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DETERMINAÇÃO DE METABÓLITOS LIPÍDICOS EM CIANOBACTÉRIA

Santa Maria, RS
2017

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Dissertação apresentada ao Curso de Mestrado do Programa de Pós-graduação em Ciência e Tecnologia dos Alimentos, Área de Concentração em Qualidade de 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: Prof. Dr. Roger Wagner
Co-orientador: Prof. Dr. Eduardo Jacob-Lopes

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"*Sábio é o ser humano que tem coragem de ir diante do espelho de sua alma para reconhecer seus erros e fracassos e utilizá-los para plantar as mais belas sementes no terreno de sua inteligência.*"

(Augusto Cury)

RESUMO

DETERMINAÇÃO DE METABÓLITOS LIPÍDICOS EM CIANOBACTÉRIA

AUTORA: Mariane Bittencourt Fagundes

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CO-ORIENTADOR: Eduardo Jacob-Lopes

O trabalho teve por objetivo o estudo exploratório do potencial de produção de esqualeno e esteróis por *Phormidium autumnale*, quando cultivado em condições heterotróficas. Para identificação e quantificação dos metabolitos lipídicos foi empregada a cromatografia gasosa. Em uma primeira fase, foi avaliada a produção de esqualeno, utilizando como meio de cultivo efluente agroindustrial. Os resultados obtidos, demonstraram que a biomassa gerada pode ser uma importante fonte de esqualeno, sendo estimada uma produção de 152.156 kg/ano a partir de uma indústria com capacidade operacional de 10.000 m^3/d. Esta biomassa caracterizou-se ainda pelo conteúdo em ácidos graxos poliinsaturados, perfazendo 51,8% do total de ácidos graxos quantificados. Em uma segunda fase, utilizando-se como fonte de carbono, efluente agroindustrial, glicose e sacarose, acrescido a produção de esqualeno, foi avaliado o conteúdo de esteróis e seus derivados. Os resultados indicaram a presença de 24 compostos, contatando-se, na fração não saponificável 22 esteróis, um hopanoide e esqualeno. Os compostos majoritários encontrados foram: esqualeno (1440,4 $\mu g.g^{-1}$) e ergosterol (1033,3 $\mu g.g^{-1}$), utilizando glicose como substrato; colesterol, (820,6 $\mu g.g^{-1}$) e estigmasterol (455,3 $\mu g.g^{-1}$) e esqualeno (225,4 $\mu g.g^{-1}$), para os cultivos em efluente industrial e (225,4 $\mu g.g^{-1}$) para a sacarose respectivamente. Os resultados indicaram ainda que na biomassa cultivada em efluente agroindustrial foi registrada a maior concentração em fitoesterois. Neste sentido, sugere-se que mediante a manipulação das fontes de carbono, possam ser obtidos diferentes compostos a serem aplicados em diversos segmentos industriais.

Palavras-chave: cultivo heterotrófico, *Phormidium autumnale*, esteróis, esqualeno.

ABSTRACT

CYANOBACTERIA LIPID METABOLITES DETERMINATION

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The objective of this work was exploratory study of the squalene and sterol potential production by Phormidium autumnale, when cultivated under heterotrophic conditions. Gas chromatography was used to identify and quantify the lipid metabolites. In a first phase, the production of squalene was evaluated, using agroindustrial wastewater as the culture medium. The obtained results, demonstrated the generated biomass can be an important source of squalene, being estimated a production of 152 156 kg/year from an industry with operational capacity of 10 000 m3/d. This biomass was also characterized by the polyunsaturated fatty acid content, making up 51.8% of the total quantified fatty acids. In a second phase, using the source of carbon, agroindustrial wastewater, glucose and sucrose, plus the production of squalene, the content of sterols and their derivatives was evaluated. The results indicated the presence of 24 compounds, in the non-saponifiable fraction 22 sterols, a hopanoid and squalene. The major compounds found were: squalene (1440.4 $\mu g.g^{-1}$) and ergosterol (1033.3 $\mu g.g^{-1}$), using glucose as a substrate; Cholesterol (820.6 $\mu g.g^{-1}$) and stigmasterol (455.3 $\mu g.g^{-1}$) and squalene (225.4 $\mu g.g^{-1}$) for industrial effluent cultures and (225.4 $\mu g.g^{-1}$) for sucrose respectively. The results indicated that the biomass cultivated in agroindustrial wastewater had the highest concentration in phytosterols. In this sense, it is suggested that by manipulating the carbon sources, different compounds can be obtained to be applied in several industrial segments.

Palavras-chave: heterotrophic culture, *Phormidium autumnale*, sterols, squalene.

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LISTA DE ABREVIATURAS

PPP	Rota das pentoses fosfato (do inglês *Pentose phosphate pathway*)
EMP	Rota Embden-Meyerhof (do inglês *Embedem-Meyerhof pathway*)
IPP	Isopentenil Pirofosfato
DMAPP	Dimetilalil pirofosfato
MVA	Rota do ácido mevalônico (do inglês *mavalonate pathway*)
MEP	Rota metil eritritol fosfato (do inglês *methyl erythritol pathway*)
DXP	1-deoxi-D-xilulose-5-fosfate
LMD	Limite de detecção (do inglês **LOD** *Limit of detection*)
LMQ	Limite de quantificação (do inglês **LOQ** *Limit of quantitation*)
GC	Cromatografia em fase Gasosa (do inglês *Gas Chromatography*)
SFA	Ácidos graxos saturados (do inglês *Saturated fatty acids*)
PUFA	Ácidos graxos polinsaturados (do inglês *Poliunsaturated fatty acids*)
MUFA	Ácidos graxos monoinsaturados (do inglês *Monounsaturated fatty acids*)
COD	Demanda química de oxigênio (do inglês *chemical oxygen demand*)
P-PO4^{-3}	Fósforo total (do inglês *total phosphorus*)
N-TKN	Nitrogênio total (do inglês *total nitrogen*)
VS	Sólidos voláteis (do inglês *volatile solids*)
FS	Sólidos fixos (do inglês *fixed solids*)
TS	Sólidos totais (do inglês *total solids*)
SS	Sólidos suspensos (do inglês *suspended solids*)
***P*x**	Produtividade de biomassa (do inglês biomass productivity)
***P*sq**	Produtividade de esqualeno (do inglês squalene productivity)
P_L	Produtividade lipidica (do inglês *Lipid productivity*)
$Y_{x/s}$	Coeficiente de rendimento da biomassa (do inglês *Biomass yield coefficient*)
$Y_{p/s}$	Coeficiente de rendimento de esqualeno (do inglês *Squalene yield coefficient*)
$Y_{p/s}$	Coeficiente de rendimento de lipideos (do inglês *Lipid yield coefficient*)
Tg	Tempo de geração (do inglês *Generation time*)

SUMÁRIO

1 INTRODUÇÃO

As cianobactérias representam um grupo amplo de micro-organismos unicelulares procariontes e fotoautotróficos, encontram-se em habitats distintos e são conhecidas como algas verde-azuladas, da classe cianofícea. Entretanto algumas cepas são capazes de metabolizar o carbono orgânico, outra característica das cianobactérias esta relacionada em realizarem a fixação de nitrogênio (PALINSKA; SUROSZ, 2014). Também possuem como particularidade a produção de químicos-finos, dentre estes os combustíveis renováveis e compostos bioativos (CHEW et al., 2017).

A *Phormidium autumnale,* entre as inúmeras cianobactérias, destaca-se como uma microalga em potencial no emprego de tecnologias de tratamento de efluentes, devido as suas características de suportar temperaturas variadas e concentrações elevadas de nutrientes (SANTOS et al., 2016).

Diversos metabólitos são produzidos pelas cianobactérias e estes situam-se em diferentes locais nas mesmas. A fração lipídica possui diversos bioativos, devido a este atributo têm sido considerada de grande importância, entretanto, trabalhos que relacionem os metabólitos lipídicos microalgais com suas biossínteses, para a compreensão das rotas metabólicas, são escassos (YAO et al., 2015).

O emprego de cultivos heterotróficos e os impactos relacionados ao metabolismo lipídico das cianobactérias, foi comprovado em estudo que ao utilizarem fontes distintas de carboidratos modificaram o perfil de ácidos graxos (FRANCISCO et al., 2014). No entanto variações na fração não saponificável, especificamente do esqualeno e dos seus derivados, os esteróis, utilizando fontes distintas de carbono não foram exploradas de forma adquada. Os perfis de esteróis adquiridos na literatura estão relacionados com as classes das microalgas e cianobactérias, para auxiliar na identificação (YAO, et al., 2015; VOLKMAN, 2016).

Em cultivos heterotróficos as cianobactérias possuem a característica de utilizar além de carboidratos fontes alternativas para obtenção de energia, os resíduos industriais, e assim contribuir de forma simultânea para o meio ambiente, este processo tecnológico auxilia na remoção material orgânica, assim como nitrogênio e fósforo (JACOB-LOPES; ZEPKA; QUEIROZ, 2014).

Outra vantagem de utilizar resíduos industriais em cultivo heterotrófico esta relacionada a redução de custos, como exemplo a *Phormidium autumnale* consegue em condições adversas uma elevada produtividade de biomassa e lipídica (OTAKAR et al., 2013;

SU; MENNERICH; URBAN, 2012). Baseado no fato de que vários metabólitos lipídicos microalgais são de interesse industrial, a obtenção de químicos finos como o esqualeno e os esteróis através de um processo biotecnológico de fonte renovável, torna-se um processo tecnológico verde e vem de acordo com inúmeros conceitos para minimizar a poluição, em conjunto com a valorização do resíduo através de uma biomassa rica.

Portanto, o presente trabalho fundamenta-se em um estudo exploratório do potencial de produção de esteróis e esqualeno por *P. autumnale* através do metabolismo heterotrófico.

2 OBJETIVOS

2.1 OBJETIVO GERAL

O objetivo do presente trabalho foi determinar e caracterizar a produção dos esteróis e seu precursor metabólico, o esqualeno, contidos na cianobactéria *Phormidium autumnale* empregando o cultivo heterotrófico.

2.2 OBJETIVOS ESPECÍFICOS

Como objetivos específicos, o estudo contempla:

a) Identificar e quantificar o esqualeno na biomassa obtida heterotroficamente a partir de efluente industrial;

b) Avaliar a produtividade do esqualeno na biomassa da cianobactéria *Phormidium autumnale* cultivada em resíduo agroindustrial;

c) Realizar a caracterização, identificação e quantificação, de alguns dos metabolitos lipídicos de alto valor comercial na cianobactéria *P. autumnale* em cultivos heterotróficos, empregando diferentes fontes de carbono;

d) Verificar possíveis relações metabólicas dos bioprodutos provenientes de diferentes fontes de carbono, em cultivos heterotróficos.

3 REVISÃO BIBLIOGRÁFICA

3.1 CIANOBACTÉRIA

As microalgas contemplam uma grande diversidade de micro-organismos autotróficos, são unicelulares e possuem dois tipos de estruturas: eucariótica e procariótica, sendo esta conhecida como cianobactéria. As fontes de energia podem ser obtidas através do metabolismo fotoautotrófico ou heterotrófico (MORALES-SANCHEZ et al., 2015; RAJNEESH et al., 2017).

As microalgas fotoautotróficas apresentam em seu metabolismo a conversão bioquímica da energia solar em energia química, sendo duas etapas essenciais responsáveis pela fotossíntese: as reações dependentes da luz, que ocorrem nos tilacóides e o ciclo de Calvin, realizados no estroma (JACOB-LOPES et al., 2009).

As diversas reações que ocorrem ao final do ciclo de Calvin, promovem a formação de gliceraldeído-3-Fosfato (GAP), e duas moléculas de GAP originam a glicose fosfato. A partir dessa molécula forma uma série de fosforilações oxidativas que ocorrem para gerar energia. Contudo para as cianobactérias a diferença esta correlacionada com o local em que ocorrem as reações metabólicas, pois estão locados no citosol. Estes micro-organismos não possuem núcleo organizado, cloroplastos e mitocôndria, apresentam pigmentos como as ficobiliproteínas, clorofila e os fotossistemas I e II, devido a presença destes constituíntes as cianobactérias são capazes de realizar a fotossíntese com liberação de oxigênio, diferenciando-se desta maneira do metabolismo das bactérias (WILLIAMS; LAURENS, 2010).

As cianobactérias por sua vez, apresentam uma grande vantagem frente a outros micro-organismos, pois possuem a habilidade de sobreviver em ambientes de temperaturas extremas, de regiões polares até regiões desérticas, demonstrando elevado grau de adaptabilidade (COMTE et al., 2007). Estes micro-organismos são majoritariamente fotoautotróficos, todavia devido a sua versatilidade metabólica algumas cepas são capazes de crescer e produzir uma biomassa de alto valor agregado através do metabolismo heterotrófico facultativo (BERLA et al., 2013; WIJFFELS; KRUSE; HELLINGWERF, 2013).

A *Phormidium autumnale* é uma cianobactéria de 3 a 4 µm de diâmetro, filamentosa e possui a habilidade de crescer em condições com concentrações elevadas de nutrientes (GUIRY & GUIRY, 2014). Devido as suas características a *Phormidium a.* pode ser empregada em tratamento de efluentes obtendo-se elevada eficiência de remoção de matéria

orgânica e metais tóxicos (PANDEY, 2017). Segundo estudos a cianobactéria destaca-se como potencial produtora de compostos bioativos como os carotenoides (RODRIGUES et al., 2015), as clorofilas (FERNANDES et al., 2016), ficobiliproteínas (SHARMA; STAL, 2014) obtendo-se uma biomassa rica.

Pesquisas comprovam que a biomassa obtida a partir de processo biotecnológico proveniente de resíduos agroindustriais demonstra ter elevada produtividade, com formação de bioprodutos de alto valor agregado. O processo torna-se passível de ser aplicado em alta escala industrial. Ademais, por se tratar uma fonte renovável, pode ser aplicada em diversos seguimentos industriais, obtendo-se um processo viável, devido ao reaproveitamento e valorização dos bioprodutos (RODRIGUES et al., 2014; FRANCISCO et al., 2015; MORALES-SANCHEZ et al., 2015).

O cultivo heterotrófico é caracterizado por total ausência de luminosidade e o uso de fonte de carbono exógena para obtenção de energia. As cianobactérias são capazes de utilizarem diferentes fontes de carbono, substâncias orgânicas, como açucares. A glicose é a fonte de mais fácil assimilação em relação aos demais demais monossacarídeos, dissacarídeos e polissacarídeos, pois devem ser hidrolisados por algumas enzimas específicas (FRANCISCO et al., 2014), bem como ácidos graxos e bem como aminoácidos (LOWREY; ARMENTA; BROOKS, 2016).

A rota metabólica mais provável para obtenção de energia, na ausência total de luminosidade, é a rota das pentose-fosfato (PPP), e a via Embden-Meyerhof (EMP), entretanto não como a principal fonte de obtenção de energia, pois esta ocorre de forma majoritária em metabolismo mixotrófico. No metabolismo heterotrofico formam-se blocos construtores químicos, moléculas responsáveis pela biossíntese de compostosque possuem potencial biotecnológico (PEREZ-GARCIA et al., 2011).

Visto que, inúmeros compostos de química fina são formados durante o processo biotecnológico e corroborando com a elevada produtividade das cianobactérias, a biotecnologia microalgal supera quaisquer alternativas utilizadas para obtenção de diversos insumos industriais, os quais demonstram ser provenientes de fontes não renováveis (WIJFFELS et al., 2013).

O óleo da cianobactéria destaca-se como um insumo de elevado valor agregado, apresentando ácidos graxos insaturados, esqualeno, esteróis e tratando-se de cianobactérias, os hopanoides e entre outros. A composição lipídica pode variar de acordo com a espécie da microalga, com as condições e o tempo de cultivo, influenciando as concentrações dos bioativos de interesse (NAVARRO-LÓPEZ et al., 2016).

3.2 LIPÍDEOS

As microalgas têm sido estudadas por possuírem capacidade de acumular grandes quantidades de lipídeos (KUMAR; SHARMA, 2014). E os extratos lipídios microalgais são passíveis de serem utilizados tanto para alimentação animal, aquicultura, quanto para produção de biometano e, principalmente conhecido como uma nova fonte de produção de biodiesel (MATA; MARTINS; CAETANO, 2010; GONZÁLEZ-FERNÁNDEZ et al., 2012; LUM; KIM; LEI, 2013; MUBARAK; SHAIJA; SUCHITHRA, 2015; ROY; PAL, 2015).

A produtividade lipídica irá variar de acordo com a disponibilidade dos nutrientes do cultivo, sendo este controle a forma tradicional para obtenção de uma elevada produtividade lipídica (SHARMIN et al., 2016). Muitas vezes o perfil lipídico pode ter grandes variações apresentando características para a produção de biodiesel, ou mesmo uma biomassa rica em compostos para fins nutricionais (WILLIAMS; LAURENS, 2010).

Na fração lipídica coexistem compostos saponificáveis e não saponificáveis, dentro da classe de compostos saponificáveis encontram-se majoritariamentente os ácidos graxos (TALEBI et al., 2013). Muitas cepas possuem a habilidade de produzir elevadas quantidades de ácidos graxos de cadeia longa, ácidos graxos de alto valor, como os ácidos eicosapentaenoico (EPA) e docosahexaenoico, ambos da família ômega 3 (ω-3) (REDDY; COUVREUR, 2009) e ácidos graxos poliinsaturados (PUFA) (PEREZ-GARCIA et al., 2011). Muitos estudos investigam que os ácidos ω-3 derivados de algas possuem efeitos de prevenção a doenças, tanto problemas de hipertensão e até prevenção de alguns tipos de câncer (HENNESSY et al., 2016; HUANG et al., 2016).

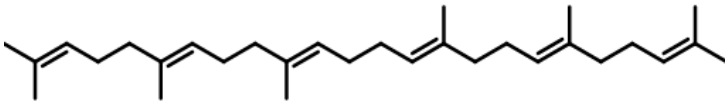
Em relação a fração não saponificável microalgal apresentam-se muitos compostos antioxidantes entre estes estão presentes alguns hidrocarbonetos, como exemplo o esqualeno, apresentam-se também os esteróis, fitol e pigmentos, como os carotenoides, além de outros compostos lipossolúveis (YAO, LINXING et al., 2015).

3.3 ESQUALENO

O esqualeno é um hidrocarboneto triterpênico, possui seis estruturas isoprenóides, (2,6,10,15,19,23-hexametil-6,6,10,14,18,20-tetracosahexane) como compreende seu nome sistemático, a estrutura química do esqualeno pode ser observada na Figura 1. Este composto foi descoberto em 1916 pelo Dr. Mitsumaru Tsujimoto, o nome esqualeno deriva de *Squalus*,

gênero do tubarão em que foi descoberta a molécula, mais especificamente no fígado do animal, onde encontra-se em grande abundância (TSUJIMOTO, 1920; SPANOVA; DAUM, 2011).

Figura 1. Imagem da estrutura quimica do esqualeno.



Diversos estudos relatam os inúmeros benefícios que este composto realiza à saúde humana, sendo esta molécula classificada como um composto bioativo (ROMERO; RUIZ-MÉNDEZ; BRENES, 2016). Dentre os benefícios, apresenta propriedades antioxidantes, devido a sua estrutura de duplas ligações em seis grupos metila (REDDY; COUVREUR, 2009), propriedades anticarcinogênicas (RONCO; STÉFANI, 2013; GHIMIRE et al., 2016), também pode ser utilizado como hidratante sendo um exemplo o seu uso como emoliente, adjuvante em vacinas (HUANG; LIN; FANG, 2009), e ainda demonstra ter propriedades de proteção cardiovascular (SABEENA FARVIN et al., 2004).

Em estudo proposto por Ravi Kumar et al. (2016), o esqualeno em sinergia com a astaxantina demonstrou apresentar uma boa capacidade antioxidante, pois foi relatado efeito sobre o estresse oxidativo *in vivo* e demonstrou modular os níveis de glicose/triacilglicerídeos. Outro estudo realizado por Sotiroudis; Kyrtopoulos (2008) sugeriu-se que o esqualeno protege a biomembrana das células imunológicas do nosso organismo durante a fagocitose e assim previne a ação de elementos do estresse oxidativo sobre as células.

De modo geral, a maior fonte de esqualeno é o óleo do fígado de tubarão pois possui aproximadamente 40% de esqualeno em sua composição, acredita-se que os tubarões de águas profundas são os animais que possuem maiores concentrações de esqualeno, pois sua densidade (0,858 g/cm^{-3}) que contribui de forma significativa para a flutuabilidade do animal (HALL et al., 2016). Entretanto devido a preservação dos animais marinhos, novas propostas e alternativas estão sendo estudadas para explorar este bioativo de modo que venha a reduzir a caça excessiva dos tubarões (HOANG et al., 2014).

Outras fontes de esqualeno podem ser encontradas na natureza, como exemplo: óleos vegetais (SHERAZI; MAHESAR; SIRAJUDDIN, 2016), azeite de oliva (XYNOS et al.,

2016), óleo francês de coentro (UITTERHAEGEN et al., 2016), óleo de camélia (XIAO et al., 2016), amaranto (DAS, 2016), contudo entre todos os usos, como já mencionado uma das funções do esqualeno esta vinculada a indústria farmacêutica, sendo ele utilizado como hidratante em cosméticos e o uso de óleos extraídos de fontes alimentícias torna o processo dispendioso (HOANG et al., 2014). Desta forma rotas biotecnológicas demonstram ser alternativas as fontes não renováveis, devido a alta produtividade e crescimento massivo, micro-organismos podem ser considerados promissores para a produção de esqualeno.

Este triterpeno ja foi isolado em cepas de *Saccharomyces cerevisiae* (GARAIOVÁ et al., 2014), *Synechocystis* (ENGLUND et al., 2014), *Candida* (LEE et al., 2014), e as algas, *Schizochytrium mangrovei* (HOANG et al., 2016) e *Botryococcus braunii* (BANERJEE et al., 2002).

O esqualeno ja têm sido comercializado como suplemento, e pesquisas relatam que elevadas doses de 500 mg/dia de esqualeno podem ser vitais para manter a saúde nutricional dos seres humanos (HOANG et al., 2014).

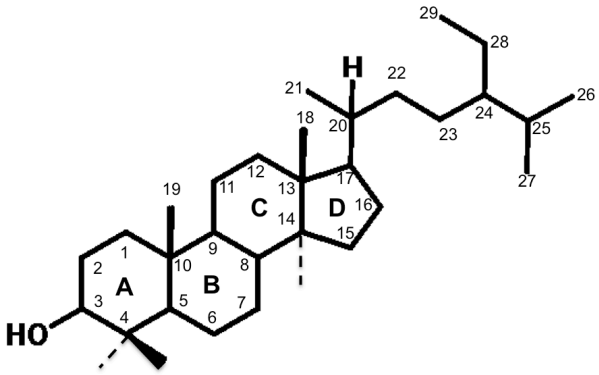
Portanto para se ter altas produções de esqualeno torna-se interessante utilizar das vias de obtenção biotecnológicas. Pois além de seus benefícios à saúde é considerado um composto chave, o precursor metabólico dos esteróis que possuem extrema importância no metabolismo de diversos organismos (WEI;YIN;WELANDER, 2016).

3.4 ESTERÓIS

Outra classe química de compostos os quais podem ser encontrados nas microalgas são os esteróis. A sua estrutura básica é composta por triterpenóides tetracíclicos, especificamente um anel de ciclopentanoperidrofenantreno com dois grupos metila angulares (NES; PARISH, 2012). Variações podem ocorrer no sistema dos anéis de acordo com as duplas ligações, estas moléculas são pertencentes a classe de isoprenóides os quais são oriundos do precursor universal dos isoprenóides o Isopentenil pirofosfato (IPP) (LU et al., 2014).

De modo geral os esteróis encontrados na natureza são conhecidos através dos seus nomes usuais, entretanto a nomenclatura segundo o IUPAC ocorre em função do anel ciclopentanoperidofenantreno de triterpenóides tetracíclicos. A divisão está estabelecida em anéis A, B, C e D conforme podemos observar na Figura 2.

Figura 2. Estrutura geral dos esteróis.



Os esteróis são classificados como um subgrupo dos esteroides, contudo com uma hidroxila ligada na posição 3 do anel A. Estes metabólitos podem ser encontrados na célula em sua forma livre, acilados, como esteres de esteróis, sulfatados, como sulfatos de esteróis, ligados a glicosídeos, como ésteres de glicosídeos e também eles podem ser encontrados acilados entre sí, assim denominando-se glicosídeos de esteróis acilados (MOREAU; WHITAKER; HICKS, 2002).

A diversidade das estruturas químicas dos esteróis localizados nas células podem ser divididas em grupos com relação a sua respectiva fonte. Os esteróis contidos em células animais denominam-se zooesterois. O esterol mais abundante é o cholesterol no metabolismo animal, é considerado um bloco construtor químico, a partir desta biomolécula temos a produção de diversas estruturas complexas, bem como os ácidos biliares, sendo importantes para o metabolismo das vitaminas lipossolúveis, incluindo A, D, E, K, têm sido relatado como o precursor metabólico principal da vitamina D e dos esteroides (hormônios) (NES, DAVID, 2011; HOSTA-RIGAU et al., 2013). Enquanto em células vegetais, encontram-se campesterol, β-sitosterol e estigmasterol, como constituintes majoritários, e são responsáveis por auxiliar na viabilidade celular, embriogênese, divisão celular e na modulação e atividade da membrana celular (LU et al., 2014).

Os fitoesteróis também apresentam benefícios a saúde humana, são responsáveis por reduzir os riscos de doenças cardiovasculares, devido as estruturas químicas serem similares ao colesterol, essas moléculas são passíveis de reduzirem a reabsorção do colesterol no intestino, e desta forma são obtidos menores níveis de colesterol no sangue (MOREAU et al., 2002). Segundo Rocha et al. (2016) a ingestão regular de fitoesteróis pode reduzir os níveis da lipoproteína de baixa densidade (LDL). Em experimentos realizados em um modelo animal

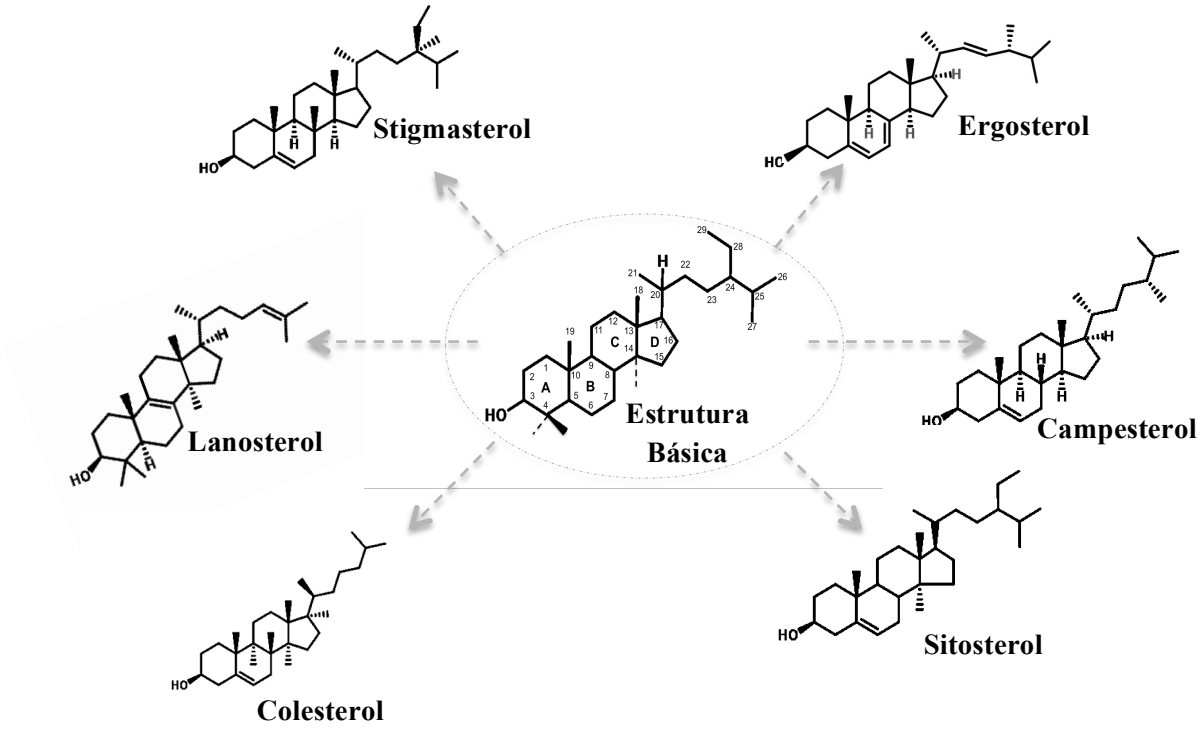
foi observado a maior excreção do colesterol através das fezes e a relação com a redução dos níveis de LDL (MOGHADASIAN et al., 2016).

Alguns trabalhos relatam que os fitoesteróis estão correlacionados com a prevenção de doenças mentais neurodegenerativas, pois os fitoesteróis são capazes de atravessar a camada hemato-encefálica e se acumular no cérebro formando uma barreira de proteção. Em um estudo realizado por Burg et al. (2013) foi observado que ratos que receberam doses de estigmasterol (0,19, 0,25, 0,39%) em sua alimentação confirmaram o efeito protetor celular, desta forma segundo a pesquisa, dietas contendo estigmasterol em sua composição podem ter benefícios na prevenção da doença Alzheimer (PARK et al., 2012).

Muitos destes compostos são investigados também na prevenção de tipos específicos de câncer, como câncer de mama (TEOH et al., 2017), podendo reduzir o desenvolvimento de tumores de próstata (DUTTA, 2004), entre outros, sem que prejudiquem as células não comprometidas.

Existem diversos esteróis e a grande variedade esta relacionada com as modificações dos radicais, o qual situa-se no carbono 17 e da hidroxila carbono 3, a mudança de radical proporciona a formação de estruturas distintas (MOREAU et al., 2002), conforme esta expresso na Figura 3, podemos observar alguns dos principais esteróis encontrados na natureza em células animais e vegetais a partir de sua estrutura básica.

Figura 3. Principais esteróis oriundos de diferentes rotas metabólicas.



3.5 PRODUÇÃO DE ESTERÓIS POR MICRO-ORGANISMOS

Muitos micro-organismos são capazes de produzir metabolitos de interesse industrial, dentre estas moléculas podemos destacar os esteróis, metabólitos secundários os quais apresentam-se como componentes vitais para as células eucariontes (VOLKMAN, 2003). Devido a diversidade de micro-organismos produtores, distintas rotas metabólicas de produção são estudadas. Em plantas superiores duas rotas de produção são responsáveis pela formação dos esteróis, a rota do ácido mevalônico (MVA) e a rota do metil eritritol-5-fosfato (MEP). Em contrapartida as células animais apresentam apenas uma forma de obtenção destas moléculas a do ácido mevalônico (BRAGAGNOLO, 2008).

Uma das funções que têm sido atribuídas aos esteróis em eucariontes é a de regulagem da membrane plasmática. Nos procariontes esta tarefa é realizada pelos hopanoides, porém muitas vezes em menores concentrações os esteróis encontram-se presentes nestes micro-organismos (BODE et al., 2003). Logo as microalgas, incluindo cianobactérias são capazes de produzir estas moléculas através do metabolismo secundário, os diversos esteróis encontrados nestes micro-organismos, suas respectivas nomenclaturas e sinônimos de acordo com os autores Lopes et al., (2011), Volkman (2016) e Martin-Creuzburg et al., (2016) podem ser observados na Tabela 1.

Tabela 1. Nomenclatura usual e sistemática dos esteróis encontrados em microalgas

Nome usual	Fórmula	Nomenclatura sistemática
Colesterol	C27H46O	colest-5en-3β-ol
Campesterol	C28H48O	campest-5en-3β-ol
Campestanol	C28H50O	24α-metil-5-colestan-3β-ol
5- Ergostenol	C28H48O	24β-metilcolest-5-en-3β-ol
7- Ergostenol	C28H48O	24β-metil-5α-colest-7-en-3β-ol
Desmosterol	C27H44O	colesta-5,24-dien-3β-ol
22-dehidrocolesterol	C27H44O	colesta-5,22E-dien-3β-ol
24-metilenecolesterol	C28H46O	24β-metilenecolesta-5,24-dien-3β-ol
Brassicasterol	C28H46O	24β-metilcolesta-5,22E-dien-3β-ol
5,7 ergostadienol	C28H46O	24β-metil-colesta-5,7-dien-3β-ol
7,22 ergostadienol	C28H46O	24β-metil-5α-colesta-7,22-dien-3β-ol
Ergosterol	C28H44O	24β-metil-colesta-5,7,22-trien-3β-ol
Fucosterol	C29H48O	24α-etilcolesta-5,24(28)-dien-3β-ol
Conasterol	C27H46O	24β-etilcolest-5-en-3β-ol
Sitostanol	C29H52O	24α-etil-5α-colestan-3β-ol
Sitosterol	C29H50O	24α-etilcolest-5-en-3β-ol
Estigmasterol	C29H48O	24α-etilcolesta-5,22E-dien-3β-ol
Fungisterol	C28H48O	24β-metilcolest-7-en-3β-ol
Condrillasterol	C29H48O	24β-etil-5α-colesta-7,22-dien-3β-ol
Dinosterol	C30H52O	4α,23,24-trimetilcolest-22E-en-3β-ol
Gorgosterol	C30H50O	22,23-metilene-23,24-dimetilcolest-5-en-3β-ol
Lanosterol	C30H50O	4,4,14α-trimetil-5α-22E-en-3β-ol
Cicloartenalol	C30H50O	4,4,14α-trimetil-9,19-ciclo-5α-colest-24-en-3β-ol

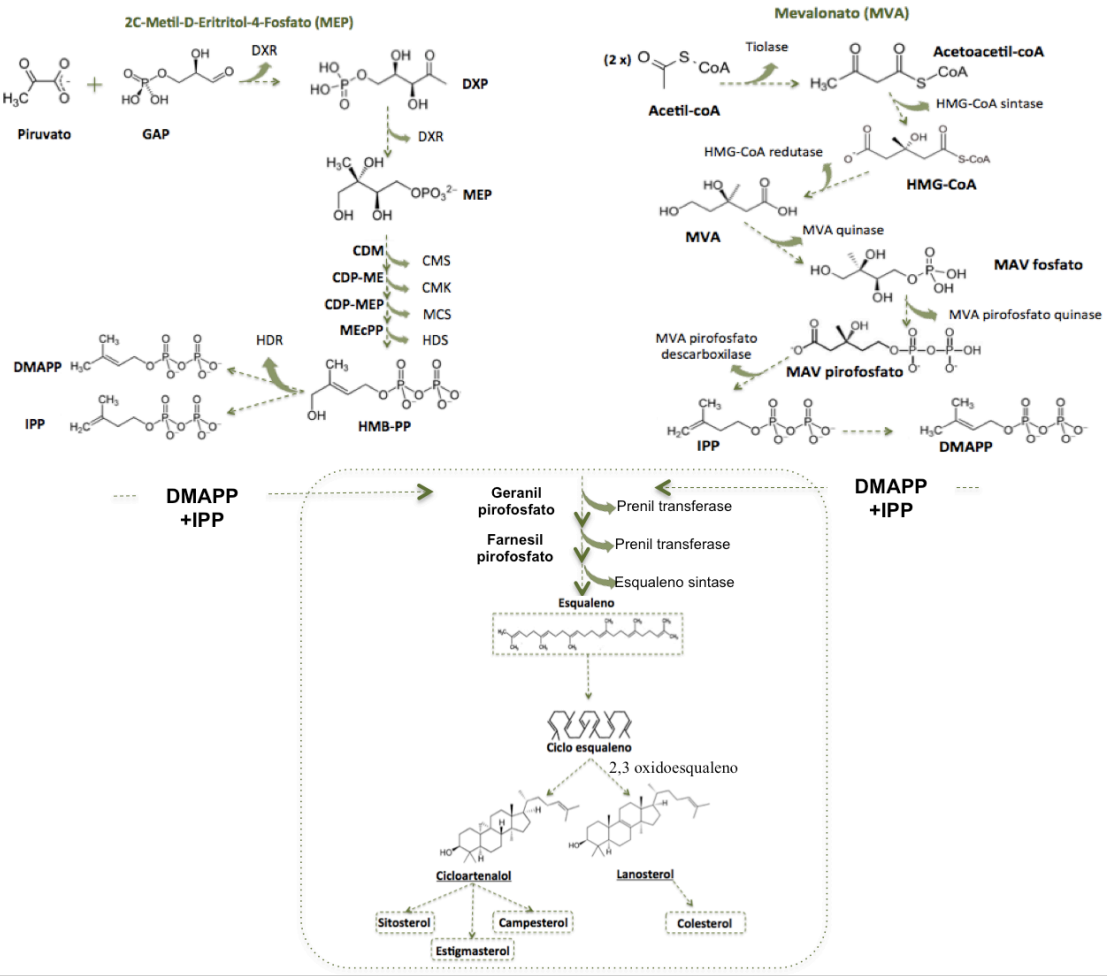
Em relação aos produtos intermediários destaca-se a rota do ácido mevalônico como responsável pela produção de lanosterol o qual é convertido a colesterol e também responsável pela formação do ergosterol, sendo a rota principal de formação dos esteróis em microrganismos eucariontes, já com relação as bactérias esta rota também é responsável pela produção, contudo não como a principal (Bode et al., 2003).

A rota do ácido mevalônico consiste na ação da enzima tiolase, acetoacetil-CoA transferase, sobre o Acetil-coenzima A, o qual catalisa a reação de ligação com outra molécula de Acetil-coenzima A e assim obtemos a molécula acetoacetil-coA. Uma condensação do acetil-coA vai ocorrer através da enzima hidroxi-metil-glucaril-coA sintase obtendo-se hidroxi-metil-glucaril- CoA (HMG) e consequentemente pela ação da enzima HMG-CoA redutase ocorre a formação do metabólito ácido mevalônico e através da transferência de energia na forma de adenosina trifosfato (ATP), chegamos ao precursor metabólico dos isoprenos, o isopentil pirofosfato (LIAO et al., 2016).

Através da ação de uma enzima isomerase, sobre a molécula de IPP obtemos a formação de um também precursor dimetilalil pirofosfato (DMAPP), como na rota metabólica expressa na Figura 4.

A união das duas moléculas formam o geranil pirofosfato (GPP), assim duas moléculas de GPP vão gerar geranilgeranil pirofostaro (GGPP), sendo que consecutivas condensações ocorrem com, dois isoprenos, e por fim com três isoprenos obtemos o farnesil pirofosfato (FPP), e através de uma polimerização dos isoprenos, obtemos o precursor esqualeno contendo 6 estruturas isoprenóides. Logo após as ciclizações forma-se o lanosterol, um metabólito intermediário para a formação do colesterol (NES, 2011) e ergosterol, moléculas estas já encontradas como compostos em algas (MILLER et al., 2012; LU et al., 2014) Outra rota metabólica, considerada a principal via de formação de fitoesteróis em bactérias e, segundo estudo é rota predominante em bactérias (GAO et al., 2016), é conhecida como MEP. A rota inicia com a combinação de moléculas de piruvato e gliceraldeido-3-fosfato (GAP), obtendo-se a formação da molécula 1-desoxi-xilulose-3-fosfato (DXP), seguindo com vários intermediários eritritol antes de formar os blocos construtores IPP e DMAPP. Consequentemente nesta rota ocorre a conversão do esqualeno a cicloartenol, do qual deriva as sínteses de estigmasterol, β-sitosterol, campesterol e outros (Figura 4) (GAO et al., 2016).

Figura 4. Rotas universais de biossíntese dos esteróis.



Biossíntese dos esteróis, rota do acido mevalônico (MVA) à direita e a rota 2C-metil-D-eritritol-4-fosfato (MEP) à esquerda. Acetil coenzima A (Acetil-coA); Acetil-coenzima A (CoA) tiolase (tiolase); 3-hidroxi-3-metilglutaril coenzima A (HMG-CoA); 3-hidroxi-3-metilglutaril coenzima A sintase (HMG-CoA sintase); 3-hidroxi-3-metilglutaril coenzima-A redutase (HMG-CoA redutase); ácido mevalônico (MVA); Mevalonato quinase (MVA quinase); Mevalonato fosfato (MVA fosfato); Mevalonato pirofosfato quinase (MVA pirofosfato quinase); Mevalonato pirofosfato (MVA pirofosfato); Mevalonato pirofosfato descarboxilase (MVA pirofosfato descarboxilase); Isopentil pirofosfato (IPP); Dimetilalil pirofosfato (DMAPP); Gliceraldeído-3-fosfato (); 1-deoxy-D-xilulose-5-fosfate (DXP); 1-deoxi-D-xilulose-5-fosfato reductoisomerase (DXR); 2C-metil D-eritritol 4-fosfato (MEP); 4-Difosfocitidil-2C-metil-d-eritritol 4-fosfato sintase (CDM); 4-Difosfocitidil-2C-metil-d-eritritol 4-fosfato sintase (CMS); 4-(citidine-5'-difosfo)-2-C-metil-d-Eritritol (CDP-ME); 4-(citidine-5'-difosfo)-2-C-metil-d-Eritritol quinase (CMK); 4-Difosfocitidil-2C-metil-d-eritritol 4-fosfate (CDP-MEP); 2C-metil-d-eritritol 2,4-ciclodifosfate sintase (MCS); 2C-metil-d-eritritol 2,4-ciclodifosfate (MecPP); 4-Hidroxi-3-metilbut2-en-il-difosfate sintase (HDS); 4-hidroxi-3-metilbut-2-enil difosfato (HMB-PP); 1-deoxi-d-xilulose 5-fosfate sintase (DXS); 4-hidroxi-3-metilbut-2-enil difosfato redutase (HDR).

3.6 ANÁLISE DE METABÓLITOS LIPÍDICOS MICROALGAIS

As extrações dos esteróis e esqualeno normalmente compreendem várias etapas, como as extrações de lipídeos com posteriores saponificações, pois estas moléculas podem ser encontradas majoritariamente na forma livre e na forma de esteres, sendo considerado o processo de saponificação essencial para eliminar possíveis interferentes (HELENO et al., 2016).

Em ambas etapas utiliza-se uma grande quantidade de solvente, desta forma, tornam-se analises dispendiosas e muitas vezes após a saponificação ainda realizam-se técnicas de derivatizações os quais utilizam reagentes de alto custo e um elevado tempo de análise (FLAKELAR et al., 2017). O uso de métodos de extrações simultâneos, os quais compreendem mais de um composto em apenas um extrato e o uso métodos com auxílio de novas tecnologias, que proporcionem uma redução de tempo de analise e solventes, são de extrema importância pois ocasionam em determinações de baixo custo (VILKHU et al., 2008).

A extração/saponificação direta da amostra por sua vez apresenta melhor eficiência, melhor precisão e menor formação de artefatos, em relação a extração de lipídeos seguida pela saponificação. O processo de extração assistida por ultrassom aumenta as taxas de recuperação dos compostos de interesse, conseguindo redução no tempo de análise, permitindo assim novas oportunidades de extração comercial e processo (BRAGAGNOLO, 2009; VILLARES et al., 2014).

Para que um método analítico seja desenvolvido ou otimizado, ele deve compreender alguns parâmetros (figuras de mérito) para assim gerar resultados de qualidade analítica e evitar falsos positivos, portanto o termo utilizado que compreende estes parâmetros denomina-se validação de método (NAZ et al., 2014).

Segundo algumas diretrizes disponíveis, requisitos devem ser seguidos para que seja realizada a validação, dentre estes: seletividade, linearidade, sensibilidade, intervalo (faixa-linear), precisão, limite de detecção (LMD), limite de quantificação (LMQ), exatidão e robustez. As diretrizes disponíveis para o aspecto analítico e bioanalítico, são as seguintes: FDA (Food and Drug Administration) (FDA, 2005), ICH (The International Conference on Harmonisation of Technical Requirements for Registration of Pharmaceuticals for Human Use) (ICH, 1993), o qual desenvolveu um guia prático para os procedimentos de validação (Q2-R1), Agência Nacional de Vigilância Sanitária (ANVISA) (ANVISA, 2003.) e INMETRO (Instituto Nacional de Metrologia, Qualidade e Tecnologia) (INMETRO, 2010).

4 ARTIGOS CIENTÍFICOS

Manuscrito 1

TOWARDS SUSTAINABLE PRODUCTION ROUTE OF SQUALENE USING CYANOBACTERIA

Submetido à revista Food Science and Biotechnology[1]

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

TOWARDS SUSTAINABLE PRODUCTION ROUTE OF SQUALENE USING CYANOBACTERIA

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Abstract

The aim of this research was to evaluate squalene production from the microalgae *Phormidium autumnale* cultivated by using agroindustrial wastewater. The use of wastewater is a sustainable technological alternative to the algae biorefinery platform for the achievement of this bioactive compound. In the present study, a derivatization method was performed to determine the squalene and fatty acids. The compounds were evaluated by using gas chromatography with flame ionization and mass spectrometry detectors. A total of 179.0 mg/kg of squalene was found in the biomass and the fatty acid profile demonstrates that the biomass is a rich source of unsaturated compounds with 51.8%. The sensitivity analysis showed that is possible to estimate a production of 1 522 to 152 156 kg/year in industries with different capacities. In this sense, *P. autumnale* can be a potential alternative to squalene production from microalgae in agroindustrial wastewater.

Keywords: Bioactive compound, Gas chromatography, Microalgae, *Phormidium autumnale*, Sensitivity analysis.

Introduction

Squalene is a natural triterpene, which is a putative condensation product of farnesyl diphosphate via head-to-head condensation and is only catalyzed by squalene synthase enzymes (1). The prokaryotes microorganisms demonstrate that their metabolism production occurs until the building blocks called isopentenyl diphosphate (IPP) and dimethylallyl diphosphate (DMAPP) (2). This compound is found in diverse types of cells and can play an important role as an intermediate in the sterol biosynthesis and it can also present an impact on human health (3).

The positive effect of this triterpene on health has been reported, such as for use in the treatment of cancer in lipid-based anticancer prodrugs for chemotherapy, antiviral treatment against hepatitis C virus, cardiovascular protection and antioxidant activity (4,5,6,7,8,9).

Traditionally, the richest source of squalene is shark liver. The highest production of shark liver oil was in Japan in 1997, with 720 tons (10). However, this practice is decreasing because of concerns over the preservation of marine wildlife and fishery (11). Another traditional source of squalene is from nuts, which are produced in a substantial amount; however, production can be affected by the soil and climatic conditions (12,13). On the other hand, the microorganism's biotechnological route has been increasing as squalene producers owing to the higher productivity and yield obtained in the process (14). Then, according to the metabolic versatility of microalgae and cyanobacteria regarding the formation of numerous bioactive compounds from food wastewater, the biomass generated can be considered to be a source of active ingredients useful for the production of supplements related to nutraceuticals and functional foods (15). Nowadays, there is still socio-economic dependence on non-renewable sources; therefore, some alternatives have been studied for the commercial exploration of many compounds that can have an industrial application (16). In this way, it is

important to demonstrate the feasibility of such bio-products in light of economic, sustainability and practical viewpoints (17,18).

Therefore, the heterotrophic cultivation process using agroindustrial wastewater can be a new technological route for the production of valuable compounds obtained from the biomass. These bio-compounds could emerge from algae biorefinery platforms, thus reducing the environmental implications (19,20). In this regard, the aim of this work is to evaluate the sustainable production route of squalene from the cyanobacteria *Phormidium autumnale* biomass.

Material and Methods

Microorganisms and culture media

Axenic cultures of *P. 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 (21). The incubation conditions used were 25 °C, a photon flux density of 15 μmol/m/s and a photoperiod of 12/12 hours light/dark.

Wastewater

The slaughterhouse wastewater was acquired from an industry located in Santa Catarina, Brazil (27º14'02"S, 52º01'40"W). The wastewater was obtained from the discharge point of an equalization tank over a period of one year, and carefully studied for hydrogenionic potential (pH), chemical oxygen demand (COD), total phosphorus ($P\text{-}PO4^{-3}$), total nitrogen (N-TKN), volatile solids (VS), fixed solids (FS), total solids (TS) and suspended solids (SS) following the Standard Methods for the Examination of Water and Wastewater (25). The wastewater average composition (mg/L) was: pH of 5.9±0.05, COD of 4100±874, $P\text{-}PO4^{-3}$ of 2.84±0.2, N-TKN of 128.5±12.1, VS of 2.9±1.4, FS of 0.9±0.3, TS of 3.8±2.7, SS of 1.9±0.8, C/N ratio of 31.9 and N/P ratio of 45.2. The nitrogen/phosphorous

ratio (N/P) and carbon/nitrogen ratio (C/N) were calculated taking into account the COD, N-TKN, and P-PO 4^{-3} parameters.

Microalgal biomass production

The biomass production was realized in heterotrophic conditions, using slaughterhouse wastewater as the culture medium. The cultivations were performed in a bubble column bioreactor (22) 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 °C, 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 (23). The biomass was separated from the wastewater by centrifugation. It was subsequently freeze-dried for 24 hours at −50 °C under -175 mmHg. The cultivations were performed twice and in duplicate. Therefore, experimental data refer to the mean value of the four repetitions.

Sampling and Kinects data analysis

The samples were collected aseptically every 24 hours during the microrganismo growth phase and the biomass data were used to calculate the biomass productivity [PX = (Xi-Xi-1)(ti-ti-1) -1, mg/L/h] and the lipid productivity [PL = PX.L, mg/L/h], in which Xi is the biomass concentration at the time ti (mg/L) and Xi-1 is the biomass concentration at the time ti-1 (mg/L), t is the residence time (h) and L is the *Phormidium autumnale* lipid content of the biomass (%). Total organic carbon concentrations were used to calculate the substrate consumption rate (rS=dS/dt, mg/L/h), and the biomass yield coefficient ($Y_{X/S}$=dX/dS, mg cell/mg substrate), where S0 is the initial substrate concentration (mg/L), S is the substrate concentration (mg/L) and t is the time (h).

Experimental

Reagents

The following reagents were of analytical grade: methanol and chloroform were from Vetec (São Paulo, SP, Brazil); anhydrous sodium sulfate, sodium methoxide, methanolic solution (1 M), methyl acetate and diethyl ether were purchased from Sigma-Aldrich (Saint Louis, MO, USA); and oxalic acid was obtained from Synth (São Paulo, SP, Brazil). Hydrochloric acid and 0.05% butyl hydroxyl toluene (BHT) from Dinâmica (São Paulo, SP, Brazil) were used in chloroform solution to avoid lipid oxidation during and after extraction. Hexane was from the last manufacturer. The squalene standard (98.9%) and a mixture of fatty acids methyl esters FAME Mix-37 was obtained from Sigma-Aldrich. The squalene stock solution with a 1 mg/mL concentration was prepared by weighing 10 mg of the standard in a volumetric flask of 10 mL and completed with hexane.

Lipids extraction

The total lipid fraction from the dry biomass was extracted by using Bligh & Dyer method (24) modified. In this method, around 0.5 g of cyanobacteria samples were submitted to a pre-treatment with a solution of hydrochloric acid (2 M) for cell hydrolysis. Afterward, the extraction was carried out in the absence of light and the lipid content was determined gravimetrically. After each extraction procedure, the chloroform–lipid extracts were evaporated at 50 °C under vacuum (-760 mm Hg) and submitted to the transesterification method.

Fatty acids derivatization

Fatty acids methyl esters were obtained according to Christie method (25). Around 50 mg of microalgal lipid extract was inserted into a flask tube and 2 mL of hexane plus 40 μL of methyl acetate were added, followed by homogenization by vortex for 30 seconds. Then, 60 μL of sodium methoxide methanolic solution (1 M) (methylation solution) was added, with a

brief shaking for 2 min. A solution of oxalic acid in diethyl ether (0.4 M) (termination solution) was responsible for solubilizing the polar lipids and hexane was added, standing alone for 1 hour at ambient temperature. Then, the extract was centrifuged at 1,775 *g* for 5 min and the supernatant was transferred into a 1.5 mL vial for further chromatographic analysis.

Fatty acids profile

The methylated samples were analyzed by using a gas chromatography instrument equipped with a flame ionization detector (GC-FID) Varian 3400 (Palo Alto, CA, USA), and autosampler Varian 8200 (Palo Alto, CA, USA). An aliquot of 1 μL of the sample was injected into a split / splitless injector, operating in split mode, with a 50:1 ratio at 240 °C. The carrier gas was hydrogen with a constant pressure of 20 psi. The FAME were separated in a capillary column SP-2560 Supelco (Bellefonte, PA, USA, 100 m × 0.25 mm × 0.20 μm). The temperature of the oven was initially 80 °C (hold time of 5 min). Afterward, the temperature was increased to 175 °C at a rate of 15 °C/min, to 190 °C at a rate of 5 °C/min and then up to 240 °C at a rate of 8 °C/min, maintaining the isothermal conditions for 15 minutes. FID temperature was held at 280 °C.

The FAME were identified by using the authentic standard FAME Mix-37 (P/N 47885-U). The standard fatty acids methyl esters were evaluated under the same conditions; consequently, their retention times were used to identify the fatty acids. The fatty acids were expressed as percentages of the total chromatographic area.

Squalene determination

The same FAME extract was used for the squalene analysis. The injection port of GC-FID operated in splitless mode (splitter valve off by 0.8 minutes; 30:1) at 280 °C. Hydrogen at a constant pressure of 15 psi was used as the carrier gas. The separation was performed in a non-polar column RTX-5MS Restek (Bellefonte, PA, USA, 30 m × 0.25 mm id × 0.25 μm).

The temperature program was initially 200 °C, with an increase to 280 °C at a rate of 15 °C min^{-1} and then up to 330 °C at a rate of 5 °C/min, maintaining the isothermal conditions for 10 minutes. The temperature of the detector was maintained at 280 °C.

The squalene was quantified by using a five-point analytical curve (10–50 mg/L) and some parameters of validation were studied, such as the linearity. For this purpose, a linear regression equation was used and the linear correlation coefficient (R^2) of the calibration curve was determined. The precision was expressed as relative standard deviation (RSD). The limit of detection (LOD) was estimated according to the concentration of the compound at a signal-to-noise ratio of 3. The quantification limit (LOQ) was achieved by injecting sequential dilutions of the standards and calculated considering a concentration that would result in a signal-to-noise ratio higher or equal to 10. Accuracy was determined by recovery assay from samples spiked with a known amount of the standard in a concentration of 20 μg/mL that was expressed as a percentage of the standard recovered.

The positive identification of the squalene in samples was carried out by a comparison of the retention time and mass spectra obtained experimentally by using an authentic standard solution. Identification was performed by using gas chromatography coupled to a mass spectrometer (GC/MS), Shimadzu QP-2010 Plus (Tokyo, Japan), at the same chromatographic conditions as those described for GC-FID, except the carrier gas that was used helium. The GC/MS interface and ion source (+70 eV) were held at 280 °C and the single quadrupole mass analyzer was operated in scan mode (35–350 m/z).

Sensitivity analysis: estimation of the squalene production

171 The estimation of the biomass and annual squalene production was based on the
172 industries of different capacities (100, 1000 and 10,000 m^3/d), operating 24 h per day,
173 336 days per year. Biomass and squalene concentration data were used to calculate the
174 biomass productivity [$P_X = (X_i - X_{i-1})(t_i - t_{i-1})^{-1}$, g/$m^3$/d] and squalene productivity
175 [$P_S = P_X$. S, g/m^3/d], where X_i is the biomass concentration at time t_i (g/m^3), X_{i-1} is the
176 biomass concentration at time t_{i-1} (g/m^3), t is the residence time (d) and S is the squalene
177 concentration in the biomass (μg/g).

178 **Results and Discussion**

179 **Squalene determination in cyanobacterial biomass**

180 Squalene was positively identified in the biomass lipid extract, and a suitable selectivity in the
181 chromatogram was observed (Fig. 1). In the linear range evaluated (1–50 mg/L) a correlation
182 coefficient of 0.998 was observed, indicating satisfactory linearity under this method. The
183 calibration curve was constructed by plotting the peak area versus the squalene concentration
184 and the calibration curve parameters were 3822.1 for the slope and 14216 for the intercept.
185 The method shows a LOD and LOQ of 0.3 and 1.0 ng/mL, respectively. The precision of the
186 method expressed as relative standard deviation (RSD) was 12.0%. Accuracy was acquired in
187 triplicate with the spike of 20 μg/mL of the standard and the average result was 101%.
188 According to European Commission (26), acceptable values of recovery range from 70% to
189 120%; hence, in this instance, our result can be considered to be acceptable.

190 **Estimation of squalene production in heterotrophic cultivation**

191 Squalene production in the cyanobacteria *Phormidium autumnale* was observed in this
192 research and the values are described in **Table 1**. The squalene was quantitatively evaluated
193 in a concentration of 0.179 g/Kg of dry biomass matter. The total lipid fraction extracted was
194 10.3 g/100 g. The growth kinetics obtained in this study showed a high biomass productivity
195 of 0.36 g/L/d. Squalene productivity is the main criterion for the selection of production

196 systems. Considering this bioactive is a lipophilic intracellular compound, the squalene
197 productivity is expressed as the squalene content multiplied by biomass productivity,
198 resulting in a squalene productivity of 0.000134 g/L/d in parallel to 0.037 g/L/d of lipid
199 productivity (27).

200 Additionally, the cultivation system is related to the conversion of the organic carbon
201 and the simultaneous production of compounds with metabolic activity. A conversion of the
202 squalene 0.000058 ($g_{Squalene}/mg_{Carbon}$), lipids 0.033 (g_{Lipids}/mg_{Carbon}) and biomass 0.32
203 ($g_{biomass}/g_{carbon}$) was observed with the cultivation system used. In other studies, *P. autumnale*
204 demonstrate a high capability to organic matter removal (28) and simultaneous production of
205 high-value compounds such as carotenoids (23).

206 Therefore, squalene extraction from cyanobacteria by using food wastewater as a
207 substrate proved to be an alternative to shark slaughter. Currently, many documents prevent
208 this social and environmental problem (e.g., plans of action to prevent this slaughter) (10).
209 The microalgae biomass demonstrates that productivity can be obtained throughout the year,
210 without a dependence of long production times to acquire the product, as it is a renewable
211 source. In addition, this compound demonstrates various applications in the food industry, and
212 for this reason, it has drawn attention as a supplement (29).

213 In this sense, taking into account that nowadays the global market increases demand
214 for compounds with bioactivity, the cyanobacterial biomass exploration rich in these
215 compounds can have a great future for industrial-scale production. In view of this possibility,
216 **Table 2** shows the results for a sensitivity analysis of squalene production in different
217 industrial capacities. These results indicate that it is possible to produce 1,522, 15,216 and
218 152,156 kg of squalene/year for industrial capability.

219 Unfortunately, deep sea shark liver oil is the most common form of squalene isolation
220 for a supplement. In general, sharks reach their prime age at 15 years old, considering their

fishery into adult age. Further, on average, 50% of the liver represents the squalene content, while the shark's extraction capacity will vary by species. In this sense, it is possible to estimate that the Bird break shark has the capability to produce 265.2 g/liver and the Black dog fish has the capability to produce 307.5 g/liver (30,31). This liver concentration promotes intense shark hunting, as this oil is used as a basis for healthcare products. In comparison with this traditional source, to obtain an amount similar to that acquired from a small industry, it is necessary to slaughter at least 79,538 bird break sharks and 86,256 of black dog fish species in one year.

In this way, cyanobacteria have higher production rates during the entire year and can be explored as a new squalene production system. Additionally, owing to the concern to protect marine animals and the presence of pollutants in the liver, limited squalene production exists, and this provides a strong motivation to search for alternative squalene sources (32). In turn distinct microbial biotechnological routes from squalene production have been explored, including the *Saccharomyces cerevisiae* strain, which presents a concentration of 41.16 μg/g (33). This is substantially lower than that of *P. autumnale* (179 μg/g), which has not been reported before in this strain growth in heterotrophic systems. On the other hand, the yeast *Torulaspora delbrueckii* has an elevated production of 430 μg/g (34). However, cyanobacteria have the ability to provide a tertiary biotreatment and can also produce this bioactive simultaneously because they can grow in diverse conditions using inorganic nitrogen and phosphorus. They also have the capacity to remove toxic organic compounds as well as heavy metals (35).

Finally, the squalene content in a microorganism is located in the cell, more specifically the configuration of squalene determine the location, for example, if the squalene structure is similar to an sterol configuration, it probably stays into the cellular membrane (2). Thus it should be considered that the squalene in cyanobacteria is an intracellular component

246 situated in the lipid fraction whose extraction occurs simultaneously with the lipid content
247 from the biomass.

248 Generally, the lipid composition of single cell oil is present in metabolites with high
249 nutritional value beyond squalene, such as fatty acids, particularly the unsaturated profile. In
250 the cyanobacteria biomass, 10 fatty acids were identified, as observed in **Table 3**. Altogether,
251 48% were saturated (SFA) and 52% were unsaturated fatty acids, of which 26.4% were
252 monounsaturated (MUFA) and 25.5% were polyunsaturated fatty acids (PUFA). Also a
253 representative amount of palmitic acid (C16:0) and oleic acid (18:1n9) were found in this
254 biomass. Besides in the use of cyanobacteria oil it is contained all lipid composition and the
255 profile demonstrate a similarity to the shark liver oil lipid profile (31).

256 In this study, squalene can be considered to be a powerful and sustainable metabolic
257 route of this compound compared with non-renewable sources. Also shows effective kinetic
258 parameters. Indeed, the sensibility analysis demonstrates a bioactive compound production of
259 ton/years in different industrial capacities. For this reason, the proposed technological route
260 demonstrates great potential and can be employed as a route of squalene production.

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267 **Conflict of Interest statements**

268 Mariane Bittencourt Fagundes declares that she has no conflict of interest and all the
269 other authors: Raquel Guidetti Vendruscolo, Mariana Manzone Maroneze, Cristiano

Ragagnin Menezes, Leila Queiroz Zepka, Juliano Smanioto Barin, Eduardo Jacob-Lopes and Roger Wagner also declares that they have no conflict of interest.

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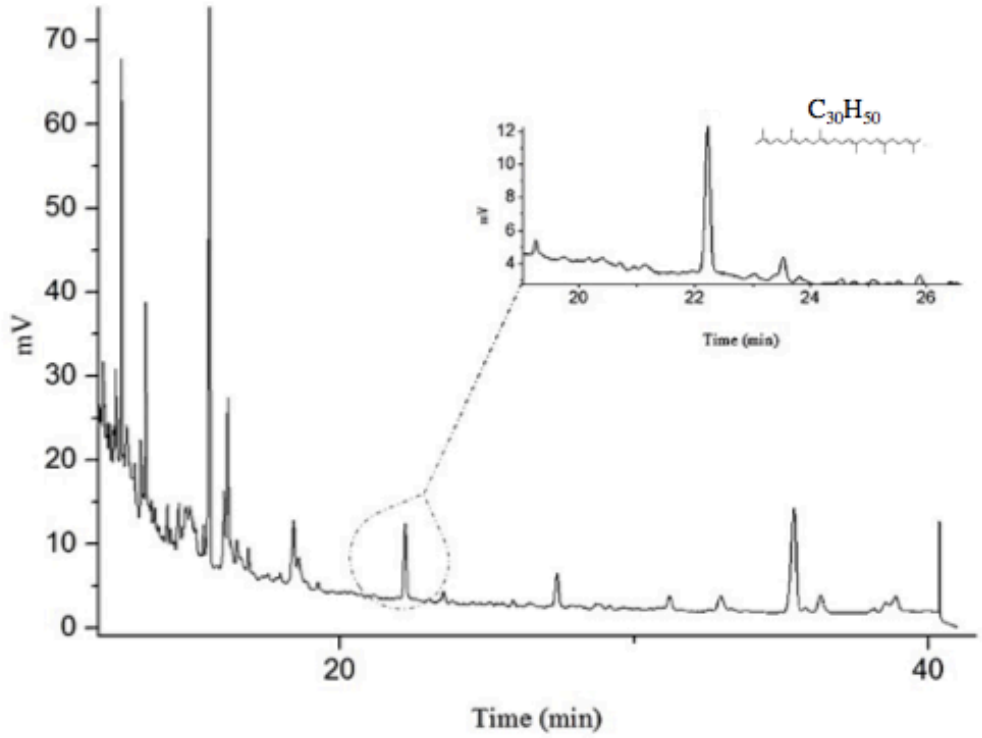

Fig. 1 GC-FID chromatogram of squalene from *Phormidium autumnale.*

Table 1. Kinetic of growth and squalene productivity of *Phormidium autumnale*

Squalene (g/kg CDW)	0.179±11.4
Total Lipids (g/100g)	10.300±0.01
P_{sq} (g/L/d)	0.000134±0.24
P_x (g/L/d)	0.360±6.40
P_L (g/L/d)	0.037±1.10
$Y_{x/s}$ (g biomass/g carbon)	0.32±0.0
$Y_{pl/s}$ (g squalene/g carbon)	0.000058±0.0
$Y_{ps/s}$ (g lipids/g carbon)	0.033±0.0
Tg (h)	48±0.0

CDW: Cellular dry weight; *P*x: biomass productivity, *P*sq: squalene productivity, P_L: lipid productivity, $Y_{x/s}$: biomass yield coefficient, $Y_{p/s}$: squalene yield coefficient, $Y_{p/s}$: lipid yield coefficient Tg: generation time

Table 2. Sensivity analysis of squalene production from cyanobacteria biomass based on industrial scale with different capacities.

Industrial capacity (m^3/d)	Production of squalene kg/year
100	1,522
1,000	15,216
10,000	152,156

Table 3. Fatty acids profile of cyanobacteria *Phormidium autumnale*

Fatty Acids	**Percentage (%)**
16:0	31.41±1.48
16:1	4.53±0.50
17:0	2.33±0.40
18:0	10.35±2.57
18:1n9c	21.96±4.72
18:2n6c	16.90±1.85
18:3n6	4.17±0.01
18:3n3	2.70 ± 0.59
20:4n6	0.45± 0.04
22:2	1.27± 0.26
SFA ∑	48.02±1.56
MUFA ∑	26.49±2.61
PUFA ∑	25.49±0.55

SFA: Saturated fatty acids; MUFA: monounsaturated fatty acids; PUFA: Polyunsaturated fatty acids.

Manuscrito 2

HETEROTROPHIC CYANOBACTERIA CULTIVATION PROSPECTS: A SOURCE OF STEROLS COMPOUNDS

Será submetido à revista Bioresource Technology[2]

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Heterotrophic cyanobacteria cultivation prospects: a source of sterols compounds

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Abstract

The aim of this research was to evaluate the sterols profile and production from the cyanobacteria *Phormidium autumnale* cultivated in a heterotrophic system by using distinct sources of carbon. In this study, a saponification ultrasound-assisted method was performed to determine the sterol and other non-saponified compounds in the dry biomass. The exogenous carbon sources used were: glucose, sucrose and agroindustrial wastewater. The experiments were conducted in a bubble column bioreactor with 2L of work volume, previously sterilized. The compounds were evaluated by using gas chromatography with flame ionization, and mass spectrometry detectors and the method was validated for cyanobacteria biomass. The results for the figures of merit confirmed that the method is satisfactory. Therefore the major compounds found were: squalene (1440.4 μg g^{-1}) and ergosterol (1033.3 μg g^{-1}), obtained from the glucose cultivation, also stigmasterol (455.3 μg g^{-1}), obtained from the wastewater cultivation, for the sucrose culture was observed a wide array of minor sterols and (225.4 μg g^{-1}) squalene as as major compound. Comprising the non-saponified identified compounds by mass spectrometer from biomasses were found 24 compounds among this molecules: hope-22, 29-en-3-one, squalene and 22 sterols, hence the wastewater cultivation propitiated the highest diversity of phytosterols. Thus the exogenous sources demonstrated that could produce distinct sterols. In this sense, the biomass from *P. autumnale* can be a possible source of sterols and other bioactive compounds to the application in diverse areas

Keywords: gas chromatography, microalgae, *Phormidium autumnale*, non-saponified extract, metabolic pathways.

1. Introduction

Sterols are biosynthesized by diverse types of microorganisms and the basic structure is a tetracyclic triterpenoid, specifically a cyclopentanoperhydrophenanthrene ring with a hydroxyl group located on the third carbon. A wide range of sterols differs according to the position and presence of double bonds in the ring systems (Goad & Akihisa, 2012). Currently, some sterols are considered compounds with health human benefits and they are established as being ingredients in functional food products, and this class of molecules is called of phytosterols. They act reducing cholesterol level in the blood, due to the phytosterols interfere with the cholesterol absorption in the intestine because of the structure similarity with this molecule (Abuajah et al., 2015). Also, some studies have shown that they can present anticarcinogenic proprieties (Ros, 2010; Singh et al., 2016).

Therefore, these compounds can be found in animal cells, being cholesterol the major final metabolic sterol (Howles, 2016), in superior plants the major compounds are, stigmasterol, β-sitosterol and ergosterol (Valitova et al., 2016). In eukaryotic microorganism, sterols are found in higher concentrations and represent an important key in the fluidity and cellular membrane regulation. On the other hand, prokaryotes present lower concentrations, and some studies show that these molecules were not observed (Volkman, 2003). The microalgae are unicellular microorganism, and they can be prokaryotes or eukaryotes (Zhu, 2015), also present two energy obtainment metabolisms, heterotrophic and photoautotrophic (Morales-Sánchez et al., 2015).

The prokaryotic microalgae are called cyanobacteria and are preferably photosynthetic, nevertheless, some species are capable of metabolizes the organic carbon, such as *Phormidium autumnale* (Siqueira et al., 2016). The cyanobacteria have many applications for the human health, but a wide range of unexplored secondary metabolites with

high-value in diverse industry areas, hence, the sterols are one of those compounds (Oms-Oliu et al., 2013).

Thus, there are few studies about the cyanobacteria sterol profile in which can be isolated a complex sterol composition, in turn, nowadays still exist a need for sterol profile exploration and correlation with the metabolic pathway from the cyanobacteria biomass (Borowitzka, 2013; Volkman, 2016). Sterols are found in the cell at esterified or free forms, in this sense, a method for total extraction is necessary. So ultrasound-assisted extraction (UAE) has attracted attention because it is an efficient method for the extraction of numerous compounds (Picó, 2013). Also, gas chromatography is a common and ideal technique to the sterol profile determination because can present a complex sterol separation and quantification (Grasso et al., 2016).

Considering the unexplored cyanobacteria sterol composition and production, the aim of this research was to explore some of those secondary metabolites and the different sources of carbon on heterotrophic cultivation influence on the sterol composition from the cyanobacteria *Phormidium autumnale* biomass.

2. Material and Methods

2.1 Reagents

The following reagents were of analytical grade: ethanol, methanol, hexane, isopropanol, potassium hydroxide (KOH) and sodium chloride (NaCl) were obtained from Vetec (São Paulo, SP, Br). The glucose and sucrose used in cultivation systems were from Dinâmica (São Paulo, SP, Br). The standards compounds squalene (99%), cholesterol (99%) and β-sitosterol (95%) were obtained from Sigma-Aldrich (Saint Luis, MO, USA). Squalene, cholesterol and

β-sitosterol stock solution with a 1 mg mL^{-1} concentration were prepared by weighing 10 mg of each standard in a volumetric flask of 10 mL and completed with hexane.

2.2 Microorganisms and culture media

Axenic cultures of *P. 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^{-1}) containing synthetic BG11 medium (Rippka et al., 1979). The cultivation conditions used were 25 °C, a photon flux density of 15 μmol/m/s and a photoperiod of 12/12 hours light/dark.

2.3 Microalgal biomass production

D-glucose and sucrose both at 12 g L^{-1} (Francisco et al., 2014), and also the slaughterhouse wastewater, acquired from an industry located in Santa Catarina, Brazil (27º14'02"S, 52º01'40"W), were evaluated as sources of carbon in the unsaponifiable lipid fraction. The wastewater was obtained from the discharge point of an equalization tank over a period of one year, and carefully studied for pH, chemical oxygen demand (COD), total phosphorus ($P\text{-}PO_4^{-3}$), total nitrogen (N-TKN), suspended solids (SS), volatile solids (VS), fixed solids (FS), and total solids (TS), following the Standard Methods for the Examination of Water and Wastewater (APHA, 2005). Also, the characterization of the cultivations systems can be observed in **Table 1.**

The biomass production was realized in heterotrophic conditions in a bubble bioreactor in agreement with Francisco et al., (2014). The cultivation experiment was performed on bubble column bioreactor for all the treatments parameters, three exogenous sources of carbon, 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^{-1}, temperature of 26 °C, 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 or medium, depending on the treatments, per minute, absence of light and a residence time of 168 h (Roso et al., 2015).

The biomass was separated from cultivation medium by centrifugation for 10 min at 10000 rpm using a Hitachi (Tokyo, Japan). It was subsequently freeze-dried for 24 hours at -50 °C under -175 mmHg. The cultivations were performed twice and in duplicate. Therefore, experimental data refer to the mean value of the four repetitions.

2.4 Sample preparation

To the sterols extraction in the biomass, a direct saponification was performed in agreement with Pereira et al. (2016) with some modifications. Briefly, 500 mg of dry biomass was weighed in a falcon tube and consequently, 20 mL of ethanolic KOH solution (10% w/v) was added. After it was transferred and submitted to an ultrasound probe (Sonifier 250, Branson- USA) with 20 kHz of frequency and 200 W of output, the amplitude of 70%, in a continuous mode for 30 minutes. Following it was added a salt-saturated solution to finished the saponification step. Afterward, the partition was acquired with the addition of 10 mL of hexane twice. The organic fraction was dried under nitrogen, and sterol compounds were suspended in 200 μL of 3:2 (hexane: isopropanol) solution. Additionally, the sterol profile from the wastewater was analyzed according to Gilli et al. (2006).

2.5 Sterol determination

The total sterol fraction obtained from the ultrasound extraction was analyzed by using a gas chromatography instrument equipped with a flame ionization detector (GC-FID) Varian 3400 (Palo Alto, CA, USA). The injection port of GC-FID operated in splitless mode (splitter valve off by 0.8 minutes; 50:1) at 280 °C. Hydrogen at a constant pressure of 15 psi was used

as the carrier gas. The separation was performed in a non-polar column BPX-5MS SGE (Sydney, NSW, AUS, 25 m × 0.22 mm id × 0.25 μm). The temperature program was initially 60 °C, with an increase to 280 °C at a rate of 15 °C min^{-1} and then up to 330 °C at a rate of 5 °C min^{-1}, maintaining the isothermal conditions for 5 minutes. The temperature of the detector was maintained at 280 °C.

The sterols were quantified by using six-point analytical curves, the curves were as following: 500–1000 mg L^{-1} for cholesterol and 50–1000 mg L^{-1} for squalene and β-sitosterol, some parameters of validation were studied. The linear range was defined according to the sterols concentration found in the samples. To the linearity study, a linear regression equation was used, and the linear correlation coefficient (R^2) of the calibration curve was determined. The precision was expressed as relative standard deviation (RSD). The limit of detection (LOD) was estimated according to the concentration of the compound at a signal-to-noise ratio of 3. The limit of quantification (LOQ) was achieved by injecting sequential dilutions of the standards and calculated considering a concentration that would result in a signal-to-noise ratio higher or equal to 10. Accuracy was determined by recovery assay from samples spiked with a known amount of the standard in a concentration of 50% of the concentration found in the biomass for the compounds. The results were expressed as a percentage of the standard recovered.

Sterols for which authentic standards were not available (stigmasterol and ergosterol) were quantified as cholesterol equivalents (Martin-Creuzburg & Merkel, 2016), using the cholesterol multipoint calibration curve, which was chosen to the calibration curve angle similarity after a sample sequence dilutions.

147 The identification of the compounds was performed by using gas chromatography coupled
148 to a mass spectrometer (GC/MS), Shimadzu QP-2010 Plus (Tokyo, Japan), at the same
149 chromatographic conditions as those described for GC-FID, except the carrier gas that was
150 used helium. The GC/MS interface and ion source (+70 eV) were held at 280 °C and the
151 single quadrupole mass analyzer was operated in scan mode (35–350 m/z). Squalene and
152 above cited compounds were positively identified by a comparison of the retention time and
153 mass spectra obtained experimentally and authentic standard. The other compounds were only
154 tentatively identified by mass spectra experimental comparison with those obtained from
155 NIST 05 library (NIST 05, Gaithersburg, MD, USA).
156

157 **3. Results and Discussion**
158

159 **3.1 Validation parameters for sterol determination in cyanobacteria biomass**

160 Cyanobacterial biomass presents a diverse range of sterols that can be found esterified in
161 phospholipids or free into the membrane cell. The sterols precursor is squalene a triterpenoid
162 compound, in which depending on the configuration can be found in the cell membrane or the
163 cytosol (Spanova & Daum, 2011). In fact for the total sterol and their precursor determination
164 has being used ultrasound to assist the extraction and recently Pereira et al, (2016) shows a
165 simultaneous extraction and saponification because it was necessary the use of ultrasound to
166 provide the sterol profile from the biomass. For these propose, a validation method was
167 performed and all the parameters of merit are shown in **Table 2.**

168 Validation was performed for the compounds squalene, cholesterol and β-sitosterol
169 and the parameters of merit linearity, precision, and accuracy were compared to the criteria
170 set by the international conference on harmonization (ICH guidelines, 2005) to observe if the
171 method was acceptable to be reproduced in cyanobacteria. Therefore it was noticed a good

linearity (**Table 2**), expressed as determination coefficient (R^2). In other studies with pork lion and vegetable oil, it was also constructed an external calibration curve, and it was observed a similar linearity 0.996 for cholesterol, however for β-sitosterol was observed a coefficient of 0.910 (M & Du, 2002). However, Grasso et al. (2016) acquired in their study a coefficient of 0.991 for β-sitosterol also indicating a good linearity and the accuracy was 102.8% for both, stigmasterol and β-sitosterol from enriched turkey. In this research was also observed accuracy values ranged from 93% to 97% that are in agreement with acceptable values.

Likewise, the study demonstrates low detection and quantification limits, indicating that the gas chromatography is a good for the separation of these secondary metabolites contained in complex matrices.

3.2 Sterol characterization in cyanobacteria biomass

Regarding the distinct profiles of the biomass non-saponifiable fraction, the variations are shown in **Figure 1.** In this figure, chromatograms present several peaks, minor compounds that were tentatively identified by the mass spectrometer and are shown in **Table 4.** Thus, among all the treatments were identified a total of 22 sterols in *P. autumnale*, their triterpenoid precursor squalene and also it was observed a hopanoid, Hope-22, 29-en-3-one.

It is well known that several sterols have nutritional effects and the microalgae present a complex group, some of them in small amounts, these molecules are formed in microalgae, as well as in the cyanobacteria metabolism, like secondary metabolites. In this way, the composition variability found can be associated with the cultivation growth, photoautotrophic or heterotrophic, algae taxonomic classification and others (Volkman, 2016). The heterotrophic cultivation may influence in the sterol composition because the respiration in the dark is supported by a carbon source replacing the light energy and it is well known that

the diverse carbon sources can imply in the formation of numerous metabolites (Perez-Garcia et al., 2011).

In the sterol, *Phormidium autumnale* composition is interesting to describe that β-sitosterol, cholesterol and squalene were positively identified on mass spectrometer and moreover were performed quantitatively. In the present study on **Table 3** can be observed the compounds concentrations after a residence time of 168 hours of residence time. Within these molecules, the major compounds detected were squalene, cholesterol and stigmasterol.

Squalene is found in diverse types of cells, is consider the sterol precursor and also exhibit several health benefits, including antioxidant and anticarcinogenic (Camera et al., 2015; Xu et al., 2004). Consequently for the squalene formation occurs a production of molecules also responsible for the sterol production pathway such as acetyl-CoA for mevalonate pathway (MVA), pyruvate and glyceraldehyde-3-phosphate (GAP) for methyl erythritol pyrophosphate (MEP), known as the non-mevalonate pathway. Both metabolic pathways are responsible for two sterols building blocks, isopentyl pyrophosphate and dimethylallyl pyrophosphate, in theory, the pentose-phosphate pathway is activated at the moment that the cyanobacteria stay in total light absence initiating the formation of acetyl-CoA, pyruvate and GAP (Perez-Garcia et al., 2011).

Squalene is an essential product from these two pathways, therefore in eukaryotic microorganisms, they are responsible for the cell membrane regulation, nevertheless in cyanobacteria the molecules responsible are the hopanoids, for this reason, many studies described that in the cyanobacteria there is no existence of sterols (Basen et al., 2012). However nowadays there are more techniques and equipment for their determination, and the genome of the homologs squalene oxide cyclase enzymes were evidenced in two cyanobacteria, this enzyme is responsible for the squalene conversion to sterols, proving the existence of this pathway (Wei et al., 2016).

The squalene found was substantially higher in the glucose cultivation than the others sources of carbon, probably because glucose is the easiest form of assimilation to cyanobacteria them disaccharides and polysaccharides since they need to be hydrolyzed (Francisco et al., 2014) and maybe the higher reactions velocity provide the squalene accumulation. This accumulation can be proved, because of the inoculum, the initial point that is the experiment of the cyanobacteria only with the propagation medium, present value substantially lower (**Table 3**).

The sucrose present value superior them the inoculum, however, was lower them glucose and the slaughterhouse wastewater culture. Also, the wastewater cultivation shows a variety of compounds including squalene and among them, their end products: β-sitosterol, cholesterol, and stigmasterol, which are present higher quantities. In other research stigmasterol was found with 0.014 $mg.g^{-1}$ as the higher concentration acquired from *Adenocystis utricularares* macroalgae, that was lower than the concentration found in our wastewater cultivation with 0.455 $mg.g^{-1}$ (Pereira et al., 2016). This molecule including β-sitosterol has been known nowadays as an important essential for the prevention and therapy of dementia diseases (Shuang et al., 2016).

These results showed that the employed wastewater cultivation affected the sterol composition and it is suggested that can be one viable alternative to the heterotrophically sterol production because can recover the contained nutrients while producing a valuable biomass (Cuellar-Bermudez et al., 2016). Therefore, the phytosterol production in the wastewater cultivation can be associated with the phosphorus involved in the phosphorylation's reaction that is required for both mevalonate and non-mevalonate pathways because sterol composition changes also depend on the phosphorus amount in the growth. Moreover for the cyanobacteria are not needed large amounts of phosphorus (Markou & Georgakakis, 2011; Volkman, 2016).

The wastewater was analyzed to observe if there was sterols presence, and it was noticed that any sterols, including cholesterol, were detected in the wastewater, a possible cholesterol source. The presence of higher amounts of some sterols demonstrated that they were formed during the wastewater cultivation. Ergosterol was present also in a significant amount in the glucose cultivation, such as cholesterol in wastewater culture, but these non-conventional sterols were also detected in microalgae in other researchers (Lopes et al., 2011; Miller et al., 2012).

Also, the presence of cholesterol in prokaryotic cells is not new, according to Bode et al. (2003). Despite it was detected the presence of ketone groups and one of the major compounds was cholestan-4-en-3-ona, that can be observed below in **Table 4.** The ketone molecule was present in both sucrose and the wastewater cultivation. This compound is important to the pharmaceutical industry. Besides in a study, enzymes of *Mycobacterium neoaurum* propitiated the cholesterol conversion to cholestan-4-en-3one (Shao et al., 2015). Thus, the cyanobacteria might present enzymes that can convert cholesterol. Indeed many microorganisms are able to convert sterols in aerobic conditions, but a few studies proved these biotransformations according to Dykstra et al. (2014). Also in aerobic conditions cholesterol is rapidly converted to cholest-5-en-3-ona, followed by the isomerization to cholest-4-en-3-ona, the enzymes involved is cholesterol oxidases and cholesterol dehydrogenases, that are responsible for the cholesterol to cholest-4-en-3-one conversion.

Taking to account the sucrose cultivation it was not observed the cholesterol presence, but cholestan-4-en-3-one (**Table 4**) was found indicating the possibility of previous cholesterol existence in the medium. On the other hand, it were present lanosterol in a little amount and also identified in other prokaryote microorganisms, being an ordinary molecule for this class of microorganism (Bode et al., 2003). Lanosterol lately also has been known as a molecule that is involved in the cataract prevention (Zhao et al., 2015).

Gives to the high cholesterol production capability from the wastewater cultivation, enables the cholesterol to be used as a building block for sterol drugs to pharmaceuticals industry. Also the presence of cholest-4-ene-3-one in line with the hypothesis of Kumari and Shamsher (2015) demonstrate to be an intermediate structure with intact side chain to androst-4-ene-3,17-dione, that is intermediary in the pathway for the drugs hormones. In this way, the presence of this ketone molecule suggests that the cyanobacteria can be able to biotransform the cholesterol into high-value chemicals to the industry. Fortunately, it was already reported that the conversion of androst-4-en-3,7-dione to testosterone, and it was realized by a microalgae *Nostoc muscorum* according to Arabi et al. (2010).

According to the exogenous source of carbon, the inoculum presents only 2 metabolites: cycloartenol and the hopanoid precursor in the oxidized form Hope-22, 29-en-3-one. Cycloartenol is the first step to the formation of phytosterols, being an important structure from the MEP pathway (Banerjee & Sharkey, 2014), in this way the inoculum propitiated the first steps of the phytosterols production and may present the phytosterols production without the presence of organic carbon source, but in small amounts.

Also, it was observed the presence of one hopanoid, hop-22(29)-en-3-one that is biosynthesized by bacteria via cyclization of squalene to diploptene (hop-22(29)-ene). The existence of this molecules is associated with the same sterols proposes for eukaryotes microorganisms, because hopanoids have similar structure and are cyclized by related enzymes (Saenz et al., 2015). The hopanoid presence means the existence of an enzyme squalene hopanoid cyclase, moreover, the presence of this enzyme doesn't change the presence of another enzyme 2,3-oxidesqualene cyclize, that is responsible for the sterols formation (Wei et al., 2016).

The glucose cultivation present 4-ergosterol's pathway products in their original form and oxidized, within them 5,6-dehydroergosterol, 7,22-ergostadienona, ergosta-4,6,8(14),22-

297 tetraen-3-one and ergosta-5,8(14)dien-3-ol. The 7,22-ergostadienona is an oxidized form from
298 the 7,22-ergostadienol, and this molecule in other study was detected in microalgae biomass
299 proving that is an ergosterol's derivative (Volkman, 2016). Also, the ergosta-5,8(14)dien-3-ol
300 that is known as ergone, that is considered a bioactive, gives to its anti-tumor activity (Sun et
301 al., 2013).

302 In this culture, it were also present two molecules from cycloartenol pathway, the first
303 product formed in the pathway, cycloartenol, and 3β-5α-stigmasta-7-25-dien-3-ol, an
304 intermediate compound for the β-sitosterol and stigmasterol synthesis (Nes, 2011). In the
305 cholesterol pathway, it was present only 4,22-cholestadien-3-one, as an intermediary
306 metabolite. Indeed the results suggest that glucose cultivation taking to account mainly the
307 metabolites products related to the ergosterol pathway, it is in line with the quantitative
308 analysis (**Table 3**) because one of the major products found was ergosterol.

309 The glucose can propitiate the higher rates of growth and it can be observed in a study
310 according to Zheng et al. (2012) in which *Chorella sorokiniana* using glucose as exogenous
311 carbon source also showed a high lipid production. Additionally, the cyanobacteria are
312 capable of accumulating substantial lipid quantities in the thylakoid membrane (Modiri et al.,
313 2015). Thus due to the higher rates of growth, physiological changes in the biomass and also
314 the lipid accumulation, the use of glucose can affect the metabolic routes, some of them
315 possibly could be associated with lipidomics (Josephine et al., 2015).

316 Therefore, the sterols are present in the unsaponifiable fraction from lipids,
317 considering the higher rates of the maximum specific growth acquired with glucose
318 (Franscisco, 2014), and that the glucose is the most energetic molecule per mol, possibly the
319 metabolic sterol pathway production occurs in a short period of time. In comparison with the
320 other carbon sources and for this reason a substantial content of squalene and ergosterol, with

several intermediary products were found in the biomass may be due to the glucose easiest form of assimilation.

For the sucrose cultivation, were found major molecules associated with the cholesterol intermediary pathway, oxidized cholesterol products as well as cholestan-2,4-diene, cholest-4-en-3-one, 4,22-cholestadien-3-one and also lanosterol. This strain also present 3 metabolites related with the ergosterol pathway, being those 5,6 dehydro ergosterol, ergosta-4,6,22-trien-3-one, 7,22-ergostadienona, and two metabolites from the cycloartenol pathway 22-stigmasten-3-one, an oxidized molecule from stigmasterol (Martin-Creuzburg & Merkel, 2016) and stigmast-4-en-3-one, that is known to possess anticancer proprieties (Choo et al., 2015).

The presence of this molecules demonstrates that phytosterols are formed in this cultivation system. Although many microorganism present enzymes capable of hydrolyzing the sucrose molecule, including the cyanobacteria, that present exogenous enzymes, nevertheless the absorption of hydrolyzed hexoses is not fast (Modiri et al., 2015), this culture presents low squalene and sterols concentrations according to **Table 3** and the results obtained suggests that the lower concentrations can be related with to carbohydrate absorption and for this reason, the metabolic pathways can happen, however slowly.

The richest sterols cultivation system was the wastewater culture that presents as a major compounds: β-sitosterol, stigmasterol, cholesterol and the sterol precursor squalene. Additionally, the minor sterols present in the culture were 3β-ergosta,5,7-dien-3-ol (brassicasterol), in which was also observed in brown macroalgae as one of the major compounds (Pereira et al., 2016), while as a minor constituent in *Choricystis minor* (Martin-Creuzburg & Merkel, 2016), lower abundance was found similarly in our study with the *P. autumnale* strain. Moreover, brassicasterol isomer was also observed ergosta-5,22-dien-3-ol (epibrassicasterol). These brassinosteroids present cytotoxic effects in various types of cancer

346 and their biosynthesis can come from campesterol, sitosterol, cholesterol (Oklestkova et al.,
347 2015), It was found in this culture also, Ergosta-4,6,8(14),22 tetraen-3-one (ergone), and two
348 metabolites from the cholesterol pathway the cholesta-4-en-3-one and 5α-14β-cholestane.
349 Cholesterol was one of the major compounds in the strain cultivated with wastewater.
350 However it was absent in the wastewater medium and only 2 metabolites that are involved in
351 cholesterol synthesis was detected, 4,4-dimethyl-5 alpha-cholesta-8,14-diene-3-beta-ol and
352 5α-,14β-cholestane. The presence of 4,4-dimethyl sterol, one of the cholesterol intermediary
353 pathways in prokaryotes, indicates that the molecule might be used by enzymes from the
354 cyanobacteria to the cholesterol production on the cultivation system (Bode et al., 2003; Nes,
355 2011).
356 In distinguishing of the other cultivations the ergosterol was found in the biomass in
357 small amounts regarding the cholesterol, but it is interesting that ergone was also found. By
358 means comprising all the sterols, the treatment that proved the production of several metabolites
359 of high-value was the cultivation using wastewater as the carbon source.
360 It is strongly emphasized that the cyanobacteria biomass composition changes according
361 to the cultivation, and can be affected, such as manipulated by various cultivation factors
362 (Markou & Georgakakis, 2011). With regard to wastewater treatment, the cyanobacteria have
363 the characteristic of removing the organic carbon. Such schemes can be associated with the
364 wastewater nutrients composition, besides the phosphorus already discussed, the wastewater
365 present also nitrogen as one of them (**Table 1**).
366 As a matter of fact, the nitrogen assimilation occurs through the glutamine synthetase
367 pathway, when nitrate is available it is reduced intracellularly by nitrate reductase to nitrite,
368 which is reduced again to ammonium by the nitrite reductase enzyme. Therefore the
369 cyanobacteria prefer to utilize already reduced nitrogen, such as ammonium because nitrate
370 reduction required energy from the cyanobacteria. In this sense, the agroindustrial wastewater

presents high ammonium and phosphate concentration, because due to the synergism between bacteria and microalgae in decomposing various organic substances, enables the production of these metabolites, which are essential to the cyanobacteria growth. The nitrogen can be used to the amino acids biosynthesis, among them, leucine, that according to Bode et al. (2003) this amino acid is a suitable precursor of isoprenoids in myxobacteria because it is degraded into 3-hydroxy-3-methylglutaryl-coA (HMG-CoA). HMG-CoA is easily incorporated in the isoprenoid pathway, specifically in the cycloartenol pathway, as related in *Stigmatella aurantiaca* (Bode et al., 2003). Therefore the presence of the free intracellular amino acid leucine, in the *Phormidium autumnale,* biomass is confirmed in another study (Vendruscolo, 2016).

The use of distinct carbon substrates promoted significant differences in the lipid profile as reported by Francisco et al., (2014). In this study with the cyanobacteria *Phormidium autumnale, it* was observed that sterols metabolic pathways also change according to the source of carbon, showing distinct lipid secondary metabolites for each treatment. Additionally in almost all treatments were found keto groups and this can be explained because similar to cholesterol, the phytosterols are susceptible to an auto-oxidation propitiating the formation of keto groups (Lin et al., 2016).

Conclusion

In conclusion, the sterol composition differs with distinct sources of carbon in a heterotrophic cultivation of *Phormidium autumnale*. In this way, the glucose and sucrose changed the sterol composition and this might be related to the carbohydrate assimilation, being glucose the most abundant sterol profile treatment between both. Therefore, the treatment of wastewater as source of carbon, present a several production of bioactive compounds and also the major compounds found in this cultivation system were: stigmasterol, cholesterol, squalene and β-sitosterol, the abundance of compounds obtained from the wastewater can be explained by the nutrients present in the medium, being phosphorus and nitrogen, one of them.

It is interesting that this study is the first attempted to link the information about the changes in the sterol profile according to the heterotrophic culture using distinct exogenous carbon sources. Moreover, the cultivation systems proved the cyanobacteria sterol production and variations according to the treatment, demonstrating that heterotrophic cultivation could be a new prospect for sterols production.

421 **Conflict of interest**

422 The authors declare that they have no conflict of interest.

423 **References**

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Table 1. Characterization parameters for the exogenous sources of carbon

Cultivation Treatments	
Exougenous sources of carbon	
Glucose	
Concentration	12 g L^{-1}
BG11 volume	2 L
Sucrose	
Concentration	12 g L^{-1}
BG11 volume	2 L
Slaughterhouse wastewater	
Wastewater volume	2 L
Wastewater characteristics [1]	
pH	5.9±0.05
COD mg L^{-1}	4100±874
P-PO_4^{-3} mg L^{-1}	2.84±0.2
N-TKN mg L^{-1}	128.5±12.1
SS mg L^{-1}	1.9±0.8
VS mg L^{-1}	2.9±1.4
FS mg L^{-1}	0.9±0.3
TS mg L^{-1}	3.8±2.7
C/N ratio	31.9
N/P ratio	45.2

[1] Wastewater characteristics: chemical oxygen demand (COD), total phosphorus (P-PO_4^{-3}), total nitrogen (N-TKN), suspended solids (SS), volatile solids (VS), fixed solids (FS), and total solids (TS).

Table 2. Parameters of merit for three standard squalene and sterols compounds.

Compounds	**Calibration curve** ($\mu g\ mL^{-1}$)	**R^2**	**Precision (%)**	**LOD** ($\mu g\ mL^{-1}$)	**LOQ** ($\mu g\ mL^{-1}$)	**Accuracy (%)**
Squalene	50-1000	0.9960	7.8	0.3	1	95
β-Sitosterol	50-1000	0.9906	15.6	1.5	5	93
Cholesterol	500-1000	0.9966	13.2	1.5	5	97

Table 3. Sterols and squalene determined in *P. autumnale* dry biomass (μg g^{-1}) obtained from the heterotrophic metabolism with distinct sources of carbon, the inoculum and the wastewater substrate performed in GC-FID.

Compounds	Inoculum	wastewater	Glucose cultivation	Sucrose cultivation	wastewater cultivation
Squalene	131.7 ± 0.1	-	1440.4 ± 3.5	225.4 ± 23.8	425.6 ± 16.9
Cholesterol	-	-	-	-	820.6 ± 8.2
*Ergosterol	-	-	1033.3 ± 3.7	-	-
*Stigmasterol	-	-	-	-	455.3 ± 2.2
β-sitosterol	-	-	-	-	279.0 ± 1.0

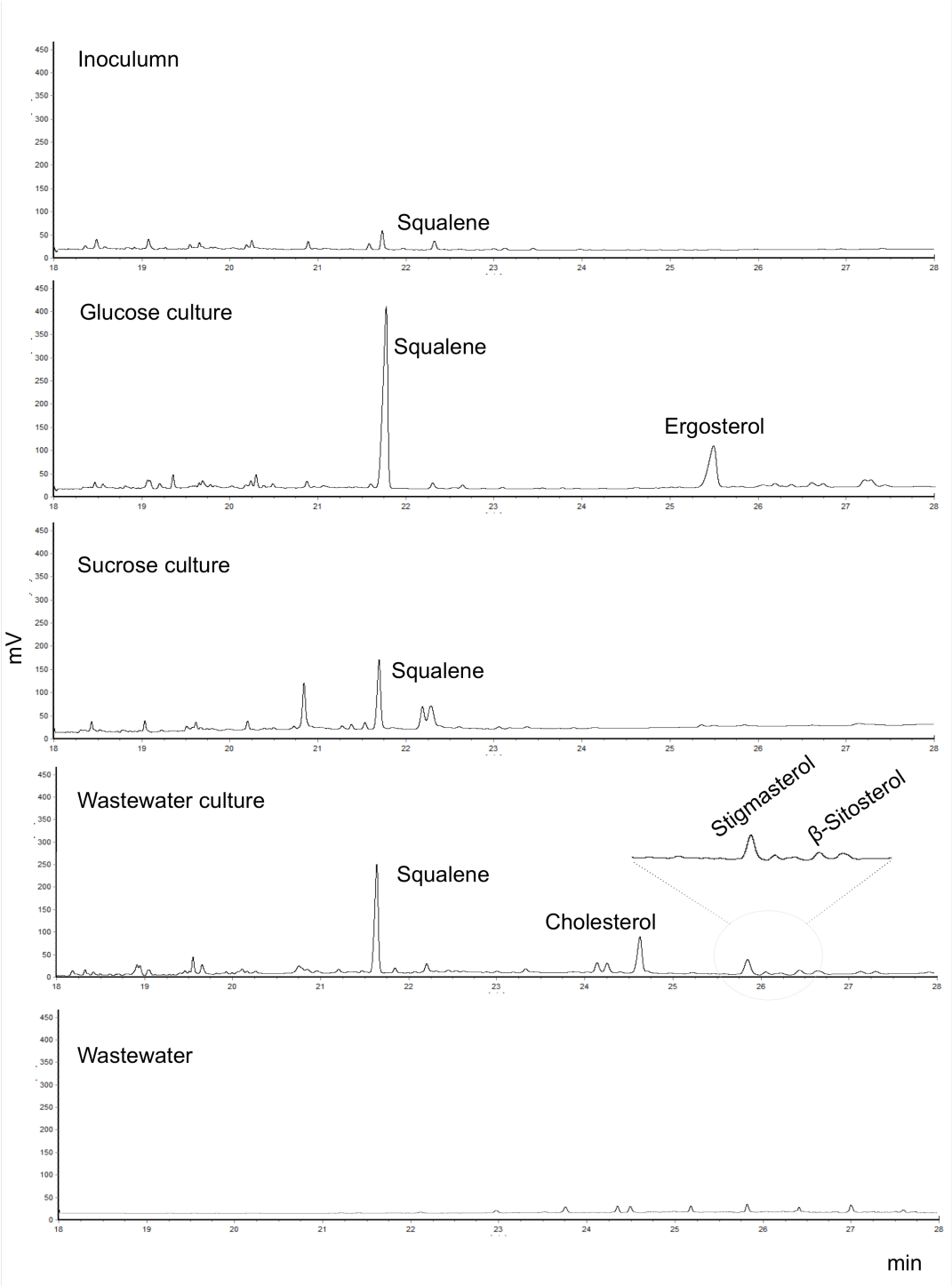
*Ergosterol and stigmasterol were quantified in cholesterol equivalents.

Table 4. Unsaponifiable compounds from cyanobacteria biomass obtained under heterotrophic metabolism.

IUPAC name (Trivial name)[1]	Inoculum	wastewater	Glucose cultivation	Sucrose cultivation	wastewater cultivation
Ergosta-5,7-dien-3-ol (dehydroergosterol)					++
5α,14β-Cholestane		+			++
(22E) 3α-Ergosta-5,22-dien-3-ol (Epibrassicasterol)					++
Cholesta-2,4-diene				++	
3β-Ergosta-5,7-dien-3-ol (Brassicasterol)					++
Cholest-4-en-3-one				++	++
3β-Lanosta-8,24-dien-3-ol (Lanosterol)				++	
5,6-Dihydroergosterol (5-dihydroergosterol)			++	++	
7,22-Ergostadienone			++	++	
3β, 5α-Stigmasta-7,25-dien-3-ol (24-ethyl desmosterol)			++		
(22E)-Cholesta-4,22-dien-3-one (4,22-Cholestadien-3-one)			++	++	
22-Stigmasten-3-one				++	
4,4-dimethyl-5 alpha-cholesta-8,14-diene-3 beta-ol		+			
(22E)-Ergosta-4,6,22-trien-3-one			++		
Ergost-5,8(14)-dien-3-ol			++		
Ergosta-4,6,8(14),22-tetraen-3-one (Ergone)			++	++	++
9β-19-cyclo-24-lanosten-3β-ol	++		++		
Hop-22(29)-en-3-one	++				
Stigmast-4-en-3-one (Sitostenone)				++	

[1] The identification was based on the agreement of the mass spectrum with NIST library; + arbitrary area raging from 1 to 6 ($x10^6$); ++ arbitrary area raging from 600 to 400000 ($x10^6$).

Figure 1. Distinct exogenous source of carbon as substrate for cyanobacteria and the major sterol production.



5 CONCLUSÃO GERAL

As cianobactérias são micro-organismos promissores para a produção de bioativos, entretanto ainda em relação a alguns metabólitos, são fontes naturais inexploradas. O cultivo heterotrófico aplicado a cianobactéria *Phormidium autumnale* demonstrou ser uma fonte de esqualeno. Pois considerando-se a elevada capacidade de produtividade lipídica da cianobactéria, altos teores de alguns bioativos contidos nesta fração lipídica podem ser obtidos. A *P. autumnale* por sua vez, confirmou ser uma nova via de produção de esqualeno, em relação a fonte de extração tradicional, pois devido sua produtividade o conteúdo torna-se superior relação ao fígado de tubarão, considerado fonte comercial. Da mesma forma a pesquisa demonstrou que fontes distintas de carbono exógenas ao serem empregados em cultivos heterotróficos podem modificar o metabolismo lipídico. Logo, com a manipulação das fontes de carbono obteve-se biomoléculas de rotas metabólicas de produção de esteróis distintas, bem como as concentrações de tais compostos, indicando possível relação com o substrato utilizado.

Elevados teores de esqualeno foram observados com o uso de glicose, assim como de ergosterol, os quais podem estar relacionados com a fonte de maior energia e de fácil assimilação. Logo também foi observado que o uso da sacarose promoveu baixos teores de esteróis, os quais estão correlacionados com a baixa velocidade de absorção de sacarose, pois existe a necessidade da hidrolise para que ocorra a absorção desta. Desta maneira, o efluente pode ser considerado uma fonte rica, pois apresenta uma variedade de fitoesteróis em elevadas concentrações e também pode ser considerado um processo tecnológico verde.

Assim o emprego de cultivos heterotróficos distintos proporcionam a compreensão das rotas de produção dos esteróis bem como de seu precursor para manipulação e aquisição destas moléculas de valor biológico, demonstrando ser novas fontes de aplicação em escala industrial.

6 SUGESTÃO DE TRABALHOS FUTUROS

- Reduzir o quantidade de amostra e volume de solventes para análise de esteróis;
- Avaliar diferentes condições de cultivo a partir da fonte exógena de carbono que foi observado maior teor de fitoesteróis, afim de relacionar a formação destes compostos às condições externas;

- Avaliar diferentes cepas de microalgas procariontes e eucariontes, afim de relacionar a formação destes compostos com os diferentes metabolismos.

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