UNIVERSIDADE FEDERAL RURAL DE PERNAMBUCO

PRÓ-REITORIA DE PÓS-GRADUAÇÃO

PROGRAMA DE PÓS-GRADUAÇÃO EM RECURSOS PESQUEIROS E AQUICULTURA

LAENNE BARBARA SILVA DE MORAES

OTIMIZAÇÃO DO CULTIVO DE *Haematococcus pluvialis* PARA MAIOR PRODUTIVIDADE EM BIOMASSA E BIOMOLÉCULAS

Recife

2023

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Tese apresentada ao Programa de Pós-Graduação em Recursos Pesqueiros e Aquicultura da Universidade Federal Rural de Pernambuco como exigência para obtenção do título de Doutora.

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Recife,

Setembro / 2023

Dados Internacionais de Catalogação na Publicação
Universidade Federal Rural de Pernambuco
Sistema Integrado de Bibliotecas
Gerada automaticamente, mediante os dados fornecidos pelo(a) autor(a)

M828o Moraes, Laenne Barbara Silva de
Otimização do cultivo de Haematococcus pluvialis para maior produtividade em biomassa e biomoléculas / Laenne Barbara Silva de Moraes. - 2023.
97 f. : il.

Orientador: Ranilson de Souza Bezerra.
Coorientador: Alfredo Olivera Galvez.
Inclui referências e anexo(s).

Tese (Doutorado) - Universidade Federal Rural de Pernambuco, Programa de Pós-Graduação em Recursos Pesqueiros e Aquicultura, Recife, 2023.

1. biomassa. 2. metabólitos. 3. astaxantina. 4. L-asparaginase. 5. biorremediação. I. Bezerra, Ranilson de Souza, orient. II. Galvez, Alfredo Olivera, coorient. III. Título

CDD 639.3

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Tese julgada adequada para obtenção do título de Doutora em Recursos Pesqueiros e Aquicultura. Defendida e aprovada em 28/09/2023 pela seguinte Banca Examinadora.

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Dedicatória

Dedico esta tese aos meus pais pelo apoio incondicional em toda minha formação e ao meu irmão pela parceria.

Agradecimentos

A Deus, pelo dom da vida e por me permitir fazer tudo o que fiz até aqui.

À minha família por todo amor e suporte, em especial, a meus pais Mauricélia Moraes e Sidnei Moraes, e meu irmão Douglas Moraes.

A todos os meus amigos, pelo apoio emocional e incentivo nos dias difíceis.

À Universidade Federal Rural de Pernambuco, por oferecer condições para uma excelente formação desde a graduação. Aos professores do Departamento de Pesca e Aquicultura (DEPAq) e Programa de Pós-graduação em Recursos Pesqueiros, por todo conhecimento compartilhado, em especial, aos professores das bancas examinadoras.

Ao orientador, Prof. Dr. Ranilson de Souza Bezerra, pelo apoio e ensinamentos.

Ao coorientador, Prof. Dr. Alfredo Olivera Gálvez, pela confiança desde o primeiro período da graduação e todo o aprendizado.

Ao Laboratório de Produção de Alimento Vivo (LAPAVI) do DEPAq e todos os que já formaram parte nesses 11 anos, deixando suas contribuições e amizades, em especial, à Géssica Cavalcanti pelo suporte em todos os momentos, e à Bruna Adriele, pela contribuição e amizade. Assim como, aos amigos dos demais laboratórios do DEPAq, por todo o tempo partilhado.

Aos laboratórios do DEPAq (LASAq, LACAR, LAPAq e DIMAR), do Departamento de Zootecnia (DZ) da UFRPE (BIOPA) e do Departamento de Bioquímica da UFPE (LABENZ) que contribuíram com equipamentos e materiais.

À planta piloto de produção de microalgas da Universidade Federal do Rio Grande do Norte / Petrobras, representada pelo professor Dr. Graco Aurelio Viana, pelo período em que participei do projeto e toda a experiência adquirida.

À Universidade de Almería (Espanha) e ao grupo de pesquisa Biotecnologia de Microalgas Marinhas pelo acolhimento no Doutorado sanduíche. De modo especial, aos professores Francisco García Camacho, Astério Sánchez Mirón, María del Carmen Cerón García e Lorenzo López Rosales, e companheiros de laboratório Yolanda Jerez, Salim Kichouh, German Escudero e Danielle Rosa. Bem como, à CAPES, através do Programa Institucional de Internacionalização (PrInt), pela concessão da bolsa de Doutorado-Sanduíche no Exterior.

Aos órgãos de fomento CAPES, CNPq e FACEPE, pela concessão das bolsas durante todo o período de formação e suporte financeiro, essenciais para o desenvolvimento desta pesquisa e formação como pesquisadora.

Resumo

A microalga *Haematococcus pluvialis* tem como bioproduto de maior valor econômico a astaxantina, além de ser capaz de produzir outros metabólitos de alto valor, como proteínas, lipídios, ácidos graxos e carboidratos. O presente estudo propõe modificações nas fontes e estratégias de alimentação de nitrogênio e carbono orgânico, bem como a utilização de efluente como meio de cultura, a fim de aumentar a produção de biomassa e de metabólitos de alto valor da *H. pluvialis*, associando ao aproveitamento do efluente da produção de tilápia em um modelo de economia circular. A primeira parte do estudo propõe uma mudança no modo de fornecimento de nitrogênio, através da estratégia de alimentação por pulsos, e o uso de diferentes fontes de nitrogênio (*i.e.*, $NaNO_3$, NH_4NO_3 e $(NH_2)_2CO$) para aumentar a produção de biomassa de *H. pluvialis*, carotenoides totais e astaxantina. Na segunda parte, utilizou-se diferentes fontes de carbono orgânico (*i.e.*, acetato de sódio, melaço e glicerol) e estratégias de alimentação (*i.e.*, sem pulsos-WPF e com pulsos-PF) a fim de aumentar a produção de biomassa e metabólitos de alto valor de *H. pluvialis*. Na terceira parte foi proposto um sistema integrado para o tratamento simultâneo de efluente do cultivo de tilápia do Nilo e produção de astaxantina utilizando a microalga *H. pluvialis*. Os cultivos foram realizados em escala laboratorial, sendo avaliadas as variáveis de crescimento. Logo, as biomassas foram processadas, através de centrifugação e liofilização, para realização de análises quantitativas e qualitativas dos metabólitos. Os resultados das variáveis de crescimento obtidos na primeira parte do estudo apresentaram diferenças significativa para densidade celular máxima, sendo maiores para $NaNO_3$-PF (176 x 10^4 células mL^{-1}) e $(NH_2)_2CO$-PF (165 x 10^4 células mL^{-1}). O rendimento e a produtividade de biomassa nas fases vegetativa e cística foram maiores para cultivos com pulsos. Por outro lado, os teores e concentrações de carotenoides totais e astaxantina foram maiores em NH_4NO_3-WPF (teor de astaxantina ~ 23 mg g^{-1}), sendo influenciados pela depleção de nitrogênio e variação de pH. Quanto ao efeito das fontes e estratégias de alimentação de carbono orgânico no rendimento em biomassa, foram obtidos maiores valores com glicerol-WPF (1,275 g L^{-1}) e acetato-PF (1,264 g L^{-1}). A composição da biomassa em termos de proteínas e lipídios apresentou influência tanto da fonte quanto da estratégia de alimentação de carbono orgânico. Os tratamentos melaço-WPF e glicerol-PF apresentaram maiores concentrações de ácidos graxos e PUFA, em média 5,5 e 2,5% do peso seco (P.S.), respectivamente. Em relação aos níveis de carotenoides, a astaxantina apresentou maior concentração sob glicerol-WPF e melaço-PF, em torno de 315 mg L^{-1} (6% P.S.). Além dos metabólitos já conhecidos em *H. pluvialis*, foi reportada pela primeira vez a enzima L-asparaginase, com atividade máxima de 256 ± 24 IU mg^{-1} de biomassa seca. Por outro lado, a utilização do efluente como meio de cultura em sistema integrado, apesar de apresentar menor densidade celular e rendimento de biomassa, resultou em biomassa com alto teor de astaxantina (10 mg g^{-1}) e atividade antioxidante (89% de inibição dos radicais DPPH). Além disso, esse sistema integrado contribuiu significativamente para a remediação do efluente (remoção de 98% de N e 91% de P). Diante disso, pode-se concluir que modificações nas fontes e estratégias de alimentação de nitrogênio e carbono orgânico incrementam a produção de biomassa e metabólitos de alto valor de *H. pluvialis*. Adicionalmente, *H. pluvialis* pode ser usada para tratamento econômico de efluentes de aquicultura com produção simultânea de astaxantina, com potencial aplicação em *aquafeed* ou outros setores industriais, como um exemplo de economia circular.

Palavras-chave: biomassa; metabólitos; astaxantina; L-asparaginase; biorremediação.

Abstract

The microalgae *Haematococcus pluvialis* has astaxanthin as its bioproduct of greatest economic value, in addition to being capable of producing other high-value metabolites, such as proteins, lipids, fatty acids and carbohydrates. The present study proposes modifications in the sources and feeding strategies of nitrogen and organic carbon, as well as the use of effluent as a culture medium, in order to increase the production of biomass and high-value metabolites of *H. pluvialis* and promote the economy circular. The first part of the study proposes a change in the nitrogen supply mode, through the pulse feeding strategy, and the use of different nitrogen sources (*i.e.*, $NaNO_3$, NH_4NO_3 and $(NH_2)_2CO$) to increase the production of biomass from *H. pluvialis* and astaxanthin. In the second part, different sources (*i.e.*, sodium acetate, molasses and glycerol) and feeding strategies (*i.e.*, without pulses-WPF and with pulses-PF) of organic carbon were used in order to increase the production of biomass and high-value metabolites *H. pluvialis*. In the third part, an integrated system was proposed for the simultaneous treatment of effluent from Nile tilapia cultivation and astaxanthin production using the microalgae *H. pluvialis*. The cultures were carried out on a laboratory scale, and the growth variables were evaluated. Therefore, the biomasses were processed, through centrifugation and freeze-drying, to carry out quantitative and qualitative analyzes of the metabolites. The results of the growth variables obtained in the first part of the study showed significant differences for maximum cell density, being greater for $NaNO_3$-PF (176×10^4 cells mL^{-1}) and $(NH_2)_2CO$-PF (165×10^4 cells mL^{-1}). Biomass yield and productivity in the vegetative and cystic phases were higher for pulsed crops. On the other hand, the contents and concentrations of total carotenoids and astaxanthin were higher in NH_4NO_3-WPF (astaxanthin content ~ 23 mg g_{-1}), being influenced by nitrogen depletion and pH variation. Regarding the effect of organic carbon sources and feeding strategies on biomass yield, higher values were obtained with glycerol-WPF (1.275 g L^{-1}) and acetate-PF (1264 g L^{-1}). The composition of the biomass in terms of proteins and lipids was influenced by both the source and the organic carbon feeding strategy. The molasses-WPF and glycerol-PF treatments showed higher concentrations of fatty acids and PUFA, on average 5.5 and 2.5% P.S., respectively. Regarding carotenoid levels, astaxanthin presented the highest concentration under glycerol-WPF and molasses-PF, around 315 mg L^{-1} (6% P.S.). In addition to the metabolites already known in *H. pluvialis*, the enzyme L-asparaginase was reported for the first time, with maximum activity of 256 ± 24 IU mg^{-1} of dry biomass. On the other hand, the use of effluent as a culture medium in an integrated system, despite presenting lower cell density and biomass yield, resulted in biomass with a high astaxanthin content (10 mg g^{-1}) and antioxidant activity (89% inhibition of DPPH radicals). Furthermore, this integrated system contributed significantly to the remediation of the effluent (removal of 98% of N and 91% of P). Therefore, it can be concluded that modifications in the sources and feeding strategies of nitrogen and organic carbon increase the production of biomass and high-value metabolites of *H. pluvialis*. Additionally, *H. pluvialis* can be used for economical treatment of aquaculture effluents with simultaneous production of astaxanthin, with potential application in aquafeed or other industrial sectors, as an example of a circular economy.

Keywords: biomass; metabolites; astaxanthin; L-asparaginase; bioremediation.

Lista de figuras

Artigo científico I

Artigo científico II

Artigo científico III

Lista de tabelas

Artigo científico I

Artigo científico II

Artigo científico III

Sumário

1- Introdução

1.1- Contextualização da pesquisa

1.1.1- Aplicações e características do cultivo de microalgas

As microalgas são seres microscópicos, fotossintetizantes, capazes de capturar energia solar, nutrientes e dióxido de carbono, convertendo em biocompostos, tais como aminoácidos, proteínas, enzimas, lipídios e carotenoides. Por isso, sua aplicação pode ser destinada à diversas áreas, como aquicultura, indústria farmacêutica, biomedicina, área de alimentos, biorremediação, biofixação de CO_2, produção de biocombustíveis, entre outras (YAP *et al*., 2021). No entanto, a capacidade de produzir tais moléculas depende da espécie e das condições de cultivo empregadas, como temperatura, pH, salinidade, luminosidade e meio de cultura (SOUSA *et al*., 2023).

O meio de cultura influencia o crescimento celular, sendo o principal responsável pela produtividade do cultivo, estimulando ou inibindo o crescimento de acordo com a disponibilidade dos nutrientes, além de determinar a composição das células e a síntese de biomoléculas (SHUBA e KIFLE, 2018; SOUSA *et al*., 2023). O carbono e o nitrogênio são uns dos principais elementos responsáveis pelo crescimento e desenvolvimento algal, podendo ser disponibilizado na forma inorgânica e orgânica (LI *et al*., 2020). O fósforo também é um elemento essencial, pois auxilia nos processos metabólicos, na conversão de energia e na fotossíntese (ZENG *et al*., 2021). Portanto, adequadas relações nitrogênio:fósforo (N:P) e carbono:nitrogênio (C:N) são importantes para promover o crescimento e a produção de metabólitos de microalgas (CHRISTIAN *et al*., 2018; NAHIDIAN *et al*., 2018).

Por outro lado, determinar estratégias de fornecimento de nutrientes na produção de microalgas (*e.g.*, contínuas, escalonadas, pulsadas) é importante para viabilizar o cultivo, pois permite incrementar a produção de biomassa e compostos bioativos, como lipídios, polissacarídeos e pigmentos (LIU *et al*., 2018; XIE *et al*., 2019). Na alimentação contínua, a fonte de carbono é fornecida continuamente ao longo do período de cultivo e mantida de forma constante ao longo do tempo de cultivo. A alimentação escalonada consiste na adição de carbono em estágios distintos, sendo aplicadas apenas em cultivos descontínuos (DEVASYA e BASSI, 2021). A alimentação por pulsos de nutrientes envolve a adição intermitente de nutrientes durante o crescimento das microalgas, podendo resultar em maior eficiência na absorção de nutrientes, redução de custos, controle metabólico, flexibilidade e menor impacto

ambiental (MA *et al*., 2023). Esse método é particularmente valioso em aplicações industriais que buscam eficiência e sustentabilidade.

1.1.2- *Haematococcus pluvialis* e produção de astaxantina

A microalga *Haematococcus pluvialis* (Chlorophyta) tem despertado grande interesse pelo seu conteúdo celular e sua capacidade de produzir astaxantina (3,3′-dihidroxi-β,β-caroteno-4, 4′-diona), sendo comumente cultivada para a extração desse carotenoide (MOTA *et al*., 2022). A produção anual global de *H. pluvialis* está estimada em aproximadamente 800 toneladas, sendo a China responsável por mais da metade da produção (TENG *et al*., 2023). A totalidade dessa produção é destinada ao mercado de astaxantina, que está em constante desenvolvimento e atualmente é avaliado em aproximadamente US$ 600 milhões (MOTA *et al*., 2022).

O mercado de astaxantina ainda é dominado pela forma sintética, com menor custo de produção (US$ 1.000 kg^{-1}) e menor valor de mercado (US$ 2.000 kg^{-1}), entretanto não é recomendada para uso humano, sendo potencialmente tóxica e cancerígena, além de possuir propriedades mais atenuadas comparada à natural (MOTA *et al*., 2022). Os custos de produção da astaxantina de *H. pluvialis* pode variar entre US$ 2.500 e 7.000 kg^{-1}, no entanto seu valor de mercado varia entre US$ 7.000 e 15.000 kg^{-1} (LI *et al*., 2011; LEU e BOUSSIBA, 2014).

Esse carotenoide é amplamente utilizado na indústria farmacêutica, nutracêutica, de cosméticos e de alimentos, por ter propriedades antioxidante, anti-inflamatória, antitumoral, antidiabética e imunomoduladora, além de ser utilizada na aquicultura, tanto para pigmentação quanto para melhorar a resposta imune e o desempenho zootécnico de camarões e peixes (MOTA *et al*., 2022; RITU *et al*., 2023).

A astaxantina destaca-se como um antioxidante de notável eficácia devido à capacidade de neutralizar radicais livres e atenuar o estresse oxidativo. Sua estrutura química caracterizada por extensas cadeias conjugadas de átomos de oxigênio possibilita a eficaz captura de radicais livres, conferindo-lhe propriedades protetoras que previnem danos celulares e preservam a integridade das membranas celulares contra processos oxidativos (DUTTA *et al*., 2023). Além disso, a astaxantina influencia positivamente a atividade de enzimas antioxidantes intracelulares, como a superóxido dismutase, e manifesta propriedades anti-inflamatórias que contribuem para a saúde celular e a mitigação de doenças relacionadas ao estresse oxidativo (WU *et al*., 2019).

Com relação a outros organismos produtores de astaxantina, como bactérias, leveduras, plantas e outras microalgas, a *H. pluvialis* detém a maior capacidade de acumular astaxantina, com cerca de 4-5% do conteúdo celular (REN *et al*., 2021). A síntese de astaxantina nessa microalga está relacionada a mudanças morfológicas, fisiológicas e bioquímicas nas células, devido a fatores ambientais, como alta luminosidade e disponibilidade de nutrientes, dentre eles, nitrogênio e carbono orgânico (DORIA *et al*., 2018; YU *et al*., 2022).

Neste contexto, modelos de alimentação descontínua e privação de nutrientes no cultivo da *H. pluvialis* são estratégias eficazes para a produção de astaxantina. A presença ou ausência de nitrogênio, por exemplo, desempenha papel fundamental nesse processo, tornando essas estratégias de manipulação metabólica eficientes (MORAES *et al*., 2023).

Além da astaxantina, a *H. pluvialis* possui em sua composição altas concentrações de proteínas, carboidratos e lipídios, variando de acordo com o modo de cultivo e a fase do ciclo de vida (PEREIRA e OTERO, 2020; MARINHO *et al*., 2021). O ciclo de vida dessa espécie é formado pelas fases vegetativa, onde há intensa reprodução celular, e cística, quando é exposta à algum fator de estresse ambiental e/ou escassez de nutrientes, sintetizando astaxantina (MARINHO *et al*., 2021). Na fase vegetativa, as células contêm, em peso seco, 29-45% de proteínas, 15-17% de carboidratos e 20-25% de lipídios, enquanto no estágio cístico possuem 17-25-% de proteínas, 36-40% de carboidratos e até 37% de lipídios (SHAH *et al*., 2016).

1.1.3- L-asparaginase: biomolécula de importância biotecnológica

L-asparaginase é uma enzima de alto valor identificada em algumas espécies de microalgas, que catalisa a asparagina, um aminoácido essencial para células leucêmicas, em amônia e aspartato. Essa enzima é usada no tratamento da leucemia linfoblástica, devido à sua capacidade de inibir a biossíntese de proteínas nos linfoblastos (BATOOL *et al*., 2016). A L-asparaginase é a primeira enzima terapêutica com propriedades antineoplásicas estudada amplamente, tendo como principal fonte para fins comerciais a bactéria *Escherichia coli*, especialmente suas versões recombinantes e geneticamente modificadas. Apesar de produzir altas concentrações dessa enzima, a *E. coli* pode estar associada a efeitos citotóxicos da enzima (MUNEER *et al*., 2020).

Essa biomolécula pode ser isolada a partir de uma gama de microrganismos, incluindo terrestres e aquáticos, entretanto muitos desses estão associados a reações

alérgicas e toxicidade. Além disso, a L-asparaginase pode apresentar propriedades físicas e bioquímicas com diferentes parâmetros cinéticos, que pode influenciar a estabilidade e a atividade da enzima, a depender da fonte de obtenção (MUNEER *et al*., 2020). Portanto, até hoje são estudadas outras fontes e formas de produzir L-asparaginase, seja através de modificações genéticas ou mudanças na forma de cultivar os microrganismos (BATOOL *et al*., 2016; DORIYA e KUMAR, 2016). A necessidade de versões novas e modificadas dessa enzima é de grande interesse tanto na biotecnologia quanto na medicina, bem como na indústria alimentícia quando é utilizada para impedir a produção de acrilamida em alimentos ricos em carboidratos (MUNEER *et al*., 2020).

A atividade de L-asparaginase foi identificada em algumas espécies de microalgas, como *Chlorella vulgaris*, *Spirulina maxima*, *Chlamydomonas* sp. e *Oscillatoria terebriformis* (PAUL, 1982; EBRAHIMINEZHAD *et al*., 2014; ABD EL-BAKY e EL-BAROTY, 2016; ELKOMY, 2018). Em *H. pluvialis* foi identificado o gene que expressa L-asparaginase quando a microalga foi submetida ao mutagênico químico N-metil-N-nitro-N nitrosoguanidina (WANG *et al*., 2005), no entanto não há registros da atividade de L-asparaginase nesta espécie de microalga. As perspectivas futuras dos estudos sobre essa biomolécula, visam aumentar a atividade da L-asparaginase e eliminar os efeitos colaterais, sendo as microalgas fontes promissoras.

1.1.4- *Haematococcus pluvialis* no contexto da bioeconomia

A *H. pluvialis* possui a capacidade de se adaptar a diversos ambientes, podendo alterar sua rota metabólica como resposta às mudanças ambientais (WANG *et al*., 2013). São capazes de crescer em sistema fotoautotrófico, ao utilizar carbono inorgânico e luz como fonte de energia; heterotrófico, quando se utilizam de carbono orgânico e fonte de energia orgânica, sem presença de luz; e mixotrófico, caracterizado pelo uso de carbono orgânico e inorgânico, luz e compostos orgânicos como fontes de energia (PEREZ-GARCÍA e BASHAN, 2015).

Tem-se demonstrado que o cultivo de *H. pluvialis* em sistema mixotrófico resulta em maiores taxas de crescimento e produtividade de biomassa (PARK *et al*., 2014; PANG *et al*., 2017); e que o acréscimo de carbono orgânico no sistema e a alta relação Carbono:Nitrogênio (C:N) induz a produção de astaxantina (CHRISTIAN *et al*., 2018; ZHANG *et al*., 2018). Contudo, a inclusão de algum fator de estresse, modificando a maquinaria da célula para estimular a produção de astaxantina, resulta na

redução ou inibição da divisão celular (PARK *et al*., 2014). Portanto, é fundamental que essa condição de estresse seja estimulada após a fase de maior crescimento, ou seja, na fase estacionária.

Essa característica de crescimento em meios mixotróficos permite que *H. pluvialis* seja produzida também em diferentes tipos de efluentes: doméstico (WU *et al*., 2013), suinocultura (LEDDA *et al*., 2016) e bioetanol (HAQUE *et al*., 2017); demonstrando efeito biorremediador ao absorver compostos nitrogenados e fosfatados do meio. Portanto, uma alternativa à produção da *H. pluvialis* é a utilização do efluente de sistemas de recirculação aquícola (RAS). O sistema RAS Oasis® é uma tecnologia social para a produção de pescado no semiárido nordestino, uma vez que preconiza produção em pequenos módulos com alta produtividade, reuso da água com praticamente zero renovação, manejo simplificado, possibilidades de cultivo com várias espécies aquáticas e hortaliças, baixo custo de implementação e manutenção, além do aproveitamento total dos resíduos gerados. Por conseguinte, esse sistema de produção aplica o conceito de economia circular, minimizando a entrada de recursos, o desperdício, a emissão e o vazamento de energia (GEISSDOERFER *et al*., 2017).

Apesar de ser favorável como agente biorremediador e produtor de astaxantina, *H. pluvialis* possui algumas características desfavoráveis em comparação com outras espécies cultivadas comercialmente, como baixas taxa de crescimento e produtividade de biomassa, aliado à dificuldade de conciliar as condições ótimas para crescimento e indução de astaxantina (WAYAMA *et al*., 2013; CHENG *et al*., 2016). Sendo assim, são necessários diversos estudos acerca de modificações de condições de cultivo, como modificações nas fontes e estratégias de alimentação de nitrogênio e carbono orgânico, para incrementar a produção de biomassa e metabólitos de alto valor de *H. pluvialis*.

Adicionalmente, a utilização de outros compostos bioativos promove o aproveitamento total da biomassa em um modelo de biorrefinaria, transcendendo a extração de astaxantina e abrangendo a valorização de outros compostos de importância biotecnológica, permitindo destiná-la a ampla gama de aplicações (MOHAN *et al*., 2020). Essa abordagem não apenas otimiza o aproveitamento dos recursos da *H. pluvialis*, como também diminui a geração de resíduos. Dessa forma, esse modelo está alinhado aos princípios da economia circular, que visam a eficaz conclusão do ciclo de vida dos produtos, estimulando práticas mais sustentáveis tanto na produção quanto no consumo (LEONG *et al*., 2021). Portanto, por meio da exploração desse modelo é possível alcançar uma abordagem holística e ecologicamente responsável para a

obtenção de recursos valiosos a partir dessa microalga (MOREIRA *et al*., 2023). Nesse contexto de produção sustentável, também pode ser empregado um sistema integrado para tratamento simultâneo de efluentes de aquicultura e produção de astaxantina por *H. pluvialis*, incentivando a economia circular.

Diante disso, este estudo foi dividido em três partes: i. Cultivo de *Haematococcus pluvialis* e produção de astaxantina utilizando diferentes fontes de nitrogênio com estratégia de alimentação por pulsos; ii. Efeitos de diferentes fontes e estratégias de alimentação de carbono orgânico na produção de metabólitos de alto valor de *Haematococcus pluvialis*; iii. Sistema integrado para tratamento simultâneo de efluentes de tilápia e produção de astaxantina por *Haematococcus pluvialis*.

1.2- Objetivos

1.2.1- Objetivo geral

Avaliar a influência de diferentes condições de cultivo na produtividade em biomassa, composição bioquímica, obtenção de metabólitos e efeito biorremediador da microalga *Haematococcus pluvialis*.

1.2.2- Objetivos específicos

- Avaliar a produtividade da *H. pluvialis* e produção de astaxantina sob diferentes fontes e estratégias de alimentação de nitrogênio;
- Analisar a composição centesimal e o perfil de carotenoides e ácidos graxos da *H. pluvialis* sob diferentes fontes e estratégias de alimentação de carbono orgânico;
- Investigar a atividade de L-asparaginase na microalga cultivada em diferentes fontes e estratégias de alimentação de carbono orgânico;
- Avaliar o crescimento, a produção de astaxantina e o efeito biorremediador da microalga *H. pluvialis* cultivada no efluente do sistema de recirculação de tilápia.

1.3- Hipóteses

- Modificações de fontes e estratégias de alimentação de nitrogênio e carbono orgânico incrementam a produção de biomassa e biomoléculas da *Haematococcus pluvialis*;
- *H. pluvialis* cultivada com diferentes fontes e estratégias de alimentação de carbono orgânico possui atividade de L-asparaginase;
- A *H. pluvialis* é capaz de utilizar nutrientes do efluente do sistema de recirculação de tilápia para crescimento e produção de astaxantina, além de atuar como agente biorremediador.

2- Artigo científico I: *Haematococcus pluvialis* cultivation and astaxanthin production using different nitrogen sources with pulse feeding strategy

Artigo publicado no periódico "Biomass Conversion and Biorefinery" (Anexo I).

Haematococcus pluvialis cultivation and astaxanthin production using different nitrogen sources with pulse feeding strategy

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Abstract

The microalga *Haematococcus pluvialis* has astaxanthin as the most economically valuable compound. However, there are challenges related to its cultivation and low biomass productivity. Therefore, the present study proposes a change in the nitrogen supply mode, through the pulse feeding strategy, and the use of different N sources to increase the biomass of *H. pluvialis* and astaxanthin production. The two-factor experimental design had as factor 1: three sources of nitrogen - $NaNO_3$, NH_4NO_3 and $(NH_2)_2CO$; and factor 2: nitrogen feeding strategy – pulse feeding (PF) and without pulse feeding (WPF). Nitrogen source of the BBM ($NaNO_3$) was replaced by NH_4NO_3 or $(NH_2)_2CO$, maintaining the original [N] in WPF; while in PF, N sources were added by pulses. The results of growth variables showed a significant difference for maximum cell density, with higher values for $NaNO_3$-PF (176×10^4 cell mL^{-1}) and $(NH_2)_2CO$-PF (165×10^4 cell mL^{-1}). Yield and biomass productivity in the vegetative and cystic phases were higher for cultures in PF. Higher N content was found in the PF medium, providing greater cell reproduction, however, excess of nitrogen after the exponential growth phase limits carotenogenesis. The contents and concentrations of total carotenoids and astaxanthin, in general, were higher in NH_4NO_3-WPF (astaxanthin content ~ 23 mg g^{-1}) being influenced by nitrogen depletion and pH variation. Thus, nitrogen pulse feeding strategy provided higher biomasses of *H. pluvialis* and the decrease in the pulses concentrations can result in higher astaxanthin production due to the lower N residue.

Keywords: microalgae; culture medium; nitrogen supply; biomass yield; carotenoid.

1. Introduction

Haematococcus pluvialis (Chlorophyta) has the ability to produce astaxanthin (3,3′-dihydroxy-β,β-carotene-4, 4′-dione) and it is commonly cultivated for this purpose [1]. This microalga's market is estimated at US$ 240 million, and the world market for astaxanthin, from different origins, is constantly growing and is valued at approximately US$ 600 million, showing the importance of increasing the production of *H. pluvialis* [2,3]. Astaxanthin is a carotenoid widely used in the pharmaceutical, nutraceutical, cosmetic and food industries [4], as it has antioxidant (10 times more than β-carotene), anti-inflammatory, antitumor, antidiabetic and immunomodulatory properties [5], in addition to being widely used in aquaculture, both for pigmentation and to improve the immune response and zootechnical performance of shrimp and fish [6].

Morphological, physiological and biochemical changes in cells are characteristics of astaxanthin production in this microalga, which are a result of the influence of environmental factors such as high luminosity and presence/absence of nutrients [7]. In relation to other astaxanthin-producing organisms, such as bacteria, yeasts, plants and other microalgae, *H. pluvialis* is considered to be the species with the greatest capacity to accumulate this carotenoid (up to 5% of dry biomass) [4]. However, feasibility of obtaining this molecule from *H. pluvialis* depends on the cultivation conditions, such as temperature, pH, salinity, luminosity and culture medium. Therefore, for production, these conditions are manipulated to achieve optimal conditions and promote cell growth as well as astaxanthin synthesis [8].

The culture medium influences cell growth, being the main responsible for the productivity obtained, since it stimulates or inhibits growth according to the availability of nutrients. Besides this, it is also able to influence and determine the cellular composition of microalgae [9]. Among the nutrients used for the preparation of culture media, nitrogen is one of the main responsible for the growth and development of microalgae, and can be made available in the form of nitrate, urea or ammonia [10]. In addition to variations in nitrogen sources and concentrations, feeding strategies (*e.g.*, continuous, staged and pulsed) can also be modified. Different feeding strategies of nitrogen sources, as well as of carbon and phosphorus, modify the physiological metabolism of microalgae, and can provide greater productivity in biomass or metabolites of high economic value [11,12]. Pulse feeding strategy has already been used for several species of microalgae, such as *Scenedesmus acuminatus*, *Chlorella sorokiniana*, *Chlamydomonas reinhardtii* and *Nannochloropsis gaditana* [13,14,15,16],

and according to Devasya and Bassi (2021), it significantly increases biomass and biomolecules production in microalgae cultivation [14].

In *H. pluvialis*, the availability of nitrogen in the medium promotes cell division, while its deprivation stimulates the synthesis of astaxanthin [17]. Therefore, one-step cultivation can occur through moderate nitrogen starvation and moderate light, promoting growth and carotenogenesis, simultaneously. Meanwhile, two-stage cultivation works as follows: first, conditions are controlled to intensify cell growth - vegetative phase (*e.g.*, high nitrogen availability and low light intensity), and then, when maximum cell density is reached, carotenogenesis is stimulated - cystic phase (*e.g.*, nitrogen starvation and high light intensity) [8].

Compared to other commercially cultivated species, *H. pluvialis* has low biomass productivity, hence, changes in the culture medium are usually used to increase the production of this biomass [18]. Based on this, an adequate feeding strategy with nitrogen sources can be a promising alternative to achieve this goal. Also, since the biomass productivity of *H. pluvialis* is extremely important to obtain a high amount of astaxanthin, finding optimal cultivation conditions for the production of biomass has been the subject of numerous studies.

Thus, in the present study, the use of different nitrogen sources under the pulsed feeding strategy was investigated in the cultivation of the microalga *H. pluvialis* to increase cell multiplication, biomass, and carotenoids and astaxanthin production.

2. Material and methods

2.1. Strain and culture conditions

H. pluvialis was obtained from the Live Food Production Laboratory, at the Fisheries and Aquaculture Department of the Federal Rural University of Pernambuco. Cultures were developed in 500 mL bottles, with fresh water previously treated with chlorine (3 ppm), filtered (22 µm) and autoclaved (120 °C), and then enriched with modified Bold's Basal Medium (BBM) (Table 1) [19].

Table 1 Composition (mg L^{-1}) of Modified Bold's Basal Medium used in the *Haematococcus pluvialis* culture

Nutrients	**Concentration (mg L^{-1})**
$NaNO_3$	255
$CaCl_2.2H_2O$	25
NaCl	25
KOH	31
EDTA $Na.2H_2O$	50
K_2HPO_4	75
KH_2PO_4	175
$FeSO_4.7H_2O$	4.98
$MgSO_4.7H_2O$	75
H_3BO_3	11.42
$ZnSO_4.7H_2O$	1.412
$MnCl_2.4H_2O$	0.232
$CuSO_4.5H_2O$	0.252
$Na_2MoO_4.2H_2O$	0.192
$Co(NO_3)_2.6H_2O$	0.08

2.2. Experimental design

The experiment was carried out on a laboratory scale using a bifactorial design (3x2), with factor 1: three nitrogen sources - Sodium nitrate ($NaNO_3$), Ammonium nitrate (NH_4NO_3) and Urea ($(NH_2)_2CO$); and factor 2: nitrogen supply strategy – with pulse feeding (PF) and without pulse feeding (WPF).

The nitrogen source of the BBM ($NaNO_3$) was replaced by NH_4NO_3 or $(NH_2)_2CO$, maintaining the original N concentration (3 mM) in the combinations without the addition of pulses (WPF). Pulse feedings were carried out on the 1st, 6th and 9th days by adding 1.5, 3.0 and 3.0 mM of nitrogen, respectively.

H. pluvialis was inoculated with an initial concentration of 2 x 10^4 cells mL^{-1}, at a temperature of 22 °C, photoperiod 12h:12h and light intensity of 40 µmol photons m^{-2} s^{-1}, under constant aeration. Cultivation occurred under photoautotrophic conditions until the stationary growth phase (vegetative phase), when organic carbon (sodium acetate - 1.98 mg L^{-1}) was introduced and there was an increase in light intensity (100 µmol photons $m^{-2}s^{-1}$). These modifications were used as factors to stimulate the process of carotenogenesis (cystic phase) as well as natural deprivation of nitrogen. The temperature and pH variables were measured on days 1, 6, 9, 14 and 24 (based on key moments of change in growth and carotenogenesis phases), using a digital pH meter (Kmoon pH/EC-983).

2.3. Growth analysis and biomass harvesting

To evaluate growth samples were taken daily and fixed in formaldehyde (2%) for quantification using a hemocytometer (Neubauer chamber). With this data, the following parameters were calculated: maximum cell density (MCD); growth rate (K, Equation 1), which represents the number of cell divisions per day performed during the total culturing time, expressed as 'division d^{-1}'; specific growth rate (µ, Equation 2), which represents the cell growth rate during the exponential phase of the growth curve as a function of time, expressed as 'd^{-1}'; and doubling time, which corresponds to the time required for doubling the initial density, expressed as 'd $division^{-1}$' (DT, Equation 3) [20].

$$K = \frac{3.322}{(t - t_0) \times \mathrm{Log}\,(N \div N_0)} \qquad (1)$$

t = last incubation day (days); t_0= beginning incubation day (days); N = final cell number (cell mL^{-1}); N_0 = initial cell number (cell mL^{-1}).

$$\mu = \frac{\ln(N(t) \div N_0)}{(t - t_0)} \qquad (2)$$

N(t) = cell number (cell mL^{-1}); N_0 = initial cell number (cell mL^{-1}); t = time (days); t_0 = beginning incubation day (days).

$$DT = \frac{1}{K} \qquad (3)$$

The growth curves with the average daily cell density were fitted by approximating the logistic curve: $Y = P_1/1 + (P_2 – N_0/N_0.e^{-kt})$ [21].

The dry biomass yield (g L^{-1}) and biomass productivity (g L^{-1} d^{-1}) for vegetative and cystic phases were determined by filtering 10 mL aliquots of suspended cells through a Whatman GF/C glass microfiber filter (1.2 µm) and drying at 75 °C for 24 h [22].

2.4. Nitrogen analysis

Nitrate-N (NO_3-N) and Ammonia-N (NH_3-N) were analyzed in the culture medium on days 1, 6, 9, 14 and 24 (based on key moments of change in growth and carotenogenesis phases). Samples of 10 mL were filtered through a Whatman GF/C glass microfiber filter (1.2 µm), then the filtered volume was collected for analysis of NO_3-N [23] and NH_3-N [23]. The concentrations found were converted into total nitrogen concentration (mM).

2.5. Quantification of total carotenoids and astaxanthin

The concentrations of total carotenoids and astaxanthin were determined at the end of cultivation using a spectrophotometer. Carotenoid analysis was performed from a 10 mL aliquot of the algae suspension that was centrifuged (1700 x g, 10 min) and the precipitate incubated (70 °C, 10 min) in 10 mL of Dimethylsulfoxide (DMSO). This suspension was analyzed in a spectrophotometer (480 nm) and the concentration of total carotenoids was calculated: 4×OD480 [18]. To obtain the concentration of astaxanthin, 1 ml of algal suspension was centrifuged (1700 x g, 10 min) and the precipitate treated with a solution of KOH (5% (w/v)) in methanol (30% (v/v)), then incubated at 70 °C for 10 min to denature chlorophyll. Then, the suspension was centrifuged (1700 x g, 10 min) and glacial acetic acid (100 µL) and DMSO (5 mL) were added to the pellet, and maintained at 70 °C for 15 min. After final centrifugation (1700 x g, 10 min), the supernatant was analyzed in a spectrophotometer (490 nm) [18]. Astaxanthin concentration and content were calculated from the expressions [4.5×OD490×(Va/Vb)] and P/W, respectively. Where Va (mL) was the volume of DMSO, Vb (mL) was the volume of microalgae samples, P (mg L^{-1}) was the concentration of astaxanthin and W (g L^{-1}) denoted the dry biomass of microalgae per unit volume of medium [18].

2.6. Statistical analysis

The experiment was performed with 6 combinations and 4 replicates, totaling 24 experimental units. All data were presented as mean ± standard deviation (n = 4). The response variables were submitted to the Shapiro-Wilk normality test and to the Bartlett's test of homogeneity of variances. The pH and N data were transformed by log(x+1). All data were submitted to a two-way ANOVA followed by Tukey's test when a significant difference was observed. Pearson's correlation test and Principal Component Analysis (PCA) were applied to the physicochemical and growth variables. P values < 0.05 were considered statistically significant for all tests. Statistical analysis was performed using the R Core Team software [24].

3. Results and discussion

3.1. Cell growth and biomass production of H. pluvialis

H. pluvialis is characterized by achieving low growth rates, cell density and biomass, compared to other microalgae species [18]. The results of growth variables showed a significant difference for MCD (Table 2), where the combinations with pulse feeding strategy (PF) obtained higher densities, demonstrating that high concentrations of nitrogen in PF result in increased cell growth. The MCD can vary between 11 and 120 x 10^4 cell mL^{-1} [25,26,27], and in this study, a MCD of up to 195 x 10^4 cell mL^{-1} was obtained. Orosa *et al*. [26], using 3 - 12 mM of nitrate, reached a MCD of 110 - 120 x 10^4 cell mL^{-1}, in 15 days of culture and μ of 0.47 – 0.50 d^{-1}, corroborating this study. The specific growth rate (μ) of this species varies between 0.10 and 0.64 d^{-1} [25,26,28], and in the present study, the highest μ value was 0.5 d^{-1}, with a mean of 0.3 for all combinations (Table 2). As regards to DT, it can vary from 2.8 – 6.9 d $division^{-1}$ [10,27,28], a higher value than that found in the present study (2.7 – 2.8 d $division^{-1}$). Furthermore, the availability of nitrogen allows a longer duration of the cell division phase (vegetative phase), consequently, the day of MCD was later for the PF cultivation units, regardless of the nitrogen source (Table 2).

Table 2 Growth variables of *Haematococcus pluvialis* cultivated with different nitrogen sources and feeding strategies

Feeding strategy	Nitrogen sources	K (d^{-1})	μ (d^{-1})	DT (days)	MCD (x 10^4 cell mL^{-1})	MCD day
WPF	$NaNO_3$	0.348 ± 0.01	0.285 ± 0.19	2.73 ± 0.26	122.03 ± 18^{Ab}	15
	NH_4NO_3	0.355 ± 0.02	0.267 ± 0.17	2.73 ± 0.08	106.50 ± 20^{Bb}	17
	$(NH_2)_2CO$	0.363 ± 0.02	0.272 ± 0.18	2.66 ± 0.01	109.22 ± 17^{Bb}	17
PF	$NaNO_3$	0.375 ± 0.01	0.282 ± 0.19	2.61 ± 0.01	175.92 ± 20^{Aa}	19
	NH_4NO_3	0.363 ± 0.01	0.266 ± 0.17	2.75 ± 0.08	140.67 ± 13^{Ba}	19
	$(NH_2)_2CO$	0.356 ± 0.03	0.273 ± 0.18	2.77 ± 0.23	164.67 ± 10^{Aa}	19
	S	ns	ns	ns	*	-
	F	ns	ns	ns	***	-
	S x F	ns	ns	ns	ns	-

Mean ± standard deviation (n=4). Mean values for the same column with different letters indicate significant differences by a two-way ANOVA followed by Tukey's test (uppercase - nitrogen sources factor, lowercase - feeding strategy factor). S = factor 1 (nitrogen sources); F = factor 2 (feeding strategy); S x F = interaction between the two factors. ns = not significant; *** ($p < 0.001$); * ($p < 0.05$). WPF: without pulse feeding; PF: pulse feeding strategy; K: growth rate; μ: specific growth rate; DT: doubling time; MCD: maximum cell density.

This trend can be seen in the growth curves (Fig. 1), with a longer duration of the exponential phase (10 to 12 days, approximately) for treatments in which nitrogen pulses were applied. WPF, regardless of the N source, had a shorter exponential phase (between the 5th and 11th day of cultivation). On the other hand, MCD was influenced by N sources, obtaining higher densities with $NaNO_3$ and $(NH_2)_2CO$ under PF (Table 2). Different N sources affect microalgae growth and metabolite production [29]. Generally, for microalgae, the order of preference for nitrogen utilization is $NH_4^+ > NO_3^- > NO_2^- > (NH_2)_2CO$ [30], being ammonia the one that requires less energy cost to be assimilated [31]. For *H. pluvialis*, lower MCD were achieved with NH_4NO_3, possibly because in the presence of NH_4^+ many genes involved in the assimilation of NO_3^- and NO_2^- are inhibited [30]. In a study carried out by Göksan *et al*. [32], $NaNO_3$ also provided the highest MCD (25.3 x 10^4 cell mL^{-1}), while $(NH_2)_2CO$ resulted in the lowest MCD (17.4 x 10^4 cell mL^{-1}).

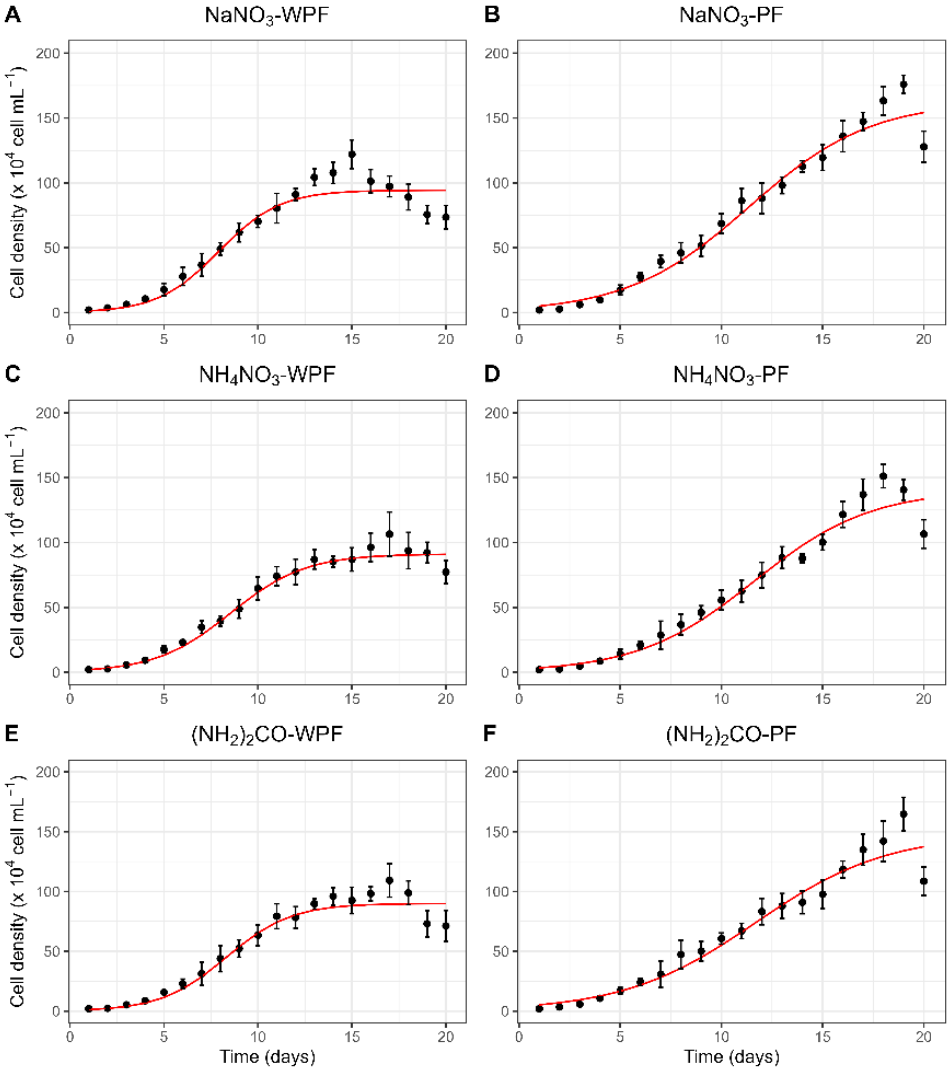

Fig. 1 Growth Curves of *Haematococcus pluvialis* cultivated with different nitrogen sources and feeding strategies

As stated by Devasya and Bassi [14], an increase in the number of cell populations due to the progression of the growth stage demands an increase in the level of substrates, hence, pulse feeding ensures the continuous availability of nutrients in the media and promotes the assimilation of nutrients that enhance cellular activities. Also, according to the same authors, pulse nitrogen feeding significantly increases biomass and lipid production in fed-batch cultures of *Nannochloropsis gaditana*. Similarly, Figueroa-Torres *et al*. [16] observed that fed-batch pulse feeding regime with organic

carbon (acetate) in the cultivation of *Chlamydomonas reinhardtii* increased the production of biomass (94%), starch (676%) and lipids (252%).

WPF cultivation units reached the stationary growth stage earlier, approximately on the 12th day (Fig. 1), which resulted in a shorter vegetative phase. The cells of *H. pluvialis* in the vegetative phase are undergoing intense division, and favorable conditions for growth must be provided, such as high nitrogen availability. Thus, a high concentration of nitrogen in the culture (up to approximately 18 mM) should provide a greater amount of biomass, mainly due to the increase in cell density [17]. Therefore, biomass yield in the vegetative phase were also influenced by the nitrogen feeding strategy, obtaining higher values in PF (Table 3).

Table 3 Yield (g L^{-1}) and productivity (g L^{-1} d^{-1}) of *Haematococcus pluvialis* biomass in the vegetative and cystic phases cultivated with different nitrogen sources and feeding strategies

Feeding strategies	Nitrogen sources	Vegetative yield (g L^{-1})	Cystic yield (g L^{-1})	Vegetative productivity (g L^{-1} d^{-1})	Cystic productivity (g L^{-1} d^{-1})
WPF	**$NaNO_3$**	0.71 ± 0.04^{Ab}	0.91 ± 0.02^{Ab}	0.050 ± 0.004^{Ab}	0.038 ± 0.001^{Ab}
	NH_4NO_3	0.75 ± 0.07^{Ab}	0.94 ± 0.03^{Ab}	0.054 ± 0.005^{Ab}	0.039 ± 0.001^{Ab}
	$(NH_2)_2CO$	0.65 ± 0.02^{Ab}	1.0 ± 0.04^{Ab}	0.047 ± 0.002^{Ab}	0.042 ± 0.002^{Ab}
PF	**$NaNO_3$**	086 ± 0.05^{Aa}	1.34 ± 0.06^{Aa}	0.061 ± 0.004^{Aa}	0.056 ± 0.003^{Aa}
	NH_4NO_3	0.89 ± 0.05^{Aa}	1.30 ± 0.14^{Aa}	0.063 ± 0.003^{Aa}	0.054 ± 0.006^{Aa}
	$(NH_2)_2CO$	0.82 ± 0.09^{Aa}	1.22 ± 0.04^{Aa}	0.059 ± 0.007^{Aa}	0.051 ± 0.002^{Aa}
	S	ns	ns	ns	ns
	F	***	***	***	***
	S x F	ns	ns	ns	ns

Mean ± standard deviation (n=4). Mean values for the same column with different letters indicate significant differences by a two-way ANOVA followed by Tukey's test (uppercase - nitrogen sources factor, lowercase - feeding strategy factor). S = factor 1 (nitrogen sources); F = factor 2 (feeding strategies); S x F = interaction between the two factors. ns = not significant; *** ($p < 0.001$). WPF: without pulse feeding; PF: pulse feeding.

Relative to dry biomass and biomass productivity, *H. pluvialis* can reach up to 0.5 – 2.8 g L^{-1} and 0.07 – 0.7 g L^{-1} d^{-1} [33,34,35,36], in the present study, the highest average biomass was 1.34 g L^{-1} and the maximum productivity was 0.18 g L^{-1} d^{-1}. According to the N-Nitrate concentration, the maximum biomass productivity can vary between 0.27 ([N] = 4.4 mM) and 0.30 g L^{-1} d^{-1} ([N] = 17.6 mM) [17]. When comparing different nitrogen sources, Göksan *et al*. [32] obtained higher dry biomass (1.2 g L^{-1}) with NH_4NO_3 ([N] = 3 mM) and lower biomass (0.65 g L^{-1}) with $(NH_2)_2CO$ ([N] = 4 mM).

The nitrogen content of the culture medium was evaluated over time, finding a higher mean concentration of N in PF (Table 4). Regarding the profile of the concentration of N during the cultivation (Fig. 2), it is possible to observe in PF greater availability of N from the 6th day onwards, due to the pulses. The feeding strategies created varied cellular activities that resulted in a heterogeneous cell growth status, resulting in variations in nitrogen depletion and residual nitrogen levels [14]. In WPF, N was significantly consumed until day 14, coinciding with the beginning of the stationary phase and, consequently, the transition to the cystic phase. Pulse feeding mode provided a short exposure to nitrogen starvation condition compared to WPF cultures, and this condition delayed the stationary phase of growth in PF. In the exponential phase of cultivation, the consumption of N is strongly linked to growth, and from the stationary phase, the cells start to absorb N, modifying the cellular composition [37]. Therefore, with the arrival of the stationary growth stage and the decrease of N concentration by the consumption of microalgae, the cultivation conditions were directed to stimulate carotenogenesis, with the insertion of an organic carbon source (sodium acetate) and an increase in light intensity. Thus, nitrogen started to participate in the metabolism of carotenogenesis, with total N consumption up to day 24 for all sources in WPF (Fig. 2).

Table 4 Physicochemical variables of *Haematococcus pluvialis* cultivation with different nitrogen sources and feeding strategies

Feeding strategies	Nitrogen sources	pH	T (°C)	Nitrogen concentration (mM)
WPF	$NaNO_3$	8.11 ± 0.07^{Aa}	22.34 ± 0.10^{Aa}	1.07 ± 0.12^{Ab}
	NH_4NO_3	7.03 ± 0.13^{Ca}	22.17 ± 0.03^{Aa}	1.39 ± 0.28^{Ab}
	$(NH_2)_2CO$	7.37 ± 0.01^{Ba}	21.98 ± 0.09^{Aa}	1.20 ± 0.15^{Ab}
PF	$NaNO_3$	8.39 ± 0.10^{Aa}	22.24 ± 0.02^{Aa}	2.64 ± 1.59^{Aa}
	NH_4NO_3	6.86 ± 0.09^{Ca}	22.07 ± 0.09^{Aa}	3.17 ± 1.82^{Aa}
	$(NH_2)_2CO$	7.70 ± 0.02^{Ba}	22.07 ± 0.13^{Aa}	3.02 ± 1.69^{Aa}
	S	***	ns	ns
	F	ns	ns	***
	S x F	*	ns	ns

Mean ± standard deviation (n=4). Mean values for the same column with different letters indicate significant differences by a two-way ANOVA followed by Tukey's test (uppercase - nitrogen sources factor, lowercase - feeding strategy factor). S = factor 1 (nitrogen sources); F = factor 2 (feeding strategies); S x F = interaction between the two factors. ns = not significant; *** ($p < 0.001$); * ($p < 0.05$). WPF: without pulse feeding; PF: pulse feeding.

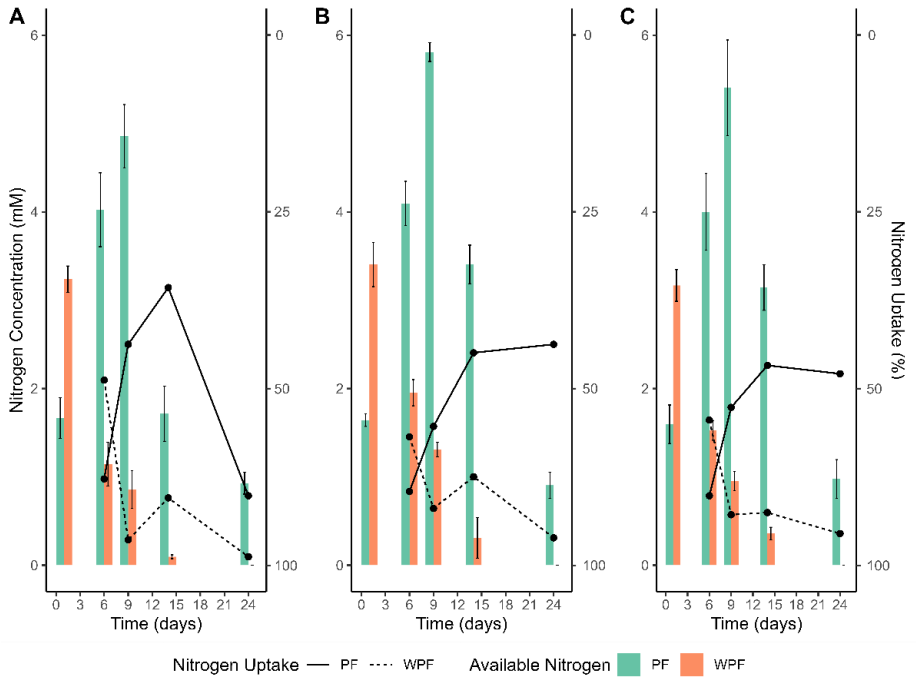

Fig. 2 Nitrogen uptake profile (rows) *versus* available nitrogen (columns) in *Haematococcus pluvialis* cultivation under different feeding strategies (PF and WPF) and nitrogen sources ($NaNO_3$ – A, NH_4NO_3 – B, $(NH_2)_2CO$ – C)

In addition to the availability of nutrients, such as nitrogen, other physical-chemical factors are important for the functioning of cellular metabolism, such as pH. Keeping the pH variation to a minimum ensures a more stable chemical environment and, consequently, better conditions for *H. pluvialis* cell division [38]. In this study, the initial pH ranged between 6.5 and 7.5; generally, at an initial pH of 7, greater growth of *H. pluvialis* is observed, while at a pH of 9 and 5, less growth and inhibition are observed, respectively [39]. Lower pH was found with NH_4NO_3 (Table 4) due to the release of H^+ in the medium [40]. In addition, pH decrease was observed after NH_4NO_3 inputs, generating pH fluctuations (Fig. 3). The stress caused by these variations may have negatively influenced the growth in NH_4NO_3-PF, reaching a lower MCD than the other sources. However, a smaller variation was observed between the initial and final pH of NH_4NO_3-PF culture due to the neutralization of OH^- (released in photosynthesis) by the release of H^+ from the N inputs, as well as by the preference of microalgae to consume ammonium ions, avoiding acidification of the medium [40].

Pulse feeding strategy increased the availability of N throughout the culture and provided an increase in cell density (CD), influencing the pH variation of the medium as a function of the N source (Fig. 3). The inputs with $NaNO_3$ and $(NH_2)_2CO$ reflected in an increase in pH due to the release of OH^- through photosynthesis and the consumption of nitrate [40,41], while the inputs with NH_4NO_3 neutralized the pH of the medium, through the production of H^+ [40]. In this scenario, pH did not correlate with the concentration of N and CD ($0.29 < r < 0.42$), differently of inputs where N concentration and CD are strongly correlated ($r > 0.97$).

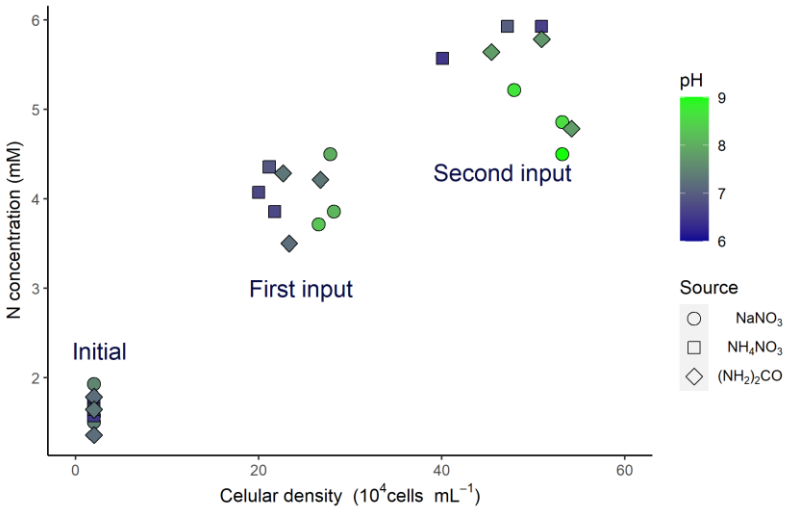

Fig. 3 Correlation among N concentration, cellular density and pH in *Haematococcus pluvialis* culture under pulse feeding strategy with different N sources

In the Principal Component Analysis (PCA), degrees of explanation of 85.8% for PF and 89.58% for WPF were found, divided between two principal components, PC1 and PC2 (Fig. 4). For the combinations in PF (Fig. 4A), all variables were positively correlated. Higher pH values were recorded for the source $NaNO_3$, higher concentrations of N for NH_4NO_3, and higher CD and µ for $(NH_2)_2CO$. As for the WPF combinations (Fig. 4B), the variable N is inversely correlated with the other variables. $NaNO_3$ source had highest pH values while NH_4NO_3 and $(NH_2)_2CO$ the highest CD, µ and N.

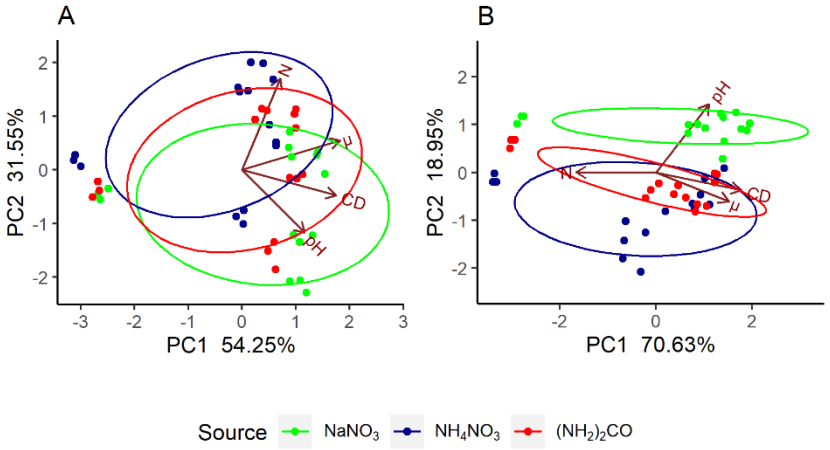

Fig. 4 Principal component analysis for physicochemical and growth variables of *Haematococcus pluvialis* culture using different nitrogen sources under pulse feeding - PF (A) and without pulse feeding - WPF (B)

Our results confirmed that the nutritional management of nitrogen sources during the growth period in fed-batch culture systems using pulse feeding mode is essential to achieve greater cell density and biomass of *H. pluvialis*.

3.2. Production of carotenoids and astaxanthin in H. pluvialis

In the cystic phase, *H. pluvialis* cells begin to synthesize and accumulate carotenoids, mainly astaxanthin. In addition, there are morphological changes - increased cell wall thickness, size, and weight [42]. In this phase, higher biomass was obtained for PF (1.22 to 1.34 g L^{-1}), regardless of the nitrogen source (Table 3). The increase in biomass in the cystic phase occurred both due to reproduction, observing an increase in cell density until the 15th (WPF) and 20th (PF) days of culture, and to the increase in cell size. When there are high concentrations of N in the medium, as in the case of PF cultures, cells can reproduce, whereas when these concentrations are low,

they synthesize carotenoids [17]. As for the content of total carotenoids obtained in the cystic phase, a significant effect was observed for the feeding strategy factor (p=0.00393), with higher concentrations for the combinations in WPF, with a significant difference being observed only between NH_4NO_3-WPF (25.18 ± 5.12 mg L^{-1}) and $NaNO_3$-PF (11.52 ± 6.03 mg L^{-1}) (Fig. 5A). When the carotenoid content was evaluated, there was also an effect of the feeding strategy factor (p= 7.88e-05), with a significant difference between NH_4NO_3-WPF (26.77 ± 5.85 mg g^{-1}) and all sources in PF ($NaNO_3$-PF = 8.67 ± 4.71 mg g^{-1}; NH_4NO_3-PF = 10.31 ± 3.58 mg g^{-1}; $(NH_2)_2CO$-PF = 11.36 ± 1.53 mg g^{-1}) (Fig. 5B). Therefore, the highest production of carotenoids occurred in WPF, which showed a shortage of nitrogen from the 14th day of cultivation (Fig. 2). The carotenoid content showed an opposite trend to the biomass, with higher content for the groups with low nitrogen concentration (WPF). This fact can be explained by nitrogen starvation and light supersaturation, both induce the degradation of chlorophylls and primary carotenoids (present in the vegetative phase) and consequent accumulation of secondary carotenoids, among which astaxanthin [43].

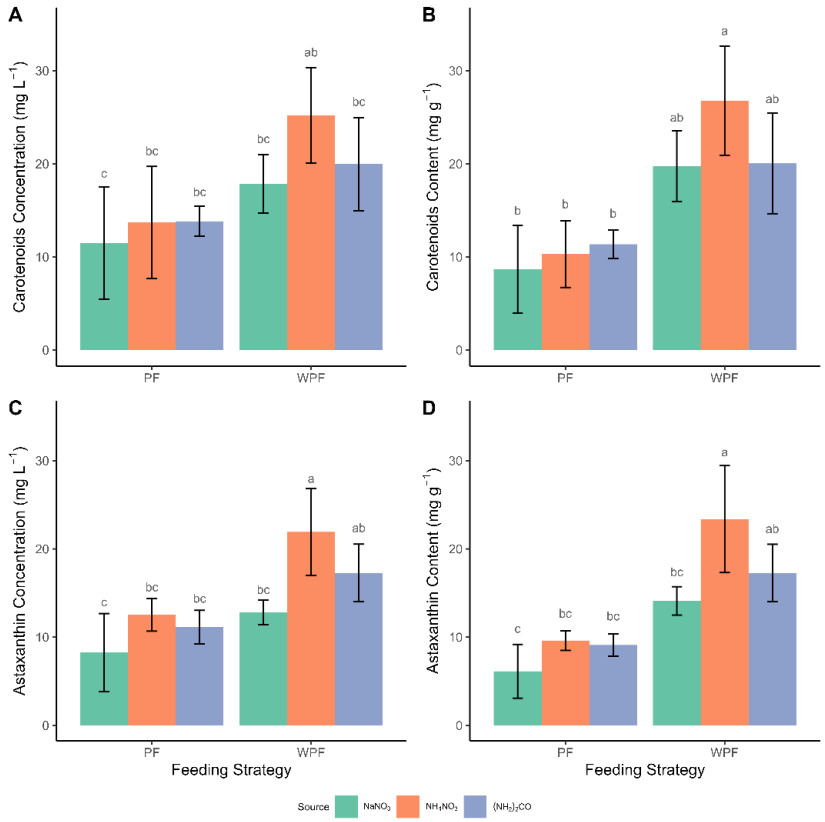

Fig. 5 Concentrations (mg L^{-1}) and contents (mg g^{-1}) of total carotenoids (A and B) and astaxanthin (C and D) produced by *Haematococcus pluvialis* cultivated in different nitrogen sources and feeding strategies (Different letters indicate significant differences between the factors by Tukey's test)

Astaxanthin is the most abundant carotenoid in cystic *H. pluvialis*, representing about 80-99% of total carotenoids [44]. In the present study, the concentration of astaxanthin was influenced both by the nitrogen source ($p=0.013009$) and by the feeding strategy ($p=0.000932$), with the highest value for NH_4NO_3-WPF (21.93 ± 4.95 mg L^{-1}), that significantly differed from all sources in PF and $NaNO_3$-WPF (Fig. 5C). Similarly, a significant effect of the two factors on the astaxanthin content was observed, with the highest value for NH_4NO_3-WPF (23.37 ± 6.06 mg g^{-1}), which was similar only to $(NH_2)_2CO$-WPF (17.27 ± 3.26 mg g^{-1}) (Fig. 5D). As with carotenoids, astaxanthin production was higher in WPF cultures, because under nitrogen depletion there is an increase in the expression of genes linked to astaxanthin biosynthesis to act in the defense metabolism to this stress condition [45].

Furthermore, under nitrogen deprivation, the carbon flux is directed towards the synthesis of astaxanthin, *i.e.*, the exogenous addition of organic carbon (sodium acetate) stimulates the utilization of carbon more quickly and efficiently, mainly through the tricarboxylic acid cycle, for the biosynthesis of this carotenoid [45,46]. In this perspective, a high C/N ratio was promoted in the cystic stage, through the insertion of sodium acetate at beginning of the stationary phase of growth. The accumulation of astaxanthin was detected through macroscopic - change in color of cultures and microscopic - pigmentation in the cells observations from the 14th day in WPF and 20th day in PF, corresponding to the times of N depletion (Fig. 2). Therefore, the greater accumulation of astaxanthin under high carbon concentration during the cystic stage is due to the change in the C/N ratio and the formation of a relative nitrogen deficiency [47].

The lower yield of carotenoids and astaxanthin by microalgae under pulse feeding strategy can be explained by the excess of nitrogen in the medium after the exponential growth phase, when there should be nitrogen limitation to promote carotenogenesis. In figure 2, it is possible to observe excess of N under PF in $NaNO_3$ (Fig. 5A), NH_4NO_3 (Fig. 5B) and $(NH_2)_2CO$ (Fig. 5C) on days 14 and 24, period in which carotenogenesis occurred in WPF cultures. Considering the greater uptake of N in PF than in WPF, the N inputs were important for the greatest increase in cells and biomass, however, when reaching the limit of cell growth (day 20, approximately), the excess of N in the medium prevented further production of carotenoids. These results indicate that a second N input with a lower concentration could be better used by the microalgae, with less N residue in the medium, without negatively affecting growth or

carotenogenesis. The longer time for carotenogenesis in PF was also confirmed by the significant difference in productivity (mg L^{-1} d^{-1}) of carotenoids and astxanthin. Carotenoid productivity was 1.24 ± 0.22 and 0.65 ± 0.06 mg L^{-1} d^{-1} and astaxanthin productivity was 1.0 ± 0.27 and 0.53 ± 0.08 mg L^{-1} d^{-1}, in WPF and PF respectively.

The production of astaxanthin is also stimulated by other stress factors, such as the presence or absence of certain nutrients, changes in salinity and variations in temperature and pH. The temperature in the present study remained constant during the cultivation period, varying on average between 21.98 ± 0.09 and 22.34 ± 0.10 °C, with no significant difference between the factors (Table 4). The average pH values ranged from 6.86 to 8.39, with a significant effect of the source and the interaction between the factors (Table 4). Among the nitrogen sources, NH_4NO_3 had the lowest average pH value, both in PF and WPF. Among the WPF cultures, the use of NH_4NO_3 resulted in a greater variation between the initial and final pH of the medium (Fig. 3), so the stress caused by this variation may have contributed to the greater carotenogenesis in NH_4NO_3-WPF.

Stress caused by high pH variation stimulates carotenogenesis, since astaxanthin is a secondary metabolite produced in response to cellular stress [8]. Under conditions of environmental stress, cells intensify the production of reactive oxygen species (*e.g.*, O_2^-, H_2O_2, OH^-), on the other hand, the synthesis of astaxanthin is promoted in order to eliminate reactive oxygen species and avoid oxidative stress [45,48]. In this sense, the combination of stress-inducing factors, such as pH variation, nitrogen depletion, high luminosity, and insertion of organic carbon can be used to increase the production of astaxanthin [48]. As a matter of fact, prolonged duration of stress may result in higher astaxanthin production and lower biomass production.

Another challenge in the production of astaxanthin is the high cost of cultivation systems related to low microalgae productivity [8]. In this scenario, the culture medium is one of the main costs, with nitrogen as the macronutrient with the highest proportion [49]. Among the sources of N, NH_4NO_3 is the fertilizer with the lowest cost [50], so, combined with the higher production of astaxanthin obtained with NH_4NO_3, its use would be the most recommended. Therefore, the cultivation of *H. pluvialis* using NH_4NO_3 as a source of N, with the natural depletion of nutrients in the stationary phase and alteration of the C/N balance by the introduction of organic carbon, can be an efficient approach to induce the accumulation of astaxanthin in systems of cultivation of *H. pluvialis*.

Depending on the final application of the biomass, it is important to analyze which is more advantageous, higher astaxanthin content (under WPF) or higher biomass (under PF). The biomass of *H. pluvialis* is very valuable, in addition to astaxanthin, it contains a high lipid content and a suitable fatty acid profile, as well as other metabolites, for applications in food, nutraceutical, pharmaceutical and biofuel industries [51]. Thus, the evaluation of the influence of the supply of different nitrogen sources on the growth and astaxanthin biosynthesis of *H. pluvialis* has important biotechnological implications.

4. Conclusions

The nitrogen pulse feeding strategy provided greater growth of *Haematococcus pluvialis*, obtaining higher yields and productivity in vegetative and cystic biomass for all nitrogen sources. The average yield of vegetative and cystic biomass was 0.8 and 1.3 g L^{-1}, respectively, for cultures under pulse feeding. On the other hand, higher concentrations and contents of total carotenoids (27 mg g^{-1}) and astaxanthin (23 mg g^{-1}), in general, were observed for the NH_4NO_3 source without pulse feeding strategy, being influenced by nitrogen depletion and pH variation. Future research can be carried out by decreasing the concentration of N pulses, so that there is less N residue in the medium in the carotenogenesis phase, in order to increase the synthesis of astaxanthin. Therefore, the proposal of the present study using the pulse nitrogen supply mode promotes greater production of *H. pluvialis* biomass and a new perspective to increase the production of astaxanthin with NH_4NO_3.

Acknowledgments

The authors would like to thank the Laboratory of Aquatic Animal Health, Laboratory of Shrimp Farming and Laboratory of Aquaculture Production at the Department of Fisheries and Aquaculture, and Laboratory of Molecular Biology Applied to Animal Production at the Department of Animal Science of the Federal Rural University of Pernambuco for providing the instruments used for the analysis.

Statements & Declarations

Ethical approval

The experiment was in accordance with Brazilian Law no. 11.794/2008.

Competing Interests

The authors have no relevant financial or non-financial interests to disclose.

Author Contributions

Writing - original draft preparation [Laenne Moraes]; Conceptualization [Laenne Moraes], [Alfredo Gálvez], [Ranilson Bezerra]; Investigation [Laenne Moraes], [Géssica Mota], [Bruna Silva]; Methodology [Laenne Moraes], [Géssica Mota], [Bruna Silva]; Formal analysis [Laenne Moraes], [Elizabeth Santos], [Clarissa Campos]; Writing - review and editing [Clarissa Campos], [Alfredo Gálvez]; Resources [Ranilson Bezerra], [Alfredo Gálvez]; Supervision [Ranilson Bezerra], [Alfredo Gálvez]; Project administration [Ranilson Bezerra].

Funding

The authors are grateful for the financial support of the Federal Rural University of Pernambuco; to the Coordination of Improvement of Higher Education Personnel (CAPES) grants to LM [88882.436230/2019-01], GM [88887.608945/2021-00], ES [88882.436231/2019-01] and CC [88882.436234/2019-01]; and the National Council for Scientific and Technological Development (CNPq) for the scholarships granted to Alfredo Olivera Gálvez [PQ 308063/2019-8] and Ranilson de Souza Bezerra [PQ 307107/2019-1].

Availability of data and materials

The datasets generated during and/or analysed during the current study are available from the corresponding author on reasonable request.

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3- Artigo científico II: Efeitos de diferentes fontes e estratégias de alimentação de carbono orgânico na produção de metabólitos de alto valor de *Haematococcus pluvialis*

Artigo a ser submetido ao periódico “Bioresource Technology”.

Resumo

A microalga *Haematococcus pluvialis* tem como bioproduto de maior valor econômico a astaxantina, além de ser capaz de produzir outros metabólitos de alto valor, como proteínas, lipídios, ácidos graxos e carboidratos. O presente estudo propõe modificações nas fontes e estratégias de alimentação de carbono orgânico, a fim de aumentar a produção de metabólitos da *H. pluvialis*. Foi utilizado um delineamento experimental bifatorial tendo como fator 1: três fontes de carbono orgânico – acetato de sódio, melaço e glicerol; e fator 2: estratégia de alimentação de carbono – com pulsos (PF) e sem pulsos (WPF); cada um com 4 réplicas, totalizando 24 unidades experimentais. A fonte de carbono orgânico (0,01M) foi inserida na fase estacionária de crescimento. A microalga *H. pluvialis* foi cultivada e processada para a obtenção de biomassa seca, subsequentemente foram realizadas as análises de composição centesimal, ácidos graxos, carotenoides e atividade de L-asparaginase. Quanto ao efeito das fontes e estratégias de alimentação de carbono orgânico no rendimento em biomassa, foram obtidos maiores valores para as fontes glicerol-WPF (1,275 g L^{-1}) e acetato-PF (1,264 g L^{-1}). A composição da biomassa em termos de proteínas e lipídios apresentou influência tanto da fonte quanto da estratégia de alimentação de carbono orgânico. Quanto aos ácidos graxos e PUFA, os tratamentos melaço-WPF (5,68% P.S.; 2,62% P.S.) e glicerol-PF (5,38% P.S.; 2,44% P.S.) apresentaram as maiores concentrações ($p < 0,05$). Em relação ao perfil de carotenoides, a astaxantina é o mais abundante e apresentou maior concentração sob glicerol-WPF (6,89% P.S.), seguido de melaço-PF (5,72% P.S.). Além desses metabólitos já conhecidos em *H. pluvialis*, foi reportada pela primeira vez a enzima L-asparaginase, com atividade média de 256 IU mg^{-1} de biomassa seca utilizando acetato-WPF. Portanto, diferentes fontes e estratégias de alimentação de carbono orgânico influenciam a produção de biomassa e metabólitos de alto valor da *H. pluvialis*.

Palavras-chave: biomoléculas, composição centesimal, astaxantina, L-asparaginase.

Effects of different sources and feeding strategies of organic carbon in the production of high-value metabolites of *Haematococcus pluvialis*

Abstract

The microalgae *Haematococcus pluvialis* has astaxanthin as its bioproduct of greatest economic value, in addition to being capable of producing other high-value metabolites, such as proteins, lipids, fatty acids and carbohydrates. The present study proposes modifications in organic carbon sources and feeding strategies in order to increase the production of *H. pluvialis* metabolites. A two-factor experimental design was used with factor 1: three sources of organic carbon – sodium acetate, molasses and glycerol; and factor 2: carbon feeding strategy – with pulses (PF) and without pulses (WPF); each with 4 replicates, totaling 24 experimental units. The organic carbon source (0.01M) was inserted in the stationary phase of growth. The microalgae *H. pluvialis* was cultivated and processed to obtain dry biomass, subsequently analyzing the metabolites. Regarding the effect of organic carbon sources and feeding strategies on biomass yield, higher values were obtained for the sources glycerol-WPF (1.275 g L^{-1}) and acetate-PF (1.264 g L^{-1}). The composition of the biomass in terms of proteins and lipids was influenced by both the source and the organic carbon feeding strategy. As for fatty acids and PUFA, the treatments molasses-WPF (5.68% P.S.; 2.62% P.S.) and glycerol-PF (5.38% P.S.; 2.44% P.S.) presented the highest concentrations ($p < 0$ .05). Regarding the carotenoid profile, astaxanthin is the most abundant and presented the highest concentration under glycerol-WPF (6.89% P.S.), followed by molasses-PF (5.72% P.S.). In addition to these metabolites already known in *H. pluvialis*, the enzyme L-asparaginase was reported for the first time, with an average activity of 256 IU mg^{-1} of dry biomass using acetate-WPF. Therefore, different organic carbon sources and feeding strategies influence the production of biomass and high-value metabolites of *H. pluvialis*.

Keywords: biomolecules, proximate composition, astaxanthin, L-asparaginase.

Introdução

Haematococcus pluvialis destaca-se das outras espécies de microalgas devido à sua capacidade de sintetizar astaxantina em altas concentrações, que correspondem a 4-5% do conteúdo celular no estágio cístico (VARDANEGA *et al*., 2022). A astaxantina é um carotenoide com alto poder antioxidante, apresenta efeitos anti-inflamatório, anticancerígeno, antidiabético e imunológico, sendo amplamente utilizada nas indústrias farmacêutica, nutracêutica, de cosméticos e de alimentos (MOTA *et al*., 2022). A produção de astaxantina está associada a alterações morfológicas, fisiológicas e bioquímicas nas células que são influenciadas por condições ambientais, como a intensidade luminosidade e a disponibilidade ou privação de nutrientes (MORAES *et al*., 2023).

A manutenção de uma alta relação Carbono:Nitrogênio (C:N), através do acréscimo de carbono orgânico no sistema, induz a produção de astaxantina, porém a introdução de um fator estressor reduz ou inibe a divisão celular (ZHAO *et al*., 2019; JOUN *et al*., 2023). Para estimular o crescimento e a produção de astaxantina, são adotadas estratégias como a inclusão de fontes de carbono orgânico na fase estacionária de crescimento celular (MORAES *et al*., 2023). O acetato de sódio é a principal fonte de carbono orgânico utilizada no cultivo de *H. pluvialis* (PAN-UTAI *et al*., 2017). No entanto, estudos também mostram uma maior catotenogênese com a utilização de glicerol como fonte de carbono orgânico (ZHANG *et al*., 2020). De forma alternativa, o melaço de cana-de-açúcar também foi identificado como promissora fonte de carbono no cultivo de microalgas e, por ser um resíduo proveniente da fabricação do açúcar, representa uma fonte de baixo custo para aumentar a produtividade de astaxantina (YEE, 2015; YEW *et al*., 2020).

Além de alterar as fontes, pode-se variar as estratégias de alimentação de carbono orgânico, como a alimentação contínua, escalonada ou pulsada. Na alimentação contínua, a fonte de carbono é fornecida continuamente ao longo do período de cultivo e mantida de forma constante ao longo do tempo de cultivo. A alimentação escalonada consiste na adição de carbono em estágios distintos, sendo aplicadas apenas em cultivos descontínuos (DEVASYA e BASSI, 2021). A alimentação por pulsos de nutrientes envolve a adição intermitente durante o crescimento das microalgas, podendo resultar em maior eficiência na absorção de nutrientes, redução de custos, controle metabólico, flexibilidade e menor impacto ambiental (MA et al., 2023). A estratégia de alimentação de carbono por pulsos modifica o metabolismo fisiológico de microalgas, como

Scenedesmus acuminatus, *Chlorella sorokiniana* e *Chlamydomonas reinhardtii*, proporcionando maiores produtividades em biomassa ou metabólitos de alto valor (*e.g.*, polissacarídeos, lipídios) (XIE *et al*., 2019; JIN *et al*., 2020; FIGUEROA-TORRES *et al*., 2022).

Apesar de ser frequentemente cultivada para produção de astaxantina, a *H. pluvialis* é capaz de produzir outras biomoléculas de alto valor, como proteínas, enzimas, lipídios, ácidos graxos e carboidratos (HU *et al*., 2020; MARINHO *et al*., 2021; MORAES *et al*., 2022; SILVA *et al*., 2022). Essa vasta diversidade de metabólitos viabiliza a utilização da biomassa de forma integral que, em um modelo de biorrefinaria, minimiza a geração de resíduos e melhora a sustentabilidade da produção (NISHSHANKA *et al*., 2022).

Nesse contexto de sustentabilidade, as microalgas são importantes fontes naturais e renováveis para a produção de compostos com aplicações biotecnológicas. Uma das biomoléculas com função terapêutica identificada em algumas espécies, como *Chlorella vulgaris*, *Spirulina maxima*, *Chlamydomonas* sp. e *Oscillatoria terebriformis*, é a enzima L-asparaginase (PAUL, 1982; EBRAHIMINEZHAD *et al*., 2014; ABD EL-BAKY e EL-BAROTY, 2016; ELKOMY, 2018). Essa enzima possui aplicação quimioterapêutica de alta potência, por sua utilização em uma ampla gama de tratamentos contra o câncer, especialmente na terapia da leucemia linfoblástica aguda (BATOOL *et al*., 2016). L-asparaginase catalisa a asparagina, um aminoácido essencial para células leucêmicas, em amônia e aspartato, inibindo a biossíntese de proteínas nos linfoblastos (BATOOL *et al*., 2016).

A L-asparaginase é a primeira enzima terapêutica com propriedades antineoplásicas estudada amplamente, tendo como principal fonte para fins comerciais a bactéria *Escherichia coli*, especialmente suas versões recombinantes e geneticamente modificadas. Apesar de produzir altas concentrações dessa enzima, a *E. coli* pode estar associada a efeitos citotóxicos da enzima (MUNEER *et al*., 2020). Portanto, buscam-se outras fontes e formas de produzir L-asparaginase, seja através de modificações genéticas ou mudanças na forma de cultivar os microrganismos, principalmente modificando o meio de cultura (EBRAHIMINEZHAD *et al*., 2014; ABD EL-BAKY e EL-BAROTY, 2016).

Portanto, a manipulação de fontes de carbono orgânico representa uma alternativa promissora para obter alta produtividade de compostos bioativos. No presente estudo, foram investigadas diferentes fontes e estratégias de alimentação de

carbono orgânico com o objetivo de incrementar a produção de astaxantina e outros compostos bioativos de alto valor em *H. pluvialis*, visando potencial aplicação em um modelo de biorrefinaria.

Material e métodos

Delineamento experimental

O experimento consistiu em duas etapas: 1. Cultivo experimental; 2. Caracterização dos metabólitos. A etapa 1 ocorreu no Laboratório de Produção de Alimento Vivo (LAPAVI) do Departamento de Pesca e Aquicultura da Universidade Federal Rural de Pernambuco. A etapa 2 foi executada no Departamento de Engenharia Química da Universidade de Almería (Espanha).

O cultivo experimental foi realizado em escala laboratorial através de um delineamento bifatorial (3x2), onde o fator 1 incluiu três fontes de carbono orgânico - Acetato de sódio, Melaço e Glicerol; e o fator 2 abordou a estratégia de alimentação do carbono orgânico, com pulsos (PF) e sem pulsos (WPF); cada um com 4 réplicas, totalizando 24 unidades experimentais.

Cultivo de Haematococcus pluvialis

A microalga *H. pluvialis* foi obtida do banco de cepas do LAPAVI. Os cultivos foram realizados em garrafas de 500 mL, utilizando água doce previamente tratada com cloro, filtrada e autoclavada. O meio foi enriquecido com Bold´s Basal Medium (BBM) modificado (MORAES *et al*., 2023), utilizando a seguinte composição em mg L^{-1}: 118 NH_4NO_3; 25 $CaCl_2.2H_2O$; 25 NaCl; 31 KOH; 50 EDTA $Na.2H_2O$; 75 K_2HPO_4; 175 KH_2PO_4; 4,98 $FeSO_4.7H_2O$; 75 $MgSO_4.7H_2O$; 11,42 H_3BO_3; 1,412 $ZnSO_4.7H_2O$; 0,232 $MnCl_2.4H_2O$; 0,252 $CuSO_4.5H_2O$; 0,192 $Na_2MoO_4.2H_2O$; 0,08 $Co(NO_3)_2.6H_2O$; 4,05 $C_4H_8N_2O_3$.

A fonte de carbono orgânico foi adicionada na fase estacionária de crescimento, mantendo uma concentração de 0,01M de carbono em todas as combinações. As alimentações de pulso de carbono foram realizadas nos 13º, 14º e 15º dias.

As microalgas foram inoculadas com concentração inicial de 2 x 10^4 cél mL^{-1}, em temperatura de 22 °C, fotoperíodo 12h:12h L/E e intensidade luminosa de 40 µmol fótons $m^{-2}s^{-1}$, sendo mantidas sob aeração constante. O cultivo foi conduzido em condições fotoautotróficas até a fase estacionária de crescimento (fase vegetativa),

quando a fonte de carbono orgânico foi introduzida e a intensidade luminosa aumentada a 100 µmol fótons $m^{-2}s^{-1}$ como estímulo ao processo de carotenogênese (fase cística), juntamente à privação natural de nitrogênio (MORAES *et al*., 2023).

Obtenção de biomassa

O rendimento da cultura em biomassa seca (g L^{-1}) e produtividade da biomassa (g L^{-1} d^{-1}) na fase cística foram determinados por filtração de alíquotas de 10 mL de células em suspensão através de filtro de microfibra de vidro Whatman GF/C (1,2 µm) e secagem a 75 °C por 24 h (CHOI *et al*., 2018).

Ao fim do cultivo, na fase cística, o volume produzido em cada unidade experimental foi coletado para obtenção da biomassa seca, através de centrifugação a 3500 x *g* por 10 min, congelamento a -80 °C por 24 h e liofilização por 48 h (SILVA *et al*., 2022). A biomassa seca foi moída em almofariz para homogeneização.

Composição centesimal da H. pluvialis

A quantificação de proteínas foi realizada através do teor de nitrogênio, utilizando fator de conversão de 4,44 (LÓPEZ *et al*., 2010). O teor total de nitrogênio na biomassa liofilizada foi medido por análise elementar (ELEMENTAR Vario Micro CHNS, Elementar Analysensysteme GmbH, Hanau, DE). Amostras de 2 mg, em triplicata, foram pesadas em cápsulas de estanho e depositadas no reator de combustão a 1150 ºC usando oxigênio puro como gás de combustão e hélio puro como gás de arraste. A quantidade dos gases N_2, CO_2, H_2O e SO_2 foram medidas usando um detector de condutividade térmica.

O conteúdo lipídico total da biomassa seca foi determinado segundo Kochert (1978). Antes disso, a biomassa liofilizada foi moída em almofariz com Óxido de alumínio 1:1 (p/p) por 5 min (CERÓN-GARCÍA *et al*., 2018). Em 100 mg de biomassa foram adicionados 2 mL de clorofórmio:metanol (2:1), agitando em vórtex e centrifugando a 5000 rpm por 5 min. Ao sobrenadante foram adicionados 3 mL de HCl 0,1N e 0,3 mL de $MgCl_2$ a 0,5% para separação de proteínas, agitando e centrifugando (5000 rpm, 5 min). A fase lipídica foi recolhida em tubo previamente pesado e colocado para evaporação em um concentrador a vácuo por rotação (RVC 2-25 CDplus, Martin Christ Gefriertrocknungsanlagen GmbH, Osterode am Harz, DE). Após evaporação total, os tubos foram pesados. Os ácidos graxos foram transesterificados *in situ* (RODRÍGUEZ-RUIZ *et al*., 1998) e quantificados por cromatografia gasosa (Agilent

Technologies 6890 N Series Gas Chromatograph, USA).

A proporção de cinzas foi obtida submetendo 100 mg de biomassa em mufla a 500 °C por 24h, até peso constante (AOAC, 2012). Enquanto, a quantidade de carboidratos foi calculada pela subtração dos demais compostos.

Caracterização de carotenoides

Todo o processo foi realizado na ausência de luz, seguindo metodologia descrita por CERÓN-GARCÍA *et al*. (2018). A biomassa liofilizada foi moída em almofariz com Óxido de alumínio 1:1 (p/p) por 5 min. Para a saponificação, 10 mg de amostra total (5 mg de biomassa) foi mesclada em vórtex (30 s) com 1 mL de solução de metanol + 20% P.S. de KOH ((g KOH/g biomassa seca) × 100) em tubo de vidro Pyrex. Posteriormente, foi centrifugado a 12.000 rpm por 3 min e o sobrenadante foi recolhido em tubo vial para análise em Cromatógrafo Líquido de Alta Performance (HPLC).

Os carotenoides foram analisados por HPLC (Shimadzu SPDM10AV High Liquid Performance Chromatograph) usando um detector de arranjo de fotodiodos e coluna LiChrospher© 100 RP-18 (5-µm) (4,6×150mm) – na qual a separação foi realizada (CERÓN-GARCÍA *et al*., 2018). O volume de injeção de cada amostra foi de 20 µL. Dois eluentes foram usados: (A) água:metanol 1:4v/v e (B) acetona:metanol 1:1v/v. O gradiente das fases móveis foi de 25% B 0–8min, 75% B 8–18min, 90% B 18–23min, 100% B 25–27min e 25% B 27–32min. Os carotenoides foram eluidos a uma taxa de 1mL min^{-1} e detectados por absorbância em 360-700nm. Os padrões foram fornecidos pela Sigma Chemical Co. (EUA). Cada amostra foi analisada em duplicata.

Atividade de L-asparaginase

A atividade da enzima L-asparaginase foi detectada mediante reação de Nessler, baseada na Nesslerização direta da amônia, segundo método descrito por EBRAHIMINEZHAD *et al*. (2014). Previamente, 10 mg de biomassa liofilizada foi sonicada com 1 mL de tampão de sonicação (50 mM TRIS + 10 mM EDTA, pH 7,5) por 10 min em gelo e centrifugada (5525 *x g*, 10 min, 4 °C), totalizando 3 ciclos de extração. 600 µL de sobrenadante foi usado como solução enzimática para medir a atividade de L-asparaginase intracelular. À solução enzimática foram adicionados 1,5 mL de Tris-HCl (50 mM, pH 8,5) e 500 µL de solução L-asparagina (10 mM, em Tris-HCl 50 mM, pH 8,5), sendo incubada a 37 °C por 20 min. Em seguida, foram adicionados 500 µL de ácido tricloroacético (15% (p/v)), 1,4 mL de H_2O destilada e 500

µL de reagente Nessler. A absorbância foi mensurada a 500 nm, após 15 min de incubação em temperatura ambiente. Uma reta padrão foi construída com várias concentrações de amônia (0, 29, 57, 114, 172, 229, 286, 429, 572 µM). De modo que, uma unidade de atividade (IU) da L-asparaginase é igual a quantidade de enzima que libera 1 µM de amônia/min a 37°C, sendo representada com relação a biomassa seca (IU mg^{-1}) e com a solução enzimática (IU mL^{-1}).

Análise estatística

As variáveis respostas foram submetidas ao teste de normalidade de Shapiro-Wilk e ao teste de homogeneidade de Bartlett, seguido de ANOVA bifatorial e teste de comparação de médias de Tukey. Valores de $p < 0,05$ foram considerados estatisticamente significativos para todos os testes. A análise estatística foi realizada com o auxílio do software R Core Team (R CORE TEAM, 2023).

Resultados e discussão

Produção de biomassa de Haematococcus pluvialis

Os resultados demonstram maior rendimento em biomassa para as fontes Glicerol e Acetato, sem pulsos e com pulsos, respectivamente, com valor médio de 1,27 e 1,26 g L^{-1}, respectivamente (Tabela 1). O cultivo mixotrófico de *H. pluvialis* normalmente é realizado utilizando acetato como substrato; no entanto o glicerol pode ser assimilado por muitas espécies de microalgas, aumentando a taxa de crescimento e induzindo mudanças bioquímicas (PEREZ-GARCIA *et al*., 2011). Adicionalmente, há indícios de preferência pelo glicerol em relação ao acetato ou glicose no metabolismo mixotrófico de outras espécies de microalgas, como *Nannochloropsis* sp., *Rhodomonas reticulate* e *Cyclotella cryptica* (WOOD *et al*., 1999). Por outro lado, o acetato pode ser tóxico em altas concentrações para muitos microrganismos, portanto mantê-lo em níveis baixos pode ser útil para promover o crescimento de muitas espécies de microalgas (PEREZ-GARCIA *et al*., 2011). Dessa forma, a alimentação de acetato por pulsos, mantendo-o em níveis baixos a cada inserção, pode favorecer a maior produção de biomassa de *H. pluvialis*.

Um dos desafios no cultivo de *H. pluvialis* é a baixa produtividade em biomassa, por isso várias condições de cultivo são modificadas com o objetivo de aumentar o rendimento. Estratégias de cultivo mixotróficas podem resultar tanto em maior

produção de biomassa, quanto em crescimento mais rápido (FIGUEROA-TORRES *et al*., 2022). Entretanto, não houve diferença significativa na produtividade em biomassa para nenhum dos fatores, com uma média entre 0,040 e 0,045 g L^{-1} d^{-1} (Tabela 1).

Tabela 1 Rendimento (g L^{-1}) e produtividade (g L^{-1} d^{-1}) final em biomassa de *Haematococcus pluvialis* na fase cística sob diferentes fontes e estratégias de alimentação de carbono orgânico

Estratégia de alimentação	Fontes de carbono orgânico	Rendimento em biomassa (g L^{-1})	Produtividade em biomassa (g L^{-1} d^{-1})
	Acetato	1,122 ± 0,04[b]	0,040 ± 0,002
WPF	**Melaço**	1,145 ± 0,02[b]	0,040 ± 0,003
	Glicerol	1,275 ± 0,03[a]	0,045 ± 0,002
	Acetato	1,264 ± 0,06[a]	0,045 ± 0,006
PF	**Melaço**	1,145 ± 0,04[b]	0,041 ± 0,005
	Glicerol	1,166 ± 0,03[b]	0,042 ± 0,003
	F	ns	ns
	E	ns	ns
	FxE	*	ns

Média de 4 réplicas ± desvio padrão. Os valores médios para a mesma coluna foram significativamente diferentes por ANOVA fatorial seguida do teste de Tukey. F = fator 1 (fontes de carbono orgânico); E = fator 2 (estratégia de alimentação); F x E = interação entre os dois fatores. ns = não significativo; * ($p < 0,05$). WPF: estratégia de alimentação sem pulsos; PF: estratégia de alimentação com pulsos.

Além da biomassa, moléculas bioativas também podem ser incrementadas com a inserção de carbono orgânico, principalmente quando combinadas com estratégias de limitação de nutrientes, como fósforo e nitrogênio (FIGUEROA-TORRES *et al*., 2021). Portanto, a inserção de carbono orgânico na fase estacionária de crescimento, quando há limitação de nitrogênio devido ao consumo pelas microalgas, combinada com o aumento da intensidade luminosa, direciona as condições de cultivo para a produção de carotenoides e outros metabólitos (MORAES *et al*., 2023).

Composição centesimal de H. pluvialis

Os resultados demonstram que tanto a fonte quanto a estratégia de alimentação de carbono orgânico influenciaram a composição da biomassa em termos de proteínas e lipídios ($p < 0,05$). Para proteínas, o tratamento melaço com pulsos (PF) apresentou maior concentração, com média de 19,8% do peso seco (P.S.). O melaço contém de 15 a 25% de nitrogênio, nas formas de nitrogênio amoniacal, betaína, alfa-amino e amida, o que resulta em maior quantidade de nitrogênio no meio influenciando diretamente a síntese de proteína (JEVTIĆ-MUČIBABIĆ *et al*., 2011; YEW *et al*., 2020). Por ser um subproduto da indústria, o melaço se apresenta como uma fonte de carbono orgânico incluída na prática da economia circular, onde através do cultivo de microalgas os nutrientes são aproveitados e assimilados antes do descarte (YEW *et al*., 2020).

Por outro lado, melaço-PF resultou em menor concentração de lipídios (12,9% P.S.), enquanto as fontes acetato de sódio (WPF e PF) e glicerol (PF) proporcionaram maior concentração lipídica, 16,6% P.S. em média. Quanto a composição de ácidos graxos da *H. pluvialis*, possuem destaque os ácidos graxos poli-insaturados (PUFAs) que representam aproximadamente 46% do total (Figura 2). Os tratamentos melaço-WPF e glicerol-PF apresentaram maiores concentrações de ácidos graxos e PUFAs, em média 5,5 e 2,5% P.S., respectivamente.

O glicerol é um subproduto da indústria do biodiesel e um substrato de carbono econômico e viável para o cultivo de microalgas em condições mixotróficas. A adição de glicerol no meio resulta em aumento dos níveis de gliceraldeído-3-fosfato (GAP), um intermediário da glicólise na formação do piruvato, e consequentemente do piruvato (XUE *et al*., 2017). O piruvato pode ser convertido em Acetilcoenzima A (Acetil-CoA), o primeiro precursor da síntese de ácidos graxos, levando ao aumento da concentração de ácidos graxos e lipídios totais em *H. pluvialis* (PEREZ-GARCIA *et al*., 2011; ZHANG e LIU, 2016; ZHANG *et al*., 2020). O uso de glicerol em pulsos intermitentes resultou em maior produção de ácidos graxos e lipídios totais, assim como ocorreu para a *Chlamydomonas reinhardtii* ao utilizar ácido acético em pulsos como fonte de carbono orgânico (FIGUEROA-TORRES *et al*., 2022). De modo semelhante ao glicerol, o melaço contém grande quantidade de monossacarídeos (*e.g.*, glicose, frutose) que podem incrementar a produção de piruvato e subsequentemente de Acetil-CoA para a produção de aminoácidos e ácidos graxos (YEW *et al*., 2020).

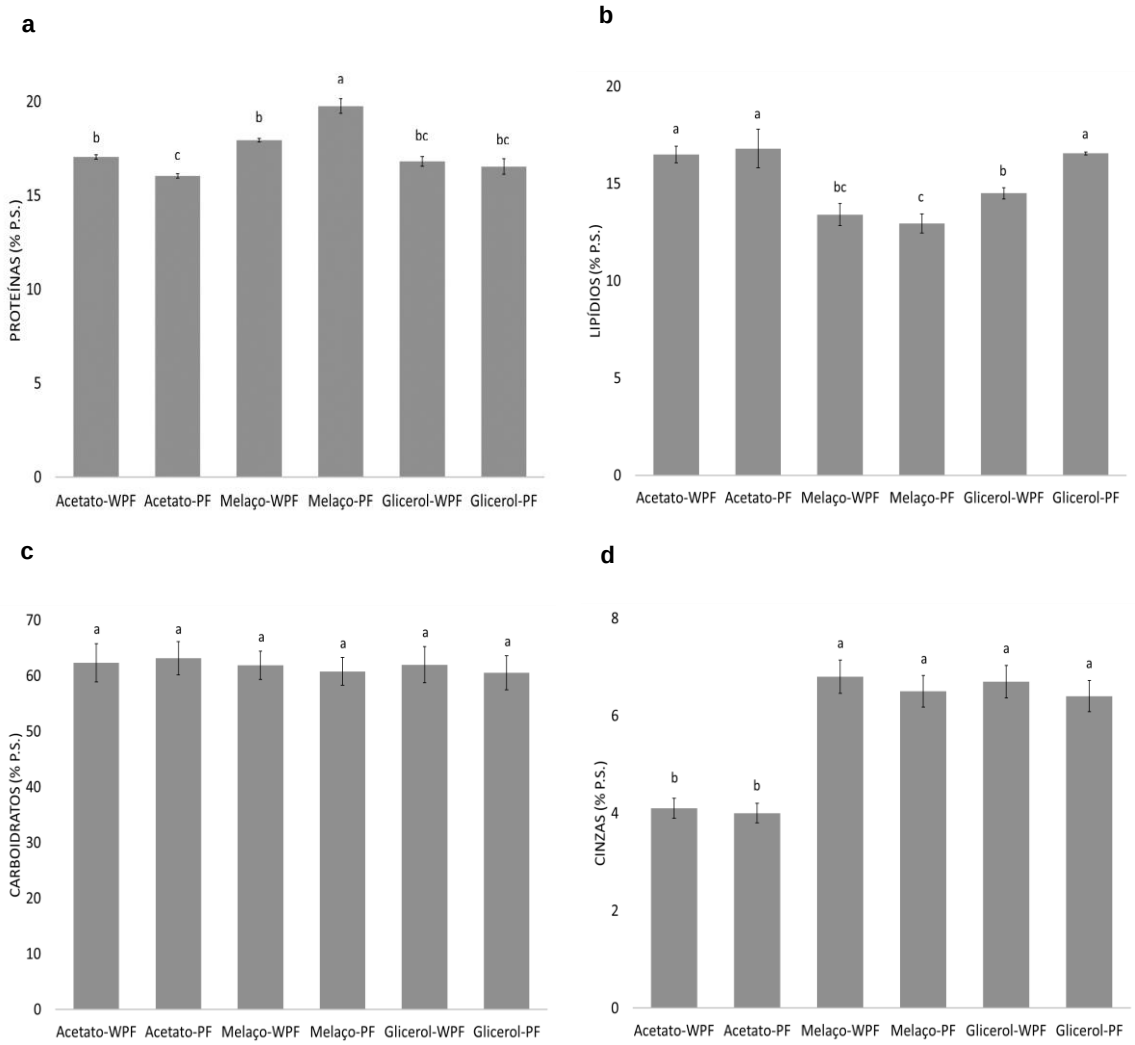

Figura 1 Composição centesimal da *H. pluvialis* cultivada sob diferentes fontes e estratégias de alimentação de carbono orgânico. Letras distintas indicam diferenças significativas entre os tratamentos pelo teste de Tukey ($p < 0,05$)

Ácidos graxos são bioprodutos de alto valor para as indústrias alimentícia, humana e animal, nutracêutica, cosmética e farmacêutica, além do grande potencial para a produção de biocombustíveis (KUMAR *et al*., 2019). Os PUFAs, parte majoritária dos ácidos graxos de *H. pluvialis* (Figura 2), possuem importância pelos seus efeitos benéficos na saúde humana e animal, especialmente pela alta disponibilidade dos ácidos graxos essenciais ômega-3 (*e.g.*, ácido α-linolênico) e ômega-6 (*e.g.*, ácido linoleico) (SAINI e KEUM, 2018). Os ácidos graxos essenciais desempenham um papel na proteção contra doenças inflamatórias e cardiovasculares, câncer e outras doenças

crônicas (SAINI e KEUM, 2018). Já os ácidos graxos monoinsaturados (MUFAs) e saturados (SFAs) apresentaram concentrações médias de 0,8 e 1,2% P.S., respectivamente (Figura 2). MUFAs e SFAs são os mais indicados para a produção de biocombustíveis, pelo baixo grau de insaturação ou ausência desta, propiciando biodiesel com alta estabilidade oxidativa e melhor qualidade (MATHIMANI e NAIR, 2016).

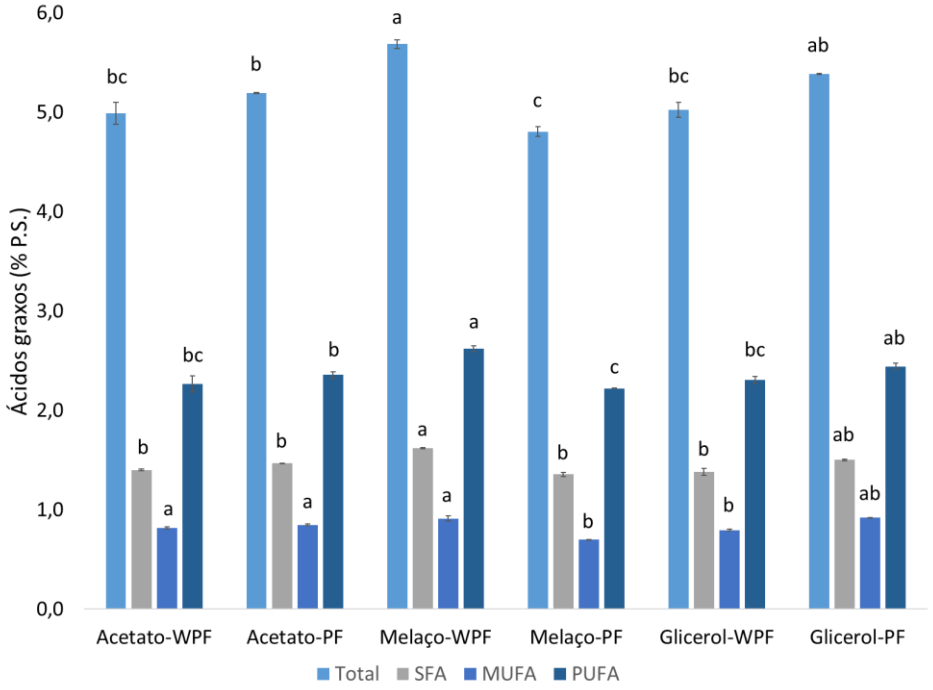

Figura 2 Composição dos ácidos graxos (% P.S.) produzidos por *H. pluvialis* cultivada sob diferentes fontes e estratégias de alimentação de carbono orgânico. SFA: Ácidos graxos saturados; MUFA: Ácidos graxos monoinsaturados; PUFA: Ácidos graxos poli-insaturados. Letras distintas indicam diferenças significativas entre os tratamentos pelo teste de Tukey ($p < 0,05$)

O perfil de ácidos graxos da *H. pluvialis* cultivada sob diferentes fontes de carbono orgânico apresentou maiores concentrações para os ácidos palmítico (C16:0), linoleico (C18:2n6), α-linolênico (C18:3n3), hexadecatrienoico (C16:3n4), oleico (C18:1n9) e vacênico (C18:1n7) (Figura 3). O ácido palmítico foi o mais abundante, seguido do linoleico, com maiores concentrações sob melaço-WPF e glicerol-PF. Após esses, o α-linolênico teve grande concentração, principalmente sob melaço (WPF e PF). A grande proporção de ácidos graxos das series ômega-6 (ácido linoleico) e ômega-3

(ácidos α-linolênico e hexadecatrienoico), faz da *H. pluvialis*, especialmente cultivada com melaço-WPF, uma fonte natural e renovável desses compostos de alto valor nas indústrias de alimentos e saúde.

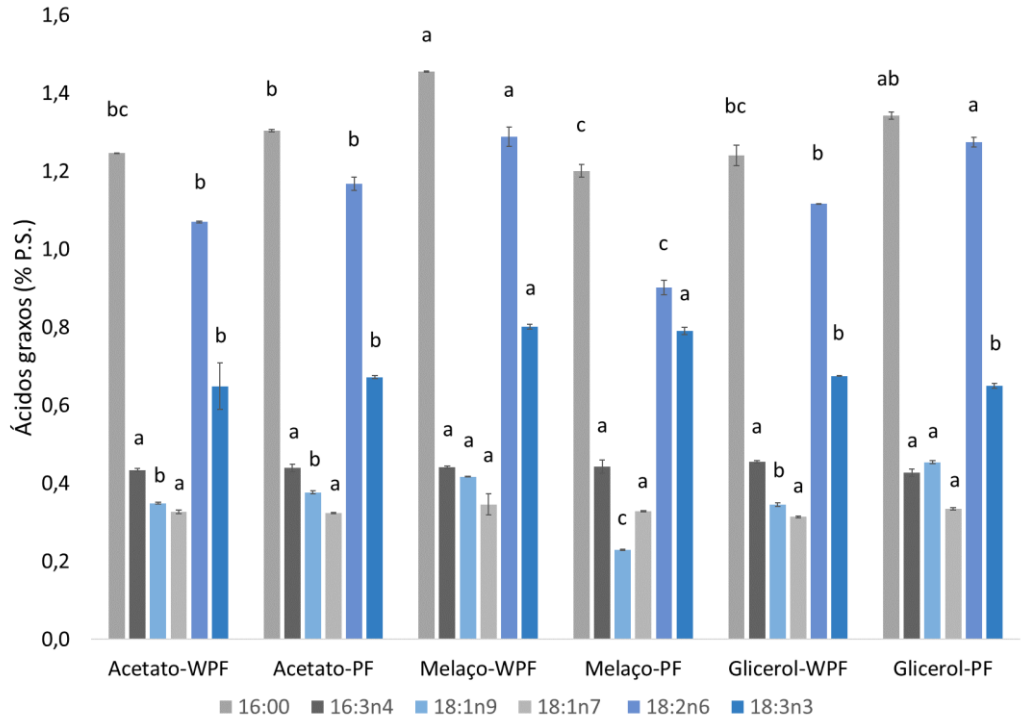

Figura 3 Principais ácidos graxos (% P.S.) produzidos por *H. pluvialis* cultivada sob diferentes fontes e estratégias de alimentação de carbono orgânico. Letras distintas indicam diferenças significativas entre os tratamentos pelo teste de Tukey ($p < 0,05$)

O crescimento mixotrófico combinado com estratégias de limitação de nutrientes, por exemplo, limitação de nitrogênio, pode melhorar a formação de lipídios e carboidratos, desde que os nutrientes sejam fornecidos de maneira ideal para sustentar o crescimento da biomassa (FIGUEROA-TORRES *et al.*, 2021). Quanto ao teor de carboidratos, não houve diferença significativa entre os tratamentos ($p > 0,05$), apresentando em média 61,8% P.S. (Figura 1c). Os carboidratos também são importantes fontes de produção de biopolímeros e bioenergia, como o biodiesel, a partir do processo de fermentação (YEW *et al.*, 2020). Em relação as cinzas, o fator fonte teve influência significativa ($p < 0,05$), sendo acetato de sódio (WPF e PF) a fonte que resultou em menor quantidade (4% P.S.) (Figura 1d).

Perfil de carotenoides e produção de astaxantina em H. pluvialis

O perfil de carotenoides de *H. pluvialis* também foi investigado, constatando-se: astaxantina, luteína, cantaxantina, β-caroteno, violaxantina, zeaxantina e neoxantina (Figura 4). Dentre esses, a astaxantina é o mais abundante, representando mais de 90% dos carotenoides totais, com média de 226,3 mg L^{-1} (4,5% P.S.) entre todos os tratamentos. A maior concentração de astaxantina foi obtida sob o tratamento glicerol-WPF, com valor médio de 344,7 ± 11,4 mg L^{-1} (6,89 ± 0,23% P.S.), seguido do tratamento melaço-PF, com média de 286,2 ± 2,92 (5,72 ± 0,06% P.S.). As menores concentrações de astaxantina foram apresentadas ao utilizar acetato de sódio (WPF e PF), aproximadamente 145 mg L^{-1} (2,9% P.S.).

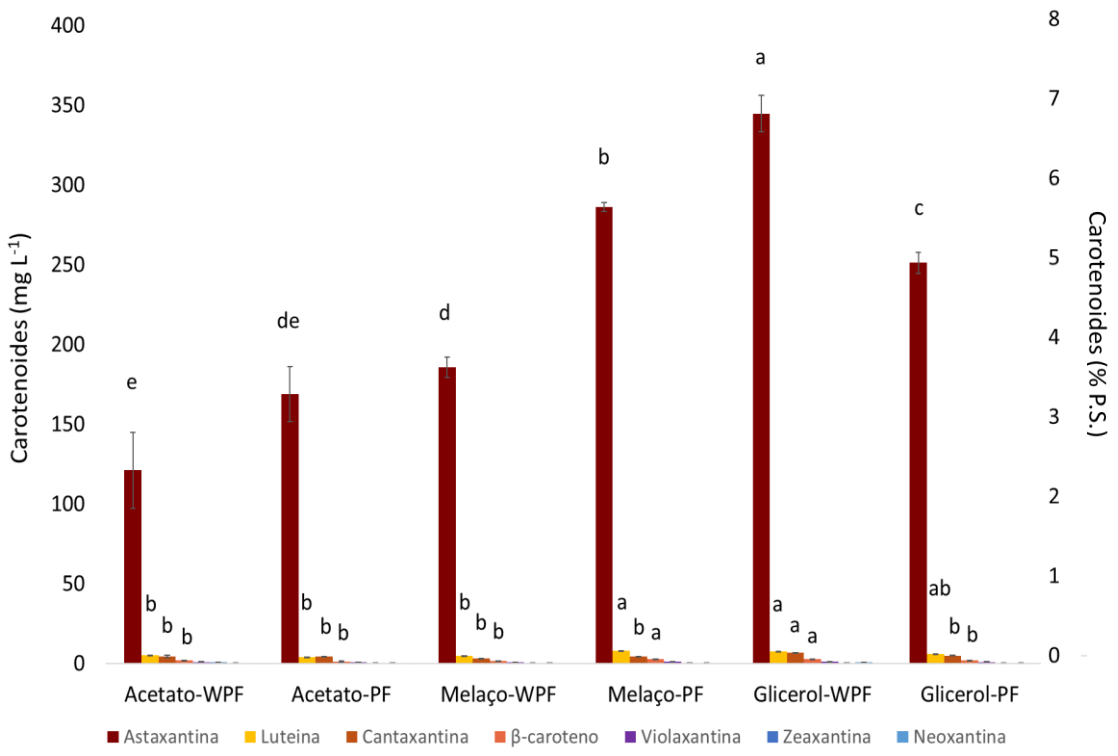

Figura 4 Perfil de carotenoides, com respectivas concentrações em mg L^{-1} e % P.S., da *H. pluvialis* cultivada sob diferentes fontes e estratégias de alimentação de carbono orgânico. Letras distintas indicam diferenças significativas entre os tratamentos pelo teste de Tukey ($p < 0,05$)

Os tratamentos glicerol-WPF e melaço-PF propiciaram concentrações de astaxantina maiores do que já reportado na literatura, acima de 5% (MOTA *et al*., 2022; VARDANEGA *et al*., 2022). O glicerol exógeno, bem como o melaço, pode incrementar a produção de astaxantina através do aumento da formação de substratos (*e.g.*, GAP e piruvato) e do nível elevado de ácidos graxos (YEW *et al*., 2020; ZHANG *et al*., 2020). A astaxantina tem como precursor o isopentenil pirofosfato (IPP) sintetizado a partir de GAP e piruvato, portanto níveis elevados dessas moléculas incrementam diretamente a biossíntese de astaxantina (LEMOINE e SCHOEFS, 2010). Adicionalmente, concentrações elevadas de ácidos graxos podem facilitar o processo de esterificação da astaxantina e, consequentemente, aumentar a produção desse carotenoide de forma indireta (CHEN *et al*., 2015).

A inserção de glicerol aumentou a concentração de astaxantina em 2x à obtida com acetato de sódio. Em estudo realizado por Zhang *et al*. (2020), o glicerol incrementou a concentração de astaxantina de *H. pluvialis* em 80,9% comparado ao controle (sem adição de carbono orgânico). Por sua vez, o uso de melaço aumentou a biossíntese de astaxantina em 1,6x comparado ao uso de acetato. O uso de melaço como fonte de carbono orgânico no cultivo de microalgas e produção de moléculas de alto valor é um método de valorização do subproduto ou resíduo proveniente da fabricação do açúcar, se tornando assim, uma fonte de carbono de baixo custo inserida na prática de economia circular. A inserção de melaço em meio de cultura também aumentou a produção de astaxantina da *Chlorella zofingiensis*, comparado ao uso de glicose, desencadeando regulação positiva de genes envolvidos na biossíntese da astaxantina (LIU *et al*., 2012). Além disso, a microalga *C. zofingiensis* atuou na biorremediação do resíduo, assimilando até 98% dos açúcares (LIU *et al*., 2012).

Diante disso, o presente estudo sugere que *H. pluvialis* tem a capacidade de utilizar glicerol e melaço para incrementar a biossíntese de astaxantina. Esse carotenoide é o composto bioativo de maior valor comercial extraído da *H. pluvialis*, entretanto por representar uma pequena fração do conteúdo celular (2-7%) se faz necessário aplicar um modelo de biorrefinaria, para aproveitamento total dos metabólitos de alto valor presentes na biomassa.

Atividade de L-asparaginase em H. pluvialis

A microalga *H. pluvialis* cultivada sob diferentes fontes e estratégias de carbono orgânico apresentou atividade da enzima L-asparaginase mínima e máxima de 62 e 256

IU mg^{-1} de biomassa seca e 26 e 109 IU mL^{-1} de solução enzimática (Figura 5), respectivamente. As maiores atividades de L-asparaginase foram apresentadas nos tratamentos acetato-WPF, glicerol-WPF e glicerol-PF ($p < 0,05$), com valores médios de 226 ± 24 IU mg^{-1} de biomassa seca e 97 ± 10 IU mL^{-1} de solução enzimática (Figura 5). O uso de melaço como fonte de carbono não favoreceu o incremento dessa enzima.

A produção de L-asparaginase em *H. pluvialis* ainda não foi documentada, apenas havendo registro da presença do gene que expressa essa enzima, quando submetida ao mutagênico químico N-metil-N-nitro-N nitrosoguanidina (WANG *et al*., 2005). No entanto, há registros de atividade de L-asparaginase em outras espécies de microalgas e cianobactérias. Para as espécies *Chlorella vulgaris*, *Spirulina maxima* e *Oscillatoria Terebriformis* foram reportadas atividades de L-asparaginase de 10 IU g^{-1}, 51,28 IU L^{-1} e 55,56 IU mL^{-1} (EBRAHIMINEZHAD *et al*., 2014; ABD EL-BAKY e EL-BAROTY, 2016; ELKOMY, 2018). Em *Escherichia coli*, a principal fonte comercial de L-asparaginase, foram registradas atividade de 20,95 IU mL^{-1} no sobrenadante bruto e atividade específica de 67 IU mg^{-1} de proteína, enquanto o extrato purificado pode alcançar 225,6 IU mL^{-1} e 190 IU mg^{-1} de proteína (KHUSHOO *et al*., 2004).

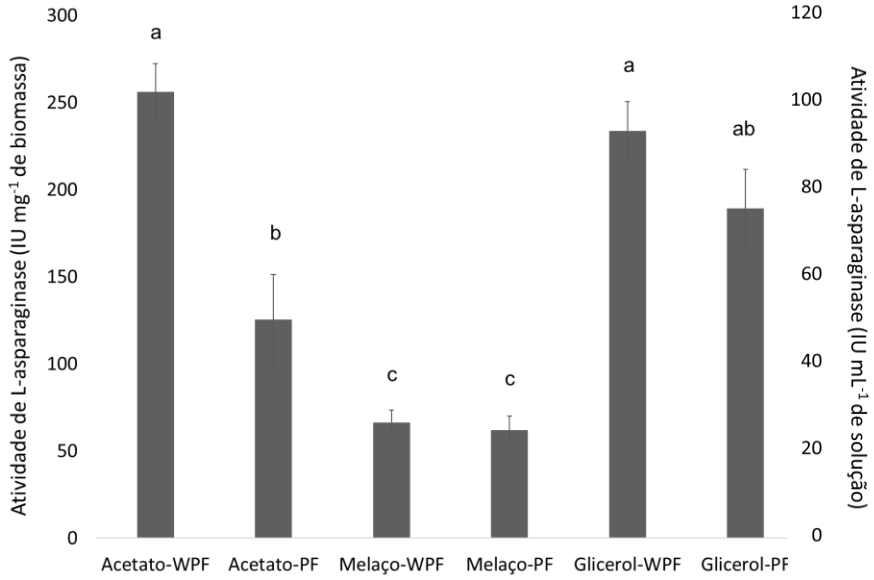

Figura 5 Atividade de L-asparaginase (IU mg^{-1} de biomassa seca e IU mL^{-1} de solução enzimátiza) da *H. pluvialis* cultivada sob diferentes fontes e estratégias de alimentação de carbono orgânico. Letras distintas indicam diferenças significativas entre os tratamentos pelo teste de Tukey ($p < 0,05$)

A L-asparaginase é uma enzima de aplicação quimioterapêutica usada em ampla variedade de terapias contra o câncer. Inúmeros microrganismos (*i.e.*, bactérias, fungos e microalgas) foram identificados como fonte de L-asparaginase, entretanto nem todos possuem propriedades bioquímicas adequadas para uso na medicina (BATOOL *et al*., 2016). Além disso, a L-asparaginase é utilizada no processamento de alimentos, reduzindo o risco de formação de acrilamida, uma neurotoxina, em alimentos ricos em amido (BATOOL *et al*., 2016). Desse modo, existe um grande interesse em encontrar novas fontes de L-asparaginase que sejam capazes de produzir em quantidade significativa. Portanto, *H. pluvialis* surge como uma fonte nova e promissora para produção de L-asparaginase.

Conclusões

Diferentes fontes e estratégias de alimentação de carbono orgânico influenciaram a produção de biomassa e metabólitos de alto valor de *Haematococcus pluvialis*. Maiores rendimentos em biomassa foram obtidos utilizando glicerol-WPF e acetato-PF, enquanto o uso das fontes glicerol e melaço, sob diferentes estratégias de alimentação, incrementou a biossíntese de proteínas, ácidos graxos e astaxantina. Além desses compostos, *H. pluvialis* surge como uma fonte nova e promissora da enzima L-asparaginase, especialmente ao utilizar acetato de sódio e glicerol. O glicerol e o melaço de cana-de-açúcar representam fontes de carbono orgânico de baixo custo, viável para o cultivo e produção de compostos bioativos de *H. pluvialis*. Finalmente, visando o aproveitamento de todas as moléculas de alto valor produzidas, é possível aplicar a biomassa de *H. pluvialis* à um modelo de biorrefinaria, utilizando a microalga em uma abordagem holística.

Agradecimentos

Os autores agradecem aos Laboratórios de Sanidade de Animais Aquáticos, de Produção Aquícola e de Dinâmica de Populações Marinhas do Departamento de Pesca e Aquicultura da Universidade Federal Rural de Pernambuco, por disponibilizarem equipamentos utilizados no experimento. Agradecimento especial ao Grupo de Investigação Biotecnologia de Microalgas Marinhas do Departamento de Engenharia Química da Universidade de Almería (Espanha), por toda a contribuição para o enriquecimento deste estudo.

Financiamento

Os autores agradecem o apoio financeiro da Pró-Reitoria de Pós-Graduação (PRPG) da Universidade Federal Rural de Pernambuco através dos recursos PROAP; à Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES) pelas bolsas concedidas a Laenne Moraes [88882.436230/2019-01]; ao Programa Institucional de Internacionalização (PrInt- CAPES) pela concessão da bolsa de Doutorado-sanduíche no Exterior [88887.717490/2022-00]; e ao Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq) pelas bolsas concedidas a Alfredo Gálvez [PQ 308063/2019-8] e Ranilson Bezerra [PQ 307107/2019-1].

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4- Artigo científico III: Integrated system for simultaneous tilapia effluent treatment and astaxanthin production by *Haematococcus pluvialis*

Artigo submetido ao periódico "Aquaculture International".

Integrated system for simultaneous tilapia effluent treatment and astaxanthin production by *Haematococcus pluvialis*

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Abstract

Haematococcus pluvialis is a microalga that can reduce the levels of inorganic and organic components in effluents, in addition to produce of the high-value carotenoid astaxanthin. The present study proposed an integrated system for the simultaneous treatment of effluent from a Nile tilapia recirculation system and astaxanthin production using the microalga *H. pluvialis*. Cultures were developed in 2 L bottles, with freshwater previously treated and enriched with a modified Bold's Basal Medium (BBM). The experiment was carried out on a laboratory scale using a completely randomized experimental design with five treatments: Control (100% BBM), E25 (25% Nile tilapia effluent + 75% BBM), E50 (50% Nile tilapia effluent + 50% BBM), E75 (75% Nile tilapia effluent + 25% BBM), E100 (100% Nile tilapia effluent), with three replicates for each condition. *H. pluvialis* could grow in all proportions of effluent from Nile tilapia cultivation. The use of effluent as culture medium (E100), despite having lower cell density and biomass, resulted in biomass with high astaxanthin content (10 mg g^{-1}) and antioxidant activity (89% inhibition of DPPH radicals). Meanwhile, this integrated system significantly contributed to the effluent remediation (98% N and 91% P removal). *H. pluvialis* can be used for cost-effective treatment of aquaculture effluent with simultaneous production of astaxanthin, with potential application as aquafeed, in a holistic bioeconomy approach.

Keywords: microalgae; high-value biomass; antioxidant activity; bioremediation; wastewater.

Statements and Declarations

Funding

The authors are grateful for the financial support of the Federal Rural University of Pernambuco; to the Coordination of Improvement of Higher Education Personnel (CAPES) grants to Laenne Moraes [88882.436230/2019-01]; to the Foundation for the Support of Science and Technology of Pernambuco (FACEPE) grants to Géssica Mota [IBPG-1574-5.04/22]; and the National Council for Scientific and Technological Development (CNPq) for the scholarships granted to Alfredo Olivera Gálvez [PQ 308063/2019-8] and Ranilson de Souza Bezerra [PQ 307107/2019-1].

Conflicts of interest

The authors declare that there are no conflicts of interest related to the publication of this article.

Ethics approval

This article does not contain any studies with animals performed by any of the authors.

Availability of data and material

The authors certify that all data support the published claims and comply with field standards.

Code availability

Not applicable

Authors' contributions

LM: writing - original draft, conceptualization, investigation, methodology, formal analysis and data curation, GM and BS: investigation, methodology and formal analysis, CO: formal analysis, data curation and writing - review and editing, JS: resources and data curation, AG: resources, supervision and writing - review and editing, RB: study conception and design, resources and project administration.

Introduction

As a result of global population increase, several environmental problems such as contamination of soil and water bodies caused by improper disposal of effluents have intensified, affecting the ecosystem functioning of aquatic and terrestrial environments (Chai *et al*. 2021). In view of the pollution and scarcity of water resources, the treatment and reuse of wastewater results in social, environmental and economic benefits (Tortajada 2020). Among the generated effluents, industrial and domestic effluents stand out, in addition to those from aquaculture, which when released into rivers and lakes lead to deterioration of water quality (Nogueira *et al*. 2022).

Fish rearing is a growing activity in worldwide due to its economic, social and nutritional importance, contributing significantly to global food security (Wang *et al*. 2021). The increase in this activity resulted, almost in the same proportion, in the increase in the production of effluents, which can cause environmental impacts due to the release of inorganic compounds, mainly nitrogen and phosphorus, from leftover feed and fish excreta (Kurniawan *et al*. 2021). The recirculation aquaculture system (RAS) is a way to reduce the disposal of effluents, however, due to the intensive mode of production, it is essential to treat waste both within the recirculation circuit and in the effluents of these systems (Rijn 2013). Such treatment can be carried out by other organisms such as plants, bacteria and algae.

To carry out photosynthesis, algae require macro and micronutrients, and can absorb these compounds from effluents (Mohsenpour *et al*. 2021). In recent years, studies have been carried out aimed at the use of microalgae for the bioremediation of different types of effluents (Daneshvar *et al*. 2019; Oliveira *et al*. 2020; Chai *et al*. 2021; Moraes *et al*. 2021). When cultivated in media rich in nitrogen, phosphorus and organic carbon, microalgae can accumulate a high amount of biomass, growing under heterotrophic, without the use of light energy, or mixotrophic metabolisms, where the metabolic routes of photosynthesis and organic carbon sources are used simultaneously (Lv *et al*. 2018; Pérez-Garcia *et al*. 2011).

These microorganisms are not only important for the environment, but also have immense potential in various industrial segments, including agriculture and human and animal nutrition (Yap *et al*. 2021). *Haematococcus pluvialis* is especially known for its high content of carotenoids, mainly astaxanthin (reaching a concentration of up to 5% of the cellular content) (Mota *et al*. 2022). Astaxanthin is a carotenoid with high antioxidant capacity, which acts to protect cells against free radical damage, combating

oxidative stress and reducing the risk of diseases in humans and animals (Mota *et al*. 2022). In recent years, astaxanthin has gained attention as a dietary supplement for animals, particularly in aquaculture (Lim *et al*. 2018).

Studies have shown that astaxanthin feed supplementation in aquaculture can improve immune function, reduce inflammation, increase resistance to environmental stressors, and improve reproductive performance, growth, and coloration (Lim *et al*. 2018). The use of *H. pluvialis* as a source of astaxanthin in animal feed offers several advantages: it is a natural and sustainable source, with greater antioxidant power than the synthetic form (Mota *et al*. 2022); it can be cultivated using wastewater as an environmentally friendly and low-cost culture medium (Pan *et al*. 2021); the inclusion of *H. pluvialis* in animal feed can lead to an improvement in product quality (Su *et al*. 2020).

Based on the circular economy concept, this study proposed an integrated system for the simultaneous treatment of effluents from a tilapia recirculation system and astaxanthin production by *H. pluvialis*, with potential application as aquafeed.

Material and methods

Strain and culture conditions

H. pluvialis was obtained from the Live Food Production Laboratory, at the Fisheries and Aquaculture Department of the Federal Rural University of Pernambuco. Cultures were developed in 2 L bottles, with freshwater previously treated with chlorine (3 ppm), filtered (22 µm) and autoclaved (120 °C), and then enriched with modified Bold's Basal Medium (BBM) (Moraes *et al*. 2023) that contain (in mg L^{-1}): 118 NH_4NO_3, 25 $CaCl_2.2H_2O$; 25 NaCl; 31 KOH; 50 EDTA $Na.2H_2O$; 75 K_2HPO_4; 175 KH_2PO_4; 4.98 $FeSO_4.7H_2O$; 75 $MgSO_4.7H_2O$; 11.42 H_3BO_3; 1.412 $ZnSO_4.7H_2O$; 0.232 $MnCl_2.4H_2O$; 0.252 $CuSO_4.5H_2O$; 0.192 $Na_2MoO_4.2H_2O$; 0.08 $Co(NO_3)_2.6H_2O$.

Experimental design

The experiment was carried out on a laboratory scale using a completely randomized experimental design with five treatments: Control (100% BBM), E25 (25% Nile tilapia effluent + 75% BBM), E50 (50% Nile tilapia effluent + 50% BBM), E75 (75% Nile tilapia effluent + 25% BBM), E100 (100% Nile tilapia effluent), with three replicates each.

H. pluvialis was inoculated in 2 L bottles with an initial concentration of 20,000 cells mL^{-1}, at a temperature of 22 °C, photoperiod of 12 h and 40 µmol photons m^{-2} s^{-1} irradiance, under continuous aeration. In the stationary phase, to stimulate the carotenogenesis (cystic phase), there was an increase in light intensity (40 to 100 µmol photons m^{-2} s^{-1}), in addition to the natural nitrogen deprivation (Moraes *et al*. 2023).

Effluent from Nile tilapia in Oasis® recirculation system

The effluent from a culture tank of Nile tilapia (*Oreochromis niloticus*) in Oasis® production system was used for *H. pluvialis* cultivation. The Oasis® is a RAS-based system that aims at high productivity, low water renewal and full use of the waste generated. In this system, Nile tilapias of about 10.88 ± 0.77 cm and 31.47 ± 2.71 g were cultivated for 45 days using a feed of 45% of crude protein offered three times a day (*ad libitum*) and presenting a feed conversion factor of 0.98. This effluent was subjected to filtration (40 µm), chlorination with sodium hypochlorite at 3 ppm for 24 hours (with aeration). After these procedures, the effluent was autoclaved at 120 °C for 15 minutes (Moraes *et al*. 2021).

Growth analysis and biomass harvesting

To evaluate growth samples were taken daily and fixed in formaldehyde (2%) for quantification using a hemocytometer (Neubauer chamber). The growth curves with the average daily cell density were fitted by approximating the logistic curve (Sprouffske and Wagner 2016). The logistic equation describes the population size N_t at time t (1):

$$N_t = \frac{K}{1+\left(\frac{K-N_0}{N_0}\right)e^{-rt}} \quad (1)$$

Where N_0 is the population size at the beginning of the growth curve, K is the maximum possible population size in a particular environment and r is the specific growth rate. With this fit, the following parameters were calculated: specific growth rate (r, day^{-1}) and doubling time (DT, division day^{-1}). The maximum specific growth rate (μ_{max}) was calculated according to the equations below.

The following asymmetric logistic equation (2) was used to fit the cell concentration (N(t)) versus time (t) data in order to accurately determine the specific growth rate:

$$N(t) = y_0 + \frac{a}{\left[1 + e^{\frac{-(t-t_0)}{b}}\right]} \quad (2)$$

Where a, b, t_0 and y_0 are constants. The cell-specific growth rate (μ, day^{-1}) was calculated using the best fit curve of Eq. (2); thus:

$$\mu(t) = \frac{1}{N(t)}\left(\frac{dN(t)}{dt}\right) \quad (3)$$

Where μ_{max} (day^{-1}) is the maximum specific growth rate in the exponential phase according to equation (3).

For maximum cell density (C_{max}, cells mL^{-1}), it was considered the day of culture in which the population reached the maximum cell density.

The dry biomass (g L^{-1}) and biomass productivity (g L^{-1} day^{-1}) were determined by filtering 10 mL aliquots of suspended cells through a Whatman GF/C glass microfiber filter (1.2 µm) and drying at 75 °C for 24 h (Choi *et al*. 2018).

Water quality and nitrogen removal

The parameters of water quality, pH, temperature, ammonia-N (NH_3-N), nitrite-N (NO_2-N) and nitrate-N (NO_3-N) were analyzed in the culture medium on days 0 (first day), 12 (exponential phase) and 20 (last day). Samples of 10 mL were filtered through a Whatman GF/C glass microfiber filter (1.2 µm), and then the filtered volume was collected for analysis of the nitrogen compounds and phosphate (APHA 2012). The pH and temperature were measured using a digital pHmeter (Kmoon pH/EC-983).

The determination of the percentages of nutrient removal from the culture media was carried out at the end of the experiment using the equation 4:

$$\text{Removal (\%)} = 100 - \left(\frac{\text{final nutrient concentration}}{\text{initial nutrient concentration}}\right) \times 100 \quad (4)$$

Quantification of total carotenoids and astaxanthin

The concentrations of total carotenoids and astaxanthin were determined at the end of cultivation. Carotenoid and astaxanthin analysis were performed from a 10 mL and 1 mL aliquot of the algae suspension, respectively, and analyzed in a spectrophotometer, according to Moraes *et al*. (2023).

The concentration of total carotenoids was calculated by [$4\times OD_{480}$], and astaxanthin concentration and content were calculated from the expressions [$4.5\times OD_{490}\times(Va/Vb)$] and [P/W], respectively. Where Va (mL) was the volume of DMSO, Vb (mL) was the volume of microalgae samples, P (mg L^{-1}) was the concentration of astaxanthin, and W (g L^{-1}) denoted the dry biomass of microalgae per unit volume of medium (Cheng *et al*. 2016).

Antioxidant activity

Extracts of *H. pluvialis* biomass were obtained using 0.1 g of dried biomass resuspended and homogenized in 2 mL of 99.9% dimethyl sulfoxide. Afterwards the mixture was centrifugated at 1000 rpm for 2 min. The supernatant was collected subjected to serial dilution (50, 25, 12.5, 6.25, and 3.12 mg mL^{-1}) for posterior antioxidant assays as proposed by Oliveira *et al*. (2022). The antioxidant activity (inhibition %) of the extracts were evaluated by 2,2'–azino–bis (3–ethylbenzothiazoline–6–sulfonic acid) ($ABTS^{+\bullet}$) (Guedes *et al*. 2013) and 2,2–diphenyl–1–picrylhydrazyl ($DPPH^{\bullet}$) (Dantas *et al*. 2019) methods. Trolox® was used as standards for calibration curve as well as positive control.

Statistical analysis

The response variables were submitted to the Shapiro-Wilk normality test and to the Bartlett's test of homogeneity of variances. All data were submitted to ANOVA followed by Tukey's test when a significant difference was observed. P values < 0.05 were considered statistically significant for all tests. Statistical analysis was performed using the R Core Team software (R Core Team 2023).

Results and discussion

Cell growth and biomass production of H. pluvialis

H. pluvialis could grow in all proportions of the Oasis® effluent from Nile tilapia rearing. The variables growth rate (*r*), doubling time (*DT*) and maximum specific growth rate (μ_{max}) were higher for treatments E75 followed by E0 (control) and E100, while the lowest values were presented by E50 and E25. On the other hand, the maximum cell density (C_{max}) was higher at E0 and E25, these treatments were significantly different from E50, E75 and E100 (Table 1).

Regarding the biomass, E0 and E25 obtained the highest values, with an average yield of 0.75 g L^{-1} and average productivity of 0.047 g L^{-1} day^{-1}. While E50 and E75 showed an average yield and productivity of 0.61 g L^{-1} and 0.038 g L^{-1} day^{-1}. E100 was significantly different for all treatments, except E75, and showed average yield and productivity of 0.53 g L^{-1} and 0.033 g L^{-1} day^{-1} (Table 1).

Table 1 Growth variables of *Haematococcus pluvialis* cultivated with different proportions of BBM culture medium and effluent from the Nile tilapia farming in Oasis® system.

	E0	**E25**	**E50**	**E75**	**E100**
r	0.554 ±	0.441 ±	0.454 ±	0.621 ±	0.539 ±
(day^{-1})	0.022^{b}	0.016^{c}	0.017^{c}	0.022^{a}	0.018^{b}
DT	1.25 ±	1.58 ±	1.53 ±	1.12 ±	1.28 ±
(division day $^{-1}$)	0.08^{b}	0.08^{c}	0.05^{c}	0.05^{a}	0.06^{b}
μ_{max}	0.287 ±	0.240 ±	0.229 ±	0.403 ±	0.267 ±
(day^{-1})	0.044^{b}	0.052^{c}	0.035^{c}	0.076^{a}	0.021^{b}
C_{max}	126.12 ±	119.88 ±	100.25 ±	89.75 ±	80.25 ±
(x10^{4} cells mL^{-1})	9.5^{a}	11^{a}	17^{b}	18^{b}	10^{b}
Biomass	0.76 ±	0.73 ±	0.63 ±	0.59 ±	0.53 ±
(g L^{-1})	0.02^{a}	0.02^{a}	0.03^{b}	0.06^{bc}	0.04^{c}
Biomass productivity (g L^{-1} day^{-1})	0.048 ± 0.001^{a}	0.046 ± 0.001^{a}	0.039 ± 0.002^{b}	0.037 ± 0.004^{bc}	0.033 ± 0.003^{c}

Different letters in the same row indicate a significant difference by the Tukey's *post–hoc* test ($p < 0.05$).
r: growth rate; DT: doubling time; μ_{max}: maximum specific growth rate; C_{max}: maximum cell density.

The growth curves of *H. pluvialis* which show the daily cell densities of each treatment, are presented in Fig. 1. The control treatment (E0) presented a longer exponential phase than the others. This may have occurred due to the availability of mixed sources of nitrogen in the effluent. Once the higher affinity nitrogen source, ammonia, is depleted, there is a metabolic readjustment to absorb the following sources, nitrate and nitrite (Su 2021). This condition justifies the 'pseudo-stationary phases' along the effluent treatment curves, which may influence the lower growth. Possibly, vegetative cells multiplied later compared to the control and treatment with lower percentage of effluent addition (*i.e.*, E25), resulting in a lower cell density in treatments E75 and E100.

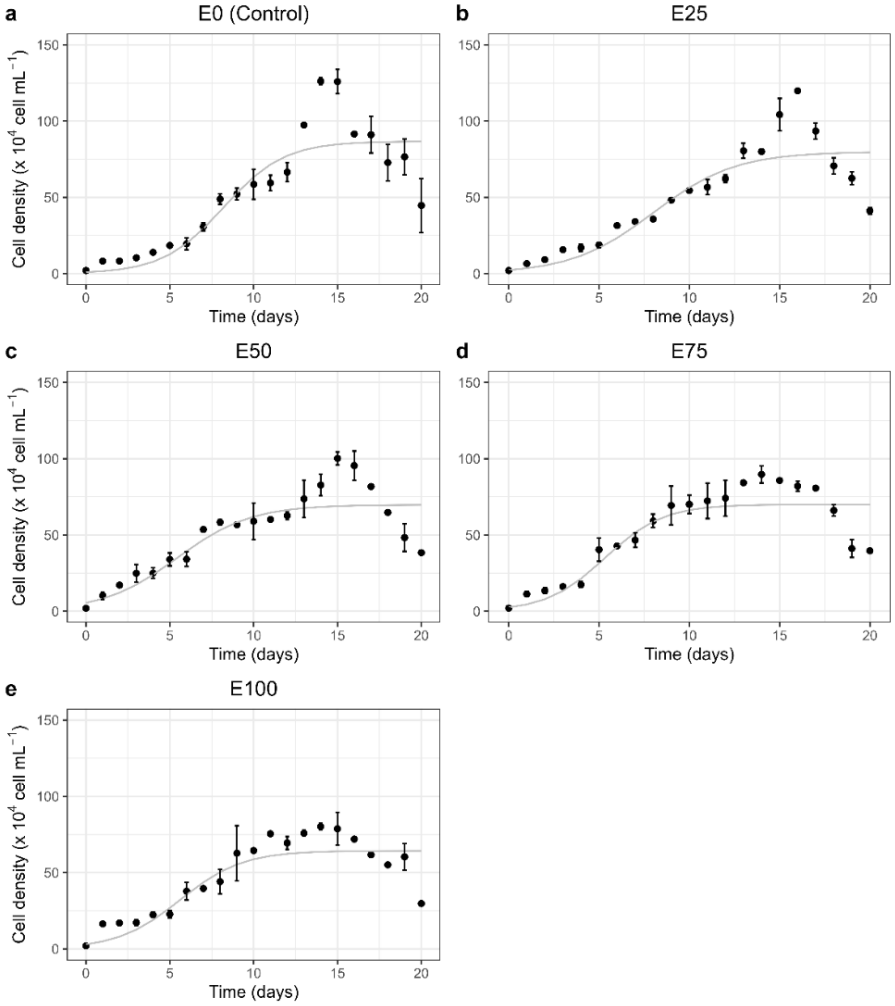

Fig. 1 Logistic growth curve of *H. pluvialis* cultivated under different proportions of BBM and effluent from the Nile tilapia Oasis® system

Besides the presence of nutrients, like nitrogen, other physicochemical aspects play a crucial role in cellular metabolism, such as temperature and pH. Maintaining minimal pH fluctuations ensures a more consistent chemical environment and, as a result, improved conditions for the division of *H. pluvialis* cells (Dos Santos and Lombardi 2017). In this study, the initial pH had no significant difference between treatments ($p > 0.05$), with an average of 7.2 ± 0.2; generally, at an initial pH of 7, greater growth of *H. pluvialis* is observed (Sarada *et al*. 2002). The pH variation until the end of cultivation was higher in treatments E50, E75 and E100 (8.4 ± 0.03, 8.26 ± 0.01, 8.37 ± 0.05, respectively), differing significantly of treatments E0 and E25 (7.8 ± 0.01, 7.9 ± 0.02). This may also have influenced the lower C_{max}, biomass and biomass productivity in treatments with a higher proportion of effluent (*i.e.*, E50, E75 and E100). The temperature had no significant difference between treatments or over time, remaining at 22.2 ± 0.4.

Removal of nitrogen and phosphate compounds by H. pluvialis

Regarding nitrogen sources, ammonia is the nitrogen compound most easily incorporated by microalgae (Su 2021). Thus, for microalgae in general, the order of preference for nitrogen utilization is NH_4^+ –> NO_3 –> NO_2, due to no redox reaction requirement and less energy cost to assimilate ammonia (Ramanna *et al*. 2014). Ammonia was more abundant (25 mg L^{-1}) in the treatments with higher amounts of BBM, *i.e.*, E0 and E25 (Fig. 2a), which showed higher C_{max}, biomass and biomass productivity (Table 1). Despite that, the total nitrogen concentration was similar for the treatments, with an average of 40 mg L^{-1}. Hence, nitrogen in the form of ammonium contributed to greater biomass, similar to previous studies (Mourya *et al*. 2023; Pan *et al*. 2021; Yap *et al*. 2022).

H. pluvialis only uses other sources of nitrogen, such as nitrate and nitrite, when ammonia is no longer available in the environment (Markou and Georgakakis, 2011). The average initial concentration of NO_2-N in treatments E75 and E100 was 0.8 mg L^{-1}, while for treatments E0 and E25 it was 0.04 and 0.2 mg L^{-1}, respectively (Fig. 2b). Meanwhile, higher concentrations of NO_3-N (27 - 40 mg L^{-1}) were found in treatments with a high proportion of effluent (E100 and E75) (Fig. 2c). The nitrogen uptake by *H. pluvialis* in the effluent was more than 95% for NH_3-N (except in E100 that reached 52%), 94% for NO_3-N and 100% for NO_2-N (Fig. 2e). Considering total nitrogen, there was an average absorption of 97.7% for all treatments, being the highest absorption in

E75 (99.5%), whose growth velocity data *r* and μ_{max} were higher.

In addition to nitrogen, phosphorus is an essential element as it assists in metabolic processes, photosynthesis and energy conversion (Marinho *et al*. 2021). The initial concentration of PO_4-P varied between 13 (E100) and 24 mg L^{-1} (E0) (Fig. 2d), being consumed by the microalgae throughout the cultivation, with a final removal efficiency of 84 to 98% (Fig. 2e). The highest phosphate absorption occurred in E75 and E100, which had a lower initial concentration of PO_4-P.

In summary, the results of the study indicate that *H. pluvialis* has a significant ability to capture nutrients from effluent, improving therefore, the levels of these compounds in the water, and this is in agreement with previous studies (Wu *et al*. 2013; Mourya *et al*. 2023; Pan *et al*. 2021; Yap *et al*. 2022).

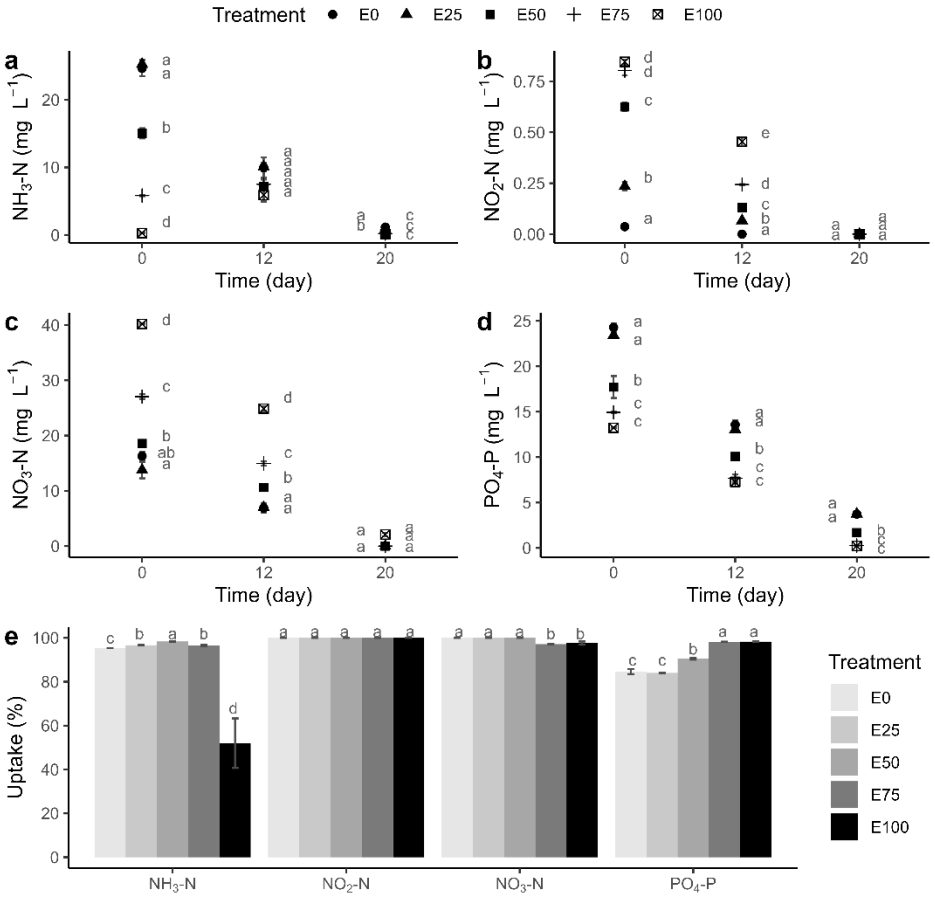

Fig. 2 Concentrations of NH_3-N (a), NO_2-N (b), NO_3-N (c) and PO_4-P (d) over 20 days and uptake of nitrogen and phosphate compounds (e) by *H. pluvialis* under different proportions of BBM and effluent from the Nile tilapia Oasis® system. Different letters indicate a significant difference among treatments by the Tukey's *post–hoc* test ($p < 0.05$)

Production of high-value metabolites from H. pluvialis

In addition to *H. pluvialis* acting in the bioremediation of effluent, it can produce high-value metabolites such as carotenoids, especially astaxanthin (80-99% of total carotenoids). As for the total carotenoids obtained in the cystic phase, higher concentrations (5.32 - 6.06 mg L^{-1}) and content (7.6 - 10.1 mg g^{-1}) were observed in the treatments E0, E25 and E100 (Fig. 3a e 3b). Following the same trend, higher concentrations (5.34 - 5.50 mg L^{-1}) and content (7.26 - 10.25 mg g^{-1}) of astaxanthin were obtained in treatments E0, E25 and E100 (Fig. 3c e 3d).

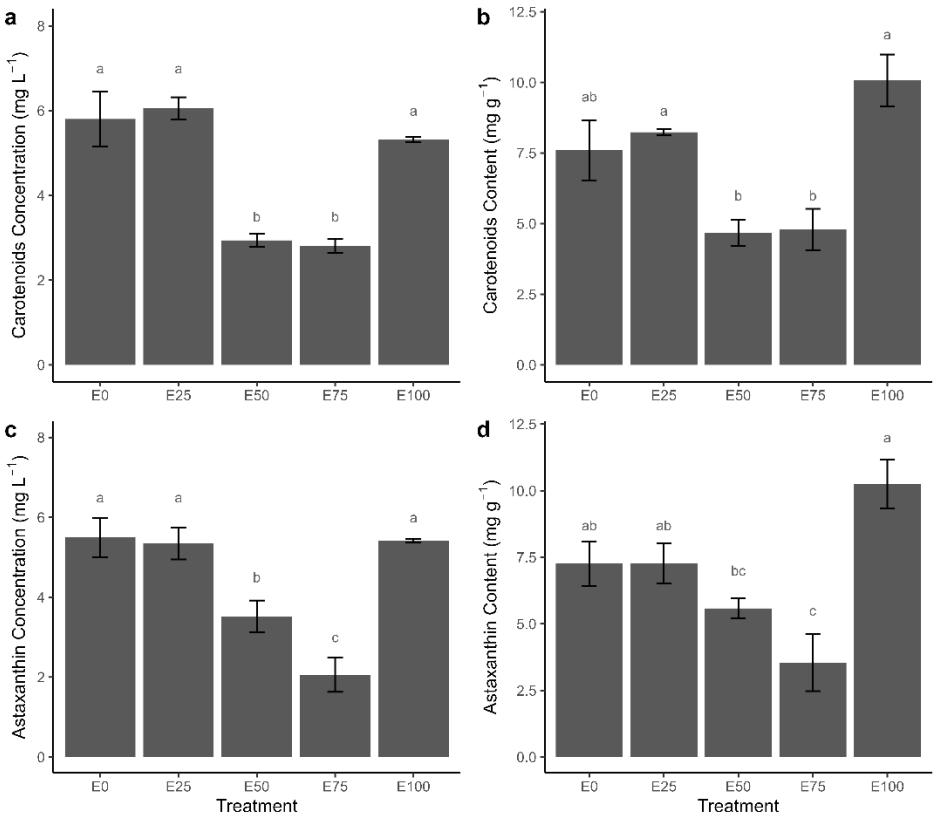

Fig. 3 Concentrations (mg L^{-1}) and contents (mg g^{-1}) of total carotenoids (a,b) and astaxanthin (c,d) produced by *Haematococcus pluvialis* cultivated in different proportions of BBM and effluent from the Nile tilapia Oasis® system (different letters indicate a significant difference by the Tukey's *post–hoc* test ($p < 0.05$)

Astaxanthin synthesis occurs under nitrogen depletion, which triggers the upregulation of genes associated with astaxanthin biosynthesis to act in defense metabolism to this stress condition (Zhao *et al*. 2019). On day 20, total nitrogen depletion was observed in all treatments, accompanied by a predominance of cystic cells, with accumulation of astaxanthin. Furthermore, under nitrogen deprivation, the carbon flux is directed towards the synthesis of astaxanthin (Lu *et al*. 2019; Moraes *et al*. 2023). A high C/N ratio stimulates the efficient and rapid carbon utilization, primarily through the tricarboxylic acid cycle, in order to biosynthesize this carotenoid (Yu *et al*. 2022). The use of effluent from the Nile tilapia recirculation system (E100) contributed to the high content of astaxanthin (10 mg g^{-1}), similar to E0 and E25, although E100 had a low biomass compared to these treatments. The use of effluent replacing the culture medium may be a stress factor for microalgae that have low growth (Pan *et al*. 2021).

Astaxanthin is a carotenoid widely used in the pharmaceutical, nutraceutical, cosmetic, and food industries, in addition to aquaculture, both for pigmentation and to improve the immune response and zootechnical performance of aquatic organisms (Lim *et al*. 2018; Su *et al*. 2020). This carotenoid has anti-inflammatory, antitumor, antidiabetic, immunomodulatory, and antioxidant properties, highlighting its antioxidant activity, which is 10 times greater than that of β-carotene (Mota *et al*. 2022). The antioxidant activity of microalgal biomass extract cultured in effluent from Oasis® system was investigated using ABTS and DPPH assays (Table 2). As noted, all treatments exhibited antioxidant effects, even at varying degrees (47.4-88.7% inhibition). The highest percentages of inhibition of ABTS and DPPH radicals were presented by treatments E50 (73.9 ± 7.9 %) and E100 (88.7 ± 1.5 %), respectively. The percentage of inhibition observed in the present study was similar to that reported by Al-Tarifi *et al*. (2020), using the same solvent (DMSO) for astaxanthin powder, approximately 85% inhibition of DPPH radical. Therefore, the biomass produced in effluent showed greater antioxidant activity.

Table 2 Antioxidant activity (% inhibition) of extracts of the *Haematococcus pluvialis* biomass under different proportions of BBM and effluent from the Nile tilapia Oasis® system.

Treatments	**$ABTS^{+\bullet}$**	**$DPPH^{\bullet}$**
E0 (control)	63.0 ± 3.3^{b}	72.6 ± 3.2^{c}
E25	49.6 ± 3.4^{d}	47.4 ± 3.1^{d}
E50	73.9 ± 7.9^{a}	79.7 ± 2.7^{b}
E75	58.6 ± 5.4^{c}	81.2 ± 1.8^{ab}
E100	54.8 ± 8.2^{c}	88.7 ± 1.5^{a}

Different letters on the same column indicate a significant difference by the Tukey's *post–hoc* test ($p < 0.05$).

The results suggest that the biomass of *H. pluvialis* cultivated with effluent from the Nile tilapia rearing, rich in astaxanthin and with high antioxidant power, it can be used in aquaculture feed. This aquafeed could be used in the production of tilapia in the Oasis® system, as well as the treated water could be returned to replenish the cultivation system, in a circular economy approach. Furthermore, the astaxanthin from *H. pluvialis* biomass has promise as a bioactive compound in nutraceutical and pharmaceutical products for human applications. Thus, the use of effluent as a culture medium contributes to the sustainable production of *H. pluvialis* biomass and astaxanthin, considering the replacement of high-cost culture media, the valorization of effluents in high-value metabolites and the treatment of effluents (potential source of contamination for the environment).

Conclusions

H. pluvialis was able to grow in all proportions of RAS effluent from the Nile tilapia rearing. The use of effluent as culture medium, despite having lower cell density and biomass, had a higher astaxanthin content. Therefore, the present study proposes an ecofriendly and low-cost medium formed only by effluent from the Nile tilapia recirculation system for astaxanthin production. Meanwhile, the integrated system contributed to the effluent remediation. *H. pluvialis* can be used for cost-effective treatment of aquaculture effluent with simultaneous production of astaxanthin with great potential for a holistic bioeconomy.

Acknowledgments

The authors would like to thank the Laboratory of Aquatic Animal Health, Laboratory of Shrimp Farming and Laboratory of Aquaculture Production at the Department of Fisheries and Aquaculture, and Laboratory of Molecular Biology Applied to Animal Production at the Department of Animal Science of the Federal Rural University of Pernambuco for providing the instruments used for the analysis.

Compliance with ethical standards

Conflict of interest

The authors declare that there are no conflicts of interest related to the publication of this article.

Ethical statement

This article does not contain any studies with animals performed by any of the authors.

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5- Considerações finais

Na primeira parte do estudo utilizou-se diferentes fontes e estratégias de alimentação de nitrogênio para aumentar a produção de biomassa de *H. pluvialis* e astaxantina. Neste estudo, a estratégia de alimentação por pulso de nitrogênio proporcionou maior crescimento de *H. pluvialis*, obtendo maiores rendimentos e produtividade em biomassa - vegetativa e cística - para todas as fontes de nitrogênio. Por outro lado, maiores concentrações e teores de astaxantina foram observados para a fonte NH_4NO_3 sem pulsos. Portanto, a alimentação de nitrogênio por pulsos, promove maior produção de biomassa de *H. pluvialis* e uma nova perspectiva para aumentar a produção de astaxantina.

Na segunda parte da pesquisa, diferentes fontes e estratégias de alimentação de carbono orgânico influenciaram a produção de biomassa e metabólitos de alto valor de *H. pluvialis*. Maiores rendimentos em biomassa foram obtidos utilizando glicerol sem pulsos e acetato com pulsos, enquanto o uso das fontes glicerol e melaço, sob diferentes estratégias de alimentação, incrementou a biossíntese de proteínas, ácidos graxos e astaxantina. Dessa forma, o melaço de cana-de-açúcar se apresenta como uma fonte de carbono orgânico de baixo custo, viável no cultivo e produção de compostos bioativos de *H. pluvialis*. Além desses metabólitos já conhecidos em *H. pluvialis*, foi reportada pela primeira vez a enzima L-asparaginase nesta espécie, surgindo como uma fonte nova e promissora.

Na terceira parte da pesquisa, o efluente de um sistema de recirculação de tilápia foi utilizado em diferentes proporções em substituição parcial ou total do meio de cultura para o cultivo de *H. pluvialis* e produção de astaxantina. A microalga foi capaz de crescer em todas as proporções do efluente do cultivo de tilápia do Nilo. A utilização do efluente como meio de cultura, apesar de apresentar menor densidade celular e rendimento de biomassa, apresentou maior teor de astaxantina. Portanto, o presente estudo propõe o cultivo de *H. pluvialis* em meio composto por efluente do cultivo de tilápia do Nilo para produção de astaxantina, contribuindo para uma aquicultura mais sustentável através da biorremediação.

Diante disso, a tese apresentada confirmou as hipóteses levantadas e atendeu a todos os objetivos propostos. De fato, modificações nas fontes e estratégias de alimentação de nitrogênio e carbono orgânico incrementam a produção de biomassa e metabólitos de alto valor de *H. pluvialis*. Paralelo a isso, a produção da *H. pluvialis* em

efluente de tilápia resulta no tratamento econômico de efluentes de aquicultura com produção simultânea de astaxantina, adotando os princípios da economia circular.

Futuras pesquisas podem ser propostas para avaliar as melhores fontes de nitrogênio e carbono orgânico utilizadas no presente estudo, tanto para produção de biomassa quanto para obtenção de metabólitos, sob diferentes concentrações e intervalos de tempo na alimentação por pulsos. Quanto aos estudos com águas residuais, há espaço para investigar diferentes tipos de efluentes no cultivo e obtenção de compostos bioativos de *H. pluvialis* com aplicação em variados seguimentos industriais.

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ANEXOS

Anexo I - Artigo I publicado no periódico “Biomass Conversion and Biorefinery”.