas quantidades de Clorofila *a*. A *Acaryochloris* é uma cianobactéria fotossintética com absorção de luz deficiente na região da luz visível absorvida pela Clorofila *a* e *b*, mas relativamente enriquecido na região de luz do infravermelho (700-750 nm) absorvida então, pela clorofila *d* (CHEN, 2011). Entretanto, nem todas

algas apresentam uma segunda clorofila, de forma que diversas espécies realizam fotossíntese apenas com participação de clorofila a e outros pigmentos não clorofilianos (LOURENÇO, 2006).

Bactérias fototróficas, cianobactérias, algas, musgos, e a maioria das gimnospérmicas podem sintetizar clorofila também na ausência de luz. Estes organismos conter uma alternativa, uma enzima luz-independente (DPOR) para a conversão de uma protoclorofilida em uma Clorofilida a. A via independente de luz é a única via de bactérias fotossintéticas anoxigênicas, levando à formação de bacterioclorofilas, e a via dependente da luz é a única via em angiospermas, levando a Clorofilas a e b. Ambos os caminhos coexistem em cianobactérias, algas, musgos, e gimnospermas; estes organismos têm a vantagem de que, após eliminação de uma via (por exemplo, por mutação) o outro pode ainda manter a síntese de clorofila. Ainda não se sabe como é que cada via contribui para a formação da clorofila em condições fisiológicas normais.

Considerando que os compostos derivados de clorofila apresentam polaridades diferentes devido à sua estrutura molecular composta principalmente pela cauda de fitol e o íon Mg^{++} , que conferem à clorofila estabilidade energética e também influenciam na polaridade da estrutura, além de ser responsável pela coloração verde, no caso do íon magnésio. A estrutura do complexo pirrólico com ligações duplas conjugadas, contribui também no caráter de absorção e emissão de energia. Todos esses fatores interferem na escolha do método antioxidante a ser usado, sendo necessário então eleger métodos que contemplem o caráter hidrofílico ou lipofílico da estrutura analisada, para avaliar de forma eficiente a capacidade destas moléculas em sequestrar radicais livres e demonstrar seu poder antioxidante.

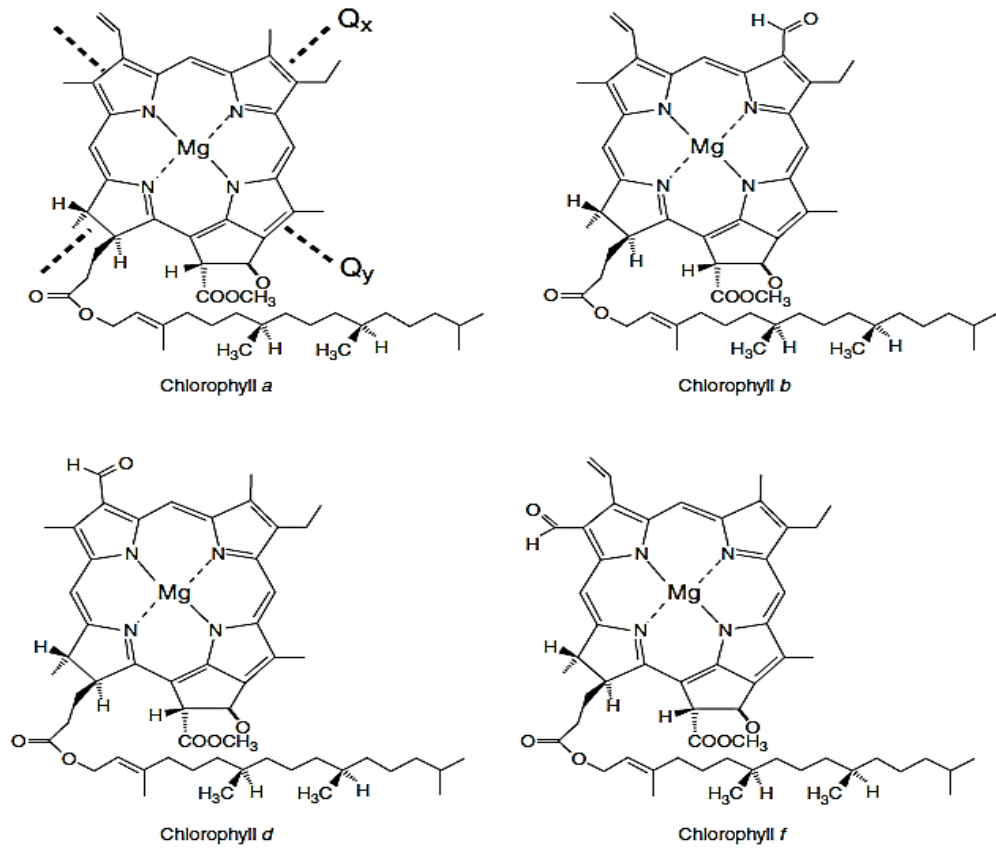


Figura 1: Estrutura química das clorofilas *a,b,d,f* (CHEN, 2011).

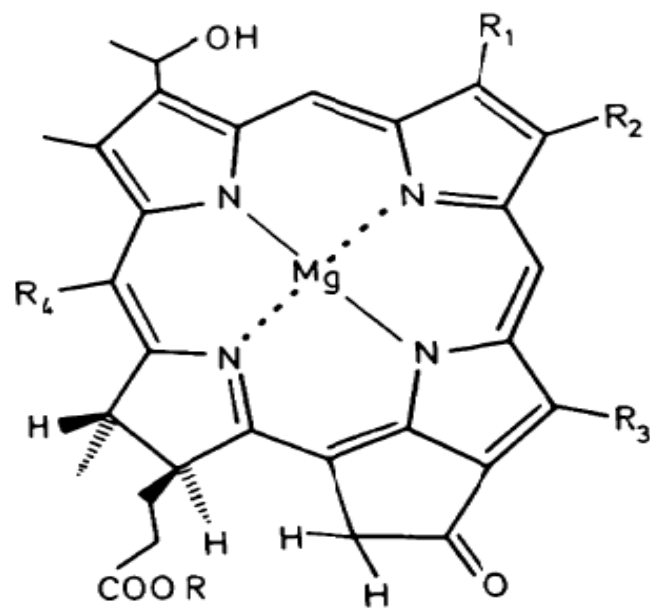


Figura2. Estrutura química da clorofila *c*

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CAPÍTULO 2

ELUCIDATION OF THE CHLOROPHYLL COMPOUNDS IN
MICROALGAL BIOMASS PHORMIDIUM AUTUMNALE AND
RESEARCH OF BIOACTIVE POTENTIAL OF THESE MOLECULES.

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Abstract

The chlorophylls were extracted by ultrasound from microalgae *Phormidium autumnale* obtaining a profile was assessed by high performance liquid chromatography coupled with a photodiode array and mass spectrometry detectors. A total of eleven chlorophylls were separated in biomass from photosynthetic cultivation. Among the majority chlorophylls were found chlorophyll *a* (1498.40 μ g/g), and pheophytin *b* (613.23 μ g/g), where the total chlorophyll pigments were found 2720.19 μ g/g in the extract of biomass to *Phormidium autumnale*. The potential for scavenging free radicals analyzed by two different methods with regard to their polarity, to *Phormidium autumnale* extract were 219.82 μ g Trolox equivalent / g extract to the method that assesses peroxy radical scavenging assay is hydrophilic extract and 200 times higher than α -tocopherol to the method evaluates the peroxy radical scavenging assay is lipophilic extract. The antioxidant activity of each extract *Phormidium autumnale* were compared with chlorophyll standards, such as chlorophyll *a* and Pheoforbide, it the chlorophyll *a* has a more lipophilic character and Pheoforbide has a more hydrophilic character. Therefore, we can evaluate the extract *Phormidium autumnale* has a considerable amount of chlorophylls species, which have antioxidant properties, extolling its bioactive potential.

Keywords: *microalgae, Phormidium autumnale, chlorophyll, bioactive potential, HPLC-PDA-MS/MS;*

1. Introduction

Microalgae are considered microscopic photosynthetic microorganisms proliferate in various environmental conditions making it attractive for biopanning and operation as commercial sources of high value. Such microorganisms constitute promising bio-catalysts to be implemented in the increasing field of biotechnology. This is valid both for the production of food, fine chemicals, and pigments. These unicellular microorganisms form a broad polyphyletic group with the mutual capability of photosynthetic fixation of CO₂. The group of microalgae include eukaryotic and, if also including the cyanobacterial representatives prokaryotic microbial species. (KOLLER, MUHR, BRAUNEGG, 2014).

The *Phormidium* is filamentous cyanobacteria and cellular content generally greenish blue, rarely brown. It is found in soil, wet rocks, mud, aquatic plants, streams, some in coastal environments. Other species are found in extreme environments such as hot springs and deserts soils (GUIRY & GUIRY, 2013; THOMAZEU et al., 2010). For this reason, have great potential biocatalyst in bioprocesses because of its robustness and simple nutritional requirements (ALTHUKAIR et al., 2007; GUIRY & GUIRY, 2013)

Small changes or variations in the chemical structure of chlorophyll allow photosynthetic organisms capture solar light at different wavelengths. These molecular changes influence on energy capture, and acting on biological properties and molecular degradation reaction mechanisms. Microalgae and cyanobacteria are achieving prominence in the biotechnology scenario, depending on the availability of funds for their metabolism products, with an emphasis on pigments as the chlorophyll and its derivatives, which participate in reactions that prevent the oxidation of molecule (TAKACHE, PRUVOST, MAREC, 2015).

Such vital function derives from its structure, chlorophylls can absorb light quanta effectively but also they can release and take up electrons reversibly by means of their aromatic. Additionally, these photochemical compounds have been also proved to possess prominent benefits to human health when consumed along with vegetable products or edible seaweeds in our daily diet, such as the

antimutagenic effect antigeno-toxic properties, and potent antioxidant capacity to scavenge free radicals, adding bioactive character (CHEN et al., 2015). However, many further studies were hampered by a lack of an overall structural analysis of all the chlorophyll derivatives that emerge in biological metabolism, tissue senescence, or during food processing (LANFER-MARQUEZ, BARROS, SINNECKER, 2005).

The chlorophyll molecule is known for its bioactive potential due to its pro-oxidant and antioxidant activity. The pro-oxidant nature is explained by the fact that this pigment promote and catalyze the photo-oxidation when exposed to light, due to the characteristics of the photosensitizing porphyrin ring of this molecule that facilitates the formation of singlet oxygen, which can trigger the autoxidation process. However, other studies show an antioxidant character of the chlorophyll molecule when it is not exposed to light, acting as a scavenger of free radicals and peroxy radicals, inhibiting autoxidation process (LANFER-MARQUEZ, 2003). The presence of phytol tail, tetrapyrrole complex of magnesium ion and contribute to the pro-oxidant and antioxidants characteristics. In addition, influence on the polarity of chlorophyll compounds, as well as their bioactive activity.

Accordingly, of microalgae can be considered biotechnologically favorable because the production of its metabolites, as well as effectively participating in the photosynthetic process, the pigments present bioactive potential, operating in scavenging free radicals and may have potential as antioxidants. The objective of this study was the operation of the microalgae biomass *Phormidium autumnale* species for the production of high industrial value compounds, such as chlorophyll, with an alternative to conventional energy sources, and evaluation of potential bioactive these structures with respect to their character antioxidant. In addition to evaluate the influence of the solvents with different polarity, the sequestration capacity of radicals, evaluating its antioxidant potential, and interaction between solvent-analyzed compound, seeking more efficient results.

2. Material and Methods

2.1 Standards

Standards of pheoforbide , chlorophyll a, were donated by Sigma Aldrich (St. Louis-MO, USA), with purities ranging from 95.0% to 99.9%, as determined by HPLC-PDA. Methanol, ethyl acetate, 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

The cultures of *Phormidium autumnale* were originally isolated from the Cuatro Cienegas desert (26°59'N, 102°03'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 °C, a photon flux density of 15 $\mu\text{molm}^{-2}\cdot\text{s}^{-1}$ and a photoperiod of 12/12 hours light/dark.

2.3 Microalgal biomass production

Biomass production was held in phototrophic conditions in bubble column photobioreactor (Jacob-Lopes, Scoparo, Queiroz, & Franco, 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 25 °C, 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 $\mu\text{mol}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$, 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.

2.4 Chlorophyll extraction by ultrasound

The chlorophylls were extracted by ultrasound-assisted extraction (UAE) using an ultrasonic processor (Sonics, Anaheim-CA, USA) with a 13 mm diameter probe. Dried samples, with moisture content close to 10%, 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 ethyl acetate followed by methanol (MANDELLI et al., 2012) 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 1500 $\times g$. The extraction procedure was repeated until the supernatant becomes colorless. The chlorophylls were transferred to petroleum ether/diethyl ether mixture [1:1 (v/v)], washed with distilled water and the extract concentrated in a rotary evaporator ($T > 30$ °C). Then, each extract was once again concentrated in a rotary evaporator ($T > 30$ °C), flushed with N₂ and kept at -18 °C in the dark until chromatographic analysis. To avoid chlorophylls degradation during analyses, the manipulation of the samples and extracts was conducted in the absence of light. The determination of the total chlorophyll concentration in the extract was determined spectrophotometrically (Molecular Devices Corp, Los Angeles-CA, USA) and calculated according Porra, Thompson, Kriedemann (1989).

2.5 HPLC-PDA-MS/MS chlorophylls analysis

The chlorophylls were analyzed by high performance liquid chromatography HPLC (Shimadzu, Kyoto, Japan) equipped with quaternary pumps (model LC-20AD), online degasser, and injection valve with a 20 μL loop (Rheodyne, Rohnert Park, CA, USA). The equipment was connected in series to a PDA detector (model SPD-M20A) and a mass spectrometer with an ion-trap analyzer and atmospheric pressure chemical ionization (APCI) source (model Esquire 4000, Bruker Daltonics, Bremen, Germany). The chlorophyll separation was performed on a C30 YMC column (5 μm , 250 \times 4.6 mm) (Waters, Wilmington, DE, USA). HPLC-PDA-MS/MS parameters were set as previously described by De Rosso and Mercadante (2007), and Rodrigues et

al., (2014). 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 C30 HPLC column, co-chromatography with authentic standards, UV–Visible spectrum (λ max, spectral fine structure, peak cis intensity), and mass spectra characteristics (protonated molecule ([M + H]⁺) and MS/MS fragments), compared with data available in the literature (De Rosso & Mercadante, 2007; Rodrigues et al., 2014; Van Breemen, Dong, & Pajkovic, 2012; Zepka & Mercadante, 2009).

The chlorophylls were also quantified by HPLC-PDA, using analytical curve of standard chlorophyll *a*, because it is the major compound present in biomass *Phormidium autumnale*. All chlorophylls pigments contents were estimated using the total chlorophylls content was calculated as the sum of the contents of each individual chlorophylls separated on the C₃₀ column.

2.6 Peroxyl radical scavenging assay for lipophilic extracts

The antioxidant capacity assay of the lipophilic extracts was carried out according to Rodrigues, Mariutti, Chisté, & Mercadante (2012). The extract chlorophylls were suspended in methanol and pooled together to compose the stock solution. Aliquots of the stock solution were taken to prepare the working solutions in five different concentrations. After evaporation under N₂ flow, they were dissolved in DMSO/MTBE (10:1, v/v) and homogenized. The assays were carried out in a microplate reader (Synergy Mx Biotek, Winooski-VT, USA). The ROO[•] scavenging capacity was measured by monitoring the effect of the chlorophylls extract or α -tocopherol standard on the fluorescence decay resulting from the ROO[•] induced oxidation of the C₁₁-BODIPY^{581/591} probe. ROO[•] was generated by thermal decomposition of AIBN at 37±0.5°C. 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[•] scavenging capacity was calculated as the ration between the slop of the curve representing the sample concentration against the net area under the curve,

and the slope of the curve representing α -tocopherol concentration against the net area under the curve.

2.7 Peroxyl radical scavenging assay for hydrophilic extracts

The antioxidant capacity of the hydrophilic extracts was carried out according to the ORAC method (Ou, Hampsch-Woodill, & Prior, 2001). The ROO scavenging capacity was measured by monitoring the effect of the chlorophylls extracts on the fluorescence decay resulting from ROO induced oxidation of fluorescein probe. ROO was generated by thermal decomposition of AAPH at 37 °C. Then they were pipetted 25 μ L of chlorophylls extract in black microplate wells containing 96 containing 150 μ L of fluorescein (81 nM in 75 μ M phosphate buffer, pH 7.4). The mixture was pre-incubated for 10 min at 37 °C followed by addition of 25 μ L of AAPH (19 μ M). The fluorescence signal was registered every min until 90 min in a SpectraMax M5 (Molecular Devices Corp, Los Angeles-CA USA) 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 μ mol trolox equivalent.g⁻¹ of dry wt.

3. Results and discussion

The HPLC chromatogram (Figure 3) demonstrates the presence of chlorophyll species in the extract of the *Phormidium autumnale* 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).

The peak 2 was tentatively identified as hidroxychlorophyll a considering the UV-visible spectrum characteristics, the protonated molecule at m/z 909, and the fragment at m/z 891 [M + H - 18]⁺ and 631[M + H - 278]⁺, results, respectively, loss of a hydroxyl and subsequently, the tail phytol.

The peak 3 It was identified as an isomer hydroxychlorophyll (hydroxychlorophyll a,) where in the protonated molecule at m/z 909, and the fragment at m/z 891 $[M + H - 18]^+$ and $631[M + H - 278]^+$, results, respectively, loss of a hydroxyl and subsequently, the tail phytol, and its isomer.

Chlorophyll a is shown as peak 5, and coelution with the chlorophyll a standard confirmed the identity. The identification was indicated by their mass spectra with the protonated molecule at m/z 893 and fragments at m/z 615 $[M + H - 278]^+$ and 583 $[M + H - CH_3O]^+$ and 555 $[M+H-278-CH_3COO]^+$. The presence of fragments resulting from the respective losses of tail phytol, CH_3O , and ester grouping in C10.

The isomer chlorophyll a, corresponds to peak 6, shows the grouping a hydrogen atom in the $R\alpha$ position and group $COOCH_3$ ester in the $R\beta$ position, different from the chlorophyll a. The identification was indicated by their mass spectra It occurs in the same procedure as chlorophyll a.

Peak 8 was tentatively identified as pheophytin *b* by comparing the UV-visible spectrum with that given in the literature. The mass spectrum showed the molecular ion at m/z 885 and the most abundant fragment ion in the MS-MS spectrum at m/z 607 $[M+H-278]^+$ 547 $[M+H-278+60]$, corresponding to the cleavage of the tail phytol and $COOCH_3$ in the carbon C10.

Peak 9, Hidroxypheophytin a, was tentatively identified considering the UV-visible and mass spectra characteristics. As expected, the mass spectrum showed the protonated molecule at m/z 887 and fragments at m/z 609 $[M+H-278]^+$, occurring in this case, only the loss of tail phytol in the structure.

Finally, the peaks 10 and 11, respectively, represent the pair of isomers Pheophytin a, and Pheophytin a' were tentatively identified, via its UV-vis spectrum and mass spectrum. The identification was indicated by their mass spectra with the protonated molecule at m/z 871 and fragments at m/z 593 $[M+H-278]^+$, 533 $[M+H-278+60]$, which occurs in that case, the loss of phytol tail and subsequent addition of the ester in its molecule $COOCH_3$, in $R\beta$, carbon 13.

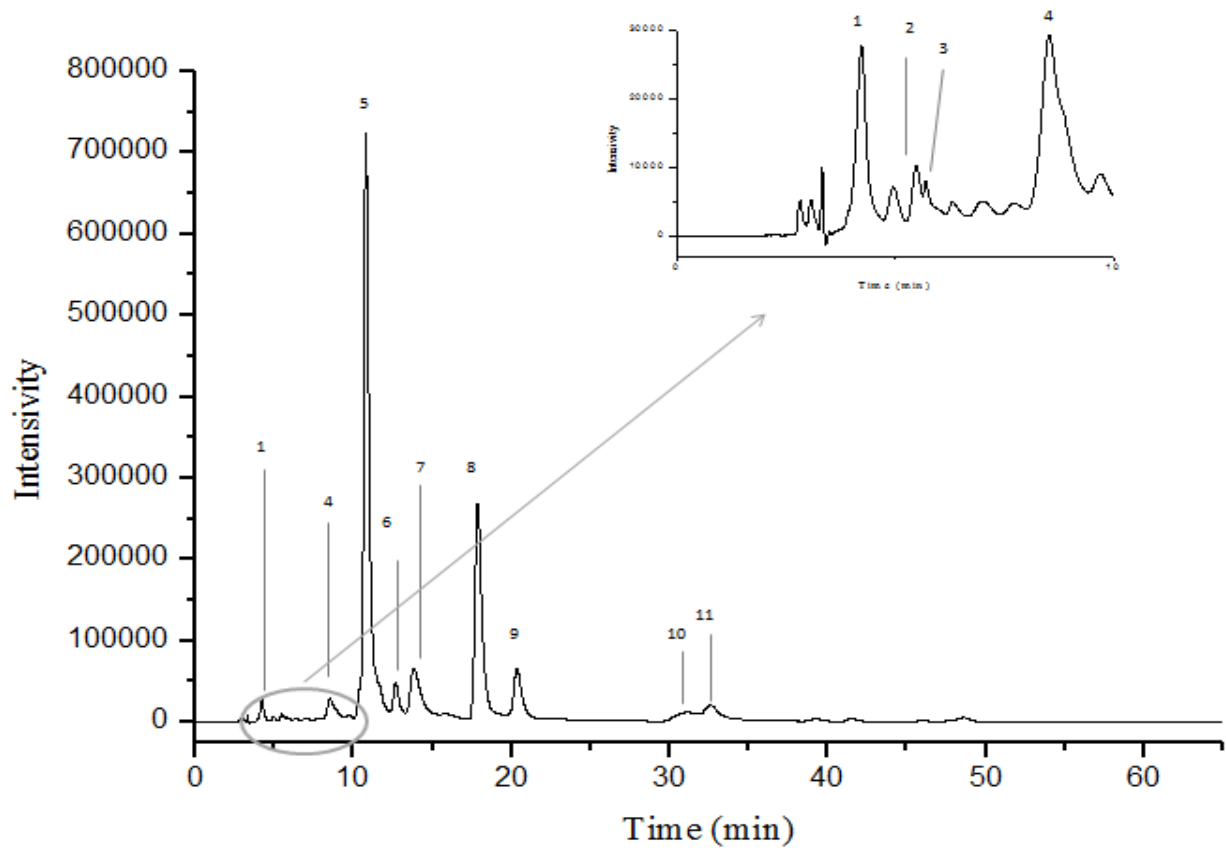


Table 1:Characterization of chlorophyll compounds present in biomass *Phormidium autumnale*

Peak ^a	Compound	t _{R(min)} ^b	λ _{max.} ^c	[M+H] ⁺ (m/z)	Fragments MS/MS	Pigment content (μg·g ⁻¹ dry wt)
1	ni ^d	5,1	409, 665	nd ^e	nd ^e	3.12
2	Hidroxychlorophyll <i>a</i>	6,2	421, 654	909	891 [M+H-18] ⁺ ; 631[M+H-278] ⁺	8.90
3	Hidroxychlorophyll <i>a'</i>	7,4	430, 664	909	891 [M+H-18] ⁺ ; 631[M+H-278] ⁺	9.99
4	Chlorophyll derivate	9,7	416, 662	Nd	nd ^e	6.81
5	Chlorophyll <i>a</i>	13,2	432, 665	893	615 [M+H-278] ⁺ ; 583[M+H-278-CH ₃ O] ⁺ ; 555 [M+H-278-CH ₃ COO] ⁺	1498.40
6	Chlorophyll <i>a'</i>	14,9	430, 655	893	615 [M+H-278] ⁺ ; 583[M+H-278-CH ₃ O] ⁺ ; 555 [M+H-278-CH ₃ COO] ⁺	60.96
7	ni ^d	16,1	420, 658		nd ^e	184.12
8	Pheophytin <i>b</i>	18,6	434, 660	885	607 [M+H-278] ⁺ ; 547 [M+H-278+60]	613.23
9	Hidroxypheophytin <i>a</i>	21,9	408, 666	887	609 [M+H-278] ⁺	139.25
10	Pheophytin <i>a</i>	31,3	408, 666	871	593 [M+H-278] ⁺ ; 533 [M+H-278+60]	75.60
11	Pheophytin <i>a'</i>	34,5	408, 665	871	593 [M+H-278] ⁺ ; 533 [M+H-278+60]	119.82
Total chlorophylls						2720.19

^aNumbered according to the chromatogram shown in Figure 3; ^bt_R: Retention time on the C30 column; ^cLinear gradient Methanol:MTBE; ^dni: not identified; ^end: not detected.

The Hydroxychlorophyll *a*, refers to the oxidation of chlorophyll by triplet oxygen in alcohol solution. Among the allomerization products identified and studied in depth are 15¹-methoxy-lactone-, 13²-methoxy-, and 13²-hydroxy-chlorophyll. It is important to highlight that 15¹-methoxy-lactone- and 13²-methoxy-chlorophyll derivatives are not produced *in vivo*, as naturally chlorophyll compounds are not in contact with alcoholic solutions, so the natural substituent in chlorophyll derivatives is the hydroxyl group, forming 13²-hydroxy- or 15¹-hydroxy-lactone chlorophyll derivatives. Coming to the oxidation to lactone group, traditional methods including exposing chlorophyll solutions in methanol to air (CHEN, 2015).

The pheophytin *a* was found as a minor pigment in the cyanobacterium. A Chlorophyll *a*/Pheophytin *a* ratio of approximately 4/2 was found in *Acc. marina* cells (AKIYAMA et al., 2001,2002) which is substantially smaller than the reported ratio of about (160–240)/2 in other cyanobacteria.

In addition, the pheophytin *b*, was the first chlorophyll 'alteration' product identified as a native constituent of photosynthetic complexes. Pheophytin *b* is found in almost all purple bacterial RC and the accepted molar ratio of chlorophyll *a* / pheophytin *a* is 4/2: a few species of purple bacteria contain chlorophyll *b* and pheophytin *b* rather than Chlorophyll *a* and Pheophytin *a* (GRIMM et al., 2006).

The total chlorophyll content of *Phormidium autumnale* grown under phototrophic condition was 2720.19 µg/g, and the majority chlorophylls were found chlorophyll *a* (1498.40 µg/g), and pheophytin *b* (613.23 µg/g), both with the presence of phytol tail, which gives a more lipophilic character to extract.

Table 2

ROO[•] scavenging capacity of chlorophylls and other analyzed compounds.

Compound	Phytol	Value M μ Trolox equivalente/g extract ^A	Value α -tocopherol relative ^B
Chlorophyll a	present	nd ^C	308
Chlorophyll b	present	nd ^C	386
Pheoforbide a	nd ^C	231.48	nd ^C
Phormidium autumnale		219.82	200

^A Calculated according Ou et al., 2001. ^B Calculated according Rodrigues et al., 2012; ^C nd: not detected.

In terms of antioxidant capacity of the pigments extracts, the Table 2 shows the scavenger capacity against peroxy radicals by hydrophilic and lipophilic extracts of *Phormidium autumnale*. The chlorophyll extract from *Phormidium autumnale* was able to scavenge ROO[•] and the net AUC values were linearly dependent to total chlorophyll concentration.

The standard of chlorophyll a proved was 308 times more potent scavenger of peroxy radicals than α -tocopherol. This value can be related to the polarity, i.e., it depends on the interaction of the solvent used with the analyzed structure. Already, the standard pheoforbide a introduced antioxidant activity of 231.48 M μ trolox equivalente/g extract. This value is related to its structure, the absence of Mg ion in pheophorbide structure may cause some reduction in antioxidant potential of the molecule. [Mg]-Chl a has a higher capacity to release electrons from the singlet excited state (WATANABE et al., 1985).

The chlorophyll extract of *Phormidium autumnale* was 200 times more potent scavenger of peroxy radicals than α -tocopherol. This value is higher to that found for the expressive sources of bioactive compounds like the following Amazonian fruits, palm peach (7.83 ± 0.21 , α -tocopherol relative) and mamey (6.90 ± 0.44 α -tocopherol relative) (Rodrigues et al., 2012). The efficiency of the chlorophylls as ROO• scavengers varied considerably according to their chemical structures and was influenced by the presence of metal, and tetrapyrrole, and presence tail phytol. The values for antioxidant activity of chlorophylls in microalgal extract were more significant than those found for carotenoids obtained from microalgal biomass. According to Rodrigues et al., 2015 for carotenoid extract obtained from biomass *Phormidium autumnale*, was found 28 times more potent than the value α -tocopherol.

According to Endo et al., (1985a) The strongest antioxidant activity was found for chlorophyll a, followed by BHT, chlorophyll b, pheophytin a and pheophytin b and the four pigments presented a linear dose-dependent response. Some factors responsible for obtaining conflicting results in measurements of antioxidant activity for the same compound are the physical structure of the test system, the nature of the substrate for oxidation and the analytical method employed (BECKER et al, 2004).

The value for the ROO• scavenging capacity chlorophyll is related to the presence or absence of phytol in chlorophyll molecule. This suggests a function of long-chain alcohols in the interactions with the environment, the ORAC test need to consider the solubility of the compounds analyzed with the reagents used, otherwise the results will not be effective. Many ORAC assays do not address the problem of lipophilic antioxidants because the test is performed in aqueous solution. The application of reagents with a relatively hydrophobic character, it is important since it allows a larger molecular interaction between them, increased solubility, allowing the occurrence of various chemical reactions (PFITZNER, FRAN CZ, BIESALSKI, 2000; SZENTE et al., 1998).

The macrocycle, the central metal, the peripheral substituents and the hydrophobic esterifying alcohol all together provide some unique physical and chemical properties. Among them, the macrocycle and the central Mg, seem to be most important, if judged by their conservation and by their known influence on the

chemical and physical parameters of the pigments. Variations in the substituents provide the necessary plasticity for chemical interactions and modulation of certain properties including light absorption and redox potential, while the functional relevance of the hydrophobic esterifying alcohol is less clear (GRIMM et al., 2006).

Among the critical factors for this situation are the solvent choice to determine the antioxidant capacity of lipophilic compounds present in the extract, especially chlorophylls (*a* and *b*), and pheophytins (*a* and *b*). The literature shows conflicting results about antioxidant activity of chlorophyll from microalgae (CHA et al., 2010; ISMAIEL, EI-AYOUTY, & PIERCEY-NORMORE, 2014; PARNIAKOV et al., 2015b), probably generated by the use of different reaction means, different probes, and different radical generators, that difficult a comparison with microalgae literature data.

The fluorescence decay resulting from ROO• induced oxidation of fluorescein probe in the absence of antioxidants (blank assay) and in the presence of standard pheoforbide *a* (fig.4A). It is possible to visualize the typical analytical curve of trolox standard (fig.4B) in the ORAC assay. The in vitro scavenging capacity against peroxy radicals of extract from *Phormidium autumnale* (fig.4C) was 219.82 $\mu\text{mol trolox equivalents.g}^{-1}$.

Besides the ORAC_{FL} assay, the most important mechanism seems to involve hydrophobic interactions and also hydrogen bonding. According to Rodrigues et al., 2015 phycobiliproteins extract obtained from *Phormidium autumnale* value presented 237.4 $\mu\text{mol trolox equivalent / g extract}$. We can see that in some natural extracts such as blueberry extracts, grape skin extracts, black tea leaves, respectively, respectively, the mean values of ORAC_{FL} were 2622; 15303; 1593 $\mu\text{mol Trolox equivalent/g extract}$. These values corroborate the low antioxidant activity pheoforbide *a*.

The absence of Mg ion in pheophorbide structure may cause some reduction in antioxidant potential of the molecule. The complex [Mg]-Chlorophyll *a* has a higher capacity to release electrons from the singlet excited state than all [Hms]-Chls, making Mg^{2+} the most favorable ion for the special-pair chlorophylls in the reaction centers. The central metal has a decisive influence on the excited state kinetics of tetrapyrroles and, therefore, on their function in photosynthesis. It

appears that nature has selected Mg as the central metal which maximizes excited state lifetime, while maintaining low intersystem crossing to the highly toxic triplet (GRIMM et al., 2006). According Lanfer-Marquez et al., (2005) pheophorbide the molecule has lower antioxidant activity when compared to pheoforbide b.

Derivatives b presented higher antioxidant capacity than derivatives a as a general trend. These results support the hypothesis that the presence of the aldehyde group ($-CHO$) in place of the methyl group provides a better antioxidant activity, although the identification of the mechanism involved needs to be investigated

In general, the antioxidant reactions involve multiple steps including the initiation, propagation, branching, and termination of free radicals. This whole process is termed a chain reaction. The antioxidants therefore fall into two mechanistic groups: those which inhibit or retard the formation of free radicals from their unstable precursors (initiation) are called the “preventive” antioxidants, and those which interrupt the radical chain reaction (propagation and branching) are the “chain-breaking” antioxidants. Specifically, a chain-breaking antioxidant (AH) donates its labile hydrogen atom to $ROO\cdot$ much more rapidly than $ROO\cdot$ reacts with substrate. The radical A is stable and is not able to continue the autoxidation of the chain (WRIGHT ET AL, 2000; VINQVIST, BARCLAY, 2000).

However the ORACFL assay cannot be considered a “total antioxidant activity assay” since the assay is performed in aqueous solution. Therefore, the ORACFL assay primarily measures hydrophilic antioxidant activity against peroxy radical. In fact, it is impossible to measure total antioxidant activity using only a single assay. To elucidate a full profile of antioxidant activity against various ROS/RNS, such as $O_2\cdot^-$, $HO\cdot$, and $NO\cdot$, the development of different methods specific for each ROS/RNS is needed (OU, HAMPSCH-WOODILL, PRIOR, 2001).

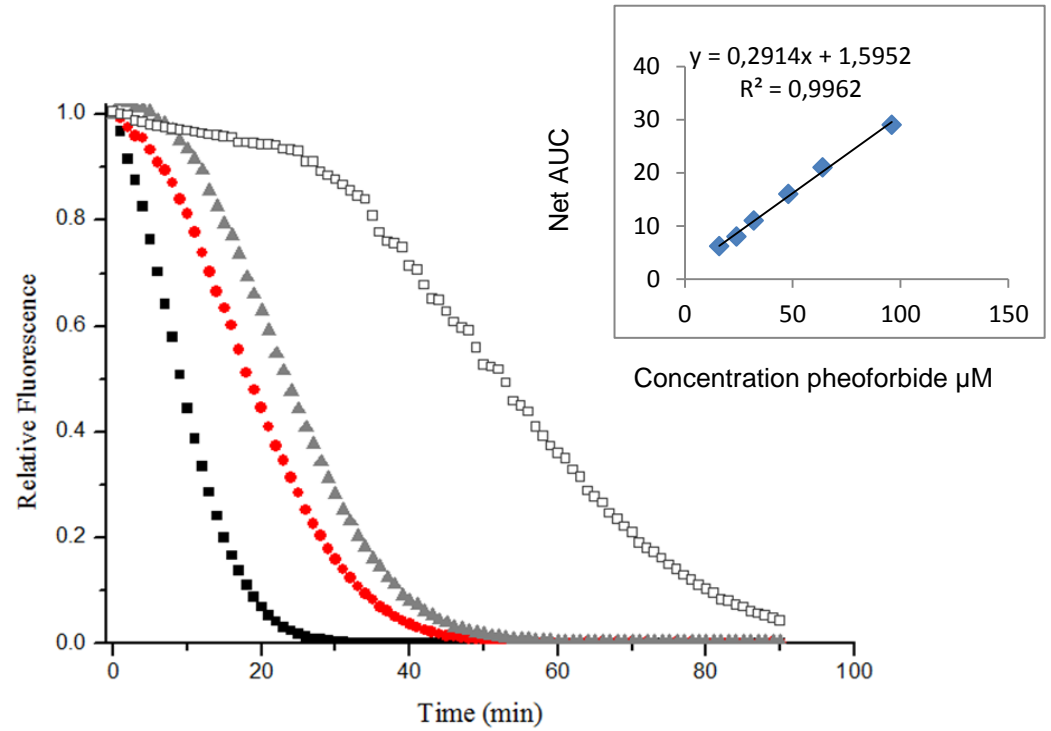


Figure 4A. Fluorescence decay of fluorescein induced by peroxy radicals in the presence of different concentrations standard Pheoforbide. Legend: Blank (filled square), 5 μ mol (red circle), 11 μ mol (gray triangle), 28 μ mol (open square) Inset: linear relationship between the standard Pheoforbide concentrations and net AUC values from the fluorescence decay curves of fluorescein oxidation.

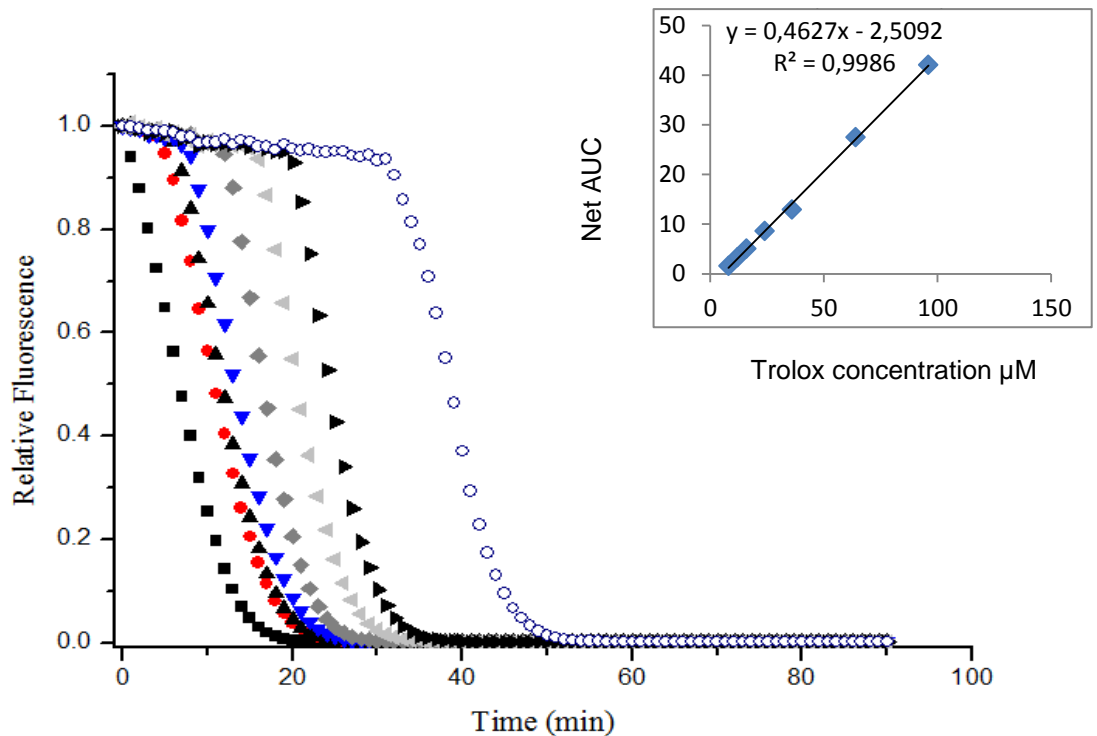


Figure 4B. Fluorescence decay of fluorescein-induced by peroxy radicals in the presence of different concentrations of trolox standard. Legend: Blank (filled square), 8 μ mol (red circle), 12 μ mol (filled triangle), 16 μ mol (blue triangle), 24 μ mol (filled diamond), 32 μ mol (gray triangle), 64 μ mol (dark triangle), 96 μ mol (open circle). Inset: linear relationship between trolox standard concentrations and net AUC values from the fluorescence decay curves of fluorescein oxidation.

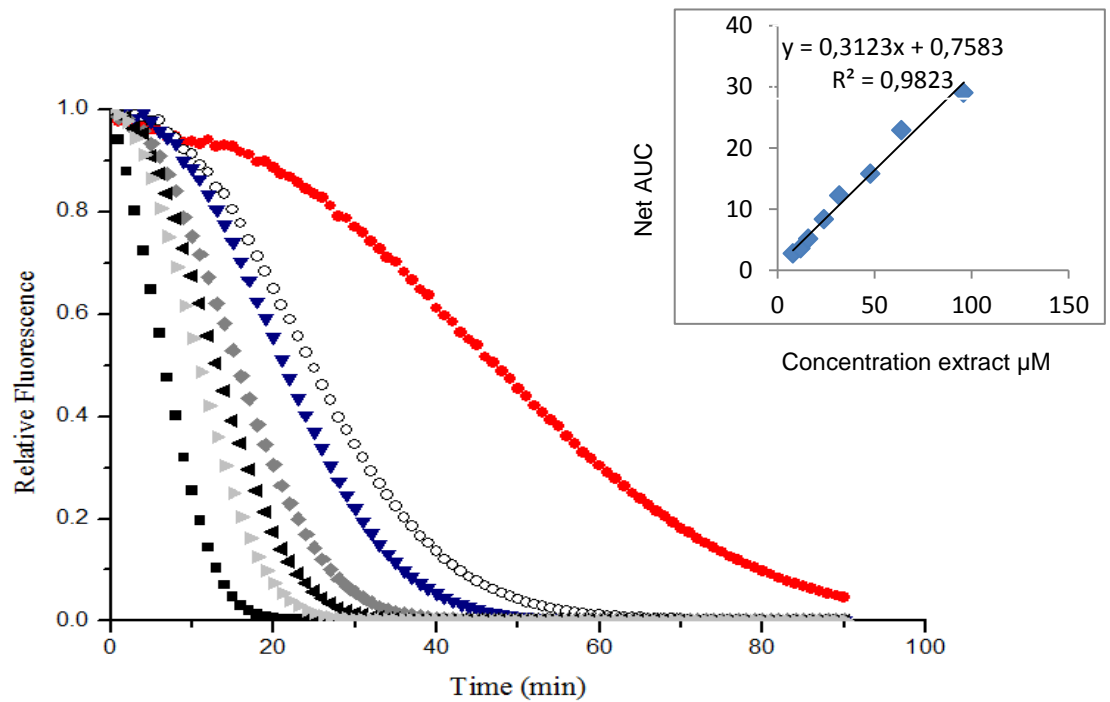


Figure 4C. Fluorescence decay of fluorescein induced by peroxy radicals in the presence of different concentrations extract BGN chlorophyll. Legend: Blank (filled square), 29 μ mol (gray triangle), 42 μ mol (dark triangle), 84 μ mol (gray diamond), 167 μ mol (blue triangle), 278 μ mol (open circle), 842 μ mol (red circle). Inset: linear relationship between the extract BGN chlorophyll concentrations and net AUC values from the fluorescence decay curves of fluorescein oxidation.

Considering the different solubility of the pigments synthesized by *Phormidium autumnale*, appropriate methods to assess the scavenging capacity of both lipophilic and hydrophilic peroxy radicals were used. Thus, this study provides an actual contribution of the chlorophylls presents in its biomass on the antioxidant capacity in microalgae against a biological relevant radical (RODRIGUES et al, 2015).

4. Conclusion

Finally, we note the *Phormidium autumnale* biomass It has considerable potential in the production of chlorophyll compounds in phototrophic conditions, proving to be an alternative source to conventional arrays of chlorophyll pigments. Considering the chlorophyll compounds having different polarity due to its molecular structure It is necessary to apply appropriate methods hydrophilic and lipophilic to assess the sequestration capacity of these free radical molecules and demonstrate

its antioxidant power improving scientific research in this respect, since chlorophylls structures are little known in its bioactive character. Thus, this study provides an actual contribution of the chlorophylls on the antioxidant capacity in microalgae against a biological relevant radical, demonstrating that the microalgae *Phormidium autumnale* can be considered a source of chlorophyll compounds.

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