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AVALIAÇÃO DA FRAÇÃO VOLÁTIL DE *Phormidium autumnale* EM BIORREATORES HETEROTRÓFICOS

DISSERTAÇÃO DE MESTRADO

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Santa Maria, RS, Brasil
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AVALIAÇÃO DA FRAÇÃO VOLÁTIL DE *Phormidium autumnale* EM BIORREATORES HETEROTRÓFICOS

Andriéli Borges Santos

Dissertação apresentada ao Curso de Mestrado do Programa de Pós-Graduação em Ciência e Tecnologia dos Alimentos, Área de Concentração em Qualidade dos Alimentos, da Universidade Federal de Santa Maria (UFSM, RS),
como requisito parcial para obtenção do grau de
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elaborada por
Andriéli Borges Santos

como requisito para a obtenção do grau de
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RESUMO

Dissertação de Mestrado
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AVALIAÇÃO DA FRAÇÃO VOLÁTIL DE *Phormidium autumnale* EM BIORREATORES HETEROTRÓFICOS

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Data e Local da Defesa: Santa Maria, 09 de Março de 2015.

As microalgas são uma potencial fonte de biomoléculas de interesse comercial devido ao seu perfil metabólico diversificado capaz de sintetizar diferentes classes de compostos orgânicos. O gênero *Phormidium* é uma cianobactéria com habilidade de crescimento em cultivos heterotróficos mediante a adição de uma fonte de carbono exógena. Visando a possibilidade de valorização dos bioprodutos gerados nos biorreatores heterotróficos microalgais, este trabalho teve como objetivo a identificação e quantificação dos compostos orgânicos voláteis nos cultivos de *Phormidium autumnale*. Na primeira etapa foi avaliado o perfil qualitativo dos compostos orgânicos voláteis formados em cultivos heterotróficos suportados com glicose em 72h de experimento. Foram identificados 54 compostos classificados como álcoois (27%), cetonas (23%), aldeídos (19%), ésteres (14%), ácidos (3,5%) e hidrocarbonetos (2,6%). No segundo trabalho foi determinado o perfil qualitativo e quantitativo dos compostos voláteis formados em cultivos heterotróficos de 144h. No total 68 compostos foram identificados e quantificados nos cultivos suportados por glicose ou frutose. O composto 3-metil-butanol foi identificado entre os majoritários 141,5 $\mu g.mg^{-1}$ no cultivo da glicose e 69,5 $\mu g.mg^{-1}$ no cultivo com frutose. Muitos compostos identificados durante o cultivo heterotrófico derivam de rotas metabólicas como a dos terpeno (β-ionona, β-ciclocitral e 5,6-epoxi-β-ionona), a dos ácidos graxos (hexanol, hexanal) ou a dos 2-cetoácidos (3-metil-butanol, propanol, butanol). Os resultados obtidos colaboraram para elucidação de uma fração de biocompostos microalgais com grande potencial de exploração comercial.

Palavras-chave: Cianobactéria, microalga, compostos orgânicos voláteis, GC-MS

ABSTRACT

Master Dissertation
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Federal University of Santa Maria

EVALUATION OF VOLATILE FRACTION OF *Phormidium autumnale* IN BIOREACTORS HETEROTROPHIC

AUTHOR: ANDRIÉLI BORGES SANTOS
ADVISOR: LEILA QUEIROZ ZEPKA
Place and Date: Santa Maria, March 09, 2015.

The microalgae are a source of potential commercial interest biomolecules due to its diverse metabolic profile able to synthesize different classes of organic compounds. The *Phormidium* gender is a cyanobacterium with growth ability in heterotrophic cultures by the addition of an exogenous source of carbon. Aiming at the possibility of recovery of byproducts generated in heterotrophic microalgal bioreactors, this study aimed to identify and quantify the volatile organic compounds in *Phormidium autumnale* crops. In the first stage we evaluated the qualitative profile of volatile organic compounds formed in heterotrophic cultures supported with glucose at 72 h experiment. We identified 54 compounds classified as alcohols (27%), ketones (23%), aldehyde (19%), ester (14%), acid (3.5%) and hydrocarbons (2.6%). In the second study we determined the qualitative and quantitative profile of volatile compounds formed in heterotrophic cultivation of 144h. In total 68 compounds were identified and quantified in cultures supported by glucose or fructose. The 3-methyl-butanol compounds was identified between the main 141.5µg.mg^{-1} in the culture of glucose and in the culture of fructose 69.5µg.mg^{-1}. Many compounds identified during heterotrophic culture derived from metabolic pathways such as the terpene (β-ionone, β-cyclocitral and 5,6-epoxy-β-ionone), of the fatty acids (hexanol, hexanal) or the 2-keto acids (3-methyl-butanol, propanol, butanol). The results helped to elucidate a fraction of biocompounds microalgal with great potential for commercial exploitation.

Keywords: Cyanobacteria, microalgae, volatile organic compounds, GC-MS.

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INTRODUÇÃO

A biotecnologia de microalgas é uma área emergente da tecnologia industrial, que vem se consolidando em função da potencialidade de exploração dos bioprodutos resultantes dos processos de produção. A manufatura de insumos intermediários e/ou produtos finais para a indústria ocorre a partir da exploração de metabólitos intracelulares e extracelulares, produzidos por estes micro-organismos durante os processos biotecnológicos. São uma nova fonte promissora de biomassa que pode complementar as culturas agrícolas para atender à crescente demanda mundial por alimentos, ração, biocombustíveis e produção de substâncias químicas (MARKOU et al, 2014).

Para a produção de biomassa microalgal, fotobiorreatores são amplamente utilizados, entretanto, em culturas de grande escala, a absorção de luz é atenuada pelo sombreamento mútuo das células, afetando seriamente a produtividade e a qualidade dos produtos da biomassa das algas (MARKOU; GEORGAKAKIS, 2011), além do custo elevado da energia elétrica (IP; CHEN, 2005). Assim, uma alternativa para este processo seria o cultivo heterotrófico, a partir do qual o fornecimento energético para os microrganismos seria a assimilação de uma fonte de carbono exógena, como por exemplo, açúcares (FRANCISCO et al, 2014).

A rota heterotrófica de produção está baseada na conversão de carbono orgânico em produtos do metabolismo, mediada pela via oxidativa da pentose fosfato, no qual os monossacarídeos são as principais fontes de carbono passíveis de utilização. Como resultado, elucidações, que contribuam com a definição destas rotas de biotransformação, podem resultar na identificação de metabólitos passíveis de exploração comercial sendo uma etapa fundamental no estabelecimento desta tecnologia. Os requisitos de crescimento simplificado das microalgas, faz com que o sistema de biorreatores para o crescimento desses microrganismos sejam atraentes para a produção de produtos de alto valor comercial (PULZ; GROSS, 2004; COUSTETS et al, 2014).

As microalgas se adaptam rapidamente à novas condições ambientais, a fim de sobreviver, produzindo uma grande variedade de metabólitos secundários com diversas estruturas (GOUVEIA et al, 2010; BATISTA et al, 2013). Essas grande

variedade de biomoléculas torna esses micro-organismos atrativos por serem uma promissora fonte de nutrientes para diversos usos. Segundo Vigani et al (2015), o mercado de produtos obtidos a partir de microalgas ainda é significantemente menor em comparação aos derivados de produtos de base agrícola e outros micro-organismos, mas o setor encontra-se em constantes descobertas e um crescimento acelerado. Devido a habilidade das microalgas em utilizar substratos orgânicos para manutenção da existência representa uma alternativa no direcionamento da obtenção de compostos não obtidos em cultivos convencionais fotossintéticos. Os compostos orgânicos voláteis (COVs) são biomoléculas pouco exploradas, que são influenciadas pelos parâmetros de cultivo podendo ser de grande importância para a elucidação bioquímica.

É conhecido a partir de estudos que as microalgas produzem uma grande variedade de COVs que podem influenciar o aroma da biomassa (DRAAISMA et al, 2013). A técnica de extração por SPME é considerada eficaz e têm sido amplamente aplicada para a extração de compostos orgânicos voláteis e semivoláteis de amostras biológicas e produtos alimentares (ZHANG et al, 2009; DURME et al, 2013). A caracterização da fração volátil dos biorreatores pode contribuir para o estabelecimento das rotas de bioconversão dos substratos, além de possibilitar a identificação de aplicações potenciais dos bioprodutos formados (JACOB-LOPES et al, 2010).

Baseado em que a elucidação de rotas de formação dos compostos orgânicos voláteis é de fundamental importância para o aprimoramento da biotecnologia microalgal, o presente trabalho fundamenta-se em um estudo exploratório sobre a formação e transformação de compostos voláteis durante o cultivo heterotrófico da cianobactéria *Phormidium autumnale* em meio enriquecido com monossacarídeos.

OBJETIVOS

Objetivo Geral

Estudar a partir de biorreatores heterotróficos, a formação e a transformação de compostos orgânicos voláteis, durante o cultivo de *Phormidium autumnale* em meio enriquecido com monossacarídeos.

Objetivos Específicos

Identificar os compostos orgânicos voláteis da biomassa de cultivo heterotrófico microalgal;

Quantificar os compostos orgânicos voláteis da biomassa de cultivo heterotrófico microalgal;

Estabelecer o potencial de exploração comercial dos bioprodutos produzidos na fase gasosa do biorreator;

Estabelecer possíveis rotas de biotransformação desses compostos.

CAPÍTULO 1

REVISÃO BIBLIOGRÁFICA

1. Microalgas

O termo microalga não apresenta nenhum valor taxonômico. Define seres microscópicos diversos presentes em sistemas aquáticos, tem hábito planctônico, embora haja também muitas espécies bentônicas e terrestres (LOURENÇO, 2006). Dez grandes grupos fazem parte da classificação geral de microalgas segundo Graham e Wilcox (2000), são eles: *Cyanophyta, Chlorarachniophyta, Glaucophyta, Euglenophyta, Cryptophyta, Prymnesiophyta, Dinophyta, Ochrophyta, Rhodophyta* e *Chlorophyta*.

As algas são as maiores removedoras de carbono da biosfera. Os oceanos sequestram anualmente 2 gigatoneladas de carbono por meio de absorção de dióxido de carbono, e a biosfera terrestre total, 1,4 gigatoneladas. As macroalgas (multicelulares) fornecem produtos como o alginato, carragena e ágar, que são valorizados por suas propriedades físicas como espessantes, emulsificantes e geleificantes. As microalgas (unicelulares), destacam-se pelo seu valor micronutricional, contendo diversos metabólitos que atuam diretamente no sistema imunológico humano (MOORE, 2001).

As cianobactérias foram identificadas como um dos grupos mais promissores de organismos a partir dos quais podem ser isolados outros produtos naturais bioquimicamente ativos devido seu perfil metabólico único e diversificado, podendo ser incorporadas em uma infinidade de aplicações comerciais, abrangendo nutrição, cosméticos e produção de biocombustíveis (BURJA et al, 2001; GOUVEIA et al, 2010; GAFFNEY et al, 2014). Cerca de 2000 espécies são conhecidas e podem ser divididas em 150 gêneros. Sua diversidade é expressa por suas propriedades morfológicas, bioquímicas e fisiológicas, que lhes permite estabelecer e persistir em uma grande variedade de habitat (PALINSKA et al, 2011). Apresentam habilidade de armazenamento de nutrientes extremamente rápidas e uma alta produção de inúmeros metabólitos (ANAGNOSTIDIS; KOMÁRIK, 1985; PULZ et al, 2001; PULZ; GROSS, 2004).

O gênero *Phormidium* sp. é uma cianobactéria filamentosa não ramificada que vivem aglomerados, com conteúdo celular geralmente azul esverdeado, raramente marrom. Pode ser encontrada em solos, rochas úmidas, lama, plantas aquáticas,

córregos, algumas em ambientes litorâneos. Outras espécies são encontradas em ambientes extremos como nascentes termais e solos de desertos (THOMAZEU et al, 2010; GUIRY; GUIRY, 2013).

As cianobactérias são assim denominadas pela ausência de organização celular e estruturas definidas, fato que as assemelha às bactérias (REVIERS, 2002). Considerando a grande biodiversidade e os recentes desenvolvimentos na engenharia genética, as cianobactérias representam uma das fontes mais promissoras para novos bioprodutos, como proteínas, amido, celulose, lipídeos, incluindo metabólitos secundários como pigmentos e alguns fármacos, devido esses micro-organismos converterem substâncias inorgânicas como carbono, nitrogênio, fósforo, enxofre, ferro em matéria orgânica fazendo com que obtenha-se uma biomassa rica em nutrientes (BATISTA et al, 2013). Fatores ambientais, como temperatura, iluminação, pH, conteúdo mineral, densidade populacional, fase de crescimento e estado fisiológico podem modificar sua composição química. Assim, as condições de crescimento podem ser otimizadas e mapeadas para maximizar a produção de biomoléculas de interesse (HU, 2013; BATISTA et al, 2013). Com o desenvolvimento da cultura e técnicas de triagem, a biotecnologia de microalgas poderá satisfazer as elevadas exigências industriais (PULZ; GROSS, 2004; HARUN et al, 2010; GUEDES et al, 2011; HU et al, 2013). É significativo na prática e em sistemas de cultura em grande escala para adaptar o meio de cultura para as necessidades de crescimento das microalgas sob condições ambientais específicas, a fim de alcançar rendimentos elevados de biomassa (MARKOU et al, 2014).

Apesar de as microalgas serem principalmente fotoautotróficas, um número considerável destes micro-organismos possui a habilidade de manutenção da estrutura na ausência de luz, assim sendo capaz de crescer também heterotroficamente, a partir da assimilação de um ou mais substratos orgânicos como fonte de carbono e energia para a manutenção de suas estruturas (WEN; CHEN, 2003; CHOJNACKA; MARQUEZ-ROCHA, 2004; SUN et al 2008; PEREZ-GARCIA et al, 2011). A utilização dessas moléculas orgânicas depende da espécie e da cepa, assim, a cianobactéria *Phormidium autumnale* faz parte deste grupo com potencial de exploração do metabolismo heterotrófico.

2. Cultivo Heterotrófico

As algas podem ser cultivadas em sistemas fechados tanto fotoautotroficamente, mixotroficamente ou heterotroficamente. O cultivo heterotrófico tem diversas vantagens: desde o sistema de cultivo bem compreendido, a elevada densidade celular podendo reduzir custos de operação (BOROWITZKA, 1999; PEREZ-GARCIA et al, 2011).

Em cultivos heterotróficos o requerimento primário para utilização de compostos orgânicos é o transporte através da membrana, a permeabilidade varia preferencialmente em função do composto. Este tipo de produção é suportada por carboidratos como glicose, frutose e sacarose. Em muitos sistemas em escala industrial glicose é adicionado ao meio para intensificação de obtenção de biomassa (PEREZ-GARCIA et al, 2011).

As microalgas podem crescer heterotroficamente usando os mesmos meios utilizados em culturas fototróficas, a diferença é a adição de carbono orgânico ao invés do fluxo contínuo de dióxido de carbono e luz (MORALES-SÁNCHEZ et al, 2013). Devem ser capaz de multiplicar e metabolizar na ausência de luz, crescer com facilidade em meio de cultura estéril e adaptar-se rapidamente às exigências do estresse hidrodinâmico do biorreator (CHEN; CHEN, 2006; CHEN et al, 2006; MOJTABA et al, 2011).

A glicose é a fonte de carbono exógena mais utilizada para as culturas heterotróficas microalgais, devido as elevadas taxas de crescimento e respiração obtidas com esse substrato. O que pode ser justificado pela alta produção de energia quando comparada com outras fontes, por exemplo, o acetato produz ~0,8 KJ/mol e a glicose ~2,8 kj/mol (BOYLE; MORGAN, 2009; PEREZ-GARCIA et al, 2011; FRANCISCO et al, 2014).

O glicogênio é o principal produto de reserva para suportar o metabolismo na fase escura (respiratória), fornece uma energia limitada para a manutenção necessária dos processos celulares essenciais (MARGHERI et al, 1991). Ele é convertido em glicose-6-fosfato e assim metabolizado pelas via respiratória. A glicose-6-fosfato é oxidada e descarboxilada em duas etapas formando ribulose-5-fosfato. Estas reações são catalisadas pela glicose-6-fosfato desidrogenase e a 6-fosfo-gluconato desidrogenase, são NADP (nicotinamida adenina dinucleótido fosfato) específicas, e gera duas moléculas de NADPH (nicotinamida adenina dinucleótido

fosfato hidreto). A subsequente oxidação do NADPH gera durante o transporte de elétrons duas moléculas de ATP (adenosina trifosfato). A via das pentoses-fosfato é ativada pela ausência de luz e inibida com a presença da mesma (FAY, 1983; PEREZ-GARCIA et al, 2011)

As cianobactérias não possuem o ciclo do ácido tricarboxílico (Ciclo de Krebs) completo. Algumas enzimas essenciais para o ciclo de Krebs, como succinil-CoA-sintetase e succínico desidrogenase, estão presentes em concentrações muito baixas ou ausentes em suas células. Esta via permite um fluxo limitado de carbono a partir de isocitrato a succinato, aumentando a síntese de porfirinas como a clorofila, o citocromo e as ficobiliproteínas (FAY, 1983; PEREZ-GARCIA et al, 2011).

A versatilidade da manutenção do metabolismo das cianobactérias consiste principalmente na habilidade e utilização de substratos orgânicos como fonte de carbono e energia na ausência de luz (FAY, 1983; CHEN et al, 2006) Comparado ao crescimento fotoautotrófico, o cultivo heterotrófico de microalgas elimina requisitos de luminosidade, e pode aumentar significativamente as taxas de crescimento da massa celular e a produtividade volumétrica em regime descontínuo (CHENG et al, 2009; MORALES-SÁNCHEZ et al, 2013; PEREZ-GARCIA et al, 2011).

A abordagem do crescimento heterotrófico elimina duas deficiências dos fotobiorreatores: permite a utilização de praticamente qualquer fermentador como um biorreator, tais como os utilizados para a produção industrial de medicamentos, bebidas, e de energia, originando um resultado importante, promove uma significativa redução de custos para a maioria dos processos devido a relação custo-eficiência e a relativa simplicidade de operação (LEE, 1997; PEREZ-GARCIA et al, 2011). As condições de cultivo estão diretamente relacionadas com o perfil qualitativo e quantitativo dos diferente produtos fracionados da biomassa microalgal.

3. Produtos de Microalgas

Um volume considerável de informações a respeito metabólitos de microalgas tem sido gerado por décadas (FAY, 1983; BECKER, 1994; LI et al, 2007; JACOB-LOPES et al, 2010; FRANCISCO et al, 2010; ZEPKA et al, 2010; SCHIRMER et al, 2010; PEREZ-GARCIA et al, 2011; QUEIROZ et al, 2013), com especial atenção voltada a variedade de metabolitos (suplementos alimentares, lipídios, enzimas, biomassa, polímeros, pigmentos e energia) que apresentam potencial de produção a

partir da biomassa microalgal (BURJA et al, 2001; RODRIGUES-MEIOZO et al, 2008; PEREZ-GARCIA et al, 2011; CHU, 2012). A análise da composição bioquímica da biomassa microbiana é uma ferramenta útil que pode fornecer uma visão sobre o comportamento de um organismo e sua resposta adaptativa às mudanças do ambiente reflete o estado fisiológico e metabólico do organismo (CHEN; VAIDYANATHAN, 2013).

O elevado teor de proteínas de várias espécies de microalgas é uma das principais razões para considerá-las como fonte convencional desse nutriente (SOLETTO et al, 2005; SPOLAORE et al, 2006). Os carboidratos em microalgas podem ser encontrados sob a forma de amido, glicose, e outros polissacarídeos. Sua digestibilidade total é elevada, razão pela qual não há limitação ao uso de microalgas em pó em alimentos e rações para animais (PULZ; GROSS, 2004).

As microalgas são reconhecidas como uma excelente fonte de pigmentos naturais, principalmente na fase exponencial de crescimento e totalmente dependente das condições de cultivo (DUFOSSÉ et al, 2005). Podem acumular vários tipos, principalmente carotenoides (por exemplo, β-caroteno, luteína, violaxantina), como resposta ao estresse das condições ambientais. A sacarose e a glicose são as formas de carbono mais comumente utilizadas na bioprodução de carotenoides (HU et al, 2013).

As cianobactérias sintetizam os mesmos carotenoides que as plantas superiores, no entanto, elas produzem alguns tipos únicos de xantofilas, como por exemplo a equinenona (PALINSKA et al, 2011). A procura por alimentos que tenham a adição de pigmentos naturais ao invés dos sintéticos é crescente, o que faz com que a indústria invista na produção através de bioprocessos (AGOCS; DELI, 2011; HU et al, 2013). Espera-se que a produção de pigmentos a partir de micro-organismos supere a produção por via sintética, isso pela sustentabilidade de produção e a natureza renovável (DUFOSSÉ et al, 2005; BATISTA et al, 2013).

As microalgas são recursos biológicos importantes devido a sua ampla gama de aplicações biotecnológicas. A diversidade evolutiva e as possibilidades metabólicas de manutenção da existência destas estruturas oportunizam a produção de frações químicas distintas as encontradas em fontes convencionais, tornando-os extremamente atraente para a exploração de biomoléculas com potencial comercial (CHU, 2012; BOROWITZKA, 2013; DESAI; ATSUMI, 2013).

As microalgas também podem ser usadas para obtenção de moléculas bioativas, porque podem ser cultivadas em alta escala produzindo em escala industrial (MOLINA GRIMA et al, 2003; CHU, 2012). Os compostos bioativos são geralmente metabólitos secundários, que incluem várias substâncias como ácidos orgânicos, aminoácidos, vitaminas, antibióticos, enzimas e até compostos tóxicos. Dos vários grupos de microalgas, as cianobactérias apresentam mais características proeminentes como fonte destes compostos (CHU, 2012). O produto mais importante da biotecnologia de microalgas em relação a quantidade de produção e valor econômico ainda é a própria biomassa (PLAZA et al, 2000). No entanto tem se observado uma tendência emergente no sentido do conhecimento de produção de compostos de baixo peso molecular a partir de fontes renováveis (WINTERS et al, 1969; SCHIRMER et al, 2010; CHOI; LEE, 2013).

Na busca de novos ingredientes funcionais com alto potencial para utilização na indústria alimentícia, os extratos de espécies desconhecidas de microalgas, tais como *Phormidium autumnale* têm sido estudados (RODRÍGUEZ-MEIZOSO et al, 2008). As aplicações vão desde a produção de biomassa para a alimentação humana e animal até obtenção de produtos químicos. Para a maioria destas aplicações, o mercado ainda está em desenvolvimento podendo se estender para novas áreas (PULZ; GROSS, 2004). Embora venha sendo estudado largamente as frações de metabolitos não voláteis, observa-se também que o rendimento em microalga não satisfaz completamente o balanço de carbono total do sistema sugerindo que parte do balanço está direcionado para produção de compostos orgânicos voláteis (JACOB-LOPES et al, 2010).

4. Compostos Voláteis

Compostos orgânicos voláteis (COV's) são comumente produzidos por micro-organismos e emitidos para o ambiente, são caracterizados por possuir baixo peso molecular e alta pressão de vapor, porém esta área do conhecimento ainda é pouco explorada (ZUO et al, 2012; POPOVA et al, 2014). Algas e cianobactérias podem apresentar descritores de terra ou mofo em água potável (WATSON; RIDAL, 2004; HEIL; BUENO, 2007; KOST; HEIL, 2008; FUJISE et al, 2010). A caracterização da fração volátil dos biorreatores pode contribuir para o estabelecimento das rotas de

bioconversão dos substratos, além de possibilitar a identificação de aplicações potenciais dos bioprodutos formados (JACOB-LOPES et al, 2010).

Segundo Nuccio et al (1995), as taxas de produção de COV's produzidos por microalgas apresentam significativo aumento durante a fase de crescimento exponencial e declive na fase de senescência, apresentando um comportamento parabólico com vértice negativo. Alguns destes compostos tem papel importante em processos químicos atmosféricos (JACOB-LOPES et al, 2010). A biossíntese destes compostos depende da disponibilidade de carbono, nitrogênio, e enxofre, bem como a energia fornecida pelo metabolismo primário. Por tanto, a disponibilidade destes "blocos de construção" tem um grande impacto na concentração de um metabólito secundário, incluindo compostos voláteis, demonstrando elevado grau de conectividade entre o metabolismo primário e secundário (DUDAREVA et al, 2013).

O método de extração mais utilizado é por microextração em fase sólida em *headspace*. Essa metodologia tem provado ser um meio bem sucedido de extração e análise de concentração de COV's em vários alimentos, bebidas e plantas, que pode ser relacionado ao número de parâmetros correlacionados desta técnica como o tipo de revestimento da fibra, temperatura e tempo de extração (LI et al, 2014). Este método se mostrou eficiente por ser altamente sensível e não destrutivo, além de ser de baixo custo, simples e livre de solventes (ARTHUR; PAWLISZYN, 1990; RISTICEVIC et al, 2010).

Hidrocarbonetos, como alcanos e alcenos, são de particular interesse devido seu alto potencial para ser usado para a produção de bioenergia, por terem 30% de energia a mais que o etanol (CHOI; LEE, 2013). Ao contrário das bactérias que necessitam de alterações genéticas para sintetizar alcanos (C13-C17), as cianobactérias possuem sua via de biossíntese, sem precisar ser engenheirada para essa obtenção. Schirmer et al (2013) identificou em cianobactérias alcanos como heptadecano, pentadecano e metil-heptadecano.

Fontes consistentes relacionam a produção de compostos voláteis por microalgas (SCARRATT; MOORE, 1996; REINER et al, 2000; KEPPLER et al, 2000; SCHIRMER et al, 2010). Os diferentes compostos orgânicos voláteis microbianos podem pertencer a distintas classes de compostos como álcool, ésteres, hidrocarbonetos, terpenos, cetonas, composto sulforados e ácidos carboxílicos. Entre as muitas aplicações viáveis destas estruturas, evidencia-se a propriedades bioativas dessas moléculas (VINING, 1990; PAPALEO et al, 2013). O uso integral da fração

volátil da biomassa microalgal pode representar um avanço em relação ao fornecimento de insumos de grande volume aos mais diferentes tipos de indústria.

A produção autotrófica é atualmente a mais explorada, em função do metabolismo preferencial fotossintético. Porém a habilidade das microalgas em utilizar substratos orgânicos para manutenção da existência representa uma alternativa no direcionamento de obtenção de compostos não obtidos nos cultivos convencionais fotossintéticos, além de vantagens como a simplificação de operações unitárias do processo (FAY, 1983; MINERDI et al, 2009; PEREZ-GARCIA et al, 2011).

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CAPÍTULO 2

BIOGENERATION OF VOLATILE COMPOUNDS FROM MICROALGAE

Capítulo aceito para o livro Flavour Science.

BIOGENERATION OF VOLATILE COMPOUNDS FROM MICROALGAE

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Abstract:

The growing interest in natural products directs the development of technologies that employ microorganisms, including microalgae, which are able to synthesize specific volatile organic compounds (VOCs). Thus, the objective of this study was to investigate the biogeneration of volatile organic compounds from microalgae *Phormidium autumnale*. The volatiles compounds were isolated from the headspace of the bioreactor and analysed by HS-SPME-GC/MS. The major products in the bioreactor were acetaldehyde (13%), 3-hydroxy-2-butanone (12%), 3-methyl-1-butanol (12%) and ethyl-3-hydroxybutanoate (8.5%).

1. Introduction:

Microalgae-based systems for chemicals production are an emergent area, representing a great promise for industrial application. However, there is little information available about the flavour biogeneration of these microorganisms. The characterization of the volatile fraction of microalgal bioreactors can establish bioconversion routes for substrates, thus enabling the identification of potential applications for volatile bioproducts formed [1].

Jacob-Lopes et al [2] reported that the VOCs are the main bioproducts formed during microalgae cultivation. The carbon balance analysis indicates that these compounds represent up to 90% of the total substrate converted in the bioreactor. The different VOCs can belong to different class of compounds such as alcohols, esters, hydrocarbons, terpenes, ketones, carboxylic acids and sulfur compounds [3].

Berger [4] reported that flavours from microorganisms can compete with the traditional sources. The screening for overproducers, elucidation of metabolic pathways and precursors and application of conventional bioengineering has resulted in a set of more than 100 commercial aroma chemicals derived via biotechnology.

Thus, the objective of this study was to investigate the biogeneration of volatile organic compounds from microalgae *Phormidium autumnale.*

2. Experimental:

Axenic cultures of *Phormidium autumnale* were used in the experiments. Stock cultures were propagated and maintained in synthetic BG11 medium [5].

The microalgae cultivation was made in a bubble column bioreactor. The system was built of borosilicate glass and had an external diameter of 12.5 cm and a height of 16 cm, resulting in a height/diameter (h/D) ratio equal to 1.28 and a nominal working volume of 2.0 L. The dispersion system of the reactor consisted of a 2.5 cm diameter air diffuser located inside the bioreactor. The air flow was monitored by flow meter (KI-Key Instruments®, Trevose-PA, USA) and the inlet of air and outlet of gases were filtered through filtering units made up of polypropylene membrane with a pore diameter of 0.22 µm and total diameter of 50 mm (Millex FG®, Billerica-MA, USA). The bioreactor including filtering units was previously sterilized by autoclaving. The cultivations were performed in a bioreactor operating under a batch regime, fed on 2.0 L of culture medium. The experimental conditions were as follows: initial concentration of inoculum of 100 mg/L, temperature of 26 °C, pH adjusted to 7.6, aeration of 0.1 VVM (volume of air per volume of culture per minute) and absence of light. The culture medium consisted of BG11 synthetic medium supplemented with D-glucose (12.5 g/L) [6].

The volatile organic compounds were isolated at 72 h of the residence time, using solid phase microextraction (SPME) with a 50/30 µm divinylbenzene/carboxen/-polydimethylsiloxane (DVB/Car/PDMS) fibre (Supelco, Bellefonte-PA, USA). The SPME fibre was inserted into the sample headspace for 45 min at temperature of 40°C, with agitation provided by a magnetic stir bar. After this period, the fibre was removed from the vial and immediately desorbed into the injector of the GC equipment.

The volatile compounds were separated on DB-wax fused silica capillary column, 60 m in length, 0.25 mm id and 0.25 µm of film thickness (Chrompack wax 52-CB) in a Shimadzu model QP 2010 Plus gas chromatograph mass spectrometer (Shimatzu, Kyoto, Japan). The initial oven temperature for the DB-wax column was 35°C for 5 min, followed by a linear increase at 5°C/min to 220°C, and held at this temperature for 5 min.

The volatile compounds were identified by a comparison of their MS spectra with those provided by the computerized library (NIST MS Search). In addition, to assist with the identification, each volatile retention index (LRI) was calculated using the retention times of a standard mixture of paraffin homologues prepared in hexane, and compared with the LRI values published in the literature for columns with the same polarity [7].

3. Results:

The volatile organic compounds produced by *Phormidium autumnale* cultivated in heterotrophic microalgal bioreactor are presented in Table 1 and Figure 1. A total of 54 compounds of various chemical structures were found. The chemical classes most representative were the alcohols (27%), ketones (23%) aldehydes (19%) and esters (14%) which together comprise 83% of the total compounds identified. Minor quantities of acids (3.5%) and hydrocarbons (2.6%) were also detected.

Among the chemical classes identified, acetaldehyde (13%), 3-hydroxy-2-butanone (12%), 3-methyl-1-butanol (12%) and ethyl-3-hydroxybutanoate (8.5%) were identified as the major contributors.

The heterotrophic microalgae cultivation is a specific niche for production of metabolic bioproducts. Heterotrophic growth in the dark, supported by an exogenous carbon source, is an important ability of some species of these organisms. The exogenous organic compounds that support the heterotrophic growth of microalgae are metabolized via oxidative pentose-phosphate pathway. This metabolic route serves as the exclusive source of energy for maintenance and biosynthesis, besides providing the carbon required as building blocks for biosynthesis [6]. The biosynthesis of volatile compounds depends mainly on the availability of carbon and nitrogen as well as on the energy provided by primary metabolism. Therefore, the availability of these building blocks has a major impact on the concentration of any secondary metabolite, including VOCs [1].

Table 1. Volatiles produced by *Phormidium autumnale* cultivated in heterotrophic microalgal bioreactor.

Compounds	LRI DB-wax	Description of odour [7]	Relative peak area (%)	
Acids				3.49
acetic acid	1572	sour	1.63	
3-methylbutanoic acid	1790	sweet, acid, rancid	1.86	
Alcohols				27.3
2-propanol	942	alcohol, pungent	3.48	
1-propanol-2-methyl	1115	wine, solvent, bitter	5.10	
3-methyl-1-butanol	1228	whiskey, malt, burnt	11.7	
1-hexanol	1466	flower	1.90	
1-heptanol	1569	chemical, green	1.02	
2-ethylhexanol	1601	rose, green	2.77	
1-octanol	1675	metal, burnt	1.33	
Aldehydes				18.6
acetaldehyde	648	pungent	13.3	
2-methylpropanal	826	pungent, malt, green	2.07	
decanal	1616	soap, peel, tallow	3.26	
Ester				13.8
ethyl acetate	905	pineapple	5.41	
ethyl-3-hydroxybutanoate	1645	marshmallow	8.45	
Ketones				22.8
2-butanone	921	ether	1.21	
2-pentanone	996	ether, fruit	3.40	
2,3-butanedione	1002	butter	2.16	
3-hydroxy-2-butanone	1411	butter, cream	12.3	
acetophenone	1796	must, flower, almond	2.17	
β-ionone	1967	violet, flower	1.54	
Hydrocarbons				2.61
octane	806	alkane	1.04	
heptadecane	1810	alkane	1.57	
Other compounds			11.4	
Total			100.0	

Phormidium autumnale biosynthesized a wide array of different VOCs. Based on their biosynthetic origin, these VOCs can be divided into terpenoids, phenylpropanoids/benzenoids, carbohydrate derivatives, fatty acids derivatives and amino acid derivatives, in addition to specific compounds not represented in those major classes [8,9]. These compounds could therefore be a source of useful chemicals products, based on a nonconventional technological route. Nine compounds produced by *Phormidium autumnale* are commercially available from other biotechnological

routes. According to Berger [4], the flavour biotechnology will be the next generation of the industrial biotechnology. The chemicals obtained from bioprocess are sold at prices up to 1000 times higher than those of synthetic origin, which show great potential for exploitation of these processes.

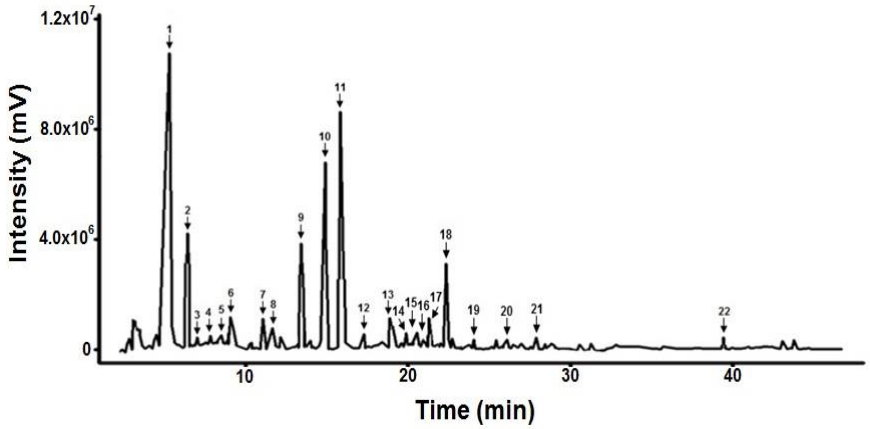

Figure 1. Gas chromatogram of volatile compounds produced in the heterotrophic microalgal bioreactor. (1) acetaldehyde, (2) ethyl acetate, (3) octane, (4) 2-methylpropanal, (5) 2-butanone, (6) 2-propanol, (7) 2-pentanone, (8) 2,3-butanedione, (9) 2-methyl-1-propanol, (10) 3-methyl-1-butanol, (11) 3-hydroxy-2-butanone, (12) 1-hexanol, (13) 2-ethylhexanol, (14) heptadecane, (15) 1-heptanol, (16) acetic acid, (17) decanal, (18) ethyl-3-hydroxybutanoate, (19) 1-octanol, (20) 3-methylbutanoic acid, (21) acetophenone, (22) β-ionone.

In conclusion, the results have shown that the heterotrophic cultivation of the *Phormidium autumnale* can be a potential biotechnological route to produce natural flavours. In view of the commercial significance, efforts should be made to elucidate the pathways of formation for these compounds.

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CAPÍTULO 3

BIOSYNTHESIS OF VOLATILE ORGANIC COMPOUNDS PRODUCED BY PHORMIDIUM AUTUMNALE IN BIOREACTOR HETEROTROPHIC

Artigo submetido para a revista Bioresource Technology.

Biosynthesis of volatile organic compounds produced by *Phormidium autumnale* in bioreactor heterotrophic

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ABSTRACT

Volatile organic compounds from biotechnology produced are important in numerous commercial applications mainly as input for chemical fine industry. Thus the objective of this study was to investigate the volatile organic compounds (VOC) produced from heterotrophic microalgae cultivation with different source of monosaccharide. The volatiles were isolated by headspace-solid phase micro-extraction in different cultivation time, separated by gas chromatography, identified by mass spectrometry (SPME-GC-MS). The volatile profile of microalgae cultivation with glucose or fructose has totals of 44 and 35 compounds, respectively. A combined total of 68 compounds were identified and 11 volatiles were common in both extracts. As consequence of the heterotrophic microalgae cultivation with glucose or fructose, the compound 3-methyl-butanol was identified among the majors volatile compounds formed, 141.5$\mu g.mg^{-1}$ and 69.5$\mu g.mg^{-1}$ respectively. Many of the compounds were detected during microalgae heterotrophic cultivation derivatives from terpenoids (β-ionone, β-cyclocytral and 5,6-epoxy-β-ionone) fatty acid (hexanol, hexanal) or 2-ketoacids pathway (3-methyl-butanol, propanol, butanol).

1. Introduction

The continual growth of commercial application of primary and secondary biotechnology metabolites and more strict environmental legislations have led to curiosity in developing renewable forms to produce these compounds to apply in fine chemistry (Havel and Weuster-Botz, 2006; Rastogi and Sinha, 2009). Microalgae are considered on the most promising feedstocks for sustainable supply of food and non-food industry (Draaisma et al., 2013).

Volatiles organics compounds (VOCs) are secondary metabolites could be important intermediates of pharmaceutical, flavor and fragrance a low cost with obtains of the alternatives source like microalgae (Havel and Weuster-Botz, 2006). However many taste and odor outbreaks have been associated with volatile organic compounds, such as geosmine and 2-methylborneol produced by microalgae, and these are typical off flavor compounds (Fujise et al., 2010). The exploration of knowledge about volatile profile from microalgae without off-flavor compound is a possibility and a scientific challenger for apply these metabolites like chemical fine feedstocks.

Among volatile organic compounds from microorganism with commercial claim are propanol, butanol, 3-methyl-butanol, hexanol, hexanal, β-cyclocytral, β-ionone and 5,6-epoxy-β-damascenone (Berger, 2009; Smith et al., 2010). Flavors from microorganism compete with the traditional agricultural source. Elucidation of metabolic pathway and precursors and application of bioengineering has resulted in a set of more than 100 commercial aroma chemical derived via biotechnology (Berger, 2009).

Biosynthesis of VOCs depends on the availability of building blocks like carbon, nitrogen as energy provide by primary metabolism. Therefore, the availability and the kind of chemical structure (glucose or fructose) of these building blocks, has a major impact on the concentration of volatile organic compounds. Based on their biosynthetic origin, VOCs are divided in several class, including terpenoids, fatty acid derivatives and 2-keto-acid pathway (Dudareva et al., 2013).

The characterization of the volatile fraction of bioreactors can contribute to the establishment of bioconversion routes of substrates, and enable the identification of potential applications of bio-products formed (Jacob-Lopes et al., 2010). The full use of the volatile fraction of microalgal biomass may represent an improvement over the supply of large volume of inputs to many different types of industry.

In this sense, there is a demand for prospective studies of the volatile fraction of microalgal bioreactors, as well as the elucidation of metabolic pathways of formation of these compounds. Thus the aim of this study was to evaluate the volatile profile from heterotrophic microalgae bioreactors of *Phormidium autumnale* using two sources of exogenous carbon, glucose and fructose.

2. Materials and Methods

2.1. Standards

The following standards 2-butanone, 3-methy-2-butanone, 2-propanol, 2-methyl-1-propanol, 1-butanol, 2-heptanone, 3-hydroxy-2-butanone, 6-methyl-5-hepten-2-one, and β-ionone from Sigma Aldrich (Saint Louis, USA) were used. The 3-octanol from Sigma Aldrich (Saint Louis, USA) was employed as an internal standard. The identities of volatile compounds were confirmed with retention indices and

comparison with the MS spectral database. The glucose and fructose were obtained from Synth (São Paulo, Brazil).

2.2. Microorganisms and culture media

Axenic cultures of *Phormidium autumnale* were originally isolated from the Cuatro Cienegas desert (26°59'N, 102°03'W-Mexico). Stock cultures were propagated and maintained in solidified agar-agar (20 $g.L^{-1}$) containing synthetic BG11 medium (Rippka et al., 1979). The incubation conditions used were 25°C, a photon flux density of 15$\mu mol.m^{-2}.s^{-1}$ and a photoperiod of 12/12 hour light/dark.

2.3. Bioreactor

The cultivations were performed in a bubble column bioreactor (Jacob-Lopes and Franco, 2013) operating under a batch regime, fed on 2.0L of culture medium. The bioreactor including filtering units was previously sterilized by autoclaving at 121°C for 40min and then for 30min containing the synthetic medium. The experimental conditions were as follows: initial concentration of inoculum of 100$mg.L^{-1}$, temperature of 26°C, pH adjusted to 7.6, aeration of 0.1 volume of air per volume of culture per minute and absence of light. The culture medium consisted of BG11 synthetic medium modified and supplemented with glucose and fructose (12.5 $g.L^{-1}$) such as exogenous carbon source.

2.4. Biomass concentration

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

The sampling of the experiment was performed during seven days at 0, 24, 48, 72, 96, 120 and 144 hours.

2.5 Isolation of the volatile organic compounds

The volatile compounds were isolated using solid phase microextraction (SPME) with a 50/30µm divinylbenzene/carboxen/polydimethylsiloxane (DVB/CAR/PDMS) fibre (Supelco, Bellefonte, USA). Sample preparation was performed using a 10mL aliquot of liquid from the bioreactor and transferred to 20mL glass vials containing 3g of NaCl and 10µL of an internal standard solution. The SPME fibre was inserted into the headspace of the vial containing the sample for 45min at temperature for 40°C, with agitation provided by a magnetic stir bar. After this period, the fibre was removed from the vial and immediately desorbed into the injector of the GC-chromatography.

2.5 GC-MS analysis

The volatile compounds were separated on DB-Wax fused silica capillary column, 60m in length, 0.25mm id and 0.25µm film thickness (Chrompack Wax 52-CB) in a Shimadzu model QP 2010 Plus gas chromatograph mass spectrometer. The initial oven temperature for the DB-Wax column was 35°C for 5 min, followed by a linear increase at 5°C/min to 220°C, and held at this temperature for 5 min. For identification, applying an electron-impact ionization voltage of 70 eV and using helium as the carrier gas.

The volatile compounds were identified by a comparison of their MS spectra with those provided by the computerized library (NIST MS Search). In addition, to assist with the identification, each volatile linear retention index (LRI) was calculated using the retention times of a standard mixture of paraffin homologues prepared in

hexane, and compared with the LRI values published in the literature for columns with the same polarity (Acree and Arn, 2015). Co-injection of the sample and the standard mixture provided experimental linear retention indices (LRI) for the compounds, which were compared with those of standards analyzed under similar conditions.

3. Results and discussion

The volatile organic compounds separated in all experiments were identified based on the positively identification obtain from chromatographic elution, retention index of authentic standards and mass spectral or tentatively identified with published mass spectra and retention index (Table 1).

Table 1. Volatile compounds detected by GC/MS in the samples from bioreactors with retention index (LRI) and odour descriptors.

Peak	LRI DB-Wax[a]	ID[b]	Compound	Medium[c]	Odour Descriptors[d]
1	648	B	acetaldehyde	g, f	fruit, apple
2	741	B	1-heptene	F	-
3	797	B	2,4-dimethylheptane	F	-
4	821	B	isobutyraldehyde	g	pungent, malt, green
5	832	B	butanal	g	pungent, green
6	835	B	hexamethylcyclotrisiloxane	F	-
7	868	B	tetrahydrofuran	F	-
8	896	B	2-methylbutanal	F	cocoa, almond
9	905	B	ethylacetate	g	pineapple
10	910	B	3-methylbutanal	F	malt
11	921	A	2-butanone	g	camphor

12	957	A	3-methyl-2-butanone	g	camphor
13	962	A	2-propanol	g, f	alcohol, pungent
14	978	B	2,3-butanedione	g	butter
15	990	B	2-butyl acetate	F	fruit
16	1015	B	4-methyl-2-pentanone	g, f	ether, fruit
17	1026	B	acetonitrile	g	-
18	1083	B	hexanal	F	grass, tallow, fat
19	1092	A	2-methyl-1-propanol	g, f	-
20	1117	B	isoamylacetate	g	fruit, banana
21	1138	B	2,3-hexanedione	g	-
22	1151	B	2-pentanol	g	green
23	1172	B	isobutylacetate	F	pungent, fruit
24	1174	A	1-butanol	F	medicine, fruit
25	1190	A	2-heptanone	g	soap
26	1228	B	3-methyl-1-butanol	g, f	whiskey, malt, burnt
27	1232	B	1-methoxy-2-propylacetate	F	fruit, herb
28	1236	B	6-methyl-2-heptanone	F	-
29	1269	B	3-methylbutenol	g	herb
30	1278	B	1-hydroxy-2-propanone	g	-
31	1325	A	3-hydroxy-2-butanone	F	butter, cream
32	1385	B	2,3-octanedione	F	-
33	1387	A	6-methyl-5-hepten-2-one	g, f	-
34	1396	B	1-hexanol	g, f	flower, green
35	1397	B	2-ethylhydroxyisovalerate	g	sweet, citrus
36	1405	B	2-butoxyethanol	g	-
37	1420	B	dihydro-2-methyl-3-furanone	g	-
38	1425	B	heptanol	g	chemical, green

39	1450	B	aceticacid	g	sour
40	1484	B	decanal	g	soap, tallow
41	1498	B	3-octenal	F	green
42	1518	B	2-hepten-1-ol	F	-
43	1522	B	ethyl-3-hydroxybutanoate	g	marshmalow
44	1530	B	3-nonen-2-one	F	pungent
45	1536	B	2-ethyl-mercaptoethanol	g	-
46	1553	B	1-octanol	g	chemical, metal, burnt
47	1574	B	isobutyricacid	g	rancid, butter
48	1590	B	2-octen-1-ol	F	-
49	1602	B	β-cyclocitral	F	mint
50	1619	B	butanoicacid	g	rancid, sweet
51	1665	B	isovalericacid	g	sweet, acid, rancid
52	1666	B	1-nonanol	g, f	fat, green
53	1687	B	2-propylheptanol	F	-
54	1697	B	3-nonen-1-ol	F	-
55	1700	B	heptadecane	g	alkane
56	1706	B	2,4-nonadienal	F	watermelon
57	1764	B	4-decen-1-ol	F	-
58	1765	B	1-decanol	g	fat
59	1767	B	2-phenyl-2-propanol	g, f	-
60	1839	B	dibutylformamide	g	-
61	1912	A	β-ionone	g, f	violet, flower
62	1915	B	benzylalcohol	g	sweet, flower
63	1935	B	1-dodecanol	g	-
64	1938	B	benzothiazole	g	gasoline, rubber
65	1952	B	2,4-decadien-1-ol	F	-

66	1958	B	5,6-epoxy-β-ionone	g, f	fruit, sweet, wood
67	1963	B	2-phenylethanol	g	honey, spice
68	2031	B	diethylesterhexanedioicacid	g	-

[a] Linear retention indices in DB-Wax column.
[b] A, mass spectrum and LRI agree with those of an authentic compound run on DB-wax column; B mass spectrum agrees with reference spectrum in the NIST mass spectral data base and LRI agree with those in the literature.
[c] Detected in medium containing glucose (g) and/or fructose (f).
[d] According to Acree and Arn (2015).

The Table 1 also includes each volatile odor description. The system with glucose or fructose has a total 44 and 35 compounds respectively. A combined total of 68 compounds were identified and 11 volatiles were common in both experiments (peaks 1, 13, 16, 19, 26, 33, 34, 52, 59, 61 and 66). About 68 compounds, these include 23 alcohols, 15 ketones, 10 aldehydes, 8 esters, 5 miscellaneous, 4 acids, and 3 hydrocarbons were identified and quantified.

3.1 Volatile organic compounds from systems with glucose:

The impact of the metabolic transformation in function of time on the composition of volatile compounds in heterotrophic microalgal bioreactor supplemented with glucose can be seen in Figure 1 and Table 2.

Table 2. Quantification of volatile compounds obtained through the bioreactor using glucose as a carbon source.

Compound	**0h µg/mg**	**24h µg/mg**	**72h µg/mg**	**144h µg/mg**
acetaldehyde	nd	34.8	63.0	1.6
isobutyraldehyde	nd	7.7	0.3	0.1
butanal	nd	nd	0.4	0.1
ethylacetate	156.9	25.5	9.0	1.5
2-butanone	81.3	11.7	5.7	1.1
3-methyl-2-butanone	nd	2.0	0.5	0.1

2-propanol	nd	nd	1.6	16.4
2,3-butanedione	86.4	180.6	14.4	2.2
4-methyl-2-pentanone	nd	2.7	0.6	0.1
acetonitrile	nd	1.3	9.3	0.8
2-methyl-1-propanol	76.5	24.1	19.6	3.7
isoamylacetate	nd	nd	0.2	0.1
2,3-hexanedione	nd	11.3	0.4	nd
2-pentanol	nd	nd	0.9	3.3
2-heptanone	nd	6.1	0.8	0.1
3-methyl-1-butanol	nd	nd	21.2	141.5
3-methylbutenol	nd	20.3	17.1	2.2
1-hydroxy-2-propanone	nd	3.3	2.6	nd
6-methyl-5-hepten-2-one	44.0	10.2	5.4	1.4
1-hexanol	117.4	9.0	2.0	nd
2-ethylhydroxyisovalerate	nd	11.6	7	1.4
2-butoxyethanol	35.4	6.8	2.1	0.4
dihydro-2-methyl-3-furanone	nd	17.5	146.8	20.5
heptanol	62.6	5.9	0.9	nd
aceticacid	nd	75.0	9.7	2.0
decanal	229.8	19.6	15.4	2.1
ethyl-3-hydroxybutanoate	nd	2.7	39.8	1.5
2-ethyl-mercaptoethanol	nd	2.9	8.7	1.7
1-octanol	124.5	10.9	5.7	0.6
isobutyricacid	nd	21.1	5.5	3.3
butanoicacid	nd	nd	29.9	12.8
isovalericacid	nd	nd	8.7	7.4
1-nonanol	183.1	16.3	7.2	1.0
heptadecane	298.5	39.2	2.7	0.6
1-decanol	42.9	3.2	Nd	nd
2-phenyl-2-propanol	80.0	11.2	3.5	0.9

dibutylformamide	33.5	2.5	0.8	nd
β-ionone	1.1	1.7	0.8	nd
benzylalcohol	26.7	7.3	0.7	0.1
1-dodecanol	75.7	8.8	2.9	0.6
benzothiazole	29.3	3.7	1.0	0.1
5,6-epoxy-β-ionone	nd	nd	0.2	2.7
2-phenylethanol	31.1	7.2	1.9	0.3
hexanedioicacid,diethylester	nd	26.9	8.2	0.7

A total of 20 different compounds were separated in the time 0h of the cultivation. Heptadecane was the major volatile (298.5µg.mg^{-1}) followed by decanal (229.8µg.mg^{-1}), 1-nonanal (183.1µg.mg^{-1}), ethyl acetate (156.9µg.mg^{-1}) in this time.

As consequence of the cultivation time (144h) 1-decanol, hexanol, heptanol, dibutylformamide, β-ionone disappeared. However five ketones (3-methyl-2-butanone, 4-methyl-2-pentanone, 2-heptanone, dihydro-2-methyl-3-furanone, 5,6-epoxy-β-ionone), five alcohols (2-propanol, 2-pentanol, 3-methyl-1-butanol, 3-methylbutenol, 2-ethyl-mercapto-ethanol), four esters (isoamyl-acetate, 2-ethyl-hydroxy-isovalerate, ethyl-3-hydroxybutanoate, di-ethyl-ester-hexanedioic-acid), four acid (acetic acid, isobutyric acid, butanoic acid, isovaleric acid), three aldehyde (acetaldehyde, isobutyraldehyde, butanal) and one micellanius (acetonitrile) were formed (Table 2). Additional, 2 ketones, 2,3-hexanedione, and 1-hydroxy-2-propanone were only formed from 24h and disappeared after 72h of experiment.

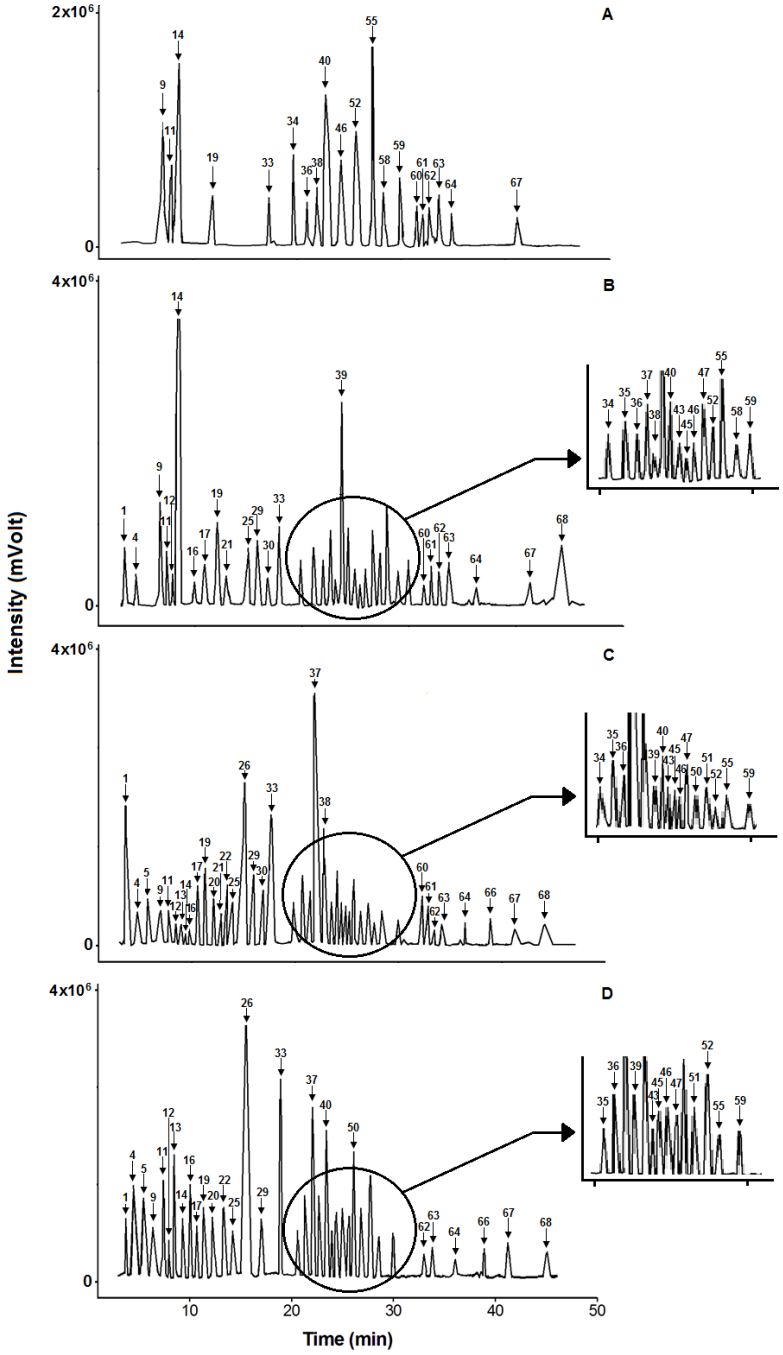

Figure 1. Chromatogram (total íon current), obtainedby GC-MS, of the volatile organic compounds from the bioreactor microalgal with addition of glucose. The letters correspond to the experimental times that chromatograms were obtained: A=0 hours, B=24 hours, C=72 hours and D=144 hours.

The Figure 1 showed that time between 24h and 72h of cultivation promoted a clear change in the volatile profile of the heterotrophic *Phormidium autumnale* cultivation, notably during the first 30 min of chromatogram, all of the 24 news compounds were formed in this period.

In the qualitative way, on the most abundant volatile group produced in the experiment was ketones the peaks 12, 16, 21, 25, 30, 66, and 68 were formed totaling 358,8µg.mg^{-1}. The 2,3-butanedione and dehydro-2-methyl-furanone were found in relative high amount, 180.6, 146.8 µg.mg^{-1} respectively between 24h until 72h of cultivation. The others ketones volatile formed was detected in relative low amounts (Table 2). Of these compounds formed 2,3-butanedione (peak 14), and 5,6-epoxy-β-carotene (peak 66) were previously identified in cyanobacteria (Rzama et al., 1995, Durme et al., 2013). Other ketones shown decreasing (2-butadione, 6-methyl-5-hepten-2-one, 2-butanone and β-ionone) were described in the literature like typical volatiles from cyanobacteria (Rzama et al., 1995; Evans, 1994; Hasegawa et al., 2012; Durme et al., 2013, Sun et al., 2012).

Ketones can be formed by many ways, aliphatic ketones might be products of lipid oxidation or degradation. Methyl ketones (C_3-C_{17}), such as 6-methyl-5-hepten-2-one one could be formed from the oxidative cleavage of carotenoids. Rodrigues et al., 2014 as identified β-carotene and echinenone like the major carotenoids in *Phormidium autumnale* biomass, observing which all these compounds have in common a neutral planar polyene chain. Acree and Arn, 2015 related the odor descriptors for following ketones identified in *Phormidium autumnale* cultivation: 2-butanone and 3-methyl-butanone (camphor), 2,3-butanedione (butter), 4-methyl-2-pentanone (fruity), 2-heptanone (soap), β-ionone (violet, flowers).

As expected a few aldehydes were formed (peaks 1, 4 and 5) with higher production (63.0µg.mg^{-1}, 7.7 µg.mg^{-1}, 0.4µg.mg^{-1} respectively) in intermediate cultivation time (24h and 72h), almost disappeared in 144h (Table 2). These aldehydes can provide several notes to food matrices depending on the number of carbon atoms and the degree of saturation. The structure formed were C_2 and C_4 compounds, the

short chain linear aldehydes are often derived from chemical lipid oxidation, and feature a green-like, fruit-like and malt-like odor (Durme et al., 2013).

Decanal (peak 40) is the second major compound in the time 0h of cultivation (229.80µg.mg^{-1}), and a long of experiment was observed losses of this compound of 99.1%. The primary barrier to over production of aldehydes in microrganisms is the rapid conversion of desired aldehydes into alcohols by numerous endogenous enzimes (Kunjapur et al., 2014).

All alcohols formed (peaks 13, 22, 26, 29, 45) are short chain linear (C_3-C_5) compounds. In the quantitative way alcohols group was the major with 496.5 µg.mg^{-1} formed. The 3-methyl-1-butanol, peak 26, was the most abundant of these alcohols (59.1%), followed by 2-propanol, peak 13, (6,9%).These compounds are a chemical signature to cyanobacteria have been confirmed for studies involving the identified of organic volatiles in these microorganisms (Durme et al., 2013; Sun et al., 2012; Ozaki et al., 2008). The biosynthesis of peak 13 and 26 are known to be generates from 2-keto acid, these keto acids are intermediates in the amino acid biosynthesis pathways and can be converted into alcohol by alcohol dehydrogenases (Dickinson, 1996).

In the time 0h, 11 compounds were alcohol corresponding a 45.1% of total organics volatile compounds in the heterotrophic *Phormidium* cultivation. All these structures (peaks 19, 34, 36, 38, 46, 52, 58, 59, 62, 63, 67) show an expressive decrease in the concentration along the time (Table 2).

The ester fraction is characterized for compounds were produced between 24h (peaks 35, 68) and 72h (peaks 20, 43) and decrease after this time of cultivation. The ethyl acetate (peak 9) shows 8.2% of total volatile compounds in the time 0h and decrease in function of experimental time. The ester were identified believes to be product of an esterification of alcohols and carboxylic acids (Sun et al., 2012).

All esters identified in volatile profile from *Phormidium autumnale* give sweet fruity flavors (Table 1), compounds are widely in food industrial application (Berger, 2009, Sun et al., 2012). The most abundant component among these compounds was ethyl acetate (156.9µg.mg^{-1}) in the time 0h, described by pineapple (Acree and Arn, 2015). Despite literature information that *Phormidium* produce, esters like dibuthyl-phthalate that is regarded as toxic component, these compound were not detected in this study, which adds business value to *Phormidium autumnale* in heterotrofic cultive (Sun et al., 2012).

The experimental time more favorable to produced ester were between 0h (1569µg.mg^{-1}), 24h (66.7µg.mg^{-1}) and 72h (64.2µg.mg^{-1}), in this period is possible observed a expressive formation this volatile group considered, in geral, esters show low odor threshold values (Durme et al., 2013).

All acids identified were produced after 24 hour of cultivation, acetic acid had the highest amount of the acids compounds identified (75.0µg.mg^{-1} at 24h), followed butanoic acid (29.9µg.mg^{-1} at 72h), isobutyric acid (21.1µg.mg^{-1} at 24h) and isovaleric acid (8.7µg.mg^{-1} at 72h). Acetic acid were identified in several microalgae (Durme et al., 2013).

Hydrocarbons compounds were not formed in headspace volatile compounds of *Phormidium*, but the major compound in the time 0h was a heptadecane (298.5 µg.mg^{-1}), this volatile as previously described like major compounds in *Chlorella* species and *Scenedesmus sp.* (Rzama et al., 1995). Heptadecane have been described as product from descarboxylation of stearic acid (Gelpi et al., 1970).

Volatile organic compounds from systems with Fructose:

The volatile profile of the *Phormidium autumnale* heterotrofic cultivation for 144h is shown in Figure 2 and Table 3.

Table 3. Quantification of volatile compounds obtained through the bioreactor using fructose as the carbon source.

Compound	0h µg/mg	48h µg/mg	96h µg/mg	144h µg/mg
acetaldehyde	13.3	41.8	3.4	4.9
1-heptene	nd	5.5	6.4	3.4
2,4-dimethylheptane	1.9	0.5	0.2	Nd
hexamethylcyclotrisiloxane	36.9	15.2	3.3	1.5
tetrahydrofuran	7.1	0.9	Nd	Nd
2-methylbutanal	nd	nd	0.2	0.6
3-methylbutanal	nd	nd	0.6	2.2
2-propanol	nd	nd	1.7	11.1
2-butyl acetate	10.4	4.2	1.7	nd
4-methyl-2-pentanone	2.5	0.7	0.4	0.2
hexanal	4.7	31.7	5.0	1.8
2-methyl-1-propanol	nd	nd	21.3	47.9
isobutylacetate	nd	nd	1.2	0.2
1-butanol	nd	0.2	1.1	0.7
3-methyl-1-butanol	nd	nd	30.7	69.5
1-methoxy-2-propylacetate	10.8	0.6	Nd	nd
6-methyl-2-heptanone	nd	nd	0.2	nd
3-hydroxy-2-butanone	nd	nd	Nd	8.4
2,3-octanedione	nd	1.5	1.5	0.6
6-methyl-5-hepten-2-one	5.9	1.7	1.4	nd
1-hexanol	1.9	15.1	45.1	26.2
3-octenal	nd	nd	1.1	2.1
2-hepten-1-ol	nd	0.3	1.9	0.4
3-nonen-2-one	nd	nd	0.8	0.3

2-octen-1-ol	nd	3.1	3.4	1.3
β-cyclocitral	nd	1.8	2.0	nd
1-nonanol	nd	1.8	0.7	0.5
2-propylheptanol	nd	1.1	0.2	0.1
3-nonen-1-ol	nd	0.5	1.7	1.2
2,4-nonadienal	nd	0.6	1.2	nd
4-decen-1-ol	0.1	0.3	1.3	4.3
2-phenyl-2-propanol	nd	nd	0.3	1.4
β-ionone	7.7	3.3	1.9	1.2
2,4-decadien-1-ol	nd	nd	0.5	1.3
5,6-epoxy-β-ionone	nd	0.3	0.7	1.0

As shown in Table 3, thirty-five volatiles were detected in heterotrophic culture with fructose by GC-MS. A total of 12 different compounds were separated in the time 0h of the cultivation. Hexamethylcyclotrisilhexane was the major volatile (36.9µg.mg^{-1}) followed by acetaldehyde (13.3µg.mg^{-1}), 1-methoxy-2-propylacetate (10.8µg.mg^{-1}), 2-butylacetate (10.4µg.mg^{-1}) in this time.

The compounds 2,4-dimethylheptane, tetrahydrofuran, 2-butylacetate, 1-methoxy-2-propylacetate, 6-methyl-5-hepten-2-one, and 2,4-nonadienal disappeared in 144h of the heterotrophic cultivation with fructose. However eleven alcohols (2-propanol, 2-methyl-1-propanol, 1-butanol, 3-methyl-1-butanol, 2-hepten-1-ol, 2-octen-1-ol, 1-nonanol, 2-propylheptanol, 3-nonen-1-ol, 2-phenyl-2-propanol, 2,4-decadien-1-ol), four ketones (3-hydroxy-2-butanone, 2,3-octadione, 3-nonen-2-one, 5,6-epoxy-β-ionone), three aldehyde (2-methylbutanal, 3-metylbutanal, 3-otenal), one ester (isobutyl-acetate) and one hydrocarbon (heptene) were formed (Table 3). Additional 6-methyl-2-heptanone, β-cyclocitral, 2,4-nonadienal were only formed from 48h and disappeared after 96h of experiment.

The Figure 2 showed that time between 48h and 96h of cultivation promoted a clear change in the volatile profile of the heterotrophic *Phormidium autumnale* cultivation, notably almost of the 20 news compounds were formed in this period, with except of 3-hydroxy-2-butanone was produced in 144h of cultivation. When compared the heterotrophic cultivations with glucose or fructose is noticeable that the cultivations with fructose needs more time to convert organic carbon in volatile organic compounds (48-96h). The results obtained agreement with literature, as demonstrated (Francisco et al., 2014), the maximum specific growth rate obtained with glucose was 0.768day^{-1}, while 0.576day^{-1} were found to fructose.

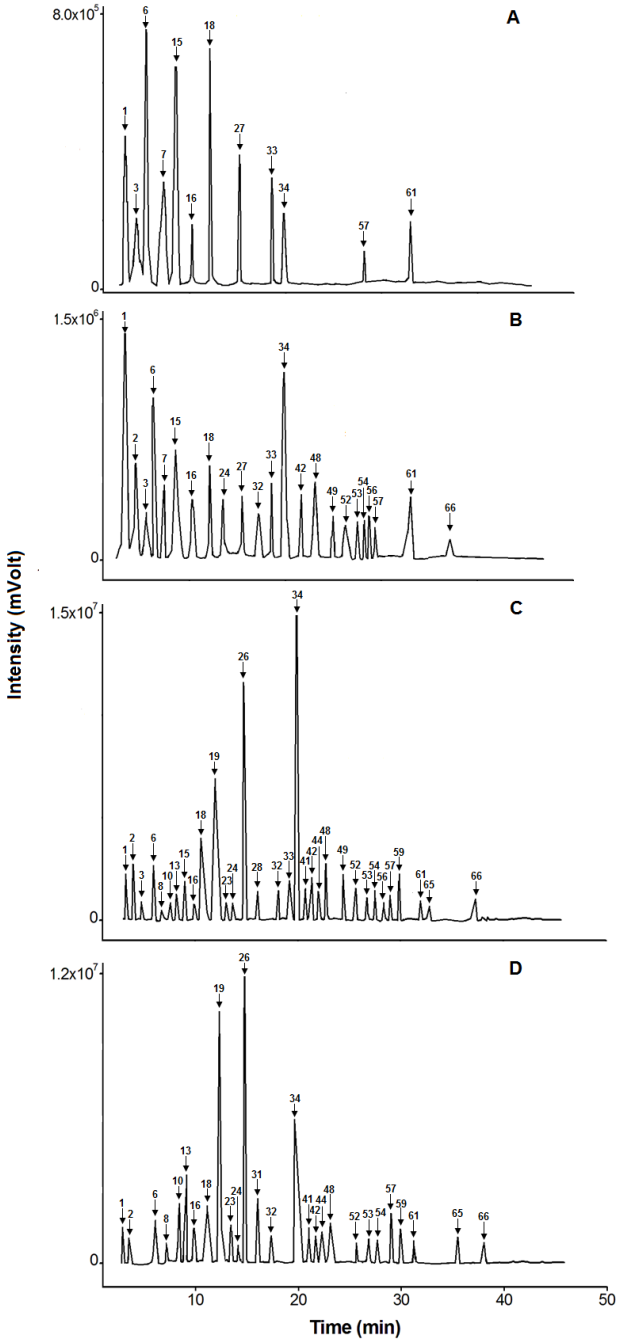

Figure 2. Chromatogram (total íon current), obtainedby GC-MS, of the volatile organic compounds from the bioreactor microalgal with addition of fructose. The letters correspond to the experimental times that chromatograms were obtained: A=0 hours, B=48 hours, C=96 hours and D=144 hours.

Based on their biosynthetic origin, several unquestionable volatile organic compounds identified in *Phormidium autumnale* derived from the different classes, including 2-keto acid derivatives, fatty acids derivatives , and terpenoids.

Almost all alcohols identified in the culture with frutose, (peaks 13, 19, 24, 26, 42, 48, 52, 53, 54, 59, and 65) were formed between 48h and 96h of experiment. The compounds like 2-propanol (peak 13), 1-butanol (peak 24), and 3-methyl-1-butanol (peak 26), are formed by deamination followed by decarboxylation of the branched-chain amino acid such L-Leucine and L-isoleucine leading to the formation a correspondent 2-ketocid (Fujise et al., 2010; Ouchi et al., 1989) . These 2-ketoacid, can be subject to decarboxylation, followed by reduction forming alcohol (Figure 3).

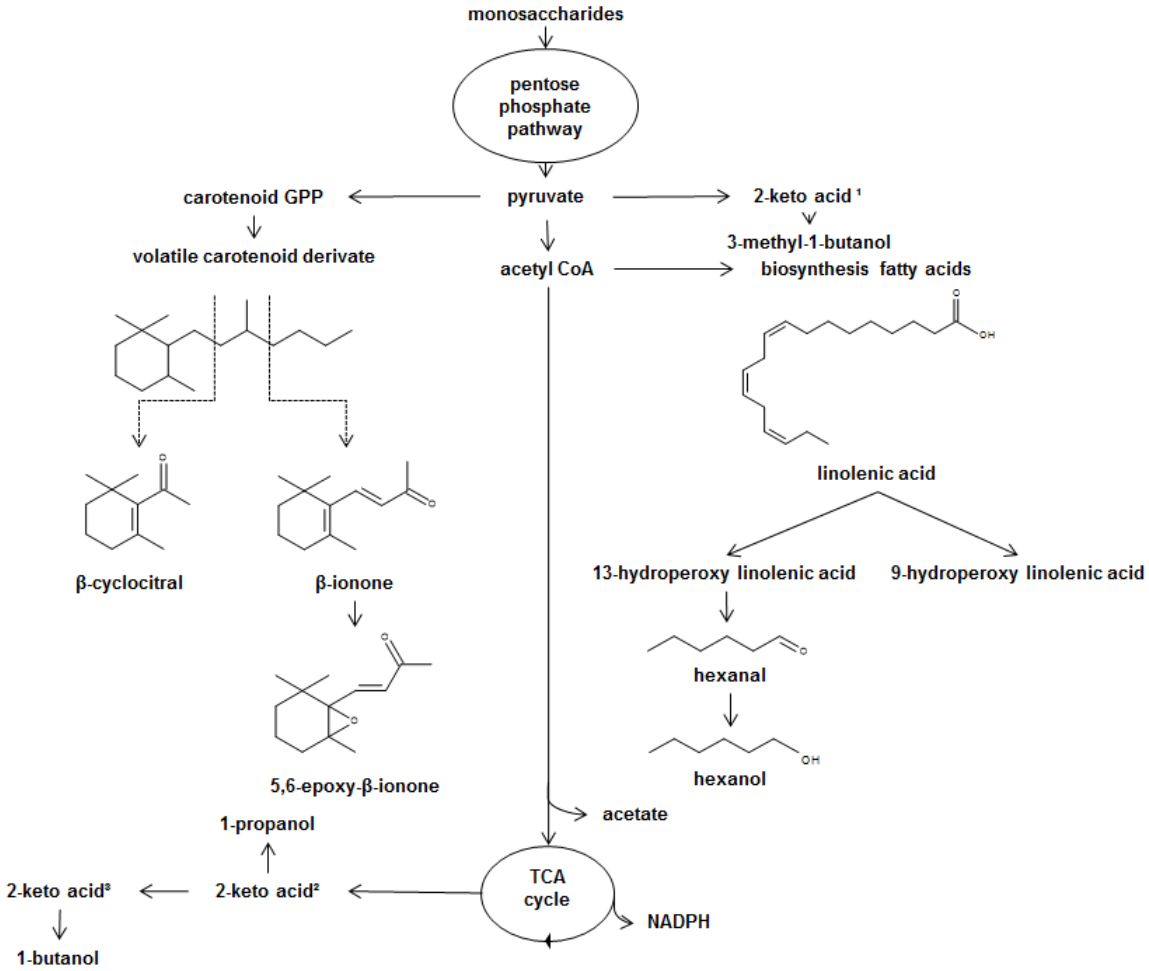

Figure 3. Overview of biosynthetic pathways leading to the emission of microalgae volatile organic compounds (VOCs).

*GPP: geranyl pyrophosphate;¹ ketoisovalerate; ² 2-ketobutyrate; ³ 2-ketovalerate.

The alcohol 1-hexanol (peak 34) show an increase in the concentration until 96h (45.1μg.mg^{-1}), in the same period that the aldehyde hexanal (peak 18) was consumed.

The volatile organic compounds like hexanol and hexanal are fatty acid derivate from C_{18} unsaturedd fatty acid, linoleic or linolenic (Figure 3). Biosynthesis of these compounds from acetyl-CoA generated from pyruvate, the final product of penthose phosphate pathway. The lipoxygenase pathway form 9-hydroperoxy and 13-hydroperoxy intermediates. The hydroperoxide lyase branch converts both hydroperoxides into C_6 and C_9 aldehydes, which are reduced to alcohols by dehydrogenases (Gigot, et al., 2010).

The other aldehyde formed (3-methylbutanal) were found in others species of microalgae (Sun et al., 2012; Durme et al., 2013), and the major aldehyde produced was acetaldehyde (41.8μg.mg^{-1}) at 48h, this compound shown an attractive odor description (fruit) for food industry.

The β-cyclocitral (peak 49), β-ionone (peak 61) and 5,6-epoxy-β-ionone (peak 66) seens to be important volatiles organics compounds in some microalgae such *Chlorella vulgaris*, *Scenedesmus sp*. (Rzama et al., 1995; Durme et al., 2013). The β-cyclocitral formed (2μg.mg^{-1}) until 96h and disappeared in 144h, while β-ionone decrease and 5,6-epoxy-β-ionone were formed in all experiment. These compounds were produced by terpenoids biosynthetic pathway. β-cyclocitral can be formed from enzymatic cleavage of the double bound between seven and eight carbons of β-carotene (Figure 3). The and β-ionone produced by enzymatic cleavage of the double bound between nine and ten carbons of the same carotenoid (Donadio et al., 2011, Dudareva et al, 2013).

The ketone 5,6-epoxy-β-ionone is a product from oxidative reaction from other ketone β-ionone, that explain the decrease concentration of β-ionone while increase of the 5,6-epoxy-β-ionone in function experimental time (Table 3).

The experimental with fructose produced less esters than glucose, just isobutyl acetate peak 23, with the highest formation at 96h (1.2μg.mg^{-1}), and odor description pungent and fruit.

In addition almost the descriptor odor of the compounds detected in experiments with glucose or fructose were classified like fruity, spice and floral compounds and no compound off flavor were identified in *Phormidium autumnale* heterotrophic cultivation.

The cultivation supported with glucose produced more kind of volatile compounds in less time when compared with supported with glucose. This information could be correlation with the total carbon balance system, considering in terms of conversion of organic carbon in biomass, the cultivations with fructose were the most efficient than glucose, 600.7mg.L^{-1}.day^{-1} and 404.1mg.L^{-1}.day^{-1} (Francisco et al., 2014).

Finally *Phormidium autumnale* proved that can produced a variety of industrially volatile compounds, and the knowledge about biosynthesis of these structures from microalgae might prove to useful to help elucidated ways to the apply the biobased feedstocks for both food and non-foods industry.

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CONCLUSÃO GERAL

O perfil de compostos orgânicos voláteis de cultivos heterotróficos suportados com glicose por 72h, determinado por cromatografia gasosa acoplada ao detector de massas, apresentou 54 compostos classificados como álcoois (27%), cetonas (23%), aldeídos (19%), ésteres (14%), ácidos (3.5%) e hidrocarbonetos (2.6%).

Para os experimentos realizados por 144h com variação do monossacarídeo como fonte de carbono exógeno, foram identificados e quantificados 68 compostos voláteis. O compostos 3-metil-butanol foi identificado entre os majoritários 141.5µg.mg^{-1} no cultivo da glicose e 69.5µg.mg^{-1} no cultivo com frutose.

Em consequência da variação de fonte de carbono, experimentos realizados com glicose apresentaram tempo experimental de formação de voláteis mais breve que os comparados aos experimentos realizados com a frutose.

Vários compostos voláteis com descritores de aroma de interesse comercial foram formados, tais como, acetaldeído, isoamilacetato, 2-etil-hidroxi-isovalerato, 6-metil-5-hepten-2-ona, 2-hepten-2-ol, 5,6-epoxi-β-ionona, 2,4-nonadienal no *headspace* dos cultivos heterotróficos de *Phormidium autumnale*. Outros compostos formados da classe dos álcoois (3-metil-1-butanol, propanol e butanol) apresentam uma possibilidade de produção sustentável com elevada densidade energética quando a produção de álcool de cadeias mais curtas.

As características estruturais das classe de compostos identificadas indicaram que foram provavelmente originados a partir de vias biosintéticas específicas. Os compostos (β-ionona, β-ciclocitral, e 5,6-epoxi-β-ionona) pela via de terpenos, (hexanol e hexanal) pela via de ácidos graxos, (3-metil-butanol, butanol, propanol) pela via dos 2-cetoácidos.

Os resultados indicaram que a fração de compostos orgânicos voláteis do headspace de cultivos heterotróficos de *Phormidium autumnale*, apresentaram um expressivo potencial de aplicação comercial nas mais diferentes áreas industriais.