SUZANA EDA HIKICHI

MICROALGAS: CULTIVO EM SUBPRODUTO DO CAFÉ, CARACTERIZAÇÃO DO METABOLOMA, AVALIAÇÃO DO EFEITO ANTIMICROBIANO E APLICAÇÃO NA SÍNTESE DE NANOPARTÍCULAS

LAVRAS – MG
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Tese apresentada à Universidade Federal de Lavras, como parte das exigências do Programa de Pós-Graduação em Microbiologia Agrícola, área de concentração Microbiologia Agrícola, para a obtenção do título de Doutor.

Prof. Dr. Whasley Ferreira Duarte
Orientador

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2020

Ficha catalográfica elaborada pelo Sistema de Geração de Ficha Catalográfica da Biblioteca Universitária da UFLA, com dados informados pelo(a) próprio(a) autor(a).

Hikichi, Suzana Eda.

Microalgas: Cultivo em subproduto do café, caracterização do metaboloma, avaliação do efeito antimicrobiano e aplicação na síntese de nanopartículas / Suzana Eda Hikichi. - 2020.

106 p.

Orientador(a): Whasley Ferreira Duarte.

Tese (doutorado) - Universidade Federal de Lavras, 2020.

Bibliografia.

1. Microalgas. 2. Subprodutos do café. 3. Metabolômica. I. Duarte, Whasley Ferreira. II. Título.

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MICROALGAE: CULTIVATION IN COFFEE BY-PRODUCTS, METABOLOMICS CHARACTERIZATION, EVALUATION OF THE ANTIMICROBIAL EFFECTS AND APPLICATION IN THE SYNTHESIS OF NANOPARTICLES

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Aprovada em 05 de Agosto de 2020.

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LAVRAS – MG
2020

Dedico este trabalho aos meus pais, professores, amigos e a todos que apoiaram e contribuíram na minha formação acadêmica e na elaboração desse trabalho.

AGRADECIMENTOS

À Deus por tornar tudo possível.

À Universidade Federal de Lavras e especialmente ao Programa de Pós-Graduação em Microbiologia Agrícola- PPGMA, pela oportunidade oferecida.

Ao meu orientador, professor Whasley Ferreira Duarte, pela oportunidade, dedicação e suporte na realização desse trabalho.

Aos professores da Universidade Federal de Lavras, em especial Patrícia Gomes Cardoso, Roberta Hilsdorf Picolli, Flávia de Freitas Coelho, Luciano Vilela Paiva e Júlio Cesar Ugucioni por disponibilizarem seus laboratórios e pela colaboração.

Às Doutoras Tatiana, Lidiany e Priscilla por gentilmente me ajudarem nas análises. E aos colegas Juliana, Ademir, Fabiana, Rafaela e Ana e a todos os alunos de Iniciação Científica pela ajuda nos experimentos.

Aos professores, técnicos e funcionários do PPGMA, em especial à Rose, por toda a paciência, disponibilidade e atenção.

Aos professores José António Couto Teixeira, por ter me supervisionado na Universidade do Minho, em Braga, Portugal e Antônio Vicente, por ter aberto as portas do Laboratório de Indústria e Processos.

Às Doutoras Michelle, Zlatina e Cristiane pelo apoio e todo auxílio dado durante a minha estadia em Portugal. E também aos meus colegas do LIP, por toda a ajuda e cooperação.

Aos meus pais, Tereza e Paulo dedicação e força em todos os momentos.

À todos os meus amigos, em especial Cibelli, Daelen, Andréa, Gabrielly, José e Rafael pelo companheirismo durante o doutorado. E também Catarina, Nayra, Cris, Marta, Anna e Leticia pela amizade em Portugal.

Aos Doutores Patrícia Gomes Cardoso, Zlatina Genisheva, Eustáquio de Souza Dias e Félix Gonçalves de Siqueira por aceitarem o convite para participaram da banca de defesa deste trabalho.

À todos que contribuíram direta ou indiretamente na realização desse trabalho.

MUITO OBRIGADA!

O presente trabalho foi realizado com apoio da Coordenação de Aperfeiçoamento de Pessoal de Nível Superior – Brasil (CAPES) – Código de Financiamento 001 e da Fundação de Amparo à Pesquisa de Minas Gerais (FAPEMIG).

RESUMO GERAL

As microalgas têm sido reconhecidas como promissores microrganismos para geração de produtos de valor agregado. A composição rica em carboidratos, lipídios, proteínas e outros compostos bioativos as tornam adequadas para diversas aplicações industriais. O presente trabalho teve como objetivo isolar microalgas de duas diferentes regiões brasileiras e avaliar seu cultivo em subprodutos do processamento do café. Além disso, foi investigado o potencial das microalgas *Desmodesmus* sp. e *Neodemus* sp. na biossíntese de nanopartículas de prata e as suas potenciais propriedades antimicrobianas. As microalgas foram isoladas de uma lagoa em Ijaci, Minas Gerais, e de duas baías em Portel e Melgaçi, Pará. O isolamento foi realizado por espalhamento em superfície usando os meios BG-11, Bold e Bold suplementado com extrato de solo, por sete dias (27ºC e fotoperíodo de 12h). Foram obtidos sete isolados, os quais foram caracterizados morfologicamente. Após triagem em mixotrofia (BG11 suplementado com glicose 1g/L, em incubadora) e água residuária de café (CWW) nas concentrações de 30, 60 e 90%, a microalga AL_UFLA06, que apresentou o maior crescimento, foi selecionada para cultivo em hidrolisados da película prateada do café (CSS), produzidos em reações térmicas com CSS e água. O crescimento de AL_UFLA06 em CWW e CSS foi comparado, e a concentração de 30% de CWW propiciou o maior crescimento, sendo selecionada para a próxima etapa. O isolado foi identificado por sequenciamento da região ITS como *Neodemsus* sp. O crescimento na condição selecionada foi repetido em maior escala e em ambientes interno (incubadora) e externo (casa de vegetação) por oito dias, com BG-11 como controle. O cultivo com CWW em ambiente externo apresentou a melhor produtividade de biomassa, resultando em 1,64 g/L em oito dias, contra 0,97 g/L produzido ao ar livre em autotrofia. O conteúdo de carboidratos (0,53 g/L), pigmentos (15,8 mg/L), lipídios (1,1%) e proteínas (0,2 g/L) em CWW foi maior do que em autotrofia também no crescimento em ambiente externo. Os metabólitos intracelulares identificados por GC-MS reforçaram as diferenças na composição da microalga entre o CWW e autotrofia, ao ar livre. Maior acúmulo de açúcares e ácidos orgânicos relacionados ao metabolismo energético foi observado no crescimento em CWW. Para avaliação das potenciais aplicações biotecnológicas, compostos extracelulares liberados por *Desmodesmus* sp. e *Neodemus* sp, no sobrenadante da cultura após 14 dias de crescimento (27ºC, fotoperíodo de 12h em BG-11), coletados por centrifugação, reduziram o nitrato de prata nas concentrações de 0,5, 0,75 e 1 mM, promovendo a biossíntese de nanopartículas de prata. Para a avaliação da atividade antimicrobiana, os extratos etanólico e de acetato de etila de *Desmodesmus* sp. e *Neodesmus* sp. foram testados em uma série de bactérias patogênicas. Compostos intracelulares presentes nos extratos etanólico e de acetato de etila de *Desmodesmus* sp. apresentaram atividade antimicrobiana contra as bactérias *Staphylococcus aureus* e *Salmonella Typhimurium*. Os resultados destacam o potencial da CWW para o crescimento de *Neodemus* sp., a adaptação das microalgas ao cultivo ao ar livre, e também o potencial dessas microalgas para o desenvolvimento de agentes antimicrobianos e nanopartículas de prata.

Palavras-chave: Mixotrofia. Subprodutos do café. GC-MS. Metabolômica. Biotecnologia.

ABSTRACT

Microalgae have been recognized as promising microorganisms due to their potential to generate value-added products. Because of their composition rich in carbohydrates, lipids, proteins and other bioactive compounds, microalgae are suitable for a variety of industrial applications. The present work aimed to isolate microalgae from two different Brazilian areas, and to evaluate their cultivation in coffee processing by-products. Additionally, the potential of *Desmodesmus* sp. and *Neodesmus* sp. to perform biosynthesis of silver nanoparticles, and also their antimicrobial properties were investigated. Microalgae were isolated from a lake located in Ijaci, in the state of Minas Gerais, and two bays in Portel and Melgaço, in the state of Pará. The isolation was performed according to the spread-plating method using BG-11, Bold and Bold medium supplemented with soil extract for seven days at 27ºC and 12h photoperiod. Seven isolates were obtained and morphologically characterized. After screening in mixotrophy of the media BG11 supplemented with 1g/L of glucose and indoor conditions and coffee wastewater (CWW) at 30, 60, and 90% concentrations, microalgae AL_UFLA06, which presented the highest growth, was selected to be cultivated in coffee silverskin (CSS) hydrolysates produced from different thermic reactions with CSS and water. The growth of AL_UFLA06 both in CWW and CSS was compared, and the concentration of 30% of CWW provided the highest growth, hence, AL_UFLA06 was selected for the next step of the experiment. The isolate was identified by sequencing its ITS region as *Neodesmus* sp. Mixotrophic growth in the selected condition was scaled-up and carried out indoors and outdoors (greenhouse) for eight days, with BG-11 as an autotrophic control. Cultivation using CWW in the outdoor environment presented the best biomass productivity, resulting in 1.64 g/L in eight days, compared to the production of 0.97 g/L under autotrophic growth and the same type of environmental condition. Carbohydrates (0.53 g/L), pigments (15.8 mg/L), lipids (1.1%), and proteins (0.2 g/L) content in CWW were higher than autotrophy also in outdoor growth. Intracellular metabolites identified by GC-MS reinforced the differences in microalgae composition between CWW and autotrophic growth outdoors. Higher accumulation of sugars and organic acids related to energy metabolism was observed in mixotrophy with CWW. In order to evaluate the potential for biotechnological applications, extracellular compounds released by *Desmodesmus* sp. and *Neodesmus* sp., in the cell-free culture supernatant after 14 days growth (27ºC, 12h photoperiod in BG-11) were collected by centrifugation, used as reducing agents of silver nitrate in concentrations of 0.5, 0.75, and 1mM, and then the biosynthesis of silver nanoparticles was performed. For antimicrobial activity screening, ethanolic and ethyl acetate extracts of *Desmodesmus* sp. and *Neodesmus* sp. were tested against pathogenic bacteria. Intracellular compounds present in the ethanolic and ethyl acetate extracts of *Desmodesmus* sp. showed antimicrobial activity against bacteria *Staphylococcus aureus* and *Salmonella Typhimurium*. Results highlight the potential of CWW for *Neodesmus* sp. growth, the microalgae adaptation to the cultivation outdoors, and also the potential of these microalgae for the development of antimicrobial agents and silver nanoparticles.

Keywords: Mixotrophy. Coffee by-products. GC-MS. Metabolomics. Biotechnology.

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PRIMEIRA PARTE

1 INTRODUÇÃO GERAL

As microalgas constituem um heterogêneo grupo de microrganismos dotados de clorofila *a*, eucarióticos ou procarióticos, com capacidade de acumular biomassa através do mecanismo fotossintético. Embora muitas espécies já tenham sido identificadas, estima-se que grande parte da diversidade de microalgas ainda permanece inexplorada, principalmente no Brasil, o que estimula a prospecção de novas cepas locais, mais facilmente adaptáveis às condições de cultivo nacionais, contribuindo para o desenvolvimento da produção de biomassa microalgal para geração de produtos de valor agregado, de forma efetivamente viável e sustentável (MENDES et al., 2012).

Recentemente, microalgas estão sendo reconhecidas como importante matéria-prima para diversas aplicações biotecnológicas. Apesar das diferenças nas condições ambientais, nos modos de cultivo e métodos de processamento, a composição química dos subprodutos do processamento é comumente rica em nutrientes como carboidratos, proteínas, lipídios e variados compostos que podem ser utilizados na geração de produtos de valor agregado. No entanto, aplicações comerciais na área de produtos farmacêuticos, antibióticos e outras estruturas biologicamente ativas ainda têm recebido pouca atenção (PRADHAN et al., 2012). Vários gêneros de algas verdes como *Chlorella*, *Spirulina*, *Desmodesmus* e *Scenedesmus* vêm se destacando como fonte de metabólitos com diversas atividades biológicas, como atividade antibacteriana, antifúngica e anticâncer (EL SEMARY, 2011; KUMAR P, 2017; MARREZ et al., 2019; PRADHAN et al., 2012).

Enquanto extratos produzidos a partir da biomassa microalgal vêm mostrando potencial atividade biológica, tal como ação sobre bactérias patogênicas (MATIAS et al., 2019), o sobrenadante dessas culturas de microalgas, previamente descartado, também pode ser fonte de compostos extracelulares com valor comercial, tal como afirmam Rahman et al. (2019). Logo, estão sendo propostas rotas biossintéticas para a produção de nanopartículas de prata, estruturas conhecidas pelas diversas aplicações, envolvendo a indústria farmacêutica e de cosméticos, utilizando materiais biológicos, tais como microalgas e suas moléculas (ARYA et al., 2018; AZIZ et al., 2014; PUGAZHENDHI et al., 2018). Esses métodos, sustentáveis e econômicos, mostram-se vantajosos com relação aos métodos físicos e químicos convencionais, nos quais produtos químicos tóxicos são usados como agentes de redução ou estabilização.

A condição fotoautotrófica é a mais amplamente utilizada no cultivo de microalgas. No entanto, devido a limitações como a baixa produtividade e longos períodos de cultivo, rotas alternativas para a obtenção de maior produtividade em escala industrial estão sendo avaliadas. Uma proposta para reduzir os custos na produção microalgal em escala industrial é testar a viabilidade do crescimento dessas microalgas em condições naturais, utilizando a luminosidade solar para seu metabolismo (FENG et al., 2014). Nessa forma de cultivo, é possível reduzir o consumo de energia elétrica na forma de luz artificial utilizada na manutenção das culturas microalgais, principalmente na produção em larga escala.

Ainda nesse contexto, a avaliação das outras condições metabólicas de cultivo microalgal, heterotrofia e mixotrofia, através da suplementação do meio de cultivo com diferentes fontes de carbono como glicose, frutose e sacarose, (ABINANDAN; SHANTHAKUMAR, 2015; ABREU et al., 2012) vêm adquirindo importância. Entretanto, a utilização de fontes nutricionais sintéticas ainda implica em um elevado custo de produção, o que inviabiliza sua aplicação na produção em grande escala. Dessa forma, a utilização de fontes alternativas de nutrientes está sendo proposta, visando conciliar o consumo dos nutrientes de materiais como resíduos agroindustriais e urbanos no crescimento das microalgas e propor uma finalidade para a crescente geração e deposição de resíduos no ambiente.

Diante desse cenário, subprodutos da produção do café, tais como a água residuária e a película prateada, com composição rica em nitrogênio, fósforo e açúcares, apresentam potencial para atuar como substrato no crescimento de microrganismos, tais como as microalgas (MUSSATTO et al., 2012; SELVAMURUGAN; DORAISAMY; MAHESWARI, 2010). Estes podem ser aplicados como fonte de nutrientes para o cultivo microalgal, constituindo um meio de cultivo de baixo custo, visando aplicações industriais, tais como a produção de alimentos.

E por fim, ainda no contexto da produção de biomassa microalgal, novas tecnologias estão sendo cada vez mais empregadas, buscando maximizar a produção de biomassa e potencializar as suas características de interesse comercial (GUIDETTI et al., 2019). Para compreender os mecanismos que regulam a biossíntese de moléculas de valor agregado em resposta a modificações no ambiente, diversas tecnologias, como a metabolômica, vêm sendo utilizadas na compreensão dos processos biológicos em microalgas (SU et al., 2014)

Diante do exposto, o presente trabalho teve os seguintes objetivos:

- Isolar microalgas provenientes de uma lagoa localizada no município de Ijaci (MG) e duas baías nos municípios de Portel e Melgaço (PA), Brasil;

- Avaliar o cultivo das microalgas em subprodutos do processamento do café e em condição ambiental, utilizando a luz natural;

- Analisar alterações no metaboloma da microalga durante o cultivo no subproduto do processamento do café em condição ambiental;

- Verificar o potencial da biomassa de *Neodemsus* sp. e *Desmodesmus* sp. na produção de compostos com atividade antimicrobiana;

- Examinar o sobrenadante do cultivo de *Neodemsus* sp. e *Desmodesmus* sp. como agente na síntese de nanopartículas de prata.

2 REFERENCIAL TEÓRICO

2.1 MICROALGAS

As microalgas representam um grupo de microrganismos taxonômica e filogeneticamente diversificado, composto por indivíduos eucarióticos ou procarióticos, unicelulares (mas que podem existir em estruturas multicelulares), fotossintetizantes, adaptados a diferentes ambientes e capazes de produzir carboidratos, lipídios e proteínas, dentre outros compostos, que podem ser utilizados para obtenção de biocompostos (HARUN; DANQUAH; FORDE, 2010; MARTINS; CAETANO; MATA, 2010; MIRANDA; PASSARINHO; GOUVEIA, 2012).

Esses microrganismos estão presentes em uma ampla variedade de ecossistemas existentes no planeta, não apenas aquáticos, mas também terrestres, representando uma grande variedade de espécies que vivem em uma ampla gama de condições ambientais e apresentam variadas formas de metabolismo (MARTINS; CAETANO; MATA, 2010). Embora a maioria das espécies exista em ambientes aquáticos, é relatada a sua presença em ambientes como geleiras, margens de fontes termais e solos de desertos (MARQUES et al., 2013a).

Segundo Richmond (2004) e Bux (2013), a classificação das algas é baseada principalmente nos seguintes critérios: tipos de pigmentos, natureza química dos produtos de armazenamento e constituintes da parede celular. As microalgas são atualmente representadas por duas classes procarióticas (*Cyanophyta* e *Prochlorophyta*) e nove eucarióticas (*Glaucophyta*, *Rhodophyta*, *Heterokontophyta*, *Haptophyta*, *Cryptophyta*, *Dinophyta*, *Euglenophyta*, *Chlorarachniophyta* e *Chlorophyta*).

2.1.2 Aspectos gerais do cultivo microalgal

As microalgas dependem, em geral, apenas de um suprimento suficiente de carbono inorgânico e luz para realizar a fotossíntese e consequentemente crescer em biomassa (apresentam composição de 40-50% de carbono), pelo modo autotrófico. Segundo Martins, Caetano e Mata (2010) e John et al. (2011), as microalgas podem se comportar, quanto ao metabolismo, das seguintes formas:

a) Fotoautotróficas: as espécies autotróficas realizam a fotossíntese para utilizar a luz solar e fixar o carbono do CO_2 atmosférico, que é assimilado na forma de reserva de nutrientes, como carboidratos;

b) Quimioheterotróficas: algumas espécies de microalgas são heterotróficas, e obtêm nutrientes por meio da absorção de moléculas orgânicas do ambiente;

c) Mixotróficas: existem ainda microalgas capazes de usar carbono a partir de CO_2 da atmosfera ou carbono orgânico do ambiente, em um processo denominado mixotrofia;

d) Fotoheterotróficas: a luz é requerida para a utilização de compostos orgânicos do ambiente como fonte de carbono para a produção de componentes como carboidratos, lipídeos ou proteínas.

O custo da produção de biomassa de microalgas é geralmente maior em relação à cultura de plantas terrestres, mas esse fato é compensado pela velocidade de crescimento dos microrganismos e pela alta produtividade em relação à área total necessária para o cultivo (CHISTI, 2007, 2008). O crescimento das microalgas geralmente possui as seguintes características (CHISTI, 2007; MARTINS; CAETANO; MATA, 2010):

a) Requerem fatores abióticos como a intensidade luminosa, temperatura, concentração dos nutrientes, oxigenação, nível de CO_2, pH, salinidade;

b) Fatores bióticos como patógenos (bactérias, fungos, vírus) e competição com outras microalgas podem afetar o crescimento;

c) Fatores operacionais, tais como a tensão de cisalhamento, taxa de diluição, profundidade e frequência de coleta dos produtos e subprodutos são importantes parâmetros no cultivo em biorreatores;

d) A temperatura deve permanecer geralmente em condições amenas entre 20 e 30ºC;

e) O meio de crescimento deve fornecer os elementos inorgânicos que constituem a célula das microalgas. Entre os elementos essenciais estão nitrogênio (N) e fósforo (P), dentre outros. As necessidades nutricionais mínimas podem ser estimadas usando a

fórmula molecular aproximada da biomassa de microalgas, que é $CO_{0,48}$ $H_{1,83}$ $N_{0,11}$ $P_{0,01}$. Esta fórmula baseia-se nos dados apresentados por Grobbelaar (2007);

f) Nutrientes como o fósforo devem ser fornecidos em quantidade adequada, visto que os fosfatos adicionados podem formar complexos com íons metálicos e, portanto, nem todo o P adicionado estará disponível;

e) Para microalgas marinhas, a água do mar, suplementada com fertilizantes comerciais contendo nitrato e fosfato e alguns outros micronutrientes, pode ser utilizada para o cultivo. Para algas de água doce, água fresca e salobra de lagos, rios e aquíferos podem ser usados. O meio de cultivo pode ser ainda suplementado com solo (ANDERSON, 2005)

f) na decisão de selecionar a cepa de microalga a ser cultivada para determinada finalidade, é importante considerar o local do seu isolamento, principalmente se o cultivo em ambiente externo for utilizado, devido à heterogeneidade de atributos fisiológicos existentes entre as microalgas (LEE et al., 2014).

O metabolismo das microalgas pode também apresentar diferentes estratégias visando maior crescimento, tais como o ajuste de sua estrutura interna (por exemplo, aclimatação bioquímica e fisiológica) em função de condições ambientais, e também a excreção de compostos com o objetivo de tornar os nutrientes disponíveis ou limitar o crescimento dos competidores (RICHMOND, 2004).

Em geral, a condição fotoautotrófica é a mais simples e amplamente aplicada ao cultivo de microalgas na produção de biomassa. No entanto, é sujeita a limitações devido ao auto sombreamento entre as células, o que dificulta a entrada de luz no estágio final do crescimento (CHEIRSILP; TORPEE, 2012). Estudos vêm sido conduzidos com o objetivo de maximizar a produção microalgal, modificando o modo de cultivo. Entre as alternativas estão submeter às culturas à condição de estresse por limitação de nutrientes, fornecendo aeração e suplementando o meio de cultivo com diferentes fontes de carbono como glicose, frutose e sacarose (ABINANDAN; SHANTHAKUMAR, 2015; ABREU et al., 2012).

2.2.1 Cultivo de microalgas em diferentes subprodutos

A geração de biomassa de microalgas em larga escala requer grandes quantidades de nutrientes, principalmente nitrogênio e fósforo, sendo que o suprimento de fósforo é limitado e a produção de nitrogênio requer alto gasto de energia fóssil (HERNÁNDEZ et al., 2015).

Adicionalmente, custos com vitaminas e metais traço ainda precisam ser considerados (PLEISSNER et al., 2013).

Comparando as três condições de cultivo, autotrófico, heterotrófico e mixotrófico, a suplementação de nutrientes no cultivo tem sido uma estratégia para aumentar a produção de biomassa microalgal (FENG et al., 2014; ZHANG et al., 2014). Entretanto, como afirmam Abinandan e Shanthakumar (2015), Caporgno et al. (2015) e Marques et al. (2013a), os custos e algumas dessas fontes ainda são impraticáveis na produção em escala industrial, impulsionando o desenvolvimento de tecnologias para a utilização de produtos agroindustriais e urbanos, que normalmente apresentam elevada concentração dos nutrientes necessários para o cultivo desses microrganismos e, portanto, podem atuar como fontes alternativas aos nutrientes sintéticos.

Assim, os subprodutos também podem atuar como uma fonte de carbono orgânico de baixo custo para o cultivo heterotrófico e mixotrófico de microalgas. A adição dessa fonte de carbono proporciona vantagens como o menor tempo de cultivo e maior produção de biomassa, lipídios, amido e proteínas (ABREU et al., 2012). Dessa forma, o cultivo utilizando as fontes de carbono inorgânico (como o CO_2) e orgânico também tem sido proposto como alternativa viável para a produção de biomassa de microalgas (YU; JIA; DAI, 2009).

Além da redução nos custos da produção, o cultivo das microalgas em subprodutos oferece uma solução para reduzir a carga nutricional, visto que as microalgas consomem esses nutrientes ao longo do seu crescimento (CAPORGNO et al., 2015). Ainda, em uma abordagem baseada no valor econômico, a biomassa produzida constitui uma matéria-prima rica em carboidratos, proteínas, lipídios, pigmentos e outros compostos potencialmente bioativos. Esses componentes podem ser utilizados para diversos fins, incluindo os biocombustíveis como bioetanol, biodiesel e metano e outros compostos de valor agregado como cosméticos, produtos nutracêuticos e suplementos para alimentação humana (FERNÁNDEZ-LINARES et al., 2017).

Na literatura, diversos trabalhos vêm sendo desenvolvidos com o intuito de cultivar microalgas em diferentes subprodutos industriais visando tratar o efluente e também reduzir os custos da produção da biomassa. Caporgno et al. (2015) realizaram o cultivo das espécies *C. kessleri* e *C. vulgaris* em resíduo doméstico para a produção de biodiesel e metano, removendo elevadas concentrações de fósforo e nitrogênio do efluente. Marques et al. (2013) cultivaram *C. vulgaris* em vinhaça, obtendo produtividades de biomassa de 70 mg/L/ dia e de lipídeos de 0,5 a 17 mg/L/dia. Salati et al. (2017) cultivaram uma cepa local de *C. vulgaris* em

meio contendo soro de queijo, subprodutos da produção de vinho branco e glicerol provenientes da indústria na produção de proteína de origem microalgal.

Com o objetivo de utilizar subprodutos como fonte de carbono orgânico para o crescimento de microalgas, Abreu et al. (2012) realizaram um cultivo mixotrófico de *C. vulgaris* em efluente hidrolisado de uma indústria de laticínios, especificamente, da produção de queijo, com alto conteúdo de matéria orgânica. Gélinas et al. (2015) verificaram que o resíduo hidrolisado da produção de milho poderia ser utilizado como fonte de carbono orgânico no favorecimento do crescimento, em regime mixotrófico, de um consórcio de *Chlorella* spp. Em relação ao cultivo heterotrófico, Pleissner et al. (2013) utilizaram resíduos hidrolisados de alimentos, rico em carboidratos, proteínas e lipídios para cultivar *Schizochytrium mangrovei* e *C. pyrenoidosa*, gerando biomassa rica em carboidratos, lipídeos, proteínas e ácidos graxos saturados e poli-insaturados. Também em um estudo com *Chlorella*, Fernández-linares et al (2017) utilizaram cepas de *C. vulgaris* (CICESE e UTEX) e um consórcio nativo em um cultivo mixotrófico com águas residuárias tratadas enriquecidas com e sem o fertilizante Bayfolan, obtendo produtividades de biomassa de respetivamente 1,167 ± 0,057, 1,575 ± 0,434 e 1,125 ± 0,250 g/L.

Entretanto, a utilização desses subprodutos ainda implica em uma série de desafios relacionados às suas características intrínsecas. Por exemplo, embora ricos em nutrientes orgânicos, geralmente eles apresentam coloração opaca, o que afeta a penetração da luz em sistemas fotossintéticos (BHATNAGAR et al., 2011). Além disso, nem todas as microalgas conhecidas são capazes de crescer em regime mixotrófico (GIOVANARDI et al., 2013).

2.2.2. Subprodutos do processamento do café

No contexto do aproveitamento de material resultante de atividades agroindustriais, resíduos do processamento do café constituem subprodutos com composição rica em polissacarídeos (celulose e hemicelulose), lignina, proteínas, lipídios e compostos fenólicos. Dentre esses principais componentes, os carboidratos e minerais (MURTHY; MADHAVA NAIDU, 2012) podem representar fonte nutricional para o crescimento de microrganismos.

O café constitui uma das bebidas mais consumidas atualmente, sendo uma das *commodities* mais comercializadas no mundo. As variedades *Coffea arabica* e *Coffea robusta* são as produzidas em maior volume, mundialmente, para fins comerciais (PANDEY; ROUSSOS; SOCCOL, 2000). Segundo o ICO (2020), a produção global de café estimada em 2018 foi de 172,452 milhares de sacas de café (60 kg), um aumento de 7,9% em relação ao

ano anterior. Também de acordo com o ICO (2020), o consumo mundial de café durante o período de 2018/2019 foi de 168,099 milhares de sacas. Esses dados vêm mostrando que a demanda por esse grão é alta e tende a crescer.

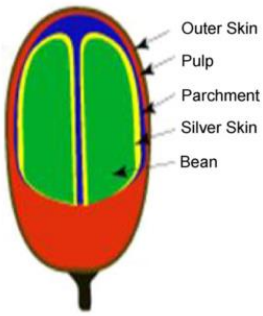
Por sua vez, o grande volume de café processado pela indústria resulta em uma gama de subprodutos que gera contaminação de corpos d'água e terras ao redor de unidades produtoras, representando um sério problema ambiental para os países produtores e consumidores (MURTHY; NAIDU, 2012). De forma geral, o gerenciamento em larga escala de subprodutos do café em continua a ser um desafio em todo o mundo devido o seu conteúdo de cafeína, fenóis e taninos livres (polifenóis), substâncias presentes principalmente na polpa, e também em outras partes do grão, como a película prateada, casca e na água residuária, e que são conhecidas por sua toxicidade à maioria dos seres vivos (MURTHY; NAIDU, 2012; SELVAMURUGAN; DORAISAMY; MAHESWARI, 2010; VON ENDEN et al., 2002).

De acordo com Murthy e Madhava Naidu (2012), os subprodutos comumente gerados durante o processamento do café variam de acordo com a técnica de processamento, torrefação e fermentação. Segundo Oliveira e Franca (2015) e Murthy e Madhava Naidu (2012), o processamento pode ocorrer de duas formas:

> 1) Processamento seco: consiste na técnica mais simples de preparação do café. Após a colheita, os frutos são secos até atingirem um teor de umidade de cerca de 10-11%, estando prontos após aproximadamente 12-15 dias de secagem natural em tempo seco. Em seguida, os grãos de café são separados e o material que cobre esses grãos é removido com o auxílio de uma máquina de descasque, gerando como subproduto as cascas de café.
>
> 2) Processamento úmido: nessa forma de processamento, a polpa que cobre a semente do fruto do café é removida mecanicamente por meio de um despolpador. Os grãos podem ser ainda fermentados para uma total remoção da camada de mucilagem. Após a fermentação, os grãos são lavados, imersos em água limpa por 12 horas, visando conferir melhor qualidade ao produto pela remoção de diterpenos e polifenóis, e secos até atingirem um índice de umidade semelhante ao grão processado via seca. Os principais subprodutos gerados são a polpa e a água residuária do café.

O processamento industrial dos grãos de café é realizado para a obtenção do café na forma de pó, por meio da remoção da casca e da porção mucilaginosa do grão, cuja estrutura esquemática pode ser observada na Figura 1.

Figura 1- Esquema simplificado do grão de café



Fonte: Murthy e Madhava Naidu (2012)

As etapas gerais das técnicas de processamento mais comumente aplicadas, seco e úmido, geram uma série de subprodutos que podem representar mais de 50% do total da massa do grão de café, mas que por sua vez, não são totalmente descartáveis, possuindo potencial para diversas aplicações industriais (CAMPOS-VEGA; OOMAH, 2015; MUSSATTO et al., 2011a). Entre os principais subprodutos gerados estão:

1) Polpa do café: a polpa do café representa cerca de 30% de peso seco do grão do café e constitui o primeiro subproduto do processamento úmido do café, apresentando em sua composição mais de 17% de celulose (BHOITE; NAVYA; MURTHY, 2013). De acordo com (PANDEY et al., 2000), apesar da natureza semelhante à casca de café, possui composição diferenciada deste subproduto;

2) Casca do café: rica em compostos orgânicos e nutrientes, também contém compostos como cafeína, taninos e outros polifenóis (PANDEY; ROUSSOS; SOCCOL, 2000);

3) Película prateada de café: é o tegumento do grão do café, obtido durante o processo de torrefação;

4) Água residuária do café: durante o processamento úmido, grandes volumes de água são utilizados nas diversas etapas, gerando um resíduo rico em sólidos totais suspensos e dissolvidos (SELVAMURUGAN; DORAISAMY; MAHESWARI, 2010);

5) Borra do café: é o resíduo sólido obtido a partir do processo de preparação do café.

A água residuária do café é responsável pela geração de grandes volumes de matéria inorgânica e orgânica insolúvel, resultantes da despolpa do grão do café e da remoção da mucilagem, realizadas no processamento úmido (VON ENDEN et al., 2002). Assim como o efluente urbano, essa água residuária também apresenta composição química rica em

nitrogênio e fósforo (TABELA 1), constituintes necessários para o metabolismo das microalgas.

Tabela 1 – Características da água residuária do processamento do café

Parâmetros	Concentração
Cor (UC)	470–640
Sólidos dissolvidos totais (mg /L)	1130–1380
Sólidos suspensos totais (mg /L)	2390–2820
Sólidos totais (mg /L)	3520–4200
pH	3,88–4,11
Condutividade elétrica (dS/m)	0,96–1,20
Oxigênio dissolvido (mg /L)	2,0–2,6
Demanda bioquímica de oxigênio (mg /L)	3800–4780
Demanda química de oxigênio (mg /L)	6420–8480
Razão DBO:DQO	0,56–0,59
Carbono orgânico total (%)	0,36–0,48
Nitrogênio (mg /L)	125,8–173,2
Fósforo (mg /L)	4,4–6,8
Potássio (mg /L)	20,4–45,8

Fonte: Selvamurugan, Doraisamy e Maheswari (2010)

Além de compostos inorgânicos, carbono orgânico também está normalmente presente nesse subproduto, proveniente da polpa e da mucilagem do grão de café (SELVAMURUGAN; DORAISAMY; MAHESWARI, 2010; VON ENDEN et al., 2002) constituindo uma possível fonte de açúcar no cultivo heterotrófico e mixotrófico de microalgas (ABREU et al., 2012). Embora a composição da água residuária do café varie em decorrência de uma série de fatores como a variedade do café e o método de processamento, a literatura relata consideráveis concentrações de açúcares em suas amostras analisadas. Segundo Bonilla-Hermosa, Duarte, Schwan (2014), porcentagens de 1,21 e 1,13% respectivamente, de açúcares totais e redutores foram encontradas em uma amostra de água residuária do café utilizada em seu cultivo fermentativo.

Outro importante subproduto do processamento do café é a película prateada, um fino tegumento que cobre o grão verde e permanece após o processamento, seja por via seca ou úmida (ALGHOONEH et al., 2017; MUSSATTO et al., 2012; NARITA; INOUYE, 2014). Ela é produzida em grande quantidade no processo de torrefação do café (cerca de 0,4 Mt por ano), sendo o único resíduo dessa etapa, e representa aproximadamente 4,2% (m/m) do grão

de café (BALLESTEROS; TEIXEIRA; MUSSATTO, 2014a; NIGLIO et al., 2019). A película prateada obtida nas indústrias de torrefação pode ser uma fonte alternativa de carboidratos para diferentes aplicações, devido à sua contínua produção durante o ano, ao contrário de outros subprodutos que são obtidos apenas durante a época da colheita, e ao conteúdo de polissacarídeos presentes no grão (HIJOSA-VALSERO et al., 2018).

O grande volume de película prateada gerado a cada ano vem motivando diversas buscas por aplicações, tanto do ponto de vista econômico quanto ambiental, incluindo a geração de combustíveis, como afirmam Mussatto et al. (2012) e Niglio et al. (2019), fertilizantes (MURTHY; NAIDU, 2012) e em alimentos funcionais, devido ao conteúdo de fibras, carboidratos e antioxidantes (BALLESTEROS; TEIXEIRA; MUSSATTO, 2014b; GOCMEN et al., 2019)

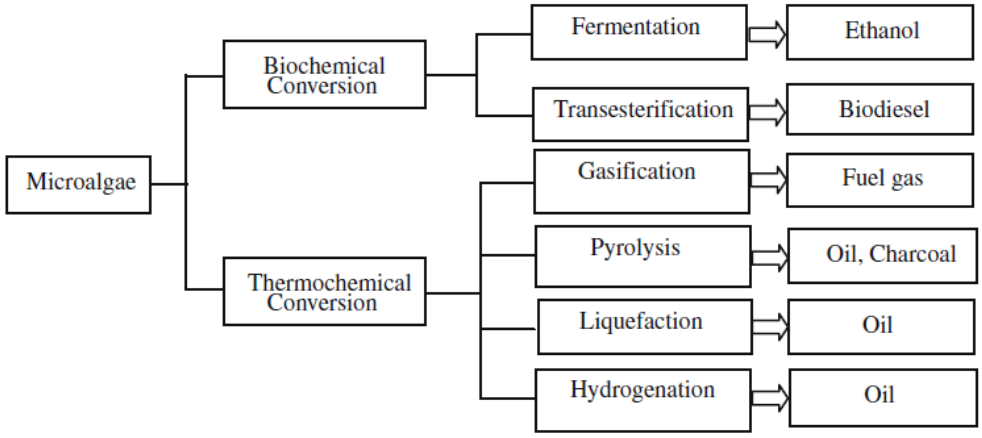
A presença de açúcares como glicose, xilose, galactose, manose e arabinose é relatada por diversos autores, tornando a película prateada uma potencial matéria-prima para o metabolismo de microrganismos, principalmente em processos de fermentação (HIJOSA-VALSERO et al., 2018; MACHADO et al., 2012; MURTHY; MADHAVA NAIDU, 2012; MUSSATTO et al., 2011b; NARITA; INOUYE, 2014). Entretanto, é conhecido que materiais de natureza lignocelulósica apresentam resistência à ação de enzimas e microrganismos, limitando a recuperação de açúcares monoméricos (NIGLIO et al., 2019; RUSSO et al., 2018). Além disso, a presença de fatores antinutricionais como compostos fenólicos comumente presentes em hidrolisados lignocelulósicos podem ser tóxicos para alguns microrganismos (MUSSATTO et al., 2012), embora já tenha sido relatado que algumas cepas podem metabolizar o substrato sem a necessidade de detoxificação (HIJOSA-VALSERO et al., 2018).

2.2 APLICAÇÕES BIOTECNOLÓGICAS DAS MICROALGAS

As microalgas apresentam em sua composição diversos nutrientes e compostos com propriedades comerciais, o que vêm motivando a realização de extensas pesquisas nas últimas décadas com o objetivo de entender como esses microrganismos podem ser usados em diferentes processos visando à obtenção de novos bioprodutos. Atualmente, sabe-se que as microalgas apresentam muitas aplicações biotecnológicas, entre as quais estão a produção de pigmentos, o tratamento de resíduos e principalmente, fornecendo matéria-prima para diversos tipos combustíveis renováveis como o biodiesel, metano, hidrogênio, etanol, entre outros (ABDELAZIZ et al., 2014; MARTINS; CAETANO; MATA, 2010; SPOLAORE et

al., 2006). Os principais combustíveis e os processos empregados na geração desses compostos podem ser observados na Figura 2.

Figura 2 – Principais bioprodutos obtidos a partir da produção de biomassa de microalgas



Fonte: Amin (2009)

Na indústria, a rica composição nutricional das algas permite sua utilização pura ou na forma de uma mistura de várias espécies na alimentação de peixes. Cerca de 30% da biomassa microalgal produzida é utilizada na alimentação animal, como fonte de proteínas, aminoácidos e ácidos graxos (DANIEL, 2016). Além da importância nutricional, a presença de pigmentos como beta-caroteno nas microalgas atua maximizando a coloração de peixes como o salmão (YUAN et al., 2011).

Na linha de compostos bioativos que podem ser aplicados à saúde, Ebrahiminezhad et al. (2014) relataram a produção de *L*-asparaginase intracelular por *C. vulgaris* na presença de *L*-asparagina. A *L*-asparaginase poduzida por *Spirulina maxima* demonstrou atividade antiviral e potencial atividade antitumoral (ABD EL-BAKY; EL-BAROTY, 2019) . Přibyl et al. (2015), Minhas et al. (2016) e Minyuk et al. (2017) avaliaram diferentes condições visando elevada produção de carotenóides de isolados de *Chlorellaceae* e *Scenedesmaceae*. Morowvat e Ghasemi (2016) sugeriram a presença de compostos fenólicos induzindo atividade antioxidante em *Scenedesmus rubescens*.

A produção de biomassa em larga escala com finalidade industrial geralmente utiliza a luz solar para a realização da fotossíntese, com o intuito de reduzir os custos do processo (CHISTI, 2007). Enquanto em escala laboratorial, as microalgas podem ser cultivadas em pequenos fermentadores, a produção em grandes volumes pode ser realizada em sistemas abertos ou fechados. Para sistemas fechados, fotobioreatores tubulares constituem o método

mais utilizado, enquanto que para sistemas abertos são comumente utilizadas lagoas artificiais (AMIN, 2009).

2.2.1 Síntese de nanopartículas utilizando microalgas

Nanopartículas constituem suspensões particuladas ou partículas sólidas com tamanhos em nanoescala (geralmente entre 10 e 100 nm) que apresentam características físicas, químicas, biológicas, mecânicas e ópticas indisponíveis em macromateriais, o que as tornam atraentes candidatas para uma série de aplicações tecnológicas.

Esses nanomateriais tem sido empregados em áreas como a produção de fármacos (AUSTIN et al., 2014; FAN et al., 2016; MAO et al., 2018), agricultura (BAO et al., 2015; RUI et al., 2016; WANG et al., 2019), formulação de cosméticos (BEN HADDADA et al., 2020; GUBITOSA et al., 2018), elaboração de biosensores (HAMDY et al., 2018; KAMIKAWA et al., 2010), produção de pontos quânticos (fontes alternativas para imagem por fluorescência) (DOOSTHOSSEINI et al., 2015; ENSA; KAZEMIFARD; REZAEI, 2015; LI et al., 2011) sensores para diagnóstico de doenças e contaminantes, biorremediação (SHI; ZHANG; CHEN, 2011; SHIPLEY; ENGATES; GUETTNER, 2011) e como agente antimicrobiano (BOGDANOVI et al., 2014; FERREIRA et al., 2016; WANG et al., 2011).

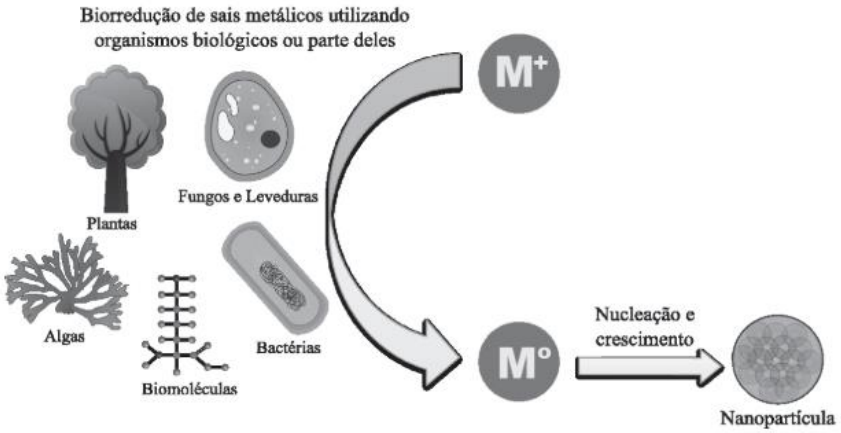
Dentre os tipos de nanopartículas, as nanoestruturas sintetizadas a partir de metais como cobre, ouro e prata vêm sendo amplamente aplicadas por terem características especiais como a grande proporção superfície-volume, alta reatividade química, o que permite a funcionalização com vários agentes biológicos, biomoléculas e agentes quimioterapêuticos, e propriedades ópticas, que permitem sua aplicação em fotocatálise, lasers e sensores (ANNAMALAI; NALLAMUTHU, 2016; ARYA et al., 2018; AUSTIN et al., 2014; AZIZ et al., 2014).

Diferentes métodos químicos são tradicionalmente utilizados na síntese de nanopartículas metálicas. No entanto, essas rotas apresentam uma série de desvantagens, tais como a utilização de substâncias nocivas, geração de resíduos tóxicos, grande consumo de energia e elevado custo de produção (PRASAD, 2013; RESENDE et al., 2017). Nesse contexto, a produção de nanoestruturas a partir de recursos biológicos naturalmente disponíveis vem sendo proposta, por meio da chamada síntese verde.

Na síntese verde, materiais biológicos como órgãos, tecidos, células, metabólitos ou resíduos de plantas, animais ou microrganismos são utilizados como precursores nas rotas de

síntese de nanopartículas metálicas como podem ser observados na Figura 3. Entre esses recursos biológicos, as microalgas se destacam pela sua diversidade e abundância, mas ainda são uma fonte pouco explorada de agentes redutores e estabilizadores que podem ser usados na síntese de nanopartículas (RESENDE et al., 2017).

Figura 3- Esquema do processo de síntese de nanopartículas a partir de organismos biológicos



Fonte: Resende et al. (2017)

Nos sistemas de produção industrial de microalgas, grandes volumes de resíduos de culturas microalgais são descartados, apesar de possuírem muitos compostos bioativos. Entre esses compostos estão enzimas e polímeros, excretados pelas células, que podem atuar como agentes redutores na geração diferentes nanomateriais (DARWESH et al., 2019). Assim, na produção de nanopartículas, as microalgas podem ser consideradas bionanofábricas, pois sua biomassa *in vivo* e morta pode usada na síntese verde. Segundo Arya et al. (2018) e Pugazhendhi et al. (2018), microalgas têm uma grande quantidade de agentes redutores como polissacarideos, lipídios, proteínas, vitaminas e polifenóis, que podem reduzir os metais à nanopartículas, boa capacidade de *uptake* de metais e não produzem subprodutos tóxicos durante a síntese, sendo, portanto um método biológico sustentável. No entanto, o mecanismo de formação de nanopartículas por microalgas ainda não é bem conhecido (PATEL et al., 2015).

Segundo Annamalai e Nallamuthu (2016), nanopartículas de prata são conhecidas há séculos pelo seu potencial antimicrobiano. Usando microalgas como fonte de agentes redutores, Arya et al. (2018) realizaram a síntese de nanopartículas de cobre e prata utilizando o extrato aquoso de *Botryococcus braunii* na redução dos íons metálicos e

estabilização das nanopartículas. Essas nanopartículas ainda demonstraram atividade antimicrobiana em bactérias Gram-positivas e negativas e poder antifúngico. Efeito antibacteriano de nanopartículas de prata produzidas a partir de microalgas também foi observado por Pugazhendhi et al. (2018), com extrato aquoso de *Gelidium amansii*, Jena et al. (2015), com as células e extrato aquoso de *Scenedesmus* sp., Muthusamy, Thangasamy e Raja, (2017) com extrato de *Spirulina platensis* e Annamalai e Nallamuthu, (2016) com extrato de *C. vulgaris*.

2.2.2 Atividade antimicrobiana de extratos microalgais

Microalgas estão presentes em quase todos os ecossistemas, sobrevivendo em ambientes altamente competitivos e enfrentando ameaças como microrganismos patógenos, disputa por espaço e nutrientes, flutuações dos parâmetros químicos e físicos e níveis inibitórios de exposição à luz solar ou radiação UV-B (AREMU et al., 2014; FALAISE et al., 2016). Como resposta, elas são estimuladas a sintetizar uma série estratégias de defesa, que incluem a síntese de compostos bioativos que atuam como uma proteção a essas condições adversas.

Nas últimas décadas, a demonstração do potencial terapêutico das microalgas tem se destacado, tendo a seleção de compostos bioativos se iniciado na década de 1950 (AMARO; GUEDES; MALCATA, 2011). Segundo Najdenski et al. (2013), recentes estudos têm levado a identificação de uma ampla variedade de metabólitos biologicamente ativos em microalgas, entre esses compostos, potenciais antimicrobianos.

De acordo com Pina-Pérez et al. (2017), entre os constituintes microalgais com potencial antimicrobiano estão proteínas e peptídeos, polissacarídeos, polifenóis e outros antioxidantes e ácidos graxos, em especial os poli-insaturados. Entretanto, os compostos específicos que determinam a atividade dos extratos de microalgas ainda não são amplamente conhecidos, assim como o mecanismo de sinergismo entre esses compostos (RICKEN et al., 2019).

Aremu et al. (2014) avaliaram a atividade antimicrobiana *in vitro* de extratos de *Scenedesmus* obtidos a partir da extração com éter de petróleo, diclorometano e etanol. Efeito antimicrobiano sobre *Enterococcus faecalis*, *Staphylococcus aureus*, *Escherichia coli*, *Pseudomonas aeruginosa* e *Candida albicans* foi observado, com concentrações mínimas inibitórias (MIC), em geral, inferiores a 1mg/mL. Efeito inibitório de extratos metanólico e

etil-acético de *Chlorella* sp. em uma série de patógenos clínicos também foi observado por Santhosh et al. (2019.

Atividade antimicrobiana em uma gama de bactérias contaminantes de alimentos foi observada em extratos hidro alcoólicos de uma série de microalgas e cianobactérias, com destaque para *Gloeocapsa* sp. e *Synechocystis* sp. e os exopolissacarídeos produzidos pelas suas células (NAJDENSKI et al., 2013). Ácidos graxos poli-insaturados, outra classe de compostos potencialmente antimicrobianos foram identificados em extratos por Pradhan et al. (2012), em extratos metanólicos e etanólicos de *Spirulina platensis* e por Marrez et al. (2019) em extratos de éter dietílico de *Scenedesmus obliquus*.

O crescimento nos casos de resistência de bactérias patogênicas a um número significativo de antibióticos tem sido uma grande preocupação. Nesse cenário, a área de alimentos está em destaque, visto que muitas bactérias patogênicas podem ser transmitidas por alimentos, infectando e causando doenças graves em humanos (FALAISE et al., 2016). Na indústria de alimentos, com o perigo da contaminação por bactérias resistentes e as maiores exigências do comércio internacional com relação à segurança dos alimentos, a busca por novos agentes antimicrobianos vêm se intensificando (PINA-PÉREZ et al., 2017).

Aliado à ocorrência de resistência bacteriana, a preocupação com segurança dos conservantes químicos está estimulando o interesse em compostos naturais que podem substituir esses antibióticos tradicionais (SCAGLIONI; BADIALE-FURLONG, 2017). O conhecimento da ocorrência de moléculas com potencial antimicrobiano em microalgas sugere a possibilidade de extrair esses compostos para aplicá-los em substituição à esses agentes sintéticos. Pina-Pérez et al. (2017) e Ricken et al. (2019), no entanto, afirmam que, embora já se conheça bastante sobre a capacidade antimicrobiana de microalgas, o desenvolvimento de antimicrobianos derivados de microalgas com foco em contaminações por patógenos, em matrizes alimentares, ainda é incipiente.

2.3 METABOLÔMICA

Metabolômica pode ser descrita um estudo abrangente, qualitativo ou quantitaivo, de diversas classes de metabólitos que constituem redes bioquímicas presentes em sistemas biológicos (SU et al., 2014; VINAIXA et al., 2012). Os metabólitos são moléculas quimicamente transformadas durante o metabolismo, e que funcionam com um registro geral do estado funcional do sistema biológico ou seja, mostram diretamente um panorama da

atividade biológica e permitem correlacionar essa informação com o fenótipo (PATTI; YANES; SIUZDAK, 2012).

Segundo Patti, Yanes e Siuzdak (2012), as três plataformas analíticas mais comumente usadas para a identificação e quantificação de metabólitos em amostras biológicas atualmente são espectrometria de massa por cromatografia gasosa (GC-MS), ressonância magnética nuclear (RMN) e espectrometria de massa por cromatografia líquida (LC-MS). Entre essas técnicas, GC-MS é atualmente a técnica mais frequentemente usada para separação e identificação de metabólitos pela sua alta eficiência e custo-benefício (AGGIO et al., 2014; MOAYEDPOUR; PARASTAR, 2019).

Koek, Jellema e Hankemeier (2011) e Courant et al. (2013) afirmam que tecnologias como a de *fingerprint* e análises estatísticas multivariadas permitem, de forma analítica, obter o panorama do estado metabólico, realizar comparações de diferenças entre metabolomas e estabelecer metabólitos relevantes para uma determinada característica.

Em metabolômica, várias estratégias diferentes são aplicadas para a análise de uma ampla gama de metabólitos. Enquanto em alguns casos, pode ser interessante avaliar o número definido de metabólitos, ou seja, estabelecer um alvo para a análise, para outros casos a melhor opção é realizar uma análise global, extraindo e identificando o maior número possível de metabólitos no sistema biológico.

a) Metabolômica alvo: nessa abordagem, o foco está em um número específico de metabólitos, geralmente relacionados à uma determinada classe bioquímica ou rota metabólica (KOEK; JELLEMA; HANKEMEIER, 2011). Nessa análise, métodos analíticos são desenvolvidos visando a análise de compostos específicos, e com isso, as etapas desses métodos podem ser mais complexas (BELINATO et al., 2019).

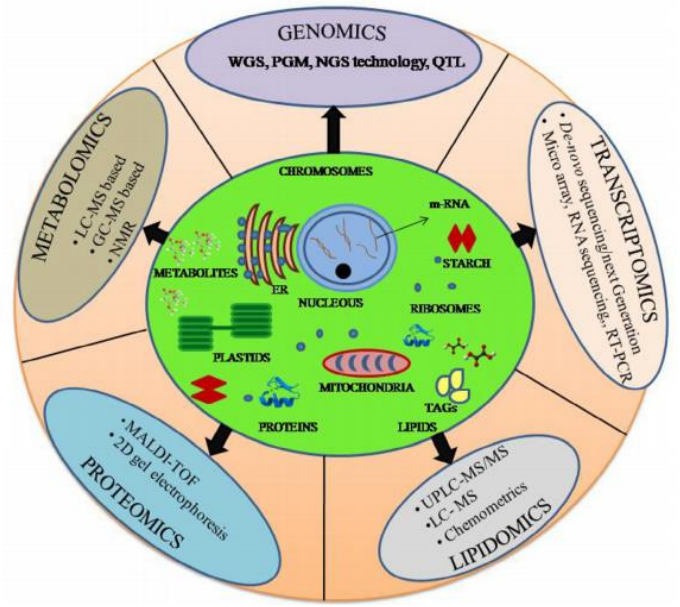
b) Metabolômica global: essa análise qualitativa concentra-se em identificar o maior número possível de metabólitos, pertencentes a diferentes classes químicas, medindo quaisquer moléculas que ionizam dentro de uma faixa específica de valores de massa (VINAYAVEKHIN; SAGHATELIAN, 2010). Segundo Belinato et al. (2019), é geralmente utilizada como uma etapa de discriminação de amostras de diferentes estados biológicos ou origem.

Entre difuculdades da metabolômica estão o fato dessas pequenas moléculas geralmente não possuirem um bloco de construção em comum, embora apresentem em majoritariamente elementos C, H, O, N, S, P em sua constituição (SCHRIMPE-RUTLEDGE et al., 2016).

2.3.1 Metabolômica de microalgas

Diante das importância biotecnológica das microalgas, são cada vez mais exploradas técnicas para otimizar a produção de biomassa e maximizar as características de interesse (GUIDETTI et al., 2019). Apesar disso, em microalgas, os mecanismos de regulação da biossíntese e degradação das moléculas de interesse comercial, assim como a resposta metabólica induzida pelas condições ambientais ainda não são totalmente conhecidos. Dessa forma, análises de metabolômica, proteômica, genômica e transcriptômica (FIGURA 4), vêm sendo propostas, com o intuito de investigar essa regulação e facilitar o entendimento de processos biológicos a nível microalgal. Essas tecnologias ômicas para microalgas, conhecidas como "algomics", ajudam a obter uma visão geral dos processos biológicos, fornecendo dados que podem ser analisados usando ferramentas e softwares computacionais (MISHRA et al., 2019).

Figura 4 – Principais abordagens ômicas para microalgas



Fonte: Mishra et al. (2019)

A metabolômica vem emergindo como uma importante ferramenta para a identificação e comparação de um grande número de metabólitos em um sistema biológico. Metabolômica global (*untarged*) ou alvo (*targed*) vem sendo usada para a obtenção de informações sobre mudanças na quantidade de metabólitos específicos ou no perfil metabólico diante de alterações em condições ambientais. Em especial, a abordagem global pode levar a

identificação de novos metabólitos com potencial funcional (VINAYAVEKHIN; SAGHATELIAN, 2010).

Sendo conhecido que a condição de escassez de nitrogênio pode induzir o biossíntse de lipídios em microalgas, Chen, Zhao e Wang (2017) e Vello (2018) analisaram o metaboloma global de espécies *Chlorella*, para identificar as principais rotas metabólicas responsáveis pela regulação da produção lipídica. Chen, Zhao e Wang (2017) verificaram que as vias de assimilação de nitrogênio e carbono relacionadas ao sistema glutamato-glutamina, metabolismo e catabolismo de aminoácidos e o ciclo do ácido cítrico/glicólise eram as principais contribuintes na redistribuição do excesso de carbono para síntese lipídica. A realocação de lipídios de membrana também foi um mecanismo de adaptação metabólica à deprivação de nitrogênio (VELLO et al., 2018)

No mesmo contexto, Kokabi et al. (2019) examinaram os efeitos da depleção de nitrogênio e fósforo no metaboloma global da microalga produtora de ácido aracdônico *Lobosphaera incisa.* A análise mostrou a existência de diferentes e complexas estratégias de regulação do metabolismo lipídico perante a escassez de cada macronutriente. Enquanto na escassez de N, a redução na abundância de aminoácidos e pirimidinas ocorreu para balancear a produção de ácidos graxos, a depleção de fósforo induziu a redução nos níveis de ATP e de nucleotídios fosforilados, com aumento no nível de intermediários do ciclo de Krebs.

Agarwal et al. (2019) realizaram um estudo metabolômico qualitativo para avaliar o crescimento em biomassa de *Asteracys* sp. em mixotrofia sob alta intensidade luminosa (900 μmol photons/m^2/s). Os perfis metabólicos indicaram que no crescimento mixotrófico, houve um desvio geral do fluxo dos metabólitos do ciclo de Krebs para a biossíntese lipídica e o redirecionamento de carbono para o uso mais eficiente do carbono orgânico com relação ao crescimento fototrófico. E por resultado, essa modulação com a presença da fonte de carbono orgânico favoreceu o aumento em biomassa.

Análises do perfil metabólico de *Haematococcus pluvialis*, produtora de astaxantina foram realizadas por Su et al. (2014), com o objetivo de maximizar a geração desse metabólito. CG-MS e LC-MS foram as técnicas aplicadas para identificar metabólitos (D-(+) altrose, ácido L-glutâmico, ácido α-cetoglutárico e d-ribose 5-fosfato) relacionados ao estresse por adição de Fe^{2+} e alta intensidade luminosa, e associados ao acúmulo do carotenóide.

Por sua vez, no contexto ecológico, Vidoudez e Pohnert (2012) analisaram alterações fisiológicas na diatomácea *Skeletonema marinoi*, um importante componente da cadeia alimentar marinha, em suas diferentes fases do crescimento e em diferentes períodos do dia.

Embora a quantidade de metabólitos identificados foi similar nas condições observadas, importantes mudanças no perfil metabólico também foram contabilizadas.

Alterações no metaboloma microalgal em função dos ciclos de luminosidade diários foram também analisadas por Willamme et al. (2015). Eles observaram que, enquanto metabólitos como proteínas, clorofila e ácidos graxos apresentam perfis de acumulação direamente ligados à divisão celular, a abundância de carboidratos estava ligada ao ciclo de dia/noite.

Segundo Mishra et al. (2019) e Wang et al. (2019), as análises metabolômicas podem ser usadas para mostrar a caracterizar a resposta de um grande número de metabólitos microalgais ao estresse induzido pela exposição à esses poluentes, por meio de mudanças na regulação da produção desses metabólitos. E assim, esses estudos metabolômicos podem auxiliar na aprimoração das abordagens de recuperação de recursos em águas residuais.

Wang et al. (2019) avaliaram a assimilação de trifenilfosfato, um agente retardador de chama e amplamente detectado na superfície aquática, por *C. vulgaris* and *S. obliquus*. A metabolômica mostrou que a redução de 65% a 95% na concentração final do contaminante por *C. vulgaris* foi acompanhada por um aumento da integridade da membrana (aumento de diacilglicoglicerolípidos, que são os principais lipídios de membrana em microalgas) e diminuição de espécies reativas de oxigênio, evidenciando possivelmente uma estratégia de defesa celular.

Alterações no metaboloma de *C. sorokiniana* quando em co-cultura com um consórcio de bactérias *Pseudomonas* foi estudado por Chen, Zhao e Wang (2017), na avaliação do potencial microbiano na remediação de águas residuárias. Foi observado um aumento na abundância em 73% dos metabólitos microalagais identificados como biomarcadores, no cultivo com o consórcio bacteriano, com destaque para uma série de ácidos graxos insaturados, com elevado valor comercial.

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SEGUNDA PARTE

ARTIGO 1

EVALUATION OF GROWTH AND INTRACELLULAR METABOLITES OF MICROALGAE CULTIVATED IN COFFEE PROCESSING BY-PRODUCTS

Artigo redigido conforme Manual de normalização e estrutura de trabalhos acadêmicos: TCCs, monografias, dissertações e teses da UFLA (versão preliminar)

ABSTRACT

Agro-industrial by-products, such as coffee wastewater and silverskin, can be used as a source of proteins, sugars, and minerals for microalgae growth. Searching for alternative culture media to cultivate microalgae, promising microorganisms have shown great potential for biotechnological applications. The present work aimed to isolate microalgae from a lake in Ijaci, in the state of Minas Gerais, and two bays in Portel and Melgaço, in the state of Pará, Brazil, and to evaluate its cultivation in coffee by-products. Additionally, changes in the content of intracellular metabolites during microalgae growth were analyzed. Isolation was performed according to the spread-plate technique using BG-11, Bold, and Bold medium supplemented with soil extract for seven days (27ºC and 12h photoperiod). Seven isolates were obtained, which were morphologically characterized. After screening in mixotrophy (BG-11 supplemented with glucose 1g/L and indoor conditions) and coffee wastewater (CWW) at 30, 60, and 90% concentrations, microalgae AL_UFLA06, which presented the highest growth, was selected to be cultivated in coffee silverskin (CSS) hydrolysates, produced from different thermic reactions with CSS and water. The growth of AL_UFLA06 in CWW and CSS was compared, and the concentration of 30% of CWW provided the highest growth, hence, AL_UFLA06 was selected for the next step. The isolate was identified by sequencing its ITS region as *Neodesmus* sp. Mixotrophic growth in the selected condition was scaled-up and carried out indoors and outdoors (greenhouse) for eight days, with BG-11 as an autotrophic control. Cultivation using CWW in the outdoor environment presented the best productivity of biomass, resulting in 1.64 g/L of biomass in eight days, compared to 0.97 g/L produced in outdoor environment under autotrophic growth. Carbohydrates (0.53g/L), pigments (15.8 mg/L), lipids (1.1%), and proteins (0.2 g/L) content in CWW were higher than autotrophy also in outdoor growth. Intracellular metabolites identified by GC-MS reinforced the differences in microalgae composition between CWW and autotrophic growth outdoors. Higher accumulation of carbohydrate reserves and organic acids related to energy metabolism and resistance to stress was observed in mixotrophy with CWW, while higher content of ethanol and 1,3-Pentanediol was quantified in autotrophy. Results highlight the potential of

CWW for biomass production of *Neodesmus* sp. and also the microalgae adaptation to the cultivation outdoors.

Keywords: Microalgae isolation. Mixotrophy. *Neodesmus.* Outdoor cultivation. Metabolomics.

1 INTRODUCTION

Microalgae are a group of eukaryotic or prokaryotic microorganisms with photosynthetic metabolism, but can also use organic compounds for their nutrition (ABINANDAN; SHANTHAKUMAR, 2015). In the last years, microalgae received increasing attention due to their potential to generate value-added products such as biofuels, pigments, foodstuffs, cosmetics, and compounds with therapeutic potential (BUX, 2013; FREITAS et al., 2017; SPOLAORE et al., 2006; XIA et al., 2013; YADIRA et al., 2014). Although many species have been identified, the great diversity of microalgae remains unexplored, especially in tropical regions such as Brazil. Due to this fact, the exploration of local strains is stimulated since these strains tend to be more fit to be cultivated under conditions similar to the isolation site and also, it contributes to the development of an effective, viable and sustainable microalgal biomass production (LEE et al., 2014; MENDES et al., 2012).

Despite the differences between species, environmental conditions, cultivation and processing methods, the microalgae chemical composition is commonly rich in metabolites, such as carbohydrates, proteins, lipids, and several bioactive compounds, showing great potential for several biotechnological applications, as a source of raw material for different types of renewable fuels and animal feed (LI et al., 2019; MENEGOL; CHISTI, 2019; PANCHA et al., 2014).

Photoautotrophy is the common metabolic condition used for biomass production of microalgae. However, the photoautotrophic growth has several limitations like availability of light, low biomass production, and long cultivation periods, to name a few (DENG et al., 2019; PANG et al., 2019). Supplementation of culture media with carbon sources such as glucose, fructose, and sucrose have been used as alternative routes to obtain higher productivity on an industrial scale (ABINANDAN; SHANTHAKUMAR, 2015; ABREU et al., 2012). As alternatives to reduce or replace the utilization of chemicals in mixotrophic processes, the microalgae-based systems using wastewater as a sustainable media for biomass production have received a great deal of attention nowadays (DANESHVAR et al., 2019). To increase the viability of the microalgae biomass production, agro-industrial waste has been tested, looking for balancing nutrient consumption by microalgae cultivation and solving a social-environmental problem caused by the increasing generation and disposal of waste as well.

According to the International Coffee Organization (ICO), the global coffee production estimated in 2018 was 172.452 thousand bags of coffee (60 kg) (ICO, 2020). For coffee ground processing, there are two methods available, wet and dry. About 40% of all coffee around the world is wet-processed (GARDE et al., 2017). Thus, a large volume of freshwater (3-5L of water per liter of coffee fruit pulped) is required in the process of mechanic remotion of fruit's skin or exocarp (MATOS, 2008). The coffee wastewater (CWW) that results from the volume of water used to carry away the pulp and mucilage that covers the fruit seed is rich in suspended and dissolved total solids and it contains nitrogen, phosphorus, and carbohydrates in its composition (MURTHY; MADHAVA NAIDU, 2012; RATTAN et al., 2015; SELVAMURUGAN; DORAISAMY; MAHESWARI, 2010). In our group, CWW and pulp were used as a substrate for yeasts due to the sugar fermentable content, leading to ethanol and aromatic volatile compounds production (BONILLA-HERMOSA; DUARTE; SCHWAN, 2014) and, recently, a distilled beverage was produced using a mixture of coffee pulp and CWW (LOPES et al., 2020).

Another by-product of the coffee bean processing is the coffee silverskin (CSS), the main residue of coffee roasting, composed primarily of cellulose, hemicellulose, and insoluble lignin (ALGHOONEH et al., 2017; MUSSATTO et al., 2012; NARITA; INOUYE, 2014). According to the Niglio et al. (2019), considering a global production of coffee beans of about 159 million of 60kg bags, CSS is produced in a large amount of 0.4 Mt per year. The CSS obtained in the roasting industries can be an alternative source of nutrients and carbohydrates for different applications, due to its continuous production during the year and the amount of polysaccharides in its composition (HIJOSA-VALSERO et al., 2018). Several added value products from CSS have been proposed, such as biofuels, according to Mussatto et al. (2012), Niglio et al. (2019) and Hijosa-Valsero et al. (2018), fertilizers (MURTHY; NAIDU, 2012), and as a functional ingredient in foods, due to the presence of fibers, carbohydrates, and antioxidants (BALLESTEROS; TEIXEIRA; MUSSATTO, 2014a; GOCMEN et al., 2019).

The rich composition of these coffee by-products makes them suitable nutrient sources for the microalgae growth, constituting low-cost cultivation media, aimed at large-scale industrial applications. Microalgae cultivation systems using coffee waste as an alternative nutrient source may represent an effective way to minimize production costs and improve biomass production (CAPORGNO et al., 2015). Still searching for minimizing production costs, in contrast to the use of artificial light, microalgae growth in solar light is another option to optimize costs in industrial-scale production (FENG et al., 2014). Under this mode

of cultivation, it is possible to reduce energy consumption in the maintenance of microalgal cultures.

In this context, novel technologies are being increasingly explored, so as to optimize the microalgae biomass production and maximize its characteristics of interest (GUIDETTI et al., 2019). Nevertheless, in microalgae, the mechanisms of regulation for biosynthesis and degradation of added value molecules, as well as the metabolic response induced by the culture medium and environmental conditions are not yet entirely understood. Thus, the use of tools that show the metabolites present in the microalgae, as well as their concentrations as a direct response to this specific culture condition is important to understand the cell's biochemistry and which components are relevant to these environmental changes.

For the purpose of comprehending the regulation of metabolites biosynthesis, metabolomics technologies have been successfully used as an approach to studying the composition of biological systems. Thus, metabolomics analysis could be useful to understand cellular processes in microalgae, providing data that can be analyzed using computational tools and softwares (MISHRA et al., 2019). Such data could evidence high-value microalgae molecules and how their metabolism could be potentially manipulated in a biorefinery strategy.

Therefore, this study aimed to isolate native microalgae from a lake located in the municipality of Ijaci (MG) and two bays in the municipalities of Portel and Melgaço (PA), Brazil, in order to evaluate the use of CWW and CSS in microalgae cultivation and characterize changes in the intracellular metabolites content of the microalgae, in response to the cultivation using the coffee by-product, under environmental conditions, with natural sunlight.

2. MATERIALS AND METHODS

2.1 MICROALGAE ISOLATION

Water samples collected during the summer of 2017 in a lake located in the municipality of Ijaci, south of the state of Minas Gerais, and two bays, located in the municipalities of Portel and Melgaço, in the state of Pará, Brazil, were used to isolate native microalgal strains. Samples were collected in duplicate, stored in sterile Falcon tubes, and taken to the Laboratory of Physiology and Genetics of Microorganisms of the Biology Department at the Federal University of Lavras - UFLA.

Water samples were initially enriched with the BG-11 medium at the proportion of 1:10. So as to obtain colonies isolated from microalgae, the surface plating method was used. The cultures (500 μL) from tubes showing microalgal growth were spread on the following culture media, according to (ANDERSON, 2005):

- Modified BG-11 medium (ALLEN; STANIER, 1968) ($NaNO_3$ 1.76 · 10^{-2} M, $MgSO_4$ 3.04 . 10^{-4} M, K_2HPO_4 2.28 · 10^{-4} M, $CaCl_2$ 2.38 · 10^{-4} M, Citric acid 3.12 · 10^{-5} M, Ammonium citrate ferric 2.23 · 10^{-5} M, Na_2EDTA 2.69 · 10^{-5} M and micronutrients solution (H_3BO_3 9.87· 10^{-7} M, $MnSO_4$ 1· 10^{-6} M, $ZnSO_4$ 9.98 · 10^{-7} M, $CuSO_4$ 1· 10^{-8} M, $(NH_4)_6Mo_7O_{24}$ 1 · 10^{-1} M));

- Bold Basal medium (BISCHOFF; BOLD, 1963; BOLD, 1949)($NaNO_3$ 2.94 · 10^{-3} M, $MgSO_4$ 3.04 · 10^{-4} M, NaCl 4.28· 10^{-4} M, K_2HPO_4 4.31 · 10^{-4} M, KH_2PO_4 1.29 · 10^{-4} M,CaCl2 1.7 · 10^{-4} M, H_3BO_3 1.85· 10^{-4} M, Na_2EDTA 1.71· 10^{-4} M, KOH 5.53· 10^{-4} M, $FeSO_4$ 1.79· 10^{-5} M, H_2SO_4 concentrated 1mL 1.79· 10^{-5} M and micronutrients solution ($ZnSO_4$ 3.07 · 10^{-5} M, $MnCl_2$ 7.28· 10^{-6} M, MoO_3 4.93· 10^{-6} M, $CuSO_4$ 6.29· 10^{-6} M, $Co(NO_3)_2$ 1.68· 10^{-6} M)).

- Bold Basal medium with soil extract: Bold Basal medium (BISCHOFF; BOLD, 1963; BOLD, 1949) supplemented with soil extract 5% (ANDERSON, 2005). According to Anderson (2005), soil extract provides the enrichment with vitamins, acts as a chelator agent and helps the cell morphology maintenance.

The algae cultures were placed under controlled conditions (incubator model LUCA-161/02), photoautotrophic regime, and light: dark cycles of 12:12 hours and temperature of 27ºC, for 7 days. Based on the colonies and cell morphology, according to microscopic observation, the square root of the number of colonies identified as possible microalgae was transferred, separately, to sterile plates containing the same medium from which they were isolated. The colonies were purified by streaking method, continuously repeated until the achievement of axenic cultures. The purity evaluation of the isolates was performed by microscopic analysis. A concentration of 75mg of the antibiotic Ampicillin Sodium (Sigma-Aldrich) was used in the control of bacterial growth.

Once the purified isolates were obtained, colony and cell morphology characteristics were evaluated. For microalgae maintenance, modified BG-11, pH 7.4 was the selected condition. Maintenance of the strains was performed by subcultures in new nutrient medium every 4-6 weeks. For the maintenance of stock cultures, cryopreservation was performed using a concentration of 10% of dimethylsulfoxide (DMSO) as the cryoprotectant agent.

For the inoculum, in triplicate, a small sample of the pure culture was inoculated into 1.0 mL of liquid modified BG-11 medium and cultivated in an incubator under the same conditions applied in the isolation step. After 3 days, the contents of the tubes were complemented, reaching a volume of 10 mL, and then incubated for another 7 days until use.

2.2 MOLECULAR IDENTIFICATION

DNA extraction was performed according to Anthony et al. (2014) with modifications, using the CTAB method. For the gene amplification of the ITS1- 5.8S - ITS2 regions, a pair of universal primers was used, ITS1 (5'– TCCGTAGGTGAACCTGCGG – 3 ') and ITS4 (5'-CCTCCGCTTATTGATATGC-3'). For PCR reaction, 25 μL of reaction mix was prepared, containing 12.5 μL of the Gotaq enzyme, 0.75 μL of each primer, 0.5 μL of the DNA sample, and the total volume was supplemented with ultrapure water. The dye used was gel-red. A total of 35 cycles was carried out, using the temperatures as it follows: 95ºC - 2 minutes, 95ºC - 45 seconds, 50ºC - 45 seconds, 72ºC - 1 minute, 72ºC - 5 minutes, and a final temperature of 4ºC after the end of the reaction. So as to visualize the extraction, gel electrophoresis was performed to extract the DNA bands. Sequencing was carried out at the Synthesis Biotechnology Company (Belo Horizonte - MG/Brazil) and analyzed using BioEdit software version 7.2.6 (HALL, 1999). The sequences were compared to the GenBank database using the Basic Local Alignment Search Tool (BLAST).

2.3 SELECTION OF MICROALGAE FOR CULTIVATION

In order to determine which isolates were able to grow in heterotrophic and mixotrophic conditions, and then to be able to assimilate organic carbon, cultivations were performed under three conditions: autotrophy, heterotrophy, and mixotrophy. For autotrophy, microalgae were maintained in BG-11 medium and, for heterotrophic and mixotrophic conditions, 1g/L of glucose was added to the BG-11 medium. Autotrophic and mixotrophic cultures were maintained under 12/12 photoperiod and for heterotrophy, the cultures were maintained under darkness.

The treatments were performed in 50 mL glass tubes containing 25 mL of each growth condition (BG-11 for autotrophy and BG-11 with glucose for heterotrophy and mixotrophy). A 10% concentration of each isolate was inoculated with OD_{680} standardized for the cultivation with the lowest optic density (OD=0.1). Incubation conditions were 27ºC and

12/12 photoperiod for seven days. In the end, the optic density of the samples was measured. The assays were performed according to Bhatnagar et al. (2011) and Wang, Yang, and Wang (2014), with the proper adaptations.

2.4 MICROALGAE CULTIVATION IN COFFEE BY-PRODUCTS

The growth of selected isolates in mixotrophy was evaluated using two coffee by-products: residual coffee wastewater (CWW) from wet processing and coffee silverskin (CSS) from the roasting process. Initially, a microalgae selection step in CWW was carried out as it was the most nutritionally rich by-product, hence, presumably more favorable to the microalgae growth. The microalgae which presented the highest growth in Log cells/mL was also evaluated in the CSS hydrolysates, with the purpose to compare the isolate growth in the two by-products. The coffee by-product that provided the best microalgae growth rate was selected for the next step of the experiment, which was the evaluation of growth in indoor and outdoor conditions.

2.5.1 Microalgae cultivation in CWW

Wastewater from coffee processing was supplied by a coffee-producing unit located in the municipality of Lavras, south of the state of Minas Gerais, Brazil, in May 2018, and immediately frozen at -20ºC until use.

In order to enable the evaluation of the CWW potential for microalgae cultivation, the microalgae that showed the highest growth in a screening step with micro-cultures was selected. Three different concentrations of CWW (30, 60 e 90% (v/v)) were tested. CWW was unfrozen at the temperature of 4ºC and centrifuged (Thermo Fisher Scientific, US) at 10,000 rpm for 5 minutes. The CWW was diluted in distilled water in the concentrations above and sterilized. The selected microalgae were inoculated into glass tubes containing 10 mL of each culture medium, with OD_{680}=0.5. The flasks were incubated at 27 °C, 12/12 photoperiod, and under light intensity of 100 μmol. BG-11 medium (autotrophy) and BG-11 medium with glucose 1g/L (mixotrophy) were used as controls.

Microalgae cell concentration was determined by optical density OD_{680} and cell count in the Neubauer chamber, as reported by Ho et al. (2013). Optical density measurements were performed at the initial time and every two days for a period of 6 days. The microalgae and

wastewater concentration that provided the highest growth were selected by the Scott-Knott test at the 5% significance level.

2.5.2 Evaluation of microalgae growth in CSS hydrolyzate

2.5.2.1 Raw material

Coffee silverskin (CSS) (a mixture of *Coffea Arabica* and *Coffea Robusta*) was supplied by NovaDelta Comércio e Indústria de Cafés S.A. (Campo Maior, Portugal), in 2018 (FIGURE 1). Dry CSS was stored in a dark and dry place and before any procedure, the moisture content was measured.

Figure 1 - Coffee silverskin sample

Source: Author (2020)

2.5.2.2 Coffee silverskin characterization and microalgae growth in CSS hydrolyzates

The chemical characterization of CSS was performed according to standard procedures proposed by the National Renewable Energy Laboratory (NREL), with the purpose of quantifying compounds such as extractives, cellulose, hemicellulose, lignin, and ashes (SLUITER et al., 2012).

1) Determination of soluble and insoluble lignin: 0.3 g (ground to 40 mesh in a coffee grinder) of CSS, was transferred in triplicate to tared glass tubes and treated with 3 mL of H_2SO_4 72% v/v, under constant agitation, in a thermostatic bath at 30ºC for 60 minutes. The samples were transferred to 200 mL reagent bottles and 84g of distilled water was added to obtain a concentration of 4% of sulfuric acid. Sealed flasks were autoclaved for 1 h at 121ºC. After the autoclave cycle, the flasks were removed and cooled to room temperature, with the solid fraction separated from the liquid fraction by vacuum filtration using a qualitative filter paper. A 5mL aliquot of the liquor was used for the quantification of soluble lignin and the

remaining was stored at -20ºC for analysis of carbohydrates, organic acids, furfural, and hydroxymethylfurfural (HMF).

In order to determine the soluble lignin content in the liquor, the absorbance of the liquor was measured at OD_{240} nm. The amount of acid-soluble lignin (ASL) present was determined according to Equation 1.

$$\%ALS = \frac{UVabs\ x Volume_{liquor} dilution}{\varepsilon\ x\ ODW_{sample}\ x\ Pathlength} x100 \quad (1)$$

Where:

UVabs = average UV-Vis absorbance for the sample at appropriate wavelength (OD = 240nm for the CSS)

$Volume_{liquor}$ = volume of filtrate, 86.73 mL

Dilution = $\frac{Volume_{sample} + Volume_{solvent}}{Volume_{sample}}$

Ɛ = absorptivity of biomass at specific wavelength (tabulated value = 30 for the CSS).

The determination of lignin in the solid fraction was made by using the Klasson method (SLUITER et al., 2012). All the solids in the flasks were transferred to the paper filter using distilled water. The filters were dried at 105ºC, cooled in a desiccator and weighed until constant mass was achieved. The percentage of acid-insoluble lignin (ALI) was calculated to the dry sample mass according to Equation 2:

$$\%ALI = \frac{(Weight_{sample} - Weight_{Filter})(Weight_{filter+ash} - Weight_{filter}) - Weight_{Protein}}{ODW_{sample}} x\ 100 \quad (2)$$

2) Determination of carbohydrates, organic acids, furfural, and HMF in the liquor: Cellulose, hemicellulose, glucose, xylose, arabinose, acetic acid, furfural, and HMF concentrations in the liquor were determined by High-Performance Liquid Chromatography (HPLC) in a chromatograph equipped with a Bio-Rad Aminex HPX-87H column (300x7.8 mm) (conditions: UV detector; flow rate 0.6 mL/min at 60ºC; H_2SO_4 at 0.005M as a mobile phase). The concentrations of sugars and acetic acid were used to calculate the cellulose and hemicellulose contents.

3) Ashes and proteins: 0.5 g (ground to 40 mesh) of dry CSS, weighed with a precision of 10mg, was transferred to previously tared porcelain crucibles. The sample was slowly

calcined to 575ºC and another 2h at 800ºC in a muffle, until constant weight. By weight difference, the total ash content was determined according to Equation 3.

$$\%\text{ashes} = \frac{\text{Weight}_{\text{ashes}}}{\text{Weight}_{\text{dry sample}}} \quad (3)$$

The raw protein content of the CSS was determined by the Kjeldahl method, according to AOAC (2016), using 0.5 g of ground sample for digestion. The nitrogen to protein conversion factor was 6.25.

2.5.2.3 Autohydrolysis

The autohydrolysis tests were performed according to Ballesteros, Teixeira, and Mussatto (2017), with adaptations. Different temperature conditions (120, 150, and 180ºC), liquid/solid ratio (1 and 2g of CSS/50mL of distilled water), and extraction time (20, 35, and 50 min) were tested, which were combined in a composite design 2^3 with three central points. The treatments can be observed in Table 1.

Table 1 - Auto hydrolysis temperatures, CSS concentrations and times of reaction

Conditions of autohydrolysis	Temperature (ºC)	Concentration of CSS (g / 50mL)	Time (min)
Treatment 1	120	1	20
Treatment 2	180	1	20
Treatment 3	120	2	20
Treatment 4	180	2	20
Treatment 5	120	1	50
Treatment 6	180	1	50
Treatment 7	120	2	50
Treatment 8	180	2	50
Treatment 9	150	1.5	35
Treatment 10	150	1.5	35
Treatment 11	150	1.5	35

Source: Author (2020)

Water and previously weighed amounts of CSS were added to stainless steel cylindrical reactors (Parr Instruments Company, US), which were properly sealed and placed

into a silicone oil bath where they stayed at the determined temperature for the time required for reaction. At the end of each reaction, the reactors were immediately cooled in an ice bath, and the resulting solid material was separated by filtration. The filtrates were immediately used as the substrate for microalgal cultures. An aliquot of the supernatant was stored at -20ºC for future analysis.

2.5.2.4 Evaluation of microalgae growth in CSS hydrolysate

The CSS hydrolysates produced in the autohydrolysis step were tested as substrates for microalgae biomass production. The microalgae that showed the highest growth in coffee residual wastewater (CWW) were selected and its cultivation was carried out in the hydrolysates, according to the treatments presented in Table 2. In 50 mL flasks with 25 mL of each hydrolysate, the microalgae in the exponential growth phase, previously centrifuged at 7,000 rpm for 6 minutes and washed twice in sterile distilled water, were inoculated, so as to obtain an initial optical density of OD_{680} = 0.5. Samples of the culture at the initial time were collected for further analysis. The flasks were incubated under the following conditions: incubator, constant lighting, temperature of 26ºC, and agitation of 120 rpm, for 20 days. The evaluated parameter was the number of cells, by cell counting in a Neubauer chamber.

2.2.5.5. Determination of total carbohydrates and sugars in CSS hydrolysate

For carbohydrates determination, the monosaccharides and acetic acid contained in hydrolysates were determined by HPLC, so as to estimate the contents of samples in cellulose (as glucan), hemicellulose (as mannan + galactan + arabinan + xylan), and acetyl groups (BALLESTEROS et al., 2015; MUSSATTO et al., 2011a). Furfural and HMF were also determined by HPLC, using a UV detector (at 280 nm). To determine the number of monosaccharides available in the microalgae metabolism, the levels of cellulose, hemicellulose, glucose, xylose, and arabinose present in the hydrolysates were analyzed by HPLC (MUSSATTO et al., 2011a).

2.6 MICROALGAE CULTIVATION: INDOOR AND OUTDOOR CONDITIONS

Larger scale cultivation was carried out using CWW, the coffee by-product selected at the previous step, at the dilution of 30% according to Santana et al. (2017) with adaptations. Microalgae inoculum in exponential growth was centrifuged at 7,000 rpm for 6 minutes, washed twice, and cultivated in glass bottles containing 1000 mL of the culture medium with CWW, with initial OD_{680} = 0.025. Samples of the culture and CWW at the initial time were collected for further analysis.

The experiments were performed in duplicate, with BG-11 medium as control, under the following conditions: 1) Indoors, in an incubator with 12/12 photoperiod at 27ºC, and 2) Outdoors (greenhouse), with temperature oscillating between 16ºC and 30ºC. Cultures were maintained for 8 days.

2.7 BIOMASS CHARACTERIZATION

2.7.1 Growth analysis

Microalgae growth was determined at the end of the 8th day by spectrophotometry by reading OD_{680} nm (UV-VIS Lab Equipment 325-1000 nm, 4nm spectrophotometer) and comparing the values with the standard curve. The microalgae growth rate was also determined according to (1) (KIM et al., 2013):

$$\text{Growth rate} = (\ln OD_{final} - \ln OD_{initial})/\text{days} \quad (4)$$

2.7.2 Determination of pigments, carbohydrates, lipids, and proteins

Pigments determination was performed according to Fernández-Linares et al. (2017) and Pancha et al. (2014). Samples of 3.0 mL of the cultures were transferred to 15.0 mL tubes and centrifuged for 6 minutes at 10,000 rpm, and the supernatant was removed. Cells were resuspended in 3.0 mL of 99% methanol and incubated at 45ºC in the dark for 30 minutes. The tubes were centrifuged (10,000 rpm/6 min) and the methanolic extract was transferred to glass cuvettes with the purpose of measuring the absorbance at wavelengths of 480, 652, 665, and 750 nm. The concentrations of carotenoids and chlorophyll a and b were estimated according to equations (5) - (7).

$$\text{Chlorophyll -A[mg/L]} = -8;10OD_{652}nm + 16.57OD_{665}nm - OD_{750}nm \quad (5)$$

$$\text{Chlorophyll -B [mg/L]} = 27.44OD_{652}nm - 12.17OD_{665}nm - OD_{750}nm \quad (6)$$

$$\text{Carotenoids [mg/L]} = 4OD_{480}nm - OD_{750}nm \quad (7)$$

For carbohydrate determination, the Anthrone method (GERHARDT *et al*., 1994) was used, with the carbohydrate extraction protocol adapted from Fernández-Linares et al. (2017). Samples of 2.0 mL culture were centrifuged for 6 minutes at 10,000 rpm and the supernatant was removed. The pellet was hydrolyzed with 2.0 mL of 1N HCl at 100ºC for 2 h. Then, 400 µL of the hydrolysate was transferred to another tube in which 800 µL of 75% ice-cold H_2SO4 was added. The solution was vortexed, received 1.5 mL of the Anthrone at 2 mg/mL concentration in 75% H_2SO_4, and incubated at 100ºC for 15 minutes. After cooling, absorbance was measured at 578 nm. The standard curve was constructed using the following glucose concentrations: 0.05; 0.25; 50; 100, 150 and 250 mg/mL.

Lipid content was determined according to Higgins et al. (2014). In a Falcon tube containing approximately 20 mg of microalgal biomass dried at 60ºC for 36 hours, 1.5 mL of the Folch solvent (2:1 chloroform/methanol) was added. Glass beads and 20-s beating intervals were used to disrupt the cells. The beads were removed by filtration and washed with 4.5 mL of Folch solvent. For phase separation, 1.2 mL of 0.9% NaCl solution was added and centrifuged at 6,000 rpm. The volume of the lower phase containing the lipids was recorded and transferred to previously weighed aluminum containers. The samples were evaporated for 24h at room temperature and the aluminum containers were again weighed. The total percentage of lipids in the dry biomass was given by (8) (DASGUPTA et al., 2015):

$$\text{Lipids (\%)} = (\text{Lipid biomass/Microalgae biomass})*100 \quad (8)$$

Protein content was determined by the Lowry spectrophotometric method (LOWRY et al., 1951). Culture samples of 1.0 mL were centrifuged for 6 min at 10,000 rpm and the supernatant was removed. The pellet was hydrolyzed with 1.0 mL 0.5M NaOH, under heating at 80ºC for 10 minutes. Then, the hydrolysate was placed in glass tubes with 2.5 mL of the Lowry reagent and the mixture was incubated for 10 minutes in the dark. Following, 500 µL of the 1:2 dilution of the Folin-Ciocalteau with distilled water was added to the tubes, and the

mixture settled for 30 minutes. The optical density of the samples was read at 750 nm. Bovine albumin at concentrations of 0.05 to 0.5 mg/mL was used for the standard calibration curve.

2.8 DETERMINATION OF CWW COMPOSITION

The composition of CWW media at the beginning and the end of microalgae cultivation was determined. The samples were collected, centrifuged for 10 minutes at 10,000 rpm and the supernatant was analyzed. For SM5210B - Biochemical Oxygen Demand (BOD), the standard method described by the American Public Health Association et al. (2005) was used. Glucose, xylose, arabinose, and glycerol concentrations in coffee by-product and at the end of cultivation were determined by High-Performance Liquid Chromatography (HPLC), so as to calculate the consumption by microalgae during cultivation. Before analyzing, the samples were centrifuged twice at 10,000 rpm, 4ºC/10 min, and filtered in a 0.22 µm filter. A Shimadzu chromatographer (Shimadzu Corp. Japan) equipped with a Supelcogel 8H (7.8 mm 9 x 30 cm) column was used for the analysis, utilizing sulfuric acid 0.005 M as mobile phase in a flow of 0.5 mL/min with the oven maintained at 30ºC and a sample volume of 20 mL (DUARTE et al., 2010). Compounds quantification was done by external calibration. Concentrations of sugars and glycerol were compared using the Scott-Knott test at the 5% significance level.

2.9 METABOLOMICS EVALUATION

In the selected condition (indoors or outdoors), a new cultivation was performed, according to Santana et al. (2017) with adaptations, so as to evaluate changes in the composition of intracellular metabolites, comparing the treatment with coffee wastewater and the BG-11 culture medium. The inoculum of the microalgae while in its exponential growth phase, was centrifuged at 7,000 rpm for 6 minutes, washed twice in sterile distilled water, and inoculated in Erlenmeyer flasks containing 1000 mL of the culture medium (coffee by-product or BG-11), with initial OD_{680} = 0.025. The experiments were performed in duplicate. Culture samples at the initial time were collected for further analysis. The flasks were maintained for 14 days.

2.9.1 GC-MS-based metabolomic analysis

For metabolomics analysis, 1 mL of microalgae culture on the initial and final day was collected and centrifuged at 8,500 rpm for 10 minutes at 4ºC. The cell pellets were immediately frozen in liquid nitrogen and then stored at -80ºC before use. The metabolomics analysis was conducted as described by Zeng et al. (2016). All chemicals used for metabolome isolation and GC-MS analysis were obtained from Sigma-Aldrich (USA). The metabolomics analysis protocol included the following steps: i) Metabolome extraction: Cells were resuspended in 1 mL of 10:3:1 (v/v/v) methanol/chloroform/H_2O solution (MCW) cold in liquid nitrogen and thawed five times. Supernatants were collected by centrifugation at 11,200 rpm for 3 minutes at 4ºC. To normalize variations throughout the samples, an internal standard (IS) solution (100 μg/mL ribitol, 10 μL) was added to 100 μL of supernatant in a microtube before it was dried by vacuum centrifugation for 2–3 h; ii) Sample derivatization: the samples were dissolved in 10 μL of methoxyamine hydrochloride (40 mg/mL in pyridine), shaken at 30ºC for 90 min, added to 90 μL of N-methyl-N-(trimethylsilyl) trifluoroacetamide (MSTFA), and incubated at 37ºC for 30 minutes to trimethylsilylate the polar functional groups. The derivate samples were collected by centrifugation at 11,200 rpm for 3 minutes, and the supernatant was placed into silanized glass vials and used directly for GC/MS analysis; iii) GC-MS analysis: Analysis was performed on a GC-MS system-GC QP2010 (Shimadzu, Japan) equipped with a SLB5ms (30m x 0.25mm x 0.25μm). One microliter of derivatized sample was injected in split ratio of 5:1 mode at 230ºC injector temperature. The GC was operated at a constant flow of 1 mL/min helium. The temperature program started isocratically at 45ºC for 2 minutes, followed by temperature ramping of 5ºC/min to a final temperature of 280ºC, and then held constant for 2 extra minutes. The range of mass scan was m/z 38–650; iv) Data processing and statistical analysis: The mass fragmentation spectrum was analyzed using the automated mass spectral deconvolution and identification system (AMDIS), in order to identify the compounds by matching the data with the mass spectral library of the National Institute of Standards and Technology (NIST). Peak areas of all identified metabolites were normalized against the internal standard and the relative abundances for each identified metabolite. The levels of the identified metabolites were clustered and displayed in a heat map using the R package *gplots* (WARNES et al., 2016). All the statistical analysis were carried out by R version 3.6.0 (R DEVELOPMENT CORE TEAM, 2020)

3 RESULTS AND DISCUSSION

3.1 MICROALGAE ISOLATION

The isolation protocol was successful in obtaining three isolates from the lake of Ijaci and four isolates from the bays of Portel and Melgaço. Table 2 shows the morphotypes and characteristics of the isolates.

Table 2 - Isolates and morphological characteristics of colony and cell

Strain	Place	Culture medium	Colony characteristics	Cell characteristics
AL_UFLA01	Melgaço bay	BG 11	Light green, irregular shape	Cells with green coloration and rounded shape.
AL_UFLA02	Melgaço bay	BG 11	Dark green, spherical	Cells with green coloration and rounded shape.
AL_UFLA03	Portel bay	BG 11	Light green, spherical	Cells with green coloration and irregular shape.
AL_UFLA04	Ijaci lake	BG 11	Dark green, spherical	Small cells with green coloration, visible at 40x, rounded shape.
AL_UFLA05	Ijaci lake	BG 11	Dark green, spherical	Cells with green coloration and rounded shape.
AL_UFLA06	Ijaci lake	Bold + Extract	Dark green, spherical	Cells with green coloration and rounded shape.
AL_UFLA07	Portel bay	BG 11	Light green, spherical	Cells with green coloration and rounded shape, small

Source: Author (2020)

Among the isolates (TABLE 2) there was a dominance of green microalgae, characteristic of members belonging to the *Chlorophyta* division, a large group of green algae commonly found in freshwater rivers and lakes and often isolated from tropical and subtropical areas (ABOU-SHANAB et al., 2011; HADI et al., 2016). *Chlorophyta* species also have been cited as components of the phytoplankton community of a tropical lake located in Minas Gerais, Brazil (FIGUEREDO; GIANI, 2009).

Isolation and prospecting of microalgae from different environmental conditions are important since differences in the cell's response to temperature and nutritional conditions vary according to the location where the strain was obtained. While searching for the microalgae that best fits the mixotrophic cultivation in coffee by-product, a condition different from the original, it is important to verify the different responses that could be observed.

According to Fouilland et al. (2014), microalgae isolated from temperate environments showed better growth in medium supplemented with urban waste and digested sludge, compared to species isolated from tropical areas, possibly due to the greater specialization (and reduced ability to grow in other conditions) of the microorganisms adapted to extreme temperatures. Teoh, Phang, and Chu (2013) and Wong et al. (2015) also noticed differences in the tolerance response to temperature and UV radiation stress from species isolated from temperate, tropical, and polar regions, with strains of temperate and tropical regions presenting greater recovery after stress compared to the Antarctic isolates.

3.2 EVALUATION OF GROWTH CONDITIONS

The optical cell density in the three conditions evaluated (autotrophy, heterotrophy, and mixotrophy) for the isolated microalgae can be seen in Table 3. The slow growth of AL_UFLA07 made it difficult to obtain the initial inoculum for evaluation of growth conditions, so a decision was made to discontinue the use of this isolate in the presented study.

Table 3 - OD_{680} mean of each microalga for each condition

Strain	**Optical density**		
	Autotrophy	Heterotrophy	Mixotrophy
AL_UFLA04	0.0710 cA	0.1163 cA	0.0860 cA
AL_UFLA01	0.1578 cA	0.1243 cA	0.1923 cA
AL_UFLA03	0.2320 cB	0.3093 cB	0.7733 bA
AL_UFLA02	0.2363 cB	0.3390 cB	0.8647 bA
AL_UFLA05	0.4590 bB	0.3417 cB	1.0207 bA
AL_UFLA06	0.4993 bB	0.3760 cB	1.6153 aA

*Means followed by the same lower case letters in a column and capital letters on the lines do not differ significantly by the Scott-Knott test ($p < 0.05$).

Source: Author (2020)

Mixotrophy provided growth greater than or equal to for all the green microalgae (TABLE 3). The results corroborate with the literature, in which several genera *Chlorophyta*, especially *Scenedesmus* and *Chlorella*, showed the ability to assimilate glucose as a carbon source in the mixotrophic growth (ABREU et al., 2012; GIRARD et al., 2014; PANCHA; CHOKSHI; MISHRA, 2015; ZHANG et al., 2014). The isolates AL_UFLA02, AL_UFLA03, AL_UFLA05 and AL_UFLA06, which presented higher mixotrophic growth compared to the other conditions, were selected for the screening step in the residual wastewater growth.

CWW was the by-product selected to initial screening due to ease of utilization and for presenting a more favorable composition to the microalgae growth.

While autotrophic growth involves only photosynthesis and heterotrophy, exclusively the use of organic carbon source, under the mixotrophic metabolism the microalgae have at its disposal light and organic carbon, which usually provides higher biomass production (ABREU et al., 2012; PANCHA; CHOKSHI; MISHRA, 2015). Zhang et al. (2017) also suggests that for some microalgae, the mixotrophic growth was superior to the sum of the heterotrophic and autotrophic growth, indicating a synergistic effect of photosynthesis and aerobic respiration, that is, a mechanism that allows the cells to use the energy and carbon more efficiently than in other modes of cultivation.

The isolates AL_UFLA04 and AL_UFLA01 presented the same optical density under the three conditions. Bhatnagar et al. (2011) suggested that the degree of assimilation of different carbon sources is species-dependent, related to the presence of specific permeases, that is, while some species may assimilate a certain carbon source, as glucose was assimilated by AL_UFLA03, AL_UFLA02, AL_UFLA05 and AL_UFLA06, others can use the organic carbon source differently or even not be able to assimilate it at all. Andrade and Costa (2007) also observed that the presence of organic carbon may alter both metabolism forms, autotrophy and heterotrophy. Moreover, although glucose is a common source of carbon assimilated by microorganisms, the precise effects of glucose on the microalgae metabolism vary in a great manner, which shows that glucose consumption depends on the amount of light and the species evaluated (PEREZ-GARCIA et al., 2011).

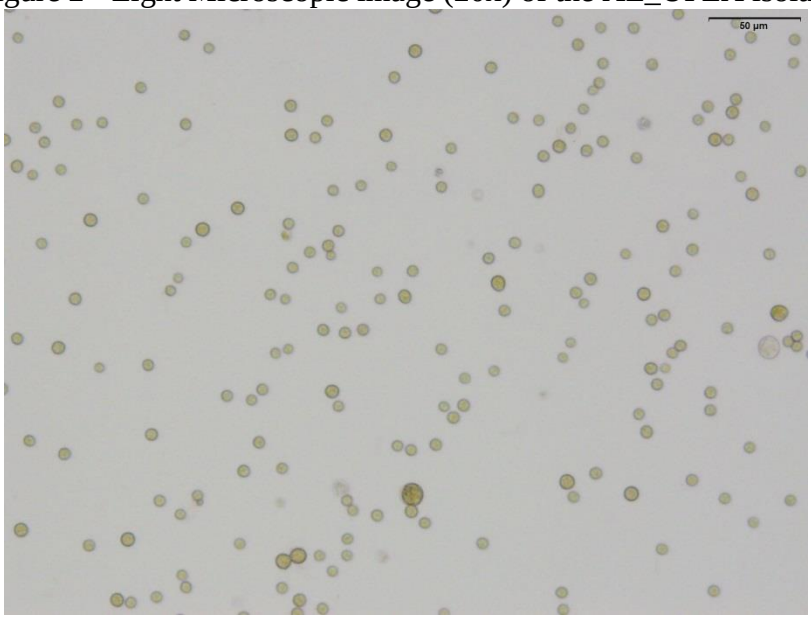
Regarding the heterotrophic growth, the optical density was equal for autotrophic growth and lower than in mixotrophy. Although some species can effectively grow in the darkness, the number of species capable of growing exclusively under heterotrophy is limited, as microalgae are naturally autotrophic (PEREZ-GARCIA et al., 2011). Also according to Cerón-García et al. (2013), the production of compounds related to photosynthesis, such as pigments, can be reduced or disrupted in heterotrophic cultures.

3.3 SELECTION AND CULTIVATION IN WASTEWATER COFFEE

The isolates AL_UFLA02, AL_UFLA03, AL_UFLA05, and AL_UFLA06, which showed higher growth under mixotrophy in BG-11 with glucose 1 g/L were selected for evaluation of growth in coffee wastewater (CWW). The analysis was carried out in glass tubes, in which the isolate AL_UFLA06, presented in Figure 2, showed the highest number of

cells in CWW, compared to synthetic medium BG-11 in both autotrophic and mixotrophic conditions.

Figure 2 - Light Microscopic image (20x) of the AL_UFLA isolate



Source: Author (2020)

Three dilutions of CWW (30, 60, and 90%) were evaluated to verify the influence of the nutrient's availability and the light incidence through the culture flask, on microalgal growth. The concentration 30% 7.32 ±0.02 Log cells/mL, the concentration 60%, of 6.94±0.02, and the concentration 90%, of 6.68±0.09. The Scott-Knott test at the level of 5% of significance showed a significant difference between the treatments, and the concentration of 30% of residual coffee wastewater, which presented the highest microalgal growth, was selected.

The highest growth at the concentration 30% may be explained by the combination of availability of nutrients in the adequate concentration, satisfying the microalgae nutritional needs and, as showed by Santana et al. (2017), due to the adequate culture light transmittance, that allowed the sunlight incidence on the mixotrophic system, with higher biomass production. Ramirez, Farenzena, and Trierweiler (2014) showed that concentrations up to 40% of vinasse in the medium were suitable to grow *Scenedesmus s*p. and, when the percentage of vinasse increased, the light intensity should be proportionately higher, to keep the microalgae growth.

Although CWW composition may vary depending on the processing mode, coffee cultivation and other factors (PANDEY et al., 2000), the most commonly found nutrients are

nitrogen, phosphorus, and potassium (AGUIAR; ANDRADE-VIEIRA; DE OLIVEIRA DAVID, 2016; RATTAN et al., 2015), which are essential for microalgal growth. Selvamurugan, Doraisamy, and Maheswari (2010) described concentrations of nitrogen (125.8-173.2 mg/L) and phosphorus (4.4-6.8 mg/L) in CWW, which could be enough for the growth of some microalgae. Xin et al. (2010) reported the use of nitrogen and phosphorus present in residues for growth and accumulation of lipids by *Scenedesmus* sp. Ebrahiminezhad et al., (2014) described high rates of nitrogen and phosphorus absorption in urban waste by *C.* sp. and *Chlamydomonas* sp. That is, integrating waste treatment with microalgal biomass production may be qualified as economically and environmentally viable alternatives.

As reported by Garde et al. (2017), the CWW is characterized by high organic load, i.e. high biochemical oxygen demand (BOD) concentration. The value of 17,740 mg/L of BOD was quantified for our samples, and the removal of 26.05% was achieved after *Neodemus* sp. cultivation. Among the organic compounds, the sugars available in pulp and mucilage (BONILLA-HERMOSA; DUARTE; SCHWAN, 2014; RATTAN et al., 2015), removed during the washing process and carried with water, suggest a source of organic carbon for mixotrophy.

Sugars and glycerol quantification by HPLC in coffee wastewater showed the presence of glucose and pentoses (xylose and arabinose), components of the hemicellulose, and glycerol (TABLE 4). From the gathered data, it can be shown that glucose from CWW was consumed by the microalgae during cultivation, as observed during mixotrophic cultivation in BG-11 medium supplemented with glucose 1g/L. However, the concentrations of pentoses and glycerol remained the same ($p < 0.05$), in the two evaluated times. Glucose is the most used carbon source in mixotrophic cultivation of microalgae, assimilated by several genera of green algae, including *Desmodesmus* (RIOS et al., 2016; ZHAO et al., 2016), *Chlorella* (KIM et al., 2013; LIN; WU, 2015; WANG et al., 2012), *Scenedesmus* (BHATNAGAR et al., 2011), *Spirulina* (CHOJNACKA; NOWORYTA, 2004) and *Nannochloropsis* (CHEIRSILP; TORPEE, 2012).

While the use of pentoses in the microalgae cultivation is still not widely studied (FREITAS et al., 2017), their metabolization by microalgae has been previously reported. Zheng et al. (2014) were the first to notice the assimilation of xylose by *C. sorokiniana*. Recently, some species can metabolize pentoses, such as xylose by *Chlamydomonas* sp. (MATA et al., 2014) and xylose and arabinose by *C. minutissima* (FREITAS et al., 2017; FREITAS; MORAIS; COSTA, 2017), *Dunaliella salina* (DE FREITAS et al., 2019) and *C. protothecoides* (MU et al., 2015). However, Zheng et al. (2014) reported the occurrence of

competition between glucose and xylose for the same carrier, thus, *C. sorokiniana* could not metabolize xylose until the depletion of glucose. Regarding glycerol, also present in CWW, its use as a carbon source has been previously reported (LIN; WU, 2015; MONDAL et al., 2017), but a few strains can utilize glycerol from the medium (PEREZ-GARCIA et al., 2011).

Table 4 - Concentrations of sugars and glycerol by HPLC in coffee wastewater before (Initial time) and after (Final time) AL_UFLA06 cultivation

Compounds	Concentration at Initial time (g/L)	Concentration at Final time (g/L)
Glucose	4.702 ± 0.028a	2.114 ± 0.036b
Xylose	4.792 ± 0.010a	4.768 ± 0.056a
Arabinose	0.153 ± 0.001a	0.155 ± 0.001a
Glycerol	0.411 ± 0.002a	0.35 ± 0.018a

*Means followed by the same letters on the lines do not differ significantly by the Scott-Knott test ($p < 0.05$).
Source: Author (2020)

3.4 MICROALGAE CULTIVATION IN HYDROLYSED COFFEE

Once selected the isolate AL_UFLA06 and 30% concentration of coffee wastewater, this microalgal, which presented the best mixotrophic growth in CWW, the most easily obtained and the most nutritionally rich by-product, was grown in hydrolysates produced with the coffee silverskin, so as to verify the feasibility of using this other coffee by-product.

3.4.1 Coffee silverskin characterization and microalgae growth in CSS hydrolyzates

Coffee silverskin (CSS) is a lignocellulosic material, composed essentially by cellulose, hemicellulose, and lignin. The chemical characterization of the CSS is shown in Table 5. The chemical composition of CSS was similar to the values reported by Ballesteros, Teixeira, and Mussatto (2014)b, Murthy and Madhava Naidu (2012), and Niglio et al. (2019): lignin (28.58%-30,5%), soluble lignin (7.61%), ashes (4.5-7%), proteins (12.6-18.69%), extractives (23.8%), cellulose (17.9%-23.77%), hemicellulose (7.7-16.68%). Differences may occur according to the variety of coffee and the composition of the grain from one country to another, due to variations in climate and soil conditions (MURTHY; MADHAVA NAIDU, 2012). In a complementary way, the characterization of macro and micronutrients showed the presence of important nutrients for microalgae growth, in the CSS by-product.

Table 4 - Chemical composition of CSS

Component	Value (%)
Lignin	28.02
Soluble lignin	3.40
Protein	18.46
Ashes	10.95
Extractives	21.97
Cellulose (Glucose)	13.48
Hemicellulose (Xylose/Arabinose)	3.72
Minerals	**mg/Kg**
Nitrogen	2600.06
Phosphorus	910
Potassium	2629
Calcium	1332
Magnesium	425
Sulfur	31
Boron	35.11
Cupper	71.57
Manganese	73.08
Zinc	12.39
Iron	333.57

Source: Author (2020)

The autohydrolysis process allows substantial liberation of components such as oligosaccharides, monosaccharides, hemicellulose acetyl groups, and lignocellulose degradation products, such as furfural and HMF (PONTES et al., 2018). According to Oosterveld et al. (2003), arabinogalactans, galactomannans, and cellulose are the most abundant polysaccharides in coffee, and then, glucose, galactose, mannose, and arabinose are the most commonly found sugars in CSS. After hydrolysis, as expected, the release of simple sugars from the lignocellulosic material was small, compared to other more severe treatments, such as acid and enzyme, but it still has advantages like the use of no other reagents than water and high temperature (MUSSATTO et al., 2011a; PONTES et al., 2018). Also, it is estimated that a high quantity of sugars was degraded during the roasting, probably as a consequence of conversion processes such as the Maillard reaction and pyrolysis (OOSTERVELD et al., 2003).

However, there were some differences in the sugar content according to the treatments, as the increase in the arabinose concentration following the increase in the severity of the treatments, as it can be seen in Table 6. This result agrees with Mussatto et al. (2011b) and Ballesteros, Teixeira, and Mussatto (2017) who found an increase in the sugars content with the increase in the severity of hydrolysis conditions, to a certain extent, in which

degradation occurred. Due to their structure, arabinose is the most easily extractable sugar from the hemicellulose structure, during extraction processes (MUSSATTO et al., 2011a; OOSTERVELD et al., 2003).

Table 6 - Characterization of coffee silverskin hydrolysates

Condition of autohydrolysis	Temperature	Time (min)	Concentration of CSS (g/50mL)	Acetic acid (g/L)	Glucose (g/L)	Mannose (g/L)	Arabinose (g/L)
Treatment 1	120	20	1	N.D.	0.046	0.028	0.154
Treatment 2	180	20	1	0.003	0.018	0.015	0.242
Treatment 3	120	20	2	0.003	0.073	0.034	0.417
Treatment 4	180	20	2	0.077	0.059	0.040	0.647
Treatment 5	120	50	1	N.D.	0.032	0.009	0.264
Treatment 6	180	50	1	0.102	0.004	0.015	0.352
Treatment 7	120	50	2	0.003	0.073	0.046	0.494
Treatment 8	180	50	2	0.301	0.059	0.070	0.779
Treatment 9	150	35	1.5	0.003	0.032	0.028	0.374
Treatment 10	150	35	1.5	0.003	0.032	0.021	0.352
Treatment 11	150	35	1.5	0.003	0.032	0.028	0.374

Source: Author (2020)

The hydrolysis of lignocellulosic materials can result in the release of acetate derivative from acetyl groups, located in the main hemicellulose chains (MIAZEK et al., 2014). Higher temperatures induced greater liberation of acetic acid in the hydrolyzed (TABLE 6). While acetic acid can be used in the metabolism of some microalgae as an organic carbon source, they may have a toxic effect on other species (ZHAO et al., 2016). Trace concentration of furfural was detected in the hydrolysate from treatment 8, in which the most severe temperature was used, simultaneous to a higher concentration of pentoses, which is expected, since furfural is a product of the degradation of pentoses, such as arabinose (MUSSATTO et al., 2011a).

Table 7 shows AL_UFLA06 growth in the CSS hydrolysates. According to the Analysis of Variance ($p < 0.05$), the factors temperature ($p=0.0216$), time ($p=0.0135$), concentration ($p= 0.0286$), and interaction between time and concentration ($p= 0.0170$) were significant, with an increase in the microalgae cell count and a reduction in the values of the autohydrolysis variables. That is, among the conditions evaluated, the mildest hydrolysis, that is, Treatment 1, was the most suitable condition for microalgal growth. Compared to the autotrophic control (BG-11), which showed a cell count of 6.88 ± 0.03 cells, most treatments performed better, and regarding mixotrophy (BG-11 medium with 1g/L of glucose), which

presented a cell count of 7.40 ± 0.02 cells, Treatment 1 provided a slightly higher cell growth, showing that the hydrolysate was a good alternative to the synthetic medium.

Table 7 - Autohydrolysis treatments and *Neodemus* sp. growth

Treatment conditions	Temperature (ºC)	Concentration of CSS (g/50mL)	Time (min)	Log cfu/mL
Treatment 1	120	1	20	7.55
Treatment 2	180	1	20	7.38
Treatment 3	120	2	20	7.26
Treatment 4	180	2	20	6.70
Treatment 5	120	1	50	7.30
Treatment 6	180	1	50	6.78
Treatment 7	120	2	50	7.31
Treatment 8	180	2	50	6.73
Treatment 9	150	1.5	35	7.33
Treatment 10	150	1.5	35	7.30
Treatment 11	150	1.5	35	7.35
Autotrophic control (BG-11)	-	-	-	6.88
Mixotrophic control (BG-11 medium with 1% of glucose)	-	-	-	7.40

Source: Author (2020)

Results showed that while the hydrolysate appears to have provided inorganic nutrients for microalgae growth, the concentration of sugars available for growth was not a decisive factor for differences in microbial growth. However, the concentration of inhibitors such as furfural seems to have strongly affected the microalgal growth. This can be seen in Table 6, where the treatments that showed the lowest growth, 4, 6, and 8, also had the more severe hydrolysis conditions applied. According to Miazek et al. (2014), although information about the furfural effect on microalgal growth is scarce, there is evidence that it may cause inhibition of photosynthesis and cell lysis.

In CSS, metabolizable sugars for microalgae are present in its complex structure, even in small quantities, but the lignocellulose rigidity difficults the access to these carbohydrates

(MIAZEK et al., 2014). Autohydrolysis of lignocellulosic materials is a complex process, dependent on many factors such as the liquid/solid ratio, temperature, extraction time, and material structure, which allows substantial liberation of components as monosaccharides, available for utilization by microalgae metabolism. Furthermore, it is a method that has several advantages such as lower cost, no use of toxic chemicals, and limited generation of undesirable products, toxic to microalgae, to name a few (ROMANÍ et al., 2010). Combination with other methods such as subsequent enzymatic hydrolysis (HIJOSA-VALSERO et al., 2018) could allow higher sugar recovery but also implies to additional costs along the process.

3.4.1 Molecular identification of AL_UFLA06

The ITS1, ITS2, and 5.8S regions of the rDNA of AL_UFLA06, the isolate that presented the highest growth in the screening step of growth in CWW were sequenced, generating an 813bp fragment. The BLAST analysis of the sequence of AL_UFLA06 showed 97% nucleotide identity in *Neodesmus* sp. The genus *Neodesmus* sp. belongs to the family *Scenedesmaceae*, the largest group of coccoid green microalgae found in freshwater. Composed of 13 phylogenetic and morphologically described genera, this group is characterized by strictly asexual reproduction by the release of auto spores from the mother cell (KRIENITZ; BOCK, 2012).

3.3.2 CULTIVATION IN BIORREATOR IN INDOOR AND OUTDOOR CONDITIONS

After testing the coffee wastewater and coffee silverskin, CWW, at the concentration of 30%, was selected for evaluation of microalgae growth under outdoor conditions. The microalgae growth in CWW was similar, in cell count, to the cultivation in CSS hydrolysate, in reduced cultivation time, (6 days for CWW and 20 days for the CSS hydrolysate). It shows that the microalgae reached the exponential phase in a reduced number of days when grown in CWW 30%, representing an advantage for the viability of large-scale production of microalgae biomass, without considering the costs and time of preparation of the coffee by-products culture medium.

Besides the source of nutrients, another factor to be considered while analyzing the feasibility of microalgae cultivation is the light dependence. Microalgal outdoor cultivation using sunlight is another alternative to increase the economic viability of the microalgae

production. However, it is important to test the viability of the selected microalgae production in outdoor conditions (XIA et al., 2013) since some microalgae are unable to grow under these conditions. Scaling up to 1000 mL flasks containing 800 mL of CWW at a concentration of 30%, with OD_{680} = 0.05, the incubation time of 8 days is justified by the microalgae growth curve, which showed that the isolate has its maximum growth after 6 days. Although longer cultivation periods could provide higher biomass production, the choice for 8 days of incubation was justified by the viability on an industrial scale.

Analyzing the cultures growth rates after 8 days, using Equation (4), presented in Table 8, it is perceptible that the mixotrophic growth was superior to the autotrophic growth under the two conditions, outdoors and indoors, with a preference for the mixotrophic outdoor cultivation. Also, the pH of the supernatant increased to 7.0-8.0, while the pH of raw CWW was 4.0.

Table 8- The growth rate of *Neodemus* sp. in CWW and BG 11 medium (Control) in outdoor and indoor conditions

Condition	Growth rate
Outdoor CWW	0.439
Outdoor BG-11	0.123
Indoor CWW	0.375
Indoor BG-11	0.158

Source: Author (2020)

Analyzing the biomass constitution, the Scott-Knott test ($p<0.05$) showed that the pigment's production for autotrophy was equal and smaller than in mixotrophy in both incubation conditions (TABLE 9). There was an increase in its content when CWW was used as a culture medium, in mixotrophy, with the highest productivity obtained for the outdoor condition.

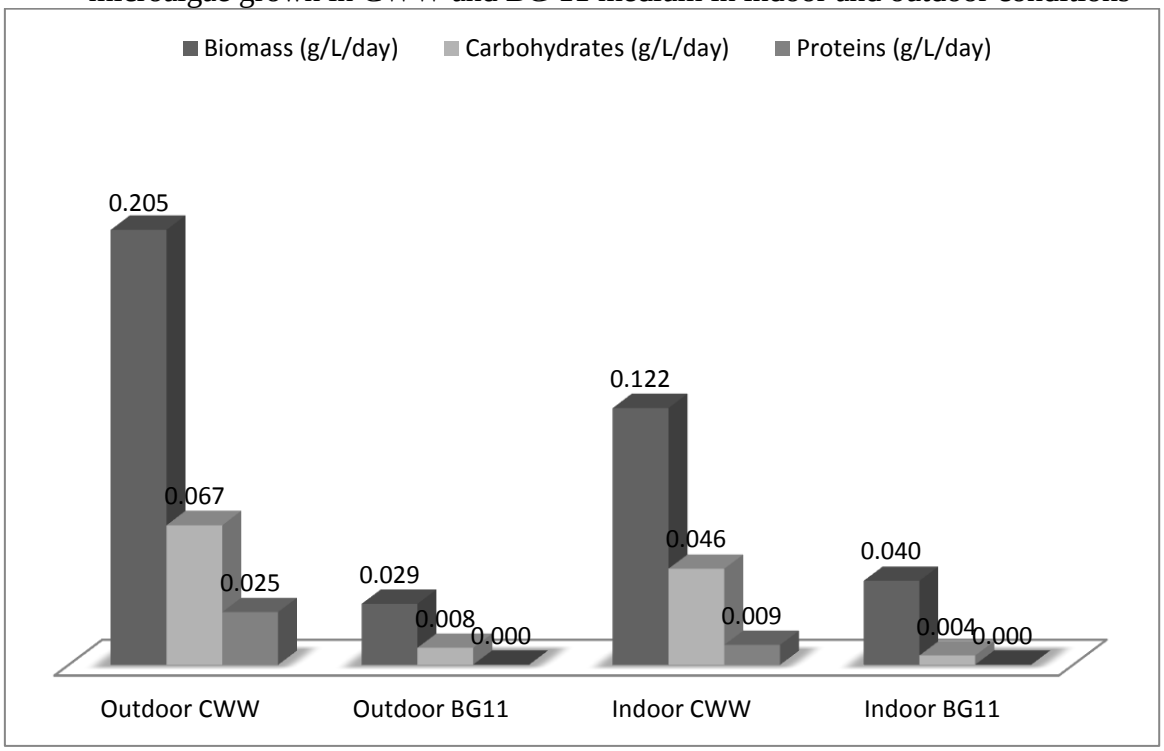
Table 9 - Pigment content in the biomass of the microalgae grown in CWW and BG 11 medium under controlled and environmental conditions

Condition	Chlorophyll A (mg/L)	Chlorophyll B (mg/L)	Carotenoids (mg/L)	Total (mg/L)
Outdoor CWW	5.961± 0.310a	7.831± 0.580a	1.992± 0.597a	15.784 ± 0.762a
Indoor CWW	3.336 ± 0.547b	3.188 ± 0.683b	1.085 ± 0.128b	7.608 ± 0.148b
Indoor BG-11	0.639 ± 0.223c	0.698 ± 0.291c	0.203 ± 0.048c	1.539 ± 0.020c
Outdoor BG-11	0.355 ± 0.118c	0.419 ± 0.164c	0.132 ± 0.021c	0.906 ± 0.066c

*Means followed by the same letters on the columns do not differ significantly by the Scott-Knott test ($p < 0.05$).
Source: Author (2020)

Figure 4 shows the biomass, carbohydrate, and protein (g/L/day) content of the microalgae under different culture conditions. Analyzing Figure 4, it can be observed that both treatments with CWW presented higher productivity than the autotrophic condition. It suggests that *Neodesmus* sp. was able to uptake nutrients such as nitrogen and phosphorus from the wastewater in order to generate biomass. Also, the organic carbon, in the form of soluble sugars, especially glucose, made possible the growth during dark periods. Therefore, under the mixotrophic condition, *Neodesmus* sp. was able to grow with photosynthesis and aerobic respiration to produce biomass of 1.64 g, corresponding to 205 mg/L/day, higher than autotrophy.

Figure 4 - Biomass productivity, carbohydrate content and protein content (g/L/day) of microalgae grown in CWW and BG 11 medium in indoor and outdoor conditions



Source: Author (2020)

Bhatnagar et al. (2011), by using a mixture of industrial and urban waste supplemented with nitrogen obtained 165mg/L of biomass for *C. globosa*, 213 mg/L for *C. minutíssima,* and 487 mg/L for *S. bijuga* after 7 days of incubation, all values higher than autotrophic growth in BG-11 medium. Also using urban waste supplemented with 37.5 mM of glycerol, Nzayisenga, Eriksson, and Sellstedt (2018) obtained productivity of 1.29 g/L of *Chlorella* sp. in mixotrophic cultivation in residue after 8 days. Productivities between 0.02

and 0.041 g/L/ day were obtained for *C. pyrenoidosa* growing mixotrophically in swine residue, greater than the produced in Bristol medium (WANG et al., 2012).

Regarding biomass production, it is important to highlight some considerations. Our cultivation was performed under static conditions and microalgae growth could be improved with the use of mechanical shaking or aeration by air enriched with CO_2. Fluctuations between the maximum and minimum temperatures throughout the day, which could have stressed the microalgae, also need to be considered.

As well as CWW, another agro-industrial waste which has been proposed for microalgae cultivation as a strategy for cost-reduction is the sugarcane vinasse. Using clarified vinasse as a nutrient source, Santana et al. (2017) obtained yields of 164.44 mg/L/day and 222.22 mg/L/day, respectively, for *Micractinium sp.* and *C. biconvex*, values similar to the obtained for *Neodesmus* sp. with CWW at 30% concentration reported on this paper. On the other hand, for uncleaned vinasse, the productivities obtained by Marques et al. (2013) were 70 mg/L/day of *C. vulgaris,* which shows the effect of increasing light transmittance in the medium through dilution or chemical clarification on microalgae growth. Also, for *Micractinium* sp., the productivity of 270 ± 1.9 mg/L/day was obtained by Engin et al. (2018), using Bold Basal medium supplemented with 10% (v/v) of vinasse. Sugarcane vinasse as a medium supplement in *Spirulina maxima* cultures accelerated the cell growth under heterotrophic conditions, compared to autotrophy (RAMIREZ, 2013). Vinasse from tequila production process, at a concentration of 10% (v/v) resulted in productivity of 49.5 ± 8.3 mg/L/ day of a microalgae-yeast system (CEA BARCIA et al., 2020).

The higher growth in outdoor conditions, under both mixotrophy and autotrophy, suggests that natural conditions are more suitable for microalgae growth than controlled conditions of temperature and luminosity. Feng et al. (2014) evaluated the growth in the incubator with controlled conditions and outdoor environment and verified that *S. obliquus* easily adapted to the outdoor condition, with a productivity of 525 mg/ L/ day at the end of 9 days of culture in BG-11 medium, which is greater than the 493 mg/ L/day obtained under controlled conditions.

Cheirsilp and Torpee (2012) reported in a study with *Chlorella* sp. that the microalgae growth increased to the same extent as the light intensity, up to the limit of 184 μmol $s^{-1}m^{-2}$. According to Pancha, Chokshi, and Mishra (2015), the growth of *Scenedesmus* sp. CCNM 1077 at a light intensity of 150 μmol/m^2/s was superior to lower light intensities. Similar results were obtained by Bohutskyi et al. (2016) with *Chlorella* and *Scenedesmus* and Feng et al. (2014) with *S. obliquus*. Ramirez, Farenzena, and Trierweiler (2014) showed that higher

productivity of *Scenedesmus* sp. was obtained with higher values of light intensity, up to 185 μmol/m^2/s. Liu et al. (2012) reported that the typical intensity of daylight in the open air is about 2000 μmol/m^2/s in the equatorial regions, which may be limiting for the growth of some microalgae.

However, in our study, the CWW dark coloration, due to the coffee compounds, reduced the translucency of the culture medium, which balanced the effect of the intense luminosity to which the microalgae culture was exposed to. Regarding the photoperiod, there was not much discrepancy between the conditions (approximately 11h of lightness to 13h of darkness).

Regarding the pigment content, although many microalgae, including *C. pyrenoidosa, C. zofingiensis* (GIOVANARDI et al., 2013), and *Neochloris oleoabundans* (BALDISSEROTTO et al., 2016) reduce their photosynthetic pigment content under mixotrophy, the photosynthetic system remained active in *Neodesmus* sp. despite the presence of an organic carbon source. The highest concentration obtained in mixotrophy can be explained as a direct consequence of the higher microalgae biomass production, in other words, each cell contains a small amount of pigment, but the total microalgal biomass, with bigger cellular concentration, was richer in pigments (BALDISSEROTTO et al., 2016). According to Pang et al. (2019), mixotrophy has been shown to increase the yields of these high-value compounds as pigments, compared to autotrophy. Pancha, Chokshi, and Mishra (2015) reported that the pigment content in *S. s*p. CCNM 1077 was proportional to the light intensity used in the mixotrophic regime, with an increase in chlorophyll-a content from 4.15 to 8.91 μg/mL, varying the intensity of light from 30 to 150 μmol/m^2/s.

About carbohydrate production, Figure 4 shows that the mixotrophic condition provided a higher carbohydrate accumulation than the autotrophy, for both types of incubation. Mixotrophic growth with glucose and galactose as organic carbon sources also induced higher levels of starch. Especially for outdoor conditions, the productivity under mixotrophy was 0.53 ± 0.03g/L in 8 days. Several studies have suggested that light intensity affects not only microalgae growth but also the metabolic pathways leading to biomass compartmentalization, producing variations on pigments, fatty acids, carbohydrates, and protein content in response to changes in illumination (AGARWAL et al., 2019; DENG et al., 2019; MENEGOL; CHISTI, 2019). Ho, Chen and Chang (2012) reported that increasing light intensity could lead to higher carbohydrate content and the concentration of glucose in *S. obliquus* increased significantly from 53.44% to 73.12% as the light intensity rose from 60 to 180 μmol/m^2/s.

As shown in Figure 1, no significant amounts of protein were quantified in the biomass generated in autotrophy, probably because of the low amount of biomass used for quantification. Unimpressive amounts of lipids were also produced under the same conditions according to the method used (HIGGINS et al., 2014). The low content of photosynthetic pigments in autotrophic cultures could explain the decrease of lipids and proteins since the energy produced by light absorption was probably not enough to synthesize big quantities of lipids and proteins, whose metabolic pathways require more energy (LI et al., 2018). The protein accumulation in mixotrophy (0.2g/L in an outdoor environment) suggests that under this condition, the cell had enough energy to keep the synthesis of this compound. For lipids, the relative percentage detected for mixotrophic cultivation in indoor and outdoor conditions was 0.313 and 1.188%, respectively. The amount of lipids obtained in outdoor conditions corroborates the results obtained by Feng et al. (2014), who also suggested that lipid storage efficiency (mainly triacylglycerol) could increase with higher light intensity, which means more energy, and Wahdin et al. (2013) suggest that higher rates of biomass production are positively correlated with higher lipid production.

3.3 METABOLOMICS ANALYSIS

In order to understand the metabolic changes behind the mixotrophic growth of microalgae in coffee wastewater (CWW) and the differences between indoor and outdoor cultivations, untargeted metabolomics was conducted. Intracellular metabolites of *Neodesmus* sp., in outdoor CWW and outdoor BG-11 medium cultivations, as well as at the initial time, an inoculum collected from indoor cultivation in BG-11 with glucose 1g/L, were analyzed by GC-MS, after extraction and derivatization. A number of 44 detectable intracellular metabolites were identified and quantified (TABLE 10). Among the metabolites identified there were sugars, organic acids, fatty acids, amino acids, and esters.

Table 10- Metabolites identified for *Neodemsus* sp. in the three treatments (Initial, Outdoor CWW, and Outdoor BG-11)

Number	Identification	Initial time ug/mg	Outdoor CWW ug/mg	Outdoor BG-11 ug/mg	Chemical class
1	1,3-Pentanediol, 2-methyl-, 1-propanoate	0.0006	0.0018	0.0172	Alcohols
2	Ethanol, 2-(trimethylsilyl)-	0.0059	0.0033	0.0235	Alcohols
3	1,4-Butanediol	0.0029	0.0000	0.0037	Alcohols

4	Ethanol, 2-(methylamino)-	0.0011	0.0000	0.0129	Alcohols
5	1,2-Ethanediamine, N-(2-aminoethyl)-	0.0011	0.0003	0.0000	Amines
6	3-Trifluoromethylbenzylamine, N,N-dinonyl	0.0000	0.0188	0.0113	Amines
7	Alanine	0.0004	0.0049	0.0026	Amino acids
8	Glycine, N-formyl-N-(trimethylsilyl)-, trimethylsilyl ester	0.0389	0.0246	0.0180	Amino acids
9	Glycine, N-(1-oxo-2-butenyl)-, trimethylsilyl ester	0.0016	0.0000	0.0239	Amino acids
10	L-Alanine, 3-[(aminocarbonyl)amino]-	0.0026	0.0067	0.0116	Amino acids
11	L-Proline, 5-oxo-1-(trimethylsilyl)-, trimethylsilyl ester	0.0200	0.0687	0.0000	Amino acids
12	2-Piperidinecarboxylic acid, 1-(trimethylsilyl)-, trimethylsilyl ester (pipecolic acid)	0.0078	0.0038	0.0000	Amino acids
13	Glutamic acid, N-(trimethylsilyl)-, bis(trimethylsilyl) ester, L-	0.0032	0.0035	0.0000	Amino acids
14	Tris(trimethylsilyl)borate	0.0024	0.0084	0.0465	Boric Acid Esters
15	1,3-Dioxolane	0.0005	0.0036	0.0055	Cyclic acetals
16	Propanoic acid, 1-methylethyl ester	0.0011	0.0013	0.0000	Esters
17	Propanoic acid, 2-[(trimethylsilyl)oxy]-, trimethylsilyl ester	0.0388	0.0765	0.0578	Esters
18	9,12-Octadecadienoic acid (Z,Z)-, trimethylsilyl ester	0.1673	0.0125	0.0000	Fatty acids
19	Butanoic acid, 4-[bis(trimethylsilyl)amino]-, trimethylsilyl ester	0.0234	0.0123	0.0624	Fatty acids
20	3H-1,2,4-Triazol-3-one, 1,2-dihydro-	0.0754	0.0534	0.3479	Heterocyclic compounds
21	Bis(trimethylsilyl)bromosuccinate	0.2406	0.2292	1.3881	Imides
22	3,5-Dimethyl-4-octanone	0.0027	0.0000	0.0321	Ketones
23	1,2,3-Propanetricarboxylic acid, 2-[(trimethylsilyl)oxy]-, tris(trimethylsilyl) ester (citric acid)	0.0732	0.1448	0.0000	Organic acids
24	2-Butenedioic acid (E)-, bis(trimethylsilyl) ester (fumaric acid)	0.0063	0.0206	0.0000	Organic acids
25	Butanedioic acid, [(trimethylsilyl)oxy]-, bis(trimethylsilyl) ester (malic acid)	0.0508	0.5339	0.1244	Organic acids
26	Glucaric acid, 2,3,4,5-tetrakis-O-(trimethylsilyl)-, bis(trimethylsilyl)	0.0158	0.0142	0.0000	Organic acids
27	Malonic acid, bis(2-trimethylsilylethyl ester	0.0123	0.0046	0.0150	Organic acids
28	Propanedioic acid, bis(trimethylsilyl) ester (methylmalonic acid)	0.0645	0.0710	0.0531	Organic acids
29	N,O,O'-Tris-(trimethylsilyl)-6-hydroxy-2-aminohexanoic acid	0.0032	0.0035	0.0000	Organic acids
30	Acetic acid, nitro-, methyl ester	0.0105	0.0000	0.0791	Organic acids

31	2,3,4-Trihydroxybutyric acid tetrakis(trimethylsilyl) deriv., (, (R*,R*)-) (threonic acid)	0.0122	0.0470	0.0000	Sugar acids
32	D-Glucuronic acid, 2,3,4,5-tetrakis-O-(trimethylsilyl)-, trimethylsilyl ester	0.0050	0.0091	0.0000	Sugar acids
33	Myo-Inositol, 1,2,3,4,5,6-hexakis-O-(trimethylsilyl)-	0.0595	0.1753	0.0000	Sugar alcohols
34	Trimethylsilyl ether of glucitol	0.4032	1.3490	0.0000	Sugar alcohols
35	D-Mannitol, 1,2,3,4,5,6-hexakis-O-(trimethylsilyl)-	0.2782	0.0250	0.0000	Sugar alcohols
36	Sucrose, octakis(trimethylsilyl) ether	0.3294	0.4719	0.0000	Sugars
37	2-O-Glycerol-.alpha.-d-galactopyranoside, hexa-TMS (floridoside)	0.0180	0.0020	0.2235	Sugars
38	D-Glucose, 2,3,4,5,6-pentakis-O-(trimethylsilyl)-, o-methyloxyme, (1Z)-	0.1504	2.2695	0.0000	Sugars
39	D-Fructose, 1,3,4,5,6-pentakis-O-(trimethylsilyl)-	1.0941	4.1509	0.0000	Sugars
40	d-Galactose, 2,3,4,5,6-pentakis-O-(trimethylsilyl)-, o-methyloxyme, (1Z)-	0.2877	0.0192	0.0000	Sugars
41	Glucopyranose, pentakis-O-trimethylsilyl-	0.0201	0.0008	0.0000	Sugars
42	Ribitol, 1,2,3,4,5-pentakis-O-(trimethylsilyl)-	0.0094	0.0009	0.0000	Sugars
43	D-(-)-Fructofuranose, pentakis(trimethylsilyl) ether (isomer 1)	0.4414	0.1138	0.0000	Sugars
44	D-(-)-Tagatofuranose, pentakis(trimethylsilyl) ether (isomer 1)	0.0078	0.0017	0.0000	Sugars

Source: Author (2020)

As shown in item 3.3.2, mixotrophy was the condition that provided higher production of biomass and intracellular components of the microalgae. The quantification of the analyzed intracellular metabolites from *Neodemus* sp. also indicated that the levels of the majority of intracellular metabolites in autotrophy, in the outdoor BG-11 condition (BG-11) were lower than the initial time (IN) and in the outdoor cultivation with coffee wastewater (CWW). Relative levels of the identified intracellular metabolites from GC–MS are displayed in the heat map in Figure 3. Looking at the heat map, for autotrophy, among the metabolites present at the lowest levels there were several sugars, while higher levels of alcohol as ethanol, 1,3-pentanediol, 2-methyl-1-propanoate, ethanol, 2- (trimethylsilyl) - and 1,4-butanediol were observed. In contrast, for mixotrophic cultivation in CWW and IN, the components that were found in higher quantities were sugars and organic acids.

Five free amino acids were detected by GC-MS after derivatization: glycine, alanine, proline, glutamic acid, and lysine, all in relatively small concentrations compared to other

metabolites analyzed, as already reported (WILLAMME et al., 2015). Vidoudez and Pohnert (2012) showed that the concentration of amino acids was correlated with cell growth intensity. Thus, the higher concentration of glycine in mixotrophy could be explained by the need to generate more amino acids for new cell production. The same was observed for pipecolic acid, acyclic amino acid, an intermediate of the catabolism L-lysine (PÉREZ-GARCÍA; BRITO; WENDISCH, 2019) and glutamic acid, generally present in low percentages in green algae (LOURENÇO et al., 2002). However, the pattern was not verified for proline and alanine.

Proline is known to be a potential non-enzymatic biomarker of the antioxidant response to stress-induced in microalgae by abiotic agents, acting as a detoxifier of reactive oxygen species, induced by exposure to p-Nitrophenol (SUBASHCHANDRABOSE et al., 2012), nitrogen starvation (CHOKSHI et al., 2017) or phosphorus starvation (DOKTOR et al., 2017), or yet as an osmoregulatory solute in a medium with high salinity (HELLEBUST, 1985). The higher proline content in CWW cultivation, compared to BG-11 can be explained as a microalgae response to a more stressful culture medium, with low pH and presence of phenolic compounds. In such manner, the higher levels of alanine under autotrophy may be related to its accumulation in a condition of hypoxic, which is limited aeration and active microbial respiration (YANG et al., 2015). In general, the metabolic analysis demonstrated that the majority of amino acids and other related compounds could not be detected at all, which would be expected, since the highest abundance of these components is generally present during the initial phase of cultivation when the levels of nitrogen available are still elevated (BLIFERNEZ-KLASSEN et al., 2018).

Regarding fatty acids, the concentration of 9,12-octadecadienoic acid (C18:2z), a fatty acid suitable for biodiesel production, was higher for mixotrophy, both in the IN and in CWW cultivation, while for butanoic acid, the condition BG-11 provided higher accumulation. The observed fatty acids composition was following those previously reported, where, for *C. vulgaris*, Abedini Najafabadi et al. (2015) and Heredia-Arroyo et al. (2011) revealed that cultivation under autotrophic conditions resulted in a higher percentage of saturated fatty acids, while unsaturated fatty acids were the largest portion for those under mixotrophic growth.

During the metabolism of respiration applied to produce energy, organic substrates as hexoses and pentoses may be assimilated by microalgae as a source of carbon and energy (SUN et al., 2018), where hexoses are mainly consumed through glycolysis and pentoses are used in the pentose phosphate pathway (PANG et al., 2019). Metabolites from both pathways

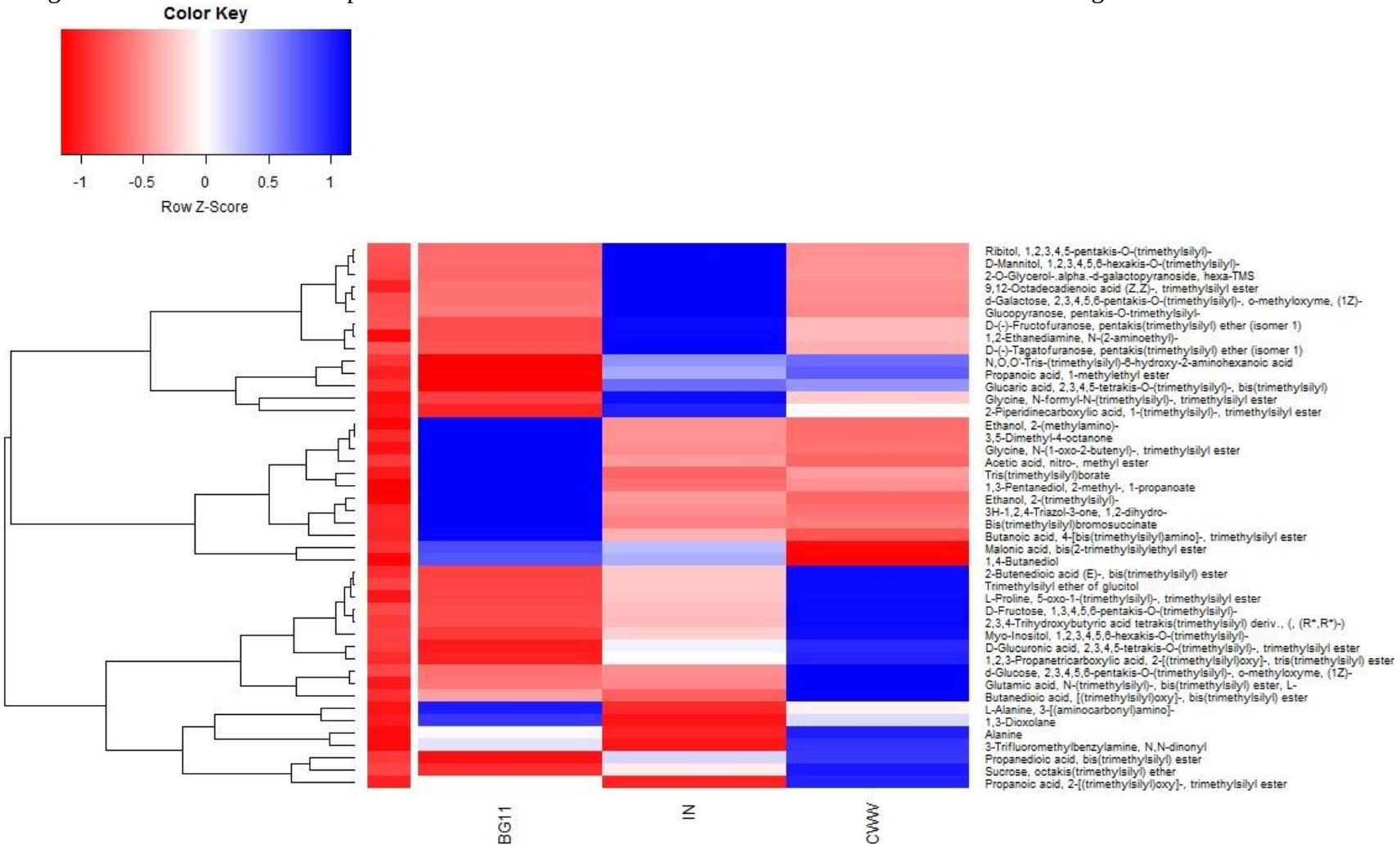
are metabolized into the citric acid cycle (TCA cycle) for the aerobic generation of energy through the oxidation of acetyl-CoA, and into the biosynthesis of amino acids, carotenoids, and fatty acids. Glucaric acid is a high-value organic acid and one of the end products of the d-glucuronic acid pathway, which is a cycle that is initiated with either d-galactose or d-glucose and interacts with the pentose phosphate pathway (MOON et al., 2009). The increase in glucaric acid and the precursors of the glycolytic pathway, d-fructose and d-glucose, under mixotrophy, suggests that glycolytic and oxidative pentose-phosphate pathways are the main routes of sugar degradation in *Neodesmus* sp. The availability of intermediates in the citric acid cycle as bis(trimethylsilyl)bromosuccinate, a derivative of succinic acid, citric, fumaric, malonic, and malic acids detected, suggests TCA cycle activity as well, possibly for the production of energy through the generation of ATP.

Differences in concentrations of threonic acid, a product of ascorbic acid catabolism, at the conditions of autotrophy and mixotrophy in the initial cultures and with CWW may be related to the mechanisms of regulation of ascorbic acid. According to Kempa et al. (2009) and Vidal-Meireles et al. (2017), ascorbic acid synthesis in microalgae, unlike plants, although also involves the conversion of d-mannose into l-galactose intermediates, is independent of photosynthesis, which would explain the differences between the conditions of autotrophy and mixotrophy. Ascorbic acid, acting as an essential scavenger of reactive oxygen species (ROS), can be converted into different species such as threonic acid, which would explain its higher content in a more stressful medium such as CWW (SMIRNOFF, 2018). D-glucuronic acid was identified as one of the cell's wall carbohydrates along to rhamnose, galactose, and glucosamine, in green freshwater microalgae *Chlorophyta* (PANKIEWICZ et al., 2016; RASHIDI; TRINDADE, 2018). The upregulation of glucuronic acid in cultivation with CWW suggests that the stressing medium could have stimulated the cell wall repair mechanism in *Neodemus* sp.

Although the oxidative phosphorylation-dependent metabolism is most commonly found in microalgae, it is reported that under certain hypoxic conditions and in the presence of substrate, microalgae can switch to fermentation as an alternative route for energy production (CATALANOTTI et al., 2013). In photosynthetic microorganisms, fermentation of reserve polysaccharides with the generation of ATP and NADH leads to the formation of different final products, being reported in genera such as *Chlorella*, *Chlamydomonas,* and *Scenedesmus* the production of acetate, ethanol, glycerol, lactic acid, H_2 and CO_2 (CHANDRA; VENKATA MOHAN, 2011; GAFFRON; RUBIN, 1942; KLEIN; BETZ, 1978; LAKATOS et al., 2019), that can be accumulated or excreted by the cell. The presence of intracellular

metabolites such as ethanol, acetic, propanoic, and lactic acid in *Neodesmus* sp. biomass suggests the occurrence of a fermentative process, due to low O_2 in the culture caused by limited aeration in the flasks or even by the imbalance between the consumption of O_2 and its production in plastids (ATTEIA et al., 2013). Under mixotrophy, the alcohols concentrations were lower than the condition of autotrophy, and a hypothesis is that the sugars metabolism, and consequently, the aerobic respiration, may have indirectly led to an increase in photosynthetic efficiency, with the generation of more O_2, allowing the maintenance of the mitochondrial respiration itself (YANG et al., 2015).

Figure 3- Hierarchical heat map of the metabolites detected for the initial time and CWW and BG-11 growth in outdoor conditions



Source: Author (2020)

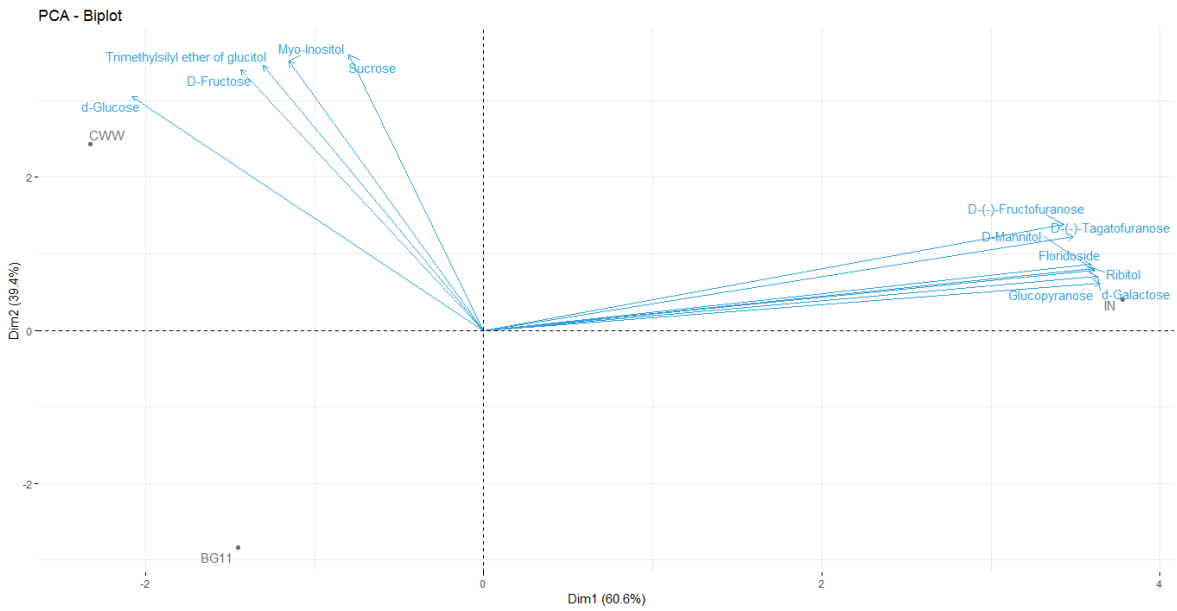
Carbohydrates may be present outside the cell wall (e.g. agar, alginate), as a component of the cell wall (e.g. cellulose and hemicellulose) and as a storage metabolite in microalgae (e.g. sucrose, starch) (ALAM; WANG, 2019; GONZALEZ-FERNANDEZ; MUÑOZ, 2017). Overall, the abundancy of the intracellular sugars observed during the mixotrophic cultivation strongly suggests that carbohydrates are the main storage product in *Neodesmus* sp. In green microalgae, D-glucose, the main source of carbon and energy during photosynthesis, is stored as starch and other forms of cellulose (β-(1,4)-glucan chains), the main component of the cell wall (GONZALEZ-FERNANDEZ; MUÑOZ, 2017; SAHOO; SECKBACH, 2015; TSEKOS, 1999). D-fructose is a monosaccharide that in the form of the derivative fructose-6-phosphate, is often bonded to UDP-glucose to synthesize sucrose, and is a precursor of starch synthesis (ALAM; WANG, 2019; PFISTER; ZEEMAN, 2016). On the other hand, sucrose is the main disaccharide stored, being reported in *Botryococcus braunii*, *C. vulgaris*, *S. obliquus*, *Chlamydomonas nivalis*, and *Chlamydomonas reinhardtii* (BLIFERNEZ-KLASSEN et al., 2018; LU et al., 2016; VIDAL-MEIRELES et al., 2017; WANG et al., 2019a).

Among the identified compounds, sugars such as sucrose, D-glucose, and D-fructose were the most abundant in the initial conditions and increased in cultivation in CWW (TABLE 9). According to Gonzalez-Fernandez and Muñoz (2017) and Khan, Shin, and Kim (2018), when the assimilation and production of the sugars along mixotrophy are elevated, exceeding the consumption rate by the cell, overproduced sugars are converted mainly into polysaccharides, with cellulose and starch being the main compounds in green microalgae. Sucrose accumulation, on the other hand, is reported to counteract the stress conditions in *S. obliquus* (WANG et al., 2019c), although Kolman et al. (2015) also reported the possibility of degradation of sucrose after osmotic stress, releasing glucose and fructose. In contrast, sugars such as ribitol, glucopyranose, D-galactose, D-tagatofuranose, and D-fructofuranose were down-regulated during CWW cultivation. Albeit these non-structural sugars can act as energy reserves, they also have the function of osmoregulation and protection against other forms of stress (HURTADO et al., 2017), so some of them may have also been assimilated during the response to environmental stress in CWW cultivation.

The principal component analysis (PCA) also displays an overview of the differences in the sugar's concentrations between mixotrophy in CWW, autotrophy (BG-11), and the initial mixotrophic condition (IN). Figure 4 explicitly indicates that there is a similarity between the variables, as well as identifies and discriminates the three conditions tested. While the first main component (PCA1) allowed the differentiation between the trophic

conditions, that is, autotrophy or mixotrophy, the second main component (PCA2) allowed the differentiation between the mixotrophy in CWW and IN. On the positive side of PCA1 and PCA2, the IN was correlated with higher levels of non-structural sugars, which were possibly assimilated while protecting the cell against stress, being down-regulated during CWW cultivation. On the negative side of PCA1 and positive ofs PCA2, an increase in structural sugars, glucose, fructose, and sucrose and polyols was correlated with CWW cultivation, which means accumulation as structure or storage in the cell. It is also possible that small quantities of these sugars are residuals from CWW. Autotrophy in BG-11, associated with a low concentration of carbohydrates and small biomass generation, contributed to none of the sugars.

Figure 4 - Principal Component Analysis - Biplot of different sugars with the two principal components



Source: Author (2020)

Regarding polyols, the increase in myo-inositol concentration under mixotrophic cultivation in CWW suggests accumulation under conditions of environmental stress, since these molecules are known for its potential in osmotic adjustment and efficient in scavenging hydroxyl radicals, or yet, sugar storage and transport alternative (BLIFERNEZ-KLASSEN et al., 2018; BOSE; RODRIGO-MORENO; SHABALA, 2014; WANG et al., 2019b). Similarly, an increase of Trimethylsilyl ether of glucitol, a derivative of sorbitol, and the isomer d-mannitol have also been reported in microalgae, with several functions as storage and mainly,

osmoregulation and scavenging of active oxygen species (BEN-AMOTZ; AVRON, 1983; IWAMOTO; SHIRAIWA, 2005). The presence of these polyols was observed in brown algae (GROISILLIER et al., 2014), red algae *Bangiopsis subsimplex* (EGGERT et al., 2007), and green algae from genera *Stichococcus* as well (SHETTY; GITAU; MARÓTI, 2019).

Finally, floridoside, a galactosyl-glycerol heteroside characteristic from photosynthetic metabolism of most red algae (LV et al., 2019), was also identified in both treatments and also in the initial time. In red algae, floridoside is used as a carbon precursor in the biosynthesis of agar, starch, and cell wall polysaccharides (GOULARD et al., 2001; MACLER, 1988) and is accumulated in acclimation to various abiotic stresses, especially salt stress (BARBIER et al., 2005; KARSTEN; BARROW; KING, 1993). Although the presence of floridoside or the isomer isofloridoside in green algae has not been reported, proteins of green algae of *Ulva pertusa* and *Ulva prolifera* have been found in the same cluster of glucosyltransferases, red algae proteins very probably correlated to floridoside metabolism (PADE et al., 2015).

FINAL CONSIDERATIONS

Isolation and selection of microalgae isolates able to grow in residual coffee by-products allowed microalgae biomass production. The microalgae cultivation using CWW in outdoor conditions with sunlight provided a higher production of biomass and accumulation of intracellular metabolites as organic acids (citric, malic, and fumaric acids), fatty acids as 9,12-octadecadienoic acid, and storage carbohydrates compared to outdoor cultivation using BG-11. These metabolites are useful in the generation of added value products as bioethanol, and biodiesel production. However, further analyses are still necessary so as to optimize the production of microalgal biomass and intracellular metabolites, such as the evaluation of the clarification of the coffee wastewater, selection of the best incubation photoperiods, and analysis of effects of balance between hypoxy/aeration supplemented with CO_2.

ACKNOWLEDGMENTS

The authors thanks CAPES (Coordenação de Aperfeiçoamento de Pessoal de Nível Superior) under the scope of the PDSE (88881.190073/2018-01, nº47/2017) and FAPEMIG (Fundação de Amparo à Pesquisa do Estado de Minas Gerais) for financial support.

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ARTIGO 2

ANTIMICROBIAL ACTIVITY AND SILVER NANOPARTICLES BIOSYNTHESIS POTENTIAL OF GREEN MICROALGAE *Neodesmus* sp. AND *Desmodesmus* sp.

Artigo redigido conforme Manual de normalização e estrutura de trabalhos acadêmicos: TCCs, monografias, dissertações e teses da UFLA (versão preliminar)

ABSTRACT

Microalgae are a promising source of intra and extracellular compounds with potential applications in aquaculture, food industry, medicine, and biofuels production. Besides the growing interest in microalgae-based compounds, specially in pharmaceuticals, antibiotics, and other biologically active structures, it is still necessary to discover the potential of new strains available in nature, in contrast to the total microalgal diversity. The main purpose of this study was to evaluate potential applications for two microalgal products, the biomass, and the cell-free culture supernatant. The cell-free culture supernatant *Desmodesmus* sp. and *Neodesmus* sp. was evaluated as a source of extracellular biomolecules capable of synthetizing silver nanoparticles. Regarding the biomass, ethanolic and ethyl acetate intracellular extracts of *Neodesmus* sp. and *Desmodesmus* sp. were tested for their antimicrobial activity against foodborne pathogens. GC-MS analysis was used to identify and quantify fatty acids, known for their innibitory effect on microbial growth in the microalgae with potential antimicrobial activity. Results showed bioreduction of silver ions, in solutions of molarities 0.5, 0.75, and 1mM by *Neodesmus* sp. and *Desmodesmus* sp. cell-free supernatants. *Desmodesmus* sp. extracts were particularly effective against *S. typhimurium* and *S. aureus*. Identification of fatty acids by GC-MS in *Desmodemus* sp. showed some compounds with antimicrobial activity already reported, as octanedioic and butanedioic acids.

Keywords: Fatty acids. Pathogenic bacteria. GC-MS. Microalgae extracts. Phyconanotechnology

INTRODUCTION

Microalgae are unicellular microorganisms which may exist individually, in chains or groups, mostly cultured photoautotrophically under solar light as an energy source to assimilate inorganic carbon, although some strains can grow in heterotrophic or mixotrophic conditions as well, using organic substrates as a carbon source (CERÓN-GARCÍA et al., 2013; KIM et al., 2013; MARREZ et al., 2019).

Usually, microalgae are rich in compounds such as carbohydrates, proteins, fatty acids, phenolics and pigments. Thus, several biotechnological applications as aquaculture, food industry, medicine, residuals treatment and biodiesel production have been proposed. In green microalgae, several genera such as *Chlorella*, *Spirulina*, *Desmodesmus* and *Scenedesmus* have drawn attention as valuable sources of metabolites with diverse biological properties such as antibacterial, antifungal, and anticancer activities (EL SEMARY, 2011; KUMAR P, 2017; MARREZ et al., 2019; PRADHAN et al., 2012). Among these metabolites, a wide variety of potential antimicrobials have been identified in microalgae's intracellular extracts, such as proteins and peptides, polysaccharides, polyphenols and specially, fatty acids (PINA-PÉREZ et al.,2017).While microalgal intracellular extracts produced by extraction of bioactive compounds in the biomass using appropriate solvents, have been showing important activity against pathogenic bacteria (RICKEN et al., 2019), the cell-free culture supernatant, collected from microalgae cultivation and previously discarded, could also represent a source of extracellular commercial valuable compounds, as stated by Rahman et al. (2019). Thus, it is interesting to explore the use of microalgae in these two pathways, including the biomolecules produced by the cells and the cell-free supernatant.

Recently, biosynthetic routes to produce silver nanoparticles, structures known for the wide range of applications, involving the pharmaceutical and cosmetics industries, using whole organisms like plants, microalgae and fungi or their derivatives are being widely studied. In an approach known as "green chemistry", these methods represent a more sustainable, simple, and cost-effective alternative when compared to conventional chemical and physical methods, that employ expensive and hazardous toxic chemicals as reducing or stabilizing agents (JENA et al. , 2014; PRASAD, 2013; PUGAZHENDHI et al., 2018). In this context, the cell-free supernatant obtained from microalgae growth and which potentially contains secondary metabolites released by the microalgae, could be a cheaper and easily obtained alternative for biogenic synthesis of nanoparticles.

The present study aimed to propose applications for the two microalgal cultivation by-products, the biomass and the cell-free culture supernatant. For the biomass, the antimicrobial activity's potential of *Neodesmus* sp. and *Desmodesmus* sp. intracellular extracts was evaluated, and the fatty acids profile of active extracts was identified using GC-MS. The cell-free supernatant was tested as a source of extracellular biomolecules for the biosynthesis of silver nanoparticles.

1. MATERIAL AND METHODS

1.1 MICROALGAE GROWTH

Neodesmus sp. and *Desmodesmus* sp. were obtained from the Culture Collection of the Laboratory of Physiology and Genetics of Microorganisms of the Federal University of Lavras - UFLA. The strains were cultivated in 250 mL flasks containing BG-11 medium supplemented with 1g/L of glucose. The initial optical density was OD_{680}=0.5, and the flasks were kept at room temperature and under constant lighting. The cell-free supernatant was collected by centrifugation (6,000 rpm, 10 minutes), on the 14^{th} day of culture.

1.2 SYNTHESIS AND CHARACTERIZATION OF MICROALGAE BASED SILVER STRUCTURES

The silver structures synthesis was carried out according to Prasad (2013) with adaptations. Twelve milliliters of the collected supernatant were added to previously prepared solutions of $AgNO_3$, molarities 0.5; 0.75; 1 and 5mM, diluted in ultrapure water. BG-11 medium supplemented with 1g/L of glucose was used as a control. The receptacles were kept in the dark at room temperature until the produced particles were stabilized.

The silver ions' bioreduction was monitored by visible changes in the sample color, from colorless to dark yellow/brown, and by ultraviolet-visible (UV-Vis) absorption measurement using a spectrophotometer, so as to take a full band scan ranging from 200 up to 1000nm. The formation of silver structures was observed by measuring the change of color of the silver-supernatant solution, due to the surface plasmon resonance (SPR) phenomenon.

1.3 EVALUATION OF THE ANTIMICROBIAL ACTIVITY OF MICROALGAL EXTRACTS

1.3.1 Culture and growth condition

Desmodesmus sp. and *Neodesmus* sp. were maintained mixotrophically in BG11 Modified medium ($NaNO_3$ 1.76 · 10^{-2} M, $MgSO_4$ 3.04. 10^{-4} M, K_2HPO_4 2.28 · 10^{-4} M, $CaCl_2$ 2.38 · 10^{-4} M, Citric acid 3.12 · 10^{-5} M, Ammonium citrate ferric 2.23 · 10^{-5} M, Na_2EDTA 2.69 · 10^{-5} M and micronutrients solution (H_3BO_3 9.87· 10^{-7} M, $MnSO_4$ 1· 10^{-6} M, $ZnSO_4$ 9.98 · 10^{-7} M, $CuSO_4$ 1· 10^{-8} M, $(NH_4)_6Mo_7O_{24}$ 1 · 10^{-1} M) (ALLEN; STANIER, 1968) supplemented with 1g/L of glucose, in 500mL flasks, at room temperature (25ºC) and constant illumination. Cultures were maintained until cells reached the late logarithmic growth phase, at 14 days, with final biomass of 1.76 ± 0.02g/L for *Neodesmus* sp. and 2.24 ± 0.11g/L for *Desmodesmus* sp.

1.3.2 Preparation of microalgae extracts

Microalgae grown as described above. They were collected by centrifugation (6,000 rpm, for 10 min, a 4ºC), and freeze-dried. Two different lipid-soluble polar solvents, ethanol 99.5% and ethyl acetate 100%, obtained from Labsynth, Brazil, were used for extraction, as described by Bulut et al. (2019).

Freeze-dried microalgal biomass (20 mg) was suspended in 5 mL of solvent in glass tubes and vigorously mixed. The tubes were sonicated in an ultrasonic water bath for 20 min and stirred on an orbital shaker for 1h at room temperature. The extracts were centrifuged at 6,000 rpm for 10 min and the extract was collected. The pellet was resuspended in the same solvent and extracted one more time following the same procedure. The extracts were combined and filtered through a 0.45µm pore size syringe filter. Extracts (10 mL) were dried using a rotary evaporator (45ºC, 180 rpm) until the solvents were completely removed. The dried residues were stored at -20ºC.

1.3.3 Antimicrobial activity

1.3.3.1 Bacterial strains and inoculum storage

The antimicrobial activity of *Desmodesmus* sp. and *Neodesmus* sp. intracellular extracts were evaluated against five pathogenic bacteria: *Staphylococcus aureus*, *Escherichia coli* (EPEC), *Listeria monocytogenes*, *Salmonella enteritidis* and *Salmonella typhimurium*. The strains were stored at -20ºC in a freezing culture medium (15 mL glycerol, 0.5 g bacteriological peptone, 0.3 g of yeast extract and 0.5 g NaCl, per 100 mL of distilled water). For strain reactivation and use, an aliquot of the freezing culture was transferred to test tubes containing 10 mL of BHI medium with two subcultures at 37 °C for 24 h.

1.3.3.2 Disc diffusion method

The 24h subcultures of each bacterial species were centrifuged at 10,000 rpm for 5 minutesutes, resuspended in saline solution, and standardized using McFarland standard (10^8 cfu/mL of 0.5 McFarland standard). Using swabs, TSA agar plates, supplemented with 0.6% yeast extract, were uniformly inoculated with the bacterial cultures. A concentration of 1 mg/mL of each extract and fraction was prepared by dissolving 1 mg in 1 mL of ultrapure water with 5% DMSO. Sterilized discs (6 mm) from Whatman No 1 filter paper were placed on the seeded plates by using a sterile tweezer, loaded with 10µL of extracts, and dried completely under sterile conditions. Ultrapure water with 5% DMSO and amoxicillin/clavulanic acid (30µg) represented the negative and positive controls, respectively. Inoculated plates with the discs were incubated at 37ºC for 24 h, and then the inhibition zones were measured and expressed as the diameter of the clear zone, considering the diameter of the paper disc (BAUER et al., 1966; MARREZ et al., 2019).

1.3.3.3 GC-MS analysis of intracellular metabolites

For GC-MS analysis, 1mL of microalgae culture used to prepare the ethanolic and ethyl-acetate extracts was collected and centrifuged at 8,500 rpm for 10 minutes at 4ºC. The cell pellets were immediately frozen in liquid nitrogen and then stored at -80ºC before use. The analysis of metabolites and data processing was conducted as described by Zeng et al. (2016). All chemicals used for metabolites extraction and GC-MS procedures were obtained from Sigma-Aldrich (USA). For metabolites extraction, cells were resuspended in 1 mL of cold 10:3:1 (v/v/v) methanol/chloroform/H_2O solution (MCW), and thawed five times. Supernatants were collected by centrifugation at 11,200 rpm for 3 minutes at 4ºC. An internal

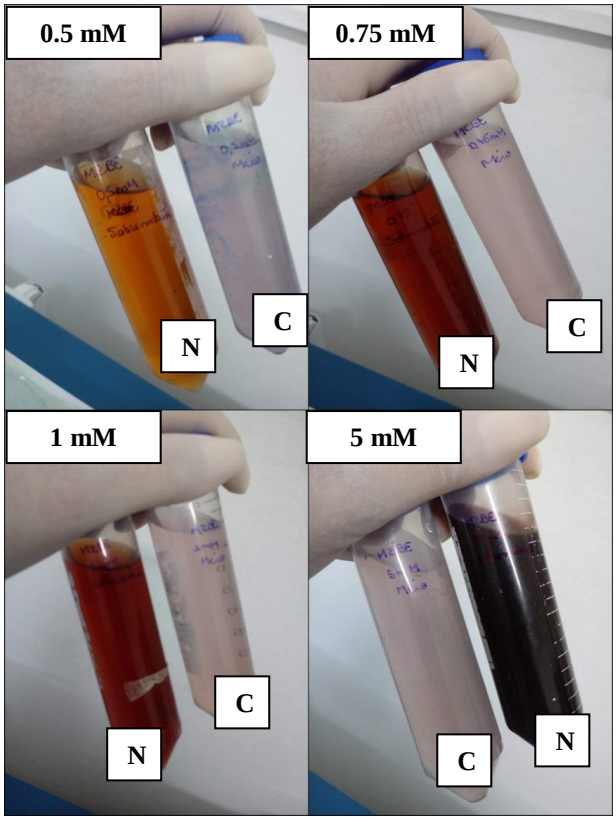
standard solution (100 µg/mL ribitol, 10 µL) was added to 100µL of supernatant in a microtube before being dried by vacuum centrifugation for 2-3 h. At the derivatization step, the samples were dissolved in 10µL of methoxyamine hydrochloride (40 mg/mL in pyridine), shaken at 30ºC for 90 minutes, added to 90µL of N-methyl-N-(trimethylsilyl) trifluoroacetamide (MSTFA), and incubated at 37ºC for 30 minutes. The derivate samples were collected by centrifugation at 11,200 rpm for 3 minutes, and the supernatant was placed into silanized glass vials and used directly for GC-MS analysis. GC-MS analysis was performed on a GC-MS system-GC QP2010 (Shimadzu, Japan) equipped with a SLB5ms (30m x 0.25mm x 0.25um). One microliter of derivatized sample was injected in split ratio of 5:1 mode at 230ºC injector temperature. The GC was operated at a constant flow of helium (1 mL/min). The temperature program started isocratically at 45ºC for 2 minutes, followed by temperature ramping of 5ºC/min up to a final temperature of 280ºC, and then held constant for 2 extra minutes. The range of mass scan was m/z 38–650. The mass fragmentation spectrum was analyzed using the automated mass spectral deconvolution and identification system (AMDIS) so as to identify the compounds by matching the data with the mass spectral library of the National Institute of Standards and Technology (NIST).

2. RESULTS AND DISCUSSION

2.1 SYNTHESIS AND CHARACTERIZATION OF SILVER STRUCTURES

The change of color in the reaction is an indicator of the silver particles formation (FIGURE 1). A stronger indication of the occurrence of silver ions reduction in silver particles can be observed in the spectra from the spectroscopy UV-Vis (FIGURE 2).

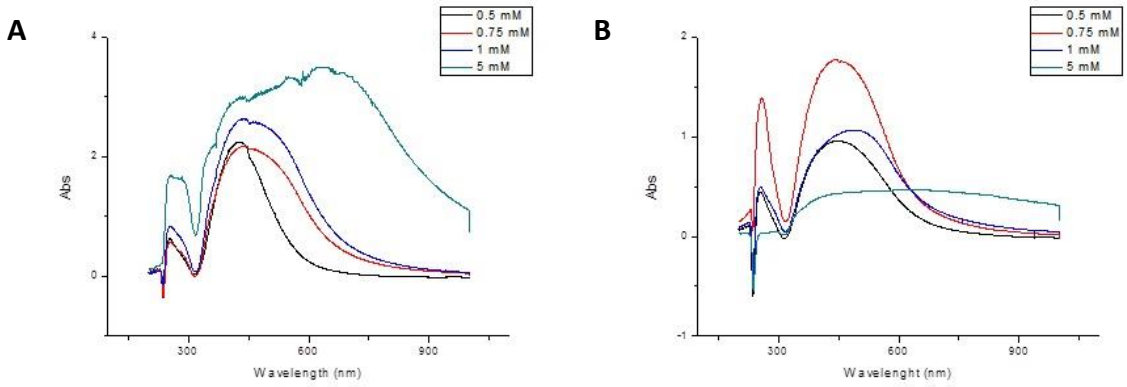
Figure 1 - Biosynthesis of $AgNO_3$ nanoparticles in different molarities with the microalgal culture supernatant (N) and the culture medium BG-11 (C)



Source: Author (2020)

The formation of synthesized metallic particles was initially observed by the change of color in the solution. The bioreduction of silver ions was monitored using a UV-Vis spectrophotometer (FIGURE 2).

Figure 2 – UV-Visible spectra of cell-free supernatant after bioreduction. (A) *Neodesmus* sp. and (B) *Desmodesmus* sp.



Source: Author (2020)

The visible UV spectrum of silver nanoparticles synthesized by *Neodesmus* sp. and *Desmodesmus* sp., with silver nitrate in the 0.5, 0.75, and 1mM molarities indicated absorption peaks around 450 nm for the 0.5 and 0.75 mM molarities and 490 nm for the 1mM molarity. An undefined peak was observed for the supernatant and $AgNO_3$ at a concentration of 5mM solution, indicating that this molarity was not adequate for the biosynthesis of silver particles. The formation of undefined peaks was also observed for higher molarities of the $AgNO_3$ in previous tests. When the BG-11 culture medium and $AgNO_3$ was tested, the absorption peak was not observed.

It is reported that silver nanoparticles with sizes between 5 and 100 nm have absorbance peaks at wavelengths between 393 and 462 nm, according to Agnihotri, Mukherji, and Mukherji (2014). In accordance with Arya et al. (2018), absorbance peaks around 460 nm were observed for nanoparticles with the range of 2-100 nm, and Muthusamy, Thangasamy, and Raja (2017) obtained nanoparticles sizing between 2 and 50 nm and maximum absorbance around 450 nm. Thus, UV-Visible spectra show that particles of this size range are likely to have been synthesized in the process.

Biosynthesis of silver nanoparticles in the microalgae culture cell-free supernatant suggests that compounds with a reducing effect are of extracellular origin and excreted by microalgae during its cultivation. Also, it reveals that the compounds responsible for the synthesis of silver particles are not present in the BG-11 culture medium, since the formation of nanostructures only occurred after the microalgae cultivation. The results corroborate with the observations made by Patel et al. (2015), who synthesized silver nanoparticles using biomass and cell-free culture liquid of various microalgae, among them *Scenedesmus* sp. and *Chlorella* sp., and by Rahman et al. (2019), who produced particles using the supernatant C. *reinhardtii*, and suggested that polysaccharides are the extracellular components responsible for the reduction reaction. Darwesh et al. (2019) suggested that reductase enzymes were the extracellular compounds responsible for $AgNO_3$ reduction in the synthesis of silver nanoparticles with the supernatant of *Scenedesmus obliquus*, while for Jena et al. (2014), proteins and peptides present in *Scenedesmus* sp. supernatant were the main responsible for the formation and stabilization of SNPs .

Fourier transform infrared spectroscopy analysis (FTIR) of nanoparticles synthesized with supernatants from microalgae culture showed peaks that may be assigned to functional groups present mainly in carbohydrates and proteins, such as hydroxyl, carboxylic, amines and carbonyl (EBRAHIMINEZHAD et al., 2016; JENA et al., 2014). Since reduction of silver ions through oxidation is necessary for the biosynthesis of nanoparticles, these groups

are probably the ones involved in the biosynthesis of nanoparticles with *Neodesmus* sp. and *Desmodesmus* sp. cell-free culture supernatants.

Traditionally, chemical methods are used for biosynthesis of metallic nanoparticles. However, these routes bring a series of disadvantages, such as the utilization of harmful substances, generation of toxic waste, high energy consumption and elevated production costs (PRASAD, 2013; RESENDE et al., 2017). In this context, the generation of nanostructures using biological materials, such as the supernatant from microalgae cultivation, through the green synthesis, represents an alternative to reduce production costs and the environmental impact related to the production of nanoparticles.

2.2 EVALUATION OF THE EXTRACTS ANTIMICROBIAL ACTIVITY

The ethanolic and ethyl acetate extractions of microalgae yielded, respectively, 4.0 and 3.3mg for *Neodesmus* sp. and 7.9 and 2.7mg for *Desmodesmus* sp. The selection of the most suitable organic solvent is generally restricted to a specific microalgae species, which shows that it is necessary to perform tests to verify the efficiency of each extraction method (DANYAL; MUBEEN; MALIK, 2013; FALAISE et al., 2016).

The antimicrobial activity of the extracts produced with the microalgae *Desmodesmus* sp., using the solvents ethanol and ethyl acetate, against the bacteria *S. aureus*, *E. coli* (EPEC), *L. monocytogenes*, *S. enteritidis*, and *S. typhimurium* are shown in Table 1. All the tested bacteria were resistant to the extracts produced with *Neodesmu*s sp.

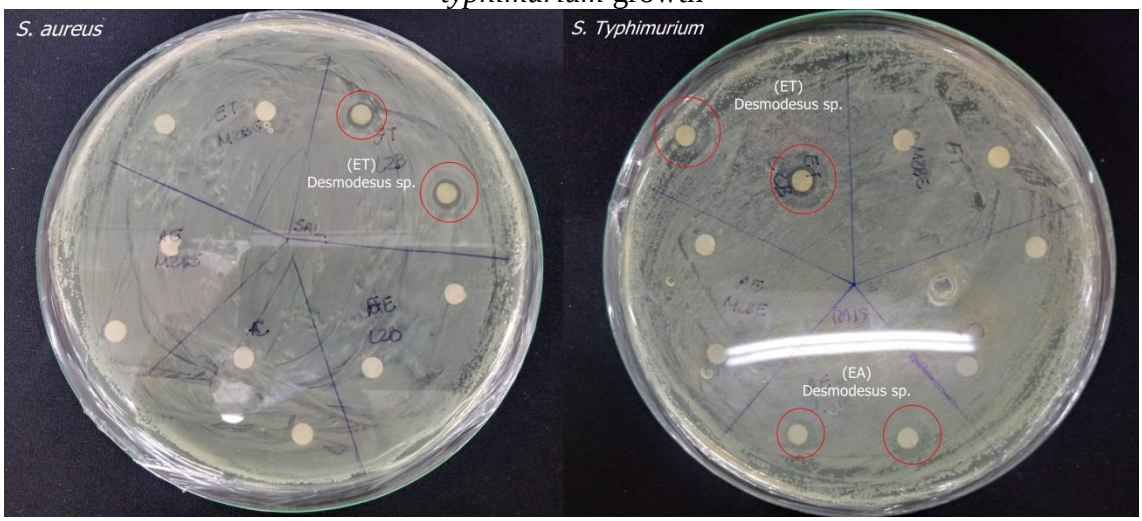
Table 1 - Diameter (mm) of the zone of inhibition of organic extracts of *Desmodesmus* sp., on different pathogenic bacteria.

		Treatment			
	Type	**Ethanolic extract**	**Ethyl acetate extract**	**Antibiotic**	**Control**
S. aureus	+	13.44 ± 0.06	0.00	30.12 ± 0.36	0.00
***E. coli* (EPEC)**	-	0.00	0.00	35.71 ± 1.59	0.00
L. monocytogenes	+	0.00	0.00	53.36 ± 0.62	0.00
S. enteritidis	-	0.00	0.00	29.17 ± 0.55	0.00
S. typhimurium	-	14.62 ± 0.32	10.69 ± 0.34	33.56±0.22	0.00

Source: Author (2020)

Results revealed that *S. aureus* and *S. typhimurium* were sensitive to the intracellular extracts of *Desmodesmus* sp. prepared with ethanol, and the extract produced with the same microalgae and ethyl acetate had an antimicrobial effect on *S. typhimurium* (FIGURE 3).

Figure 3 - Evaluation of the effect of *Desmodesmus* sp. extracts on *S. aureus* and *S. typhimurium* growth



Source: Author (2020)

The formation of inhibition zones for *S. aureus* and *S. typhimurium* by methanolic and aqueous extracts of the microalgae *Poterioochromonas malhamensis* was also observed by Ricken et al. (2019). The methanolic extract was the most efficient, producing an inhibition zone of 10.86 ± 1.71mm for *S. typhimurium* and 5.41 ± 0.91mm for *S. aureus*. Regarding *S. aureus*, Aremu et al. (2014) also verified the effect of petroleum ether, dichloromethane and ethanol extracts of *Scenedesmus* isolates on *S. aureus* growth, obtaining minimum inhibitory concentrations between 0.313 and 2.5 mg/mL. According to Marrez et al. (2019), extracts of *S. obliquus*, obtained using a series of solvents, among them, ethanol, produced inhibition zones of 9.7 ± 1.04mm. Ethanolic extract of *C. vulgaris* also presented an inhibitory effect, forming an inhibitory zone of 8.0 ± 0.4mm (KUMAR P, 2017).

Compounds with potential biological activity are scarcely found in aqueous extracts, and compounds with antimicrobial activity against contaminating food pathogens are reported to be normally hydrophobic and can be more easily extracted using organic solvents (DANYAL; MUBEEN; MALIK, 2013; FALAISE et al., 2016; PRADHAN et al., 2012). Thus, ethanol (NAJDENSKI et al., 2013; PRADHAN et al., 2012) and ethyl acetate (EL SHAFAY; ALI; EL-SHEEKH, 2016; SANTHOSH et al., 2019), described as better solvents to extract bioactive compounds from microalgae, showed to have extracted compounds with antimicrobial activity.

Among the microalgal compounds with antimicrobial activity are different classes of proteins, polysaccharides, amino acids, and antioxidants, (PINA-PÉREZ et al., 2017), but

especially fatty acids (FALAISE et al., 2016; SHANNON; ABU-GHANNAM, 2016). El Semary (2011) suggested that the presence of fatty acids such as palmitoleic acid, known for its toxic action against bacteria, in fractions of the methanol extract of *Desmodesmus* spp. is responsible for the antimicrobial effect detected on *E. coli*, *P. aeruginosa* e *Bacillus subtilis*.

2.3 GC-MS ANALYSIS OF FATTY ACIDS IN *Desmodesmus* sp.

Since the literature frequently claims that fatty acids, especially with medium and long-chains (containing more than 7 carbons), have inhibitory activity towards pathogenic microorganisms, *Desmodesmus* sp., that showed antibacterial performance against *S. aureus* and *S. typhimurium*, was chemically profiled for those compounds.

The results of the GC-MS analysis of *Desmodesmus* sp. are displayed in Table 2. The identified compounds included a series of fatty acids, whose antimicrobial activity has been previously described.

Table 2 - Fatty acids of *Desmodesmus* sp. identified by GC-MS

Fatty acid	Concentration µg/mg	Molecular formula
9,12-Octadecadienoic acid (Z,Z)- Linoleic acid, TMS(9Z,12Z)-, trimethylsilyl ester	0.0079	$C_{18}H_{32}O_2$
3-Deoxy-ribo-hexonic acid, pentakis-TMS	0.0693	$C_{21}H_{52}O_6Si_5$
4-Pentenoic acid, trimethylsilyl ester	0.0054	$C_9H_{18}O_2Si$
Butanedioic acid, [(trimethylsilyl)oxy]-, bis(trimethylsilyl) ester	0.0269	$C_{13}H_{30}O_5Si_3$
Octanoic acid trimethylsilyl ester	0.0057	$C_{11}H_{24}O_2Si$
Octatriacontyl pentafluoropropionate	0.0027	$C_{41}H_{77}F_5O_2$

Source: Author (2020)

Regarding fatty acids, Zheng et al. (2005) and Yoon et al. (2018) claim that long-chain fatty acids, such as 9,12-octadecadienoic acid (Z, Z) -, are known for their antimicrobial activity, being used as additives in the food industry. Thus, according to Zheng et al. (2005), analyzing the effect of several fatty acids and their derivatives, linoleic acid showed an antimicrobial effect on three strains of the *S. aureus*, through the inhibition of enoyl-acyl carrier protein reductase (FabI), a component of the synthesis of fatty acids, and consequently affecting cellular components, as the membrane. Recently, Tabashsum et al. (2020) also

showed that linoleic acid had an inhibitory effect on the growth and pathogenesis of *S. typhimurium*.

The compound 3-deoxy-ribo-hexonic acid, pentakis-TMS is a derivative of hexonic acid, a fatty acid known for its antimicrobial property and low toxicity in small concentrations (ZHANG et al., 2012). Among other fatty acids, hexonic acid's antimicrobial activity against *S. aureus* has also been reported by Zhang et al. (2012).

There are few reports of quantitative analyzes of the antimicrobial effect of butanedioic acid (COBAN, 2020). A mixture of butanedioic acid and oregano essential oil has been reported by Purohit and Mohan (2019) as being efficient to reduce the *Salmonella* growth. Gao (2012) also verified the antimicrobial effect of butanedioic acid, at minimum inhibitory concentrations of 1,667, 6,667, and 32,000 mg/mL against, respectively, *E. coli*, *B. subtilis*, and *S. suis* detected in Japanese apricot fruits. There is also few information about the antimicrobial effect of octatriacontyl on pathogenic bacteria. El Shafay, Ali e El-Sheekh (2016) reported the presence of this fatty acid in red algae extracts, which showed antimicrobial activity in *S. aureus*.

Octanedioic acid is a food-grade chemical, approved by the Food and Drug Administration (CFR 184.1025) and considered safe (JOHNY et al., 2009). Díaz De Rienzo et al. (2016) showed that octanoic acid, a short-chain saturated fatty acid, had an antimicrobial effect against a series of gram-positive and gram-negative bacteria, among them *S. aureus*. Nair et al. (2005) investigated the effect of octanoic acid against bacteria that cause bovine mastitis and found that it was effective against *S. aureus*, although in a less pronounced manner. According to Vasudevan et al. (2005), concentrations of 50 and 100 mM octanoic acid were able to reduce the population of *S. enteritidis* by ~5.0 log10cfu/mL in 1 minute and inactivate the pathogen in 24h. Although this fatty acid mechanism of action has not yet been fully elucidated, changes in the permeability of the bacterial membrane and acidification of the cytoplasmic medium due to the presence of dissociated forms of octanoic acid are hypotheses suggested to explain the antimicrobial effect.

FINAL CONSIDERATIONS

The strategy resulted in the indication of the occurrence of the bioreduction of silver ions present in solutions of molarities 0.5, 0.75, and 1 mM, by *Neodesmus* sp. and *Desmodesmus* sp. cell-free culture supernatants. The reaction synthesized silver particles, as

observed in the resulting spectra of the spectroscopy UV-Vis. Regarding the microalgal biomass, ethanolic and ethyl acetate intracellular extracts of *Desmodesmus* sp. demonstrated antimicrobial effect against *S. typhimurium* and *S. aureus* (ethanolic). The identification of fatty acids by GC-MS in *Desmodesmus* sp. revealed a series of compounds with antimicrobial activities already reported. Further studies can be carried out to evaluate the possible antimicrobial effect of the silver nanoparticles themselves and also the utilization of other solvents in the extracting process of the intracellular metabolites from microalgae.

ACKNOWLEDGMENTS

This study was financed by the Coordenação de Aperfeiçoamento de Pessoal de Nível Superior – Brasil (CAPES) – Finance Code 001 and Fundação de Amparo à Pesquisa de Minas Gerais (FAPEMIG)

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