UNIVERSIDADE FEDERAL DO RIO GRANDE DO SUL
ESCOLA DE ENGENHARIA
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PROGRAMA DE PÓS-GRADUAÇÃO EM ENGENHARIA QUÍMICA

# Aplicação de tecnologias emergentes no crescimento de microalgas e na extração de compostos de interesse

- Tese Doutorado -

Renata Nunes Pereira

Porto Alegre
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Tese de doutorado apresentada como requisito parcial para a obtenção do título de Doutora em Engenharia.

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# Resumo

Microalgas são organismos capazes de sintetizar lipídeos, proteínas e carotenoides que podem ser matéria-prima para produção de biocombustíveis, bioestimulantes agrícolas, alimentos e rações. Alguns estudos sugerem que o estresse celular como o uso de tecnologias emergentes, como o ultrassom (US), poderia aumentar a produção de biomassa e de seus compostos, melhorando sua competitividade no mercado. Além disso, os métodos de extração mais comuns desses compostos envolvem solventes tóxicos, como éter de petróleo, metanol e outros, prejudiciais ao meio ambiente. Assim, métodos alternativos de extração, como o uso da tecnologia de campo elétrico pulsado (PEF, do inglês *Pulsed electric field*), é relevante por ser considerada uma tecnologia limpa. Neste contexto, o presente trabalho tem como objetivo avaliar a aplicação de US durante o crescimento de microalgas, e avaliar o processo extrativo com uso de PEF dos compostos sintetizados. No estudo do uso de US durante o crescimento das microalgas *Pseudoneochloris marina* e *Chlorella zofingiensis* foi observado comportamentos distintos entre as espécies que reagiram de forma diferente aos estímulos físicos. Para *P. marina*, o tratamento com US não aumentou a proliferação celular, e ainda reduziu a densidade celular quando utilizado por 60 min (fase exponencial, durante 5 dias), indicando uma possível ocorrência de danos celulares. Para *C. zofingiensis*, a aplicação de US descontínuo durante 10 min resultou num aumento de 65 % na concentração de biomassa (2,19 ± 0,10 g $L^{-1}$) em comparação com o controle (1,33 ± 0,16 g $L^{-1}$). No estudo do uso de PEF como pré-tratamento extrativo utilizando etanol como solvente, foi avaliada a extração da biomassa fresca de *C. zofingiensis* em duas fases de crescimento: verde (sem stress) e laranja (com stress). O melhor tratamento PEF (20 kV $cm^{-1}$, 150 μs) mostrou um aumento de 46 % de carotenoides (0,76 mg $L^{-1}$) em comparação com a extração do controle. Em geral, os resultados do presente estudo mostraram o potencial do US descontínuo para aumentar a proliferação de células de microalgas e a aplicação de PEF combinada com etanol como sendo uma abordagem adequada para a extração de carotenoides.



**Palavras-chave:** ultrassom; campo elétrico pulsado; crescimento; extração; carotenoides; lipídeos.

# Abstract

Microalgae can synthesize lipids, proteins, and carotenoids, which can serve as raw material for biofuels and agricultural biostimulants, food, and animal feed. Studies have been suggesting that stressing microalgae cells applying emerging technologies, such as ultrasound (US), might increase biomass and compounds production, improving its competitiveness. In addition, the most common extraction methods for these compounds involve toxic solvents such as petroleum ether, methanol and others, which are harmful to the environment. Thus, alternative extraction methods, such as the use of pulsed electric field (PEF) technology, is relevant because it is considered a clean technology. In this context, the present work aims to evaluate the application of US during the growth of microalgae, and to evaluate the extractive process using PEF of the synthesized compounds. In the study of the use of US during the growth of the microalgae *Pseudoneochloris marina* and *Chlorella zofingiensis*, distinct behaviors were observed between the species that reacted differently to physical stimuli. For *P. marina*, US treatment did not increase cell proliferation, and even reduced cell density when used for 60 min (exponential phase for 5 days), indicating a possible occurrence of cell damage. For *C. zofingiensis*, discontinuous US application for 10 min resulted in a 65 % increase in biomass concentration ($2.19 \pm 0.10$ g $L^{-1}$) compared to the control ($1.33 \pm 0.16$ g $L^{-1}$). In the study of the use of PEF as an extractive pretreatment using ethanol as solvent, the extraction of fresh biomass of *C. zofingiensis* at two growth stages: green (unstressed) and orange (stressed) was evaluated. The best PEF treatment (20 kV $cm^{-1}$, 150 μs) showed a 46 % increase in carotenoids (0.76 mg $L^{-1}$) compared to the control extraction. Overall, the results of the present study showed the potential of discontinuous US to enhance microalgae cell proliferation and the application of PEF combined with ethanol to be a suitable approach for carotenoid extraction.



**Keywords:** ultrasound; pulsed electric field; growth; extraction; carotenoids; lipids.

# Lista de figuras

# Lista de tabelas

# Lista de siglas, abreviaturas e símbolos

| | |
|---|---|
| US | ultrassom |
| PEF | campo elétrico pulsado, do inglês *pulsed electric field* |
| nsPEF | campo elétrico pulsado de nanosegundos, do inglês *nanoseconds pulsed electric field* |
| AG | ácidos graxos |
| TAG | triacilgliceróis |
| PUFAs | ácidos graxos poli-insaturados, do inglês *polyunsaturated fatty acids* |
| $\Psi m$ | potencial transmembrana |
| f | fator de forma |
| E | força do campo elétrico em função do tempo |
| $a_m$ | raio da célula |
| $\theta$ | ângulo em relação na direção do campo elétrico |
| *t* | tempo de tratamento |
| $\tau_m$ | tempo de carregamento da membrana |
| $C_m$ | capacitância da membrana por unidade de área |
| $\sigma_e$ e $\sigma_i$ | condutividade extracelular e intracelular |
| R | resistência |
| $\sigma$ | condutividade do meio |
| d | distância do eletrodo |
| A | área de superfície do eletrodo |
| $Z_{tot}$ | impedância total |

| | |
|---|---|
| $Y_c$ | sistema de entrada |
| W | energia específica de entrada |
| $\tau_p$ | largura do pulso |
| *n* | número de pulsos |
| *f* | frequência |
| *t* | tempo de permanência ou tratamento |
| *Y* | variável de resposta a ser modelada |
| *Xi* e *Xj* | fatores independentes |
| $\beta_0$ | intercepto |
| $\beta_i$ | coeficiente linear |
| $\beta_{ii}$ | coeficiente quadrático |
| $\beta_{ij}$ | coeficiente de produto vetorial |
| k | número total de fatores independentes |

# Sumário

# Capítulo 1 - Introdução

Novos desafios em relação ao suprimento de energia, à segurança alimentar, à saúde humana e à biodiversidade têm surgido com o constante crescimento da população. A implementação de tecnologias inovadoras e o aumento do uso de produtos de origem natural, como os produzidos por plantas e microrganismos, são possíveis soluções para enfrentar esses desafios (Buchmann et al., 2019).

Dentre as alternativas de origem natural, tem-se as microalgas, que são organismos eucariotos ou procariotos, fotoautotróficos, podendo ser encontradas em oceanos e no ambiente terrestre. Essas são capazes de sintetizar compostos de interesse, como proteínas, lipídeos, carboidratos e pigmentos (carotenoides, ficocianinas, entre outros), que podem ser matéria-prima para diferentes produtos. Os lipídeos podem ser utilizados na indústria de alimentos, em suplementos alimentares, ou mesmo na indústria de biocombustíveis, para obtenção de biodiesel. Os carotenoides são pigmentos naturais que têm sido utilizados nas indústrias farmacêutica, química, de alimentos e rações, devido à sua coloração e à sua capacidade antioxidante (Liu et al., 2014). Os pigmentos antioxidantes, astaxantina, β-caroteno e luteína, são os principais carotenoides de valor comercial encontrados nas microalgas (Gong e Bassi, 2016). Aumentar a capacidade produtiva desses organismos favorece a produção desses compostos de interesse comercial, agregando valor à cadeia produtiva das microalgas, o que aumenta sua competitividade no mercado.

As microalgas possuem algumas vantagens quando comparadas as plantas, como maior taxa de crescimento, facilidade de gerenciamento, e não utilização de terras aráveis

para seu cultivo. Além disso, também são capazes de fixar $CO_2$ da atmosfera, contribuindo para a redução do efeito estufa (Buchmann et al., 2019; Han et al., 2016). No entanto, os custos dos seus produtos permanecem relativamente altos, prejudicando sua viabilidade econômica quando comparados a outros produtos de origem vegetal (Haberkorn et al., 2019). Dessa forma, é importante buscar alternativas para otimizar sua capacidade produtiva, favorecendo sua competitividade no mercado.

O estresse celular tem sido utilizado para aumentar a produção de biomassa e compostos produzidos por microrganismos. Ambientes de estresse com esgotamento de nutrientes, alta temperatura, presença de luz e alta salinidade são os mais comumente empregados (Han et al., 2016). Alguns estudos também mencionam os efeitos de tecnologias emergentes, como ultrassom, campo elétrico moderado e campo elétrico pulsado, no crescimento de microalgas e na síntese de compostos intracelulares (Batghare et al., 2018; Han et al., 2016; Sivaramakrishnan et al., 2019; Singh et al., 2019).

O ultrassom tem sido utilizado para a intensificação de diversos processos físicos e químicos, para melhorar os rendimentos e modificar as vias em reações químicas e biológicas. No entanto, poucos estudos podem ser encontrados a respeito do uso desta tecnologia para estímulo do crescimento celular. Alguns estudos indicam que essa aplicação pode ser uma boa alternativa, embora pouco se saiba sobre como o seu uso afeta as células vivas.

Após a produção de compostos de interesse pelas microalgas, é necessária a aplicação de métodos de extração e purificação para a obtenção de ingredientes desejáveis às indústrias alimentícia, farmacêutica e cosmética. Os métodos de extração comumente utilizados para obtenção de carotenoides e lipídeos de microalgas são baseados no uso de

solventes tóxicos, como hexano, clorofórmio, metanol, éter de petróleo e acetona (Bligh & Dyer, 1959; Rodriguez et al., 2014). O uso desses solventes torna difícil a aplicação dos extratos obtidos pelas indústrias. As tecnologias emergentes podem ser utilizadas para extração de compostos de interesse a partir de células biológicas, permitindo o uso de processos extrativos menos agressivos ambientalmente, com minimização do gasto energético.

Neste contexto, o presente trabalho visou avaliar o uso das tecnologias emergentes em duas etapas do processo produtivo para obtenção de compostos de interesse a partir de microalgas: 1) durante o crescimento das microalgas, visando melhorar a produção de biomassa e compostos de interesse; e 2) durante a extração dos compostos, visando a otimização de um processo ambientalmente amigável e com altos rendimentos. Num primeiro momento, a tecnologia de ultrassom foi empregada no crescimento de duas microalgas, *Pseudoneochloris marina* e *Chlorella zofingiensis*, para induzir mudanças no metabolismo celular. Numa segunda etapa, foi avaliado o processo de extração assistido pela tecnologia de campo elétrico pulsado, visando a obtenção de carotenoides e clorofilas a partir da microalga *C. zofingiensis.*

Esse trabalho está estruturado conforme descrito a seguir. No Capítulo 2 são apresentados os objetivos gerais e específicos. No Capítulo 3 é apresentada uma revisão bibliográfica sobre a importância das microalgas, bem como sobre os principais compostos sintetizados por esses microrganismos. Além disso, serão apresentados os principais fatores que influenciam o cultivo de microalgas e a aplicação de tecnologias emergentes durante o cultivo desses microrganismos e para a extração de compostos.

O Capítulo 4 apresenta o estudo da avaliação da influência do ultrassom no crescimento das microalgas *P. marina* e *C. zofingiensis*. Nesse capítulo, são apresentadas as metodologias empregadas e os resultados, que estão apresentados na forma de um artigo publicado na revista *Bioresource Technology*. O Capítulo 5 apresenta o estudo da aplicação de campo elétrico pulsado na extração de carotenoides e clorofilas a partir da microalga *C. zofingiensis*. Os resultados também estão apresentados na forma de artigo científico.

No Capítulo 6 são apresentadas as considerações finais e futuros desafios para continuidade do trabalho. Por fim, anexo a esse trabalho, é apresentado um artigo de revisão sobre o crescimento de microalgas assistido por tecnologias emergentes, publicado na *Brazilian Journal of Chemical Engineering*.

# Capítulo 2 – Objetivos

## 2.1. Objetivo geral

Este trabalho tem como objetivo geral estudar a influência de tecnologias emergentes em duas etapas do processo de obtenção de compostos de interesse a partir de microalgas: 1) avaliar o uso ultrassom durante o crescimento das microalgas *Pseudoneochloris marina* e *Chlorella zofingiensis* para obtenção de maior produção de biomassa e concentração de carotenoides, lipídeos e proteínas; 2) avaliar o uso de campo elétrico pulsado na extração de carotenoides e clorofilas produzidos pela microalga *C. zofingiensis.*

## 2.2. Objetivos específicos

Considerando os objetivos gerais, o presente trabalho tem como objetivos específicos:

- cultivar as microalgas *P. marina* e *C. zofingiensis* e identificar as fases de crescimento, assim como as melhores condições de cultivo;
- aplicar a tecnologia de ultrassom de forma contínua e intermitente em diferentes fases do crescimento da microalga *P. marina* para avaliar a influência dessa tecnologia sobre a produção de biomassa, carotenoides, lipídeos e proteínas;
- aplicar o tratamento ultrassônico contínuo e intermitente no crescimento da microalga *C. zofingiensis* e comparar com os resultados obtidos para a microalga *P. marina*;

- ambientar a cepa da microalga *C. zofingiensis* em novas condições de cultivo e padronizar o crescimento das células estressadas (biomassa laranja) e não estressadas (biomassa verde);
- aplicar a tecnologia de campo elétrico pulsado no processo de extração de carotenoides e clorofilas a partir da microalga *C. zofingiensis* para as biomassas verde e laranja, avaliando a influência do tempo de tratamento, intensidade do campo elétrico e presença de etapa difusiva.

# Capítulo 3 - Fundamentos teóricos e revisão bibliográfica

Esse capítulo apresenta uma revisão bibliográfica sobre a importância das microalgas e os principais compostos de interesse sintetizados por esses organismos; são abordados, também, aspectos relacionados ao crescimento e produção de microalgas, assim como, à extração de compostos, assistidos por tecnologias emergentes como ultrassom e campo elétrico pulsado.

## 3.1. Microalgas

As microalgas são organismos eucariotos ou procariotos, fotoautotróficos, podendo ser encontradas em oceanos e no ambiente terrestre. Estes microrganismos são capazes de captar $CO_2$ da atmosfera, contribuindo para a redução de gases do efeito estufa, convertendo em biomassa a partir do processo de fotossíntese (Kim, 2015). Esta característica é uma das vantagens da utilização desses microrganismos em processos biotecnológicos. As cores distintas encontradas nestes organismos se devem às clorofilas (pigmento que absorve a luz) e outros pigmentos acessórios envolvidos na fotossíntese, como os carotenoides (Tortora; Funke; Case, 2012).

As microalgas são capazes de utilizar eficientemente $CO_2$ para a produção de vários produtos, como proteínas, polissacarídeos, lipídeos, vitaminas, carotenoides e outros compostos biologicamente ativos. Portanto, podem ser utilizadas em uma ampla variedade de aplicações tecnológicas para o desenvolvimento ingredientes e alimentos para consumo humano e animal (Liu et al., 2014).

A produção de carotenoides a partir de microalgas tem sido intensivamente estudada nos últimos anos, como β-caroteno de *Dunaliella salina*, zeaxantina de *Synechocystis*, luteína de *Chlorella protothecoides* e astaxantina de *Haematococcus pluvialis* e *Chlorella zofingiensis*. Além disso, muitos estudos visam identificar maneiras de aumentar a produtividade destes compostos (Gonçalves et al., 2019; Li et al., 2019; Ördög et al., 2012; Yeesang e Cheirsilp, 2011).

### *3.1.1. Pseudoneochloris marina*

As microalgas *Pseudoneochloris marina* são algas verdes unicelulares, que possuem células vegetativas esféricas, de 3 a 20 µm de diâmetro, com parede celular lisa quando maduras, sendo sua reprodução assexuada, por zoósporos. Estes são piriformes, obovoides, apresentam dois flagelos de tamanhos iguais, mas não possuem parede celular verdadeira (Watanabe et al., 2000).

Essa espécie apresenta a capacidade de produzir lipídeos e carotenoides intracelulares, sendo os majoritários luteína e β-caroteno. Poucos estudos avaliaram o crescimento dessa microalga, assim como sua capacidade de produzir compostos. Gonçalves et al. (2019) avaliaram as melhores condições de cultivo para essa espécie, variando a concentração de nitrogênio no meio, a intensidade de luz e a temperatura, obtendo maior produtividade com 74,1 mg $L^{-1}$ de $N\text{-}NO_3$ a 28 °C e 252 µmol $m^{-2}$ $s^{-1}$. Os autores também observaram um efeito negativo da intensidade de luz sobre a produção de carotenoides e lipídeos, enquanto a produção de proteínas foi afetada pelo incremento de todos os fatores.

### *3.1.2. Chlorella zofingiensis*

*Chlorella zofingiensis* é uma microalga verde de água doce, unicelular, esférica, não móvel, uma vez atribuída ao gênero *Chlorella*, mas alguns autores classificam essa alga nos gêneros *Muriella*, *Mychonastes* ou *Chromochloris*, indicando uma distância evolutiva da "verdadeira *Chlorella*". As células de *C. zofingiensis* podem apresentar tamanho de 2 a 15 μm de diâmetro, e se reproduzem assexuadamente através da formação de autósporos. A reprodução assexual geralmente envolve três fases: crescimento, amadurecimento e divisão (Liu et al., 2014).

As microalgas verdes *Chlorella zofingiensis* e *Haematococcus pluvialis* são os produtores mais promissores de astaxantina, carotenoide relevante para indústria alimentícia e farmacêutica. A vantagem de se utilizar a microalga *C. zofingiensis* é que esta cresce mais rápido tanto fototroficamente, como heterotroficamente ou mixtroficamente. Além disso, essa espécie é fácil de cultivar e escalonar, em ambientes internos ou externos, e pode atingir altas densidades celulares (Liu et al., 2014). Essas características biotecnológicas fornecem a *C. zofingiensis* um alto potencial para a produção em massa de astaxantina.

Além disso, de acordo com Chen e Wang (2013), a microalga *C. zofingiensis* também pode crescer bem e acumular astaxantina em condições de cultura heterotrófica sem a presença de luz. Os autores ainda observaram que, nessa condição, a presença de glicose, nitrato e magnésio foram os principais nutrientes que afetaram a cinética do crescimento celular e o acúmulo de astaxantina. Essa diversidade adaptativa que a microalga apresenta durante o crescimento permite sugerir que uso desse microrganismo em processos biotecnológicos possa ser promissor.

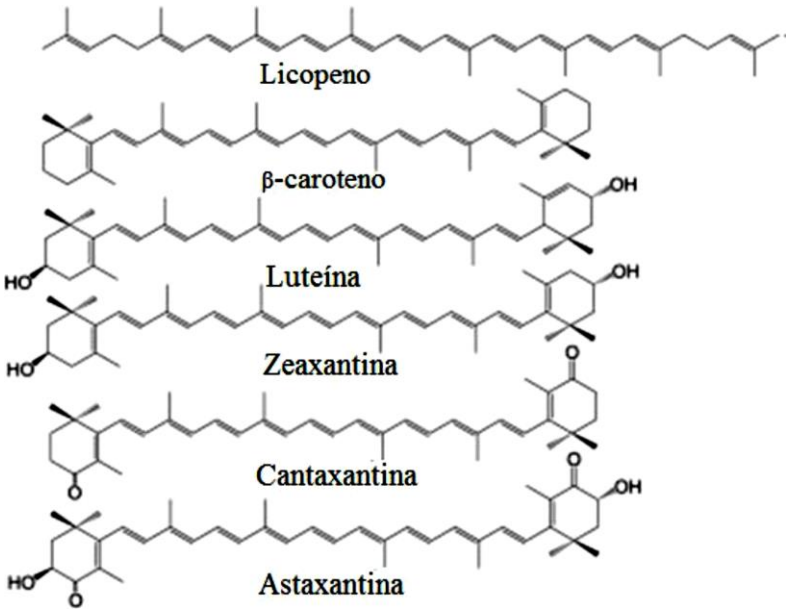
Ademais, os carotenoides produzidos por esta microalga, principalmente astaxantina e cetoluteína, existem na forma de ésteres com ácidos graxos e são armazenados em corpos lipídicos sob condições de estresse, ou seja, *C. zofingiensis* tem a capacidade de acumular grandes quantidades de astaxantina e lipídeos simultaneamente. Essas propriedades tornam esta microalga capaz de atender às demandas comerciais de múltiplos bioprodutos, devido a sua alta taxa de crescimento em modo trófico orgânico para atingir altas densidades celulares e acúmulo de carotenoides (Chen et al., 2020).

## 3.2. Compostos de interesse de alto valor agregado

### *3.2.1. Carotenoides*

Os carotenoides são compostos lipofílicos, geralmente de cor amarela, laranja ou vermelha, sendo os pigmentos mais diversos e amplamente encontrados na natureza. Algumas estruturas mais comuns de carotenoides estão apresentadas na Figura 1. Estes representam um grupo de pigmentos terpenoides estruturalmente diversos, sendo a maioria uma cadeia de 40 carbonos conjugada por ligações duplas, e são divididos em dois grupos: carotenos e xantofilas (Gong e Bassi, 2016). Os carotenos são compostos apenas por hidrocarbonetos em sua estrutura, enquanto as xantofilas apresentam compostos oxigenados, o que permitem que esses carotenoides sejam relativamente hidrofílicos em função da presença dos grupos hidroxila e grupos ceto nos anéis terminais (Liu et al., 2014).

Figura 1 - Estrutura química dos carotenoides mais comumente encontrados. Adaptado de Liu et al. (2014).




Devido à estrutura de polieno conjugado, os carotenoides desempenham um papel importante como pigmentos acessórios na fotossíntese em termos de transporte de energia e oxigênio, e na proteção dos organismos fotossintéticos contra a lesão causada por fotoxidação. Além disso, extinguem o oxigênio singlete ($^1O_2$) e os radicais livres que são gerados pelo metabolismo e danificam os tecidos metabolizantes, sendo essenciais para a vida. Em razão disso, os carotenoides possuem propriedades que auxiliam na prevenção e tratamento contra muitos tipos de doenças, como doenças cardiovasculares, catarata e câncer (Tapiero et al., 2004).

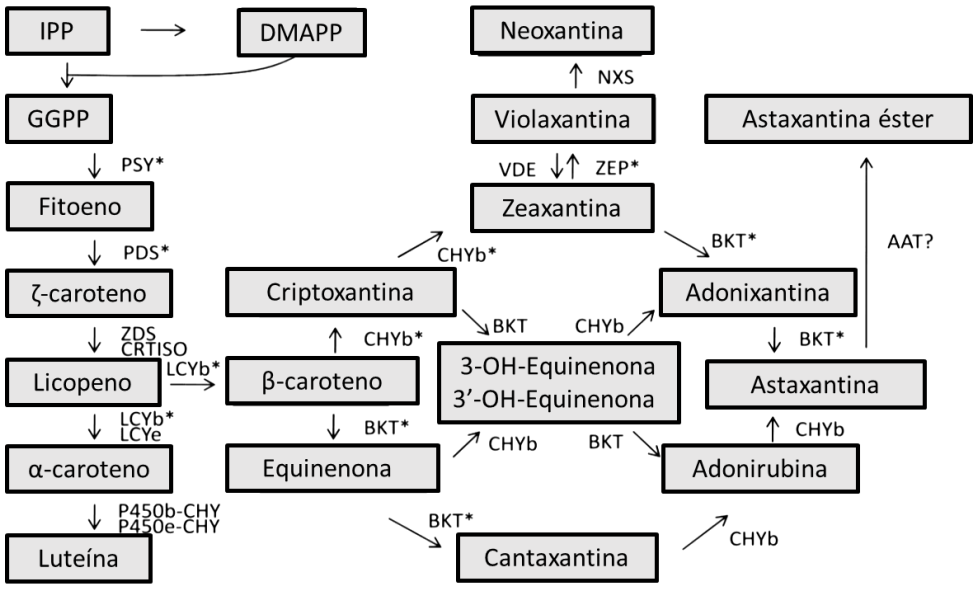
A recente preferência dos consumidores por produtos naturais, saudáveis e coloridos resultam no uso universal de carotenoides como aditivos naturais para alimentos e cosméticos (Ye et al., 2008). Ademais, como a maioria dos animais não

consegue sintetizar esses pigmentos naturalmente, os carotenoides são amplamente utilizados na alimentação animal, como corantes e para a nutrição (Tapiero et al., 2004). Entretanto, devido à sua capacidade antioxidante, estes pigmentos são geralmente sensíveis à luz, oxigênio e calor, o que pode levar a dificuldades no armazenamento e manuseio (Gong e Bassi, 2016).

Como as plantas, as microalgas sintetizam e acumulam carotenoides primários, como β-caroteno, luteína e zeaxantina, no cloroplasto. Por outro lado, carotenoides secundários, como astaxantina, cantaxantina e adonixantina, acumulam-se nos corpos lipídicos fora do cloroplasto. O acúmulo de carotenoides secundários está associado a condições de estresse (limitação de nitrogênio, alta irradiância, ou alta salinidade), sendo esses pigmentos-acessórios o mecanismo de proteção contra danos oxidativos (Lee, 2008; Liu et al., 2014; Rise et al., 1994).

Os aspectos bioquímicos e moleculares da biossíntese de carotenoides em microalgas é aceito como sendo semelhante às plantas superiores, envolvendo, geralmente, cinco tipos de reações (Figura 2): formação de difosfato de isopentenila (IPP); condensação de unidades de isopreno resultando em fitoeno; reações de dessaturação convertendo fitoeno incolor em licopeno rosa; ciclização de licopeno, formando β-caroteno e α-caroteno; e síntese de xantofilas pela introdução de grupos de oxigênio aos carotenos (Liu et al., 2014).

Figura 2 - Diagrama esquemático da possível biossíntese de carotenoides em microalgas.
Adaptado de Liu et al. (2014).




IPP, pirofosfato de isopentenilo; DMAPP, pirofosfato de dimetilalilo; GGPP, pirofosfato de geranilgeranilo; PSY, fitoeno sintase; PDS, fitoenodesaturase; ZDS, ζ-caroteno dessaturase; CRTISO, caroteno isomerase; LCYb, licopeno β-ciclase; LCYe, licopeno ε-ciclase; P450b-CHY, citocromo P450β-hidroxilase; P450e-CHY, citocromo P450ε-hidroxilase; CHYb, hidroxilase de β-caroteno; BKT, β-carotenocetolase; ZEP, zeaxantinepoxidase; VDE, violaxantina des-epoxidase; NXS, neoxantina sintase; AAT, astaxantina acil transferase; * denotam os genes caracterizados em *C. zofigiensis*.

Os principais carotenoides encontrados em microalgas são β-caroteno, luteína e astaxantina. O β-caroteno é o pigmento natural mais abundante do grupo dos carotenoides, podendo ser encontrado em vegetais e frutas de cor amarelo-alaranjada, e em vegetais folhosos de cor verde-escura. Nestes, a cor natural do carotenoide é mascarada pela clorofila, presente nos cloroplastos. O β-caroteno pode ser encontrado em várias estruturas, pois a configuração de cada ligação dupla pode existir naturalmente em *trans* ou *cis* (Ye et al., 2008). Como outros carotenoides, o β-caroteno desempenha um papel importante na medicina, nutrição e corante, e é muito procurado no mercado global.

Em relação a luteína, esta é um pigmento amarelo que existe em plantas superiores e outros organismos fotoautotróficos como as algas. Este carotenoide faz parte da família das xantofilas, sendo também encontrado em flores, grãos, frutas e vegetais, como espinafre e couve (Yang et al., 2018). O mercado de luteína é segmentado em indústrias farmacêuticas, de suplementos dietéticos, de alimentos e de rações para animais e peixes. Sua principal aplicação é para iluminar as cores das penas das aves e aprofundar o amarelo da gema do ovo (Lin et al., 2015).

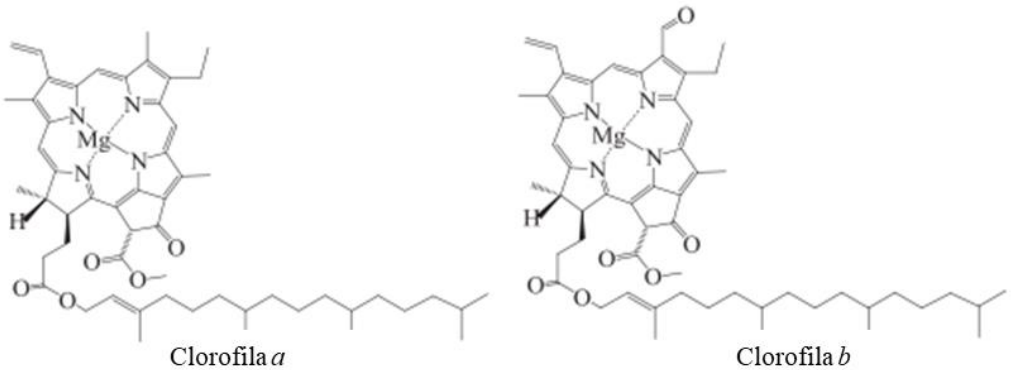
Por fim a astaxantina, um cetocarotenoide de alto valor, com uma ampla gama de aplicações nas indústrias de alimentos, rações, nutracêuticos e farmacêuticos, vem ganhando grande atenção da ciência e do público nos últimos anos. Este pigmento de cor laranja-avermelhada tem sido utilizado como corante natural na indústria. Esse composto também apresenta uma poderosa atividade antioxidante, capaz de melhorar o sistema imunológico, com potencial para tratamento de câncer e diabetes, sendo muito mais eficaz na eliminação de radicais livres do que outros carotenoides e vitamina E (Chen e Wang, 2013; Liu et al., 2014).

A pigmentação forte da astaxantina permite que ela sirva como complemento alimentar para a aquicultura, visto que os animais não podem sintetizar carotenoides, mas precisam obtê-los através de sua cadeia alimentar ou ração (Liu et al., 2014). Os carotenoides da dieta conferem aos organismos como salmonídeos e crustáceos a cor laranja avermelhada, considerada pelos consumidores como um dos principais atributos de qualidade (Niamnuy et al., 2008).

*3.2.2. Clorofilas*

As clorofilas são pigmentos verdes lipossolúveis, apresentam um anel de porfirina em sua estrutura (Figura 3), e são responsáveis por converter a energia solar em energia química durante a fotossíntese (D'Alessandro e Antoniosi, 2016). Na natureza, a clorofila *a* e *b* predominam nas plantas superiores, enquanto a clorofila *c*, *d* e *e* são encontrados em vários fotossintéticos como algas e espécies diatômicas (Ferruzzi e Blakeslee, 2007).

Figura 3 - Estrutura molecular das clorofilas.
Adaptado de D'Alessandro e Antoniosi (2016).




Um derivado da clorofila é a clorofilina, no qual o magnésio é substituído por sódio ou cobre e as cadeias de fitol são perdidas. As clorofilinas têm sido usadas para controlar o odor corporal de pacientes geriátricos e como suplementos dietéticos. Alguns estudos demonstraram que a clorofila e a clorofilina também podem ter ação antimutagênica e anticarcinogênica (Chimploy et al., 2009; Ferruzzi e Blakeslee, 2007).

*3.2.3. Lipídeos*

Os lipídeos são insolúveis em água, mas solúveis na maioria dos solventes orgânicos. Estes podem ser gorduras, óleos, algumas vitaminas e hormônios. As gorduras

e os óleos são derivados de ácidos graxos (AG), sendo constituídos por ácidos carboxílicos com grupos laterais de hidrocarbonetos de cadeia longa (VOET; VOET, 2013). Além disso, os lipídeos podem ser classificados em polares e neutros. Os polares incluem fosfolipídeos e glicolipídeos, enquanto os neutros incluem triacilgliceróis (TAGs), esteróis e ácidos graxos livres. Microalgas utilizam lipídeos neutros como fonte de energia e lipídeos polares para formar as membranas celulares (Halim et al., 2012).

Dependendo da espécie de microalga, a síntese de lipídeos polares e TAGs pode ocorrer nos cloroplastos ou no retículo endoplasmático. Os lipídeos polares normalmente são acumulados nas membranas plasmáticas (do citoplasma, retículo endoplasmático, tilacoides, cloroplastos e mitocôndrias), enquanto os TAGs são comumente armazenados nos plastídios ou no citoplasma na forma de gotículas de lipídeos (Schüler et al., 2017).

Vários autores avaliaram a composição de AG em microalgas (Gonçalves et al., 2019; Jaeschke et al., 2016; Pereira, 2019; Tsai et al., 2016). Os AG que não apresentam insaturação em sua cadeia carbônica são chamados de saturados (SFA), aqueles com apenas uma ligação insaturada são chamados de monoinsaturados (MUFA), aqueles com duas são di-insaturados (DUFA), três são tri-insaturados (TUFA) e mais de três são poli-insaturados (PUFA). A maioria dos AG de microalgas são de C14:0 a C22:6, sendo a maioria monoinsaturada e saturada. Os mais comuns são C16:0, C18:0, C16:1, C18:1, C16:2, C18:2 e C18:3 (D'Alessandro e Antoniosi, 2016).

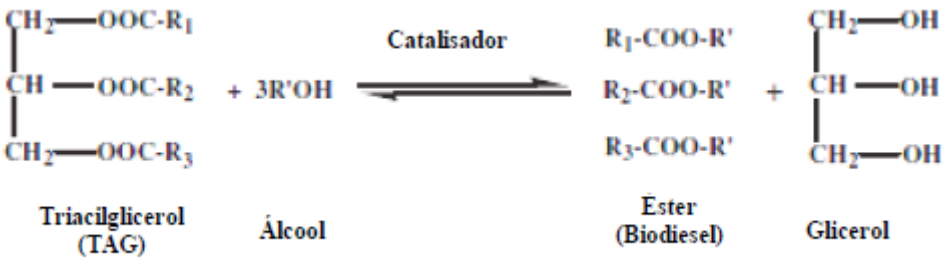
Os AG poli-insaturados são muito utilizados, por exemplo, como suplemento alimentar, podendo ser obtidos a partir de plantas, como também de micro-organismos. Os mais importantes são o ácido docosaexaenoico (DHA) e o ácido araquidônico (ARA),

os quais são encontrados em tecidos do cérebro e, também, estão presentes no leite materno, sendo importantes para o desenvolvimento neural e para funções da retina. O DHA também pode ser utilizado para ajudar a prevenir problemas cardíacos, perturbações degenerativas (Alzheimer), ou ainda para o tratamento da síndrome de déficit de atenção em crianças (RATLEDGE, 2004).

Além disso, outra aplicação para os lipídeos é na produção de biodiesel, finalidade bastante estudada para os óleos (TAGs) sintetizados por microalgas. Os TAGs são constituídos de ésteres de AG e glicerol (1,2,3-propanotriol ou glicerina), contendo diferentes tipos de AG ligados à cadeia do glicerol. Estes diferentes AG compõe o perfil graxo de cada óleo (KNOTHE et al., 2006). A partir da reação de transesterificação dos óleos, demonstrada na Figura 4, é possível obter ésteres metílicos de ácidos graxos, ou mais comumente chamado de biodiesel (KNOTHE et al., 2006; LIANG; JIANG, 2013). Dentre os combustíveis, o biodiesel constitui uma alternativa viável para o mercado de combustíveis, que atualmente se baseia em matéria-prima de origem fóssil.

Figura 4 - Reação de síntese de biodiesel a partir de triacilgricerol (TAG).

Adaptado de LIANG e JIANG (2013).



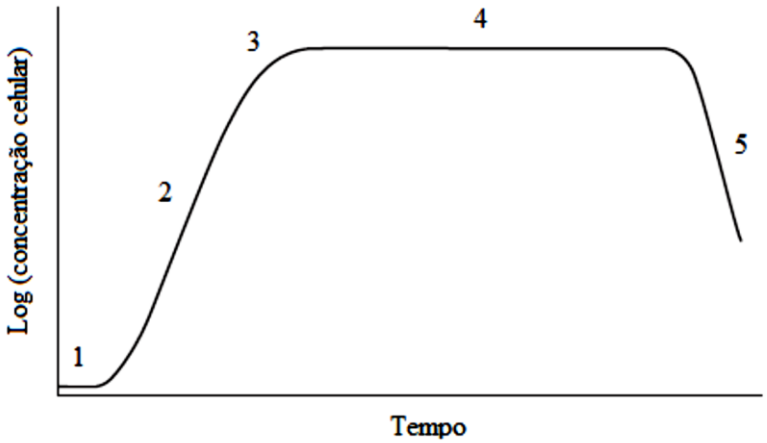
### 3.3. Crescimento e produção de microalgas

As microalgas podem ser cultivadas fotossinteticamente, a partir da luz solar e dióxido de carbono da atmosfera como fonte de carbono, produzindo biomassa e oxigênio. Além disso, podem ser cultivadas heterotroficamente, utilizando alguma fonte de carbono orgânico; ou ainda mixotroficamente, uma combinação dos cultivos fototróficos e heterotróficos (Kim, 2015).

O crescimento de microalgas é caracterizado por cinco diferentes fases: fase lag, fase exponencial, fase de declínio do crescimento, fase estacionária e fase de morte, conforme a Figura 5. A fase lag corresponde à fase de adaptação da microalga (Kim, 2015).

Os principais fatores que afetam o crescimento de microalgas incluem condições ambientais (luz, temperatura, pH, fornecimento de nutrientes e salinidade) e físicas (agitação), parâmetros biológicos como espécies de algas, e o formato do biorreator. Esses parâmetros podem afetar a atividade fotossintética, a produtividade da biomassa e a composição fisiológica e bioquímica das microalgas.

Figura 5 - Fases de crescimento durante um cultivo de microalgas: (1) fase lag; (2) fase exponencial; (3) fase de declínio do crescimento; (4) fase estacionária; (5) fase de morte.
Adaptado de Kim (2015).




Kim (2015) sugere que as condições mais comumente usadas durante os cultivos de microalgas são temperatura entre 18 – 24 °C, salinidade de 20 – 24 g $L^{-1}$, intensidade de luz de 2,5 – 5 klux, podendo variar de acordo com o volume, e pH de 8,2 – 8,7. Em relação aos nutrientes do meio de cultivo, os mais comuns são: nitrogênio, fósforo, vitaminas, e elementos traços (como ferro, manganês, selênio, cobalto, zinco e níquel).

Além dos nutrientes, a disponibilidade de luz também é essencial para o crescimento, visto que favorece a atividade fotossintética. Entretanto, quando em excesso, pode causar danos aos receptores de luz nos cloroplastos. A exposição contínua de luz pode propiciar um aumento de biomassa e da concentração de carotenoides, contudo pode diminuir a produção de clorofila (Kim, 2015).

Além disso, alguns autores sugerem que condições de estresse adequadas, como diminuição de nitrogênio no meio e alta salinidade, podem estimular a síntese e o acúmulo de lipídeos e carotenoides (Gonçalves et al., 2019; Oliveira et al., 2020; Zarrinmehr et al.,

2020). Portanto, várias estratégias de estresse celular têm sido desenvolvidas para manipular e otimizar os processos de cultivo para aumentar a eficiência da bioprodução.

Entre essas estratégias, o uso de tecnologias emergentes para promover estresse celular tem atraído o interesse de diversos autores (Batghare et al., 2018; Han et al., 2016; Sivaramakrishnan et al., 2019; Singh et al., 2019). Assim, tecnologias como o ultrassom, campo elétrico moderado e campo elétrico pulsado, podem ser uma nova alternativa para estimular a proliferação celular e síntese de compostos.

### 3.4. Extração de compostos de microalgas

A extração de compostos produzidos durante o crescimento das microalgas são uma etapa relevante dos processos biotecnológicos. Durante o processo de extração, um ou mais compostos são transferidos a partir da biomassa para o solvente. Esse processo ocorre devido à diferença de potencial químico entre o soluto dissolvido no solvente e o soluto na matriz biológica (Lebovka et al., 2011).

Nas biorrefinarias à base de microalgas, os processos extrativos de produtos intracelulares são considerados os principais gargalos técnico-econômicos (Kim et al., 2016). Muitas vezes, devido à robustez da parede celulares de algumas microalgas, como *Chlorella* e *Haematococcus* spp., solventes orgânicos convencionais podem ser inibidos, impedindo assim o contato adequado entre o solvente orgânico e o componente intracelular, sendo necessária etapas adicionais de extração (Brennan e Owende, 2010; Safi et al., 2014).

Vários métodos de extração são amplamente conhecidos, sendo que a maioria utiliza solventes tóxicos no processo. Para lipídeos, os métodos mais utilizados são

extração Soxhlet com n-hexano (Halim et al., 2011), método de Bligh e Dyer com uma mistura de solvente clorofórmio/metanol (Bligh and Dyer, 1959), e método sulfo-fosfo-vanilina (SPV) (Mishra et al., 2014).

Com relação aos carotenoides, a recuperação desses pigmentos requer uma série de unidades de operação *downstream* nas quais a extração é crítica. A extração por solvente convencional requer a utilização de uma grande quantidade de solventes, em várias etapas de extração, como acetona, éter de petróleo, éter etílico e metanol (Mandelli et al., 2011; Rodriguez et al., 2014). Após a extração, os solventes tóxicos são evaporados e os carotenoides são ressuspensos em outros solventes, gerando uma quantidade considerável de poluentes. Muitos desses solventes demonstram ser altamente tóxicos e prejudiciais ao meio ambiente (Martínez et al., 2020).

Comparado aos métodos químicos tradicionais envolvendo a adição de solvente, a extração sem solvente é mais ecológica e mais amigável ao meio ambiente. Além disso, pode ser considerada mais econômica, por não precisar de energia suplementar para separar fases e eliminar solventes (Adam et al., 2012). Neste contexto, estudos com o uso de tecnologias emergentes, como ultrassom e campo elétrico pulsado, como pré-tratamento em processos extrativos, vêm atraindo o interesse de diversos autores (Adam et al., 2012; Jaeschke et al.; 2019; Martínez et al., 2020; Parniakov et al., 2015; Rodrigues et al.; 2018).

## 3.5. Tecnologias emergentes

### *3.5.1. Ultrassom*

Ultrassom (US) consiste em ondas sonoras com frequência na faixa de 20 kHz - 500 MHz, acima do limite da audição humana. O US pode ser classificado em duas categorias: US de baixa potência, com alta frequência (1 - 10 MHz); e US de alta potência, com baixa frequência (20 - 100 kHz) (Sillanpää et al., 2011; Terefe et al., 2009).

O US se propaga por ondas de compressão e rarefação induzidas nas moléculas do meio através do qual é aplicado. Os ciclos de compressão unem as moléculas, enquanto a rarefação as separa. Quando os ciclos de rarefação excedem as forças atrativas das moléculas do líquido, em alta potência, as bolhas de cavitação são formadas. O colapso da bolha produz aquecimento local intenso (~ 5000K) e alta pressão (~ 1000 atm), fornecendo à região interfacial a energia de ativação necessária para a quebra de ligação, dissociação de solventes e outros vapores ou gases, levando à formação de radicais livres ou espécies excitadas (Sillanpää et al., 2011).

A aplicação de US tem sido utilizada em muitos processos para redução do tamanho das partículas, filtração de partículas em suspensão, estabilizações de emulsões, homogeneização, processamento de alimentos e aplicações médicas (Sillanpää et al., 2011; Mason e Lorimer, 2002). Na área química, o US pode melhorar a conversão e o rendimento, e modificar as rotas de reações químicas, reações biológicas e sistemas eletroquímicos (Singh et al., 2015). No processamento de alimentos, o US pode também ser aplicado para inibição e inativação microbiana (Joyce et al., 2010; Greenly e Tester, 2014; Rajasekhar et al., 2012). Zhang et al. (2006), por exemplo, observaram que o uso do US a 0,32 W $mL^{-1}$ (25 kHz) por 5 min inibiu o crescimento de *Microcystis aeruginosa*.

Estudos demonstram que o US pode ser utilizado assistindo processos de extração de componentes bioativos e processos envolvendo hidrólise enzimática (Adam et al., 2012; Jaeschke et al., 2017; Martínez et al., 2020; Rodrigues et al., 2018). Park et al. (2014) sugeriram a homogeneização em alta velocidade assistida por US como um método eficiente de rompimento da parede celular de *Chlorella vulgaris*. Neto et al. (2013) realizaram a extração lipídica com solvente assistida por US das biomassas de *Chlorella minutíssima*, *Thalassiosira fluviatilis* e *Thalassiosira* pseudonana. Os autores observaram um maior rendimento de extração de lipídeos com o pré-tratamento ultrassônico que métodos tradicionais (15,5 %, 40,3 % e 39,5 %, respectivamente).

Da mesma forma, Araujo et al. (2013) reportaram que o método de Bligh e Dyer assistido por US resultou em uma maior extração de lipídeos de *Chlorella vulgaris*.

Recentemente, o tratamento ultrassônico tem sido sugerido como alternativa para potencializar o crescimento de microrganismos e a produção de compostos. Essa tecnologia pode intensificar diversos processos físicos e químicos, podendo ser aplicada em células vivas com o objetivo de favorecer a proliferação celular.

*3.5.1.1. Efeito do ultrassom no crescimento de microrganismos*

A Tabela 1 apresenta os principais estudos realizados que utilizaram o US para estimular o crescimento celular e a produção de compostos de interesse. Como pode ser observado, em todos os estudos citados, o uso desta tecnologia favoreceu a síntese de biomassa, ou mesmo a produção de compostos (Dahroud et al., 2016; Huang et al., 2018; Pawar e Rathod, 2018; Sun et al., 2017). Batghare et al. (2018) relataram que o ultrassom foi capaz de induzir um aumento de 26 % na produção de astaxantina no cultivo em

batelada de *Phaffia rhodozyma* MTCC 7536. Han et al. (2016) revelaram que o tratamento ultrassônico aumentou a produção de lipídeos em 23 % na microalga *Anabaena variabilis*. Singh et al. (2019) encontraram resultados que corroboram com essas afirmações ao observar um aumento na produção de lipídeos e β-caroteno em 34 % e 31 %, respectivamente, após a aplicação de US no crescimento da microalga *Tetradesmus obliquus* SGM19.

O estímulo celular observado nos estudos citados pode ser consequência da turbulência gerada no meio com aplicação de US, ou pode estar relacionado com o aumento da energia de ativação na região interfacial, responsável pela formação de radicais livres e espécies excitadas. Esses efeitos podem desencadear outros processos corresponsáveis pela proliferação celular e síntese de compostos, como: alterações em enzimas intracelulares, aumento da aeração do meio de cultura, aumento da permeabilidade da membrana, redução da inibição do substrato, liberação de aglomerados de células formados durante o cultivo, e efeitos nos componentes celulares e funções genéticas.

Tabela 1- Estudos envolvendo aplicação de tratamento ultrassônico durante o cultivo de diferentes microalgas para produção de biomassa e compostos.

| Espécie | Tipo | Frequência (kHz) | Intensidade (W) | Tempo de US | Intervalo | Fase de crescimento | Principais resultados | | Referências |
|---|---|---|---|---|---|---|---|---|---|
| | | | | | | | Biomassa | Produtos | |
| *Tetradesmus obliquus* | banho | 33 | 100 | 10 min | 1 min ON/9 min OFF a cada12 h | início estacionária | 3,12 g $L^{-1}$ (22,8 % de acréscimo) | 0,39 g $g^{-1}$ de lipídeos e 1,17 mg $g^{-1}$ de β-caroteno (34,5 % e 31,5 % de acréscimo, respectivamente) | Singh et al. (2019) |
| *Scenedesmus* sp. | sonda | 18 | 20 | 10 min | - | logarítmica | 1,56 g $L^{-1}$ | 240 mg $L^{-1}$ de lipídeos | Ren et al. (2019a) |
| *Scenedesmus* sp. | sonda | 20 | 20 | 2s | - | logarítmica | 2,78 g $L^{-1}$ (26,9 % de acréscimo) | 28,5 % de lipídeos (37 % de acréscimo) | Ren et al. (2019b) |
| *Scenedesmus* sp. | sonda | - | 20 | 4 min | 2s | contínuo | 2,68 g $L^{-1}$ (1,4 vezes de acréscimo) | 49 % de lipídeos (72,4 % de acréscimo) | Sivaramakrishnan and Incharoensakdi (2019) |
| *Anabaena variabilis* | banho | 40 | 200 | 5 min | - | início estacionária | 1,36 g $L^{-1}$ | 46,9 % de lipídeos (1,46 vezes de acréscimo) | Han et al. (2016) |

De acordo com Han et al. (2016), a influência do tratamento ultrassônico no acúmulo de lipídeos em *Anabaena variabilis* se deve ao ambiente hostil gerado pelas alterações físicas e químicas do meio, como altas temperaturas, altas pressões e produção de radicais livres. Os autores explicam que as microalgas priorizam a síntese de compostos lipídicos em ambientes adversos.

O metabolismo lipídico é uma ferramenta fundamental para reajustar o sistema ao estresse, permitindo às algas escaparem de consequências potencialmente graves. Após o estresse celular, que pode, por exemplo, ser causado por uma deficiência de nutrientes minerais ou irradiação excessiva, muitas espécies de algas continuam a sintetizar ativamente ácidos graxos enquanto a taxa de crescimento desacelera (Roessler, 1990; Sharma et al., 2012; Thompson, 1996). Sem foco no crescimento, as células produzem ácidos graxos na forma de TAG, desviando grandes quantidades de ATP e NADPH, que poderiam ser canalizados para o crescimento celular em outras circunstâncias. Esses triglicerídeos sintetizados podem ser usados como uma fonte de carbono rica em energia quando as condições de cultivo melhoram (Roessler, 1990).

Além de armazenar o excesso de lipídeos como reserva para crescimento futuro, algumas espécies de algas também utilizam essa reserva para reduzir a fotossíntese, absorvendo diretamente parte da luz incidente. Assim, os carotenoides, que normalmente funcionam como pigmentos acessórios, permitem um aumento na absorção de luz pelo aparato fotossintético, agindo como escudos para absorver a luz que, de outra forma, poderia causar dano fotoxidativo (Thompson, 1996).

Portanto, o aumento na produção de metabólitos secundários, como lipídeos e carotenoides, em uma situação estressante, como a causada pela aplicação do ultrassom

durante o crescimento celular, pode ser explicado por essa alteração na via metabólica celular.

Além disso, outro fator que pode influenciar na síntese desses compostos, assim como na proliferação celular, é a formação de radicais livres e outras espécies reativas. As espécies reativas podem formar poros na membrana celular, permitindo um aumento na permeabilidade do material extra e intracelular (Huang et al., 2017; Batghare et al., 2018). Dai et al. (2016) sugeriram que um tratamento ultrassônico adequado poderia aumentar a taxa de crescimento de *Saccharomyces cerevisiae*, melhorando a fluidez e a permeabilidade da membrana. Além disso, Avhad e Rathod (2015) afirmaram que a transferência de massa através do meio de fermentação é potencializada pela aplicação de US que acelera a absorção do substrato pelas células, resultando em maior rendimento na produção de *Bacillus sphaericus* MTCC 3672. Assim, um aumento da permeabilidade da membrana pode resultar na difusão eficiente de substratos, nutrientes e produtos metabólicos através da membrana celular.

Além disso, de acordo com Batghare et al. (2018), turbulência intensa gerada pelo US pode induzir mudanças conformacionais na estrutura secundária das enzimas, o que pode aumentar a cinética do metabolismo. O desdobramento das enzimas com a redução do conteúdo da α-hélice e aumento do conteúdo des folhas β e de bobina pode melhorar a atividade e a cinética das enzimas intracelulares, aumentando o metabolismo. Tizazu et al. (2018) observaram uma redução nas constantes de saturação Monod (concentração de substrato necessária para atingir a metade da taxa máxima de crescimento específico da massa celular) envolvidas no metabolismo do xilitol induzido por US. Esse resultado foi

explicado pelo aumento da afinidade enzima-substrato como consequência das mudanças na estrutura secundária das enzimas intracelulares.

O US também pode induzir uma turbulência interfacial adicional que quebra as bolhas em particulas menores, aumentando a área de troca entre o gás e o líquido e o tempo de residência das bolhas de ar no meio de cultura (Kumar et al., 2004). Assim, as trocas entre o gás e o líquido são suficientes, e a taxa de transferência de oxigênio aumenta. Batghare et al. (2018) sugeriram que a melhoria da produção de astaxantina por células *Phaffia rhodozyma* MTCC 7536 foi um resultado da transferência de massa gás-líquido aprimorada e um aumento no consumo de oxigênio no meio de fermentação, corroborando os achados citados anteriormente.

A intensa mistura provocada pelo US pode também eliminar gradientes de concentração no meio de cultura, melhorando a disponibilidade de nutrientes aos microrganismos. O uso de US também pode resultar na redução dos efeitos de inibição pelo excesso de substratos durante o crescimento celular. Tizazu et al. (2018) observaram um aumento simultâneo na afinidade enzima-substrato e efeito de inibição reduzido, causado pela glicose, no metabolismo do xilitol, resultando em uma cinética mais rápida. Singh et al. (2015) estudaram a síntese de etanol assistida por US a partir da fermentação de *Parthenium hysterophorus* e observaram que o uso dessa tecnologia aumentou a afinidade enzima-substrato e reduziu os efeitos de inibição por excesso de substrato e formação de produtos.

Além disso, a mistura também favorece a dispersão dos aglomerados de células, que muitas vezes reduzem o contato com a luz, $CO_2$ e nutrientes. De acordo com Han et al. (2016), os efeitos de vibração e cavitação do tratamento ultrassônico foram

importantes para separar as microalgas e beneficiar o crescimento da *Anabaena variabilis*. Ren et al. (2019a) também afirmam que o tratamento ultrassônico adequado pode ajudar a separar as células de algas agrupadas e beneficiar o seu crescimento.

Outro fator envolvido nos efeitos da tecnologia de US nas células foi reportado por Huang et al. (2017). Os autores propuseram que o US pode alterar a expressão das proteínas de transporte da membrana, possivelmente proporcionando um efeito intensificado no crescimento celular. Dessa forma, sugere-se que mudanças genéticas também possam ocorrer com o tratamento ultrassônico, mas mais estudos serão necessários para melhor compreensão desses efeitos.

Embora algumas questões ainda não tenham sido totalmente abordadas, como mutações genéticas, viabilidade econômica e aplicação industrial, alguns parâmetros de processamento têm sido sugeridos para utilização do US visando aumento da proliferação celular. Conforme demonstrado na Tabela 1, baixas intensidades de US (15 - 200 W) e baixas frequências (18 - 40 kHz) resultam no aumento da densidade celular e produção de compostos. Entretanto, mais estudos são necessários para contribuir com a literatura e implementar esses conceitos em larga escala.

*3.5.1.2. Efeito do ultrassom na extração de compostos*

Em contraposição aos métodos convencionais, a extração assistida por US tem chamado a atenção por ser uma tecnologia mais limpa e menos agressiva ao meio ambiente. Ainda, com a utilização do US, é possível realizar extrações em condições brandas de temperatura, proporcionando a manutenção de compostos bioativos termolábeis (Soria e Villamiel, 2010) e a redução do consumo de energia.

A Tabela 2 apresenta os principais estudos envolvendo a extração assistida por US de lipídeos e carotenoides a partir de microalgas. De modo geral, o uso desta tecnologia em processos de extração apresenta efeitos positivos, com rendimentos superiores se comparada com métodos convencionais de extração.

Deenu et al. (2013) avaliaram a extração de luteína da microalga *Chlorella vulgaris* usando uma combinação de tratamento enzimático e US como pré-tratamento e etanol como solvente. Os resultados indicaram maior rendimento de extração em presença de US, sendo possível extrair aproximadamente 3,16 mg $g^{-1}$ de luteína.

Parniakov et al. (2015) avaliaram a extração assistida por US de compostos fenólicos e clorofilas da microalga *Nannochloropsis* spp, com diferentes combinações de solventes, comparados com os métodos de extração convencional. Os melhores rendimentos de extração foram obtidos quando o US foi utilizado como pré-tratamento por 5 minutos com água/DMSO (50 %) e água/etanol (25 – 30%).

Tabela 2 - Estudos envolvendo aplicação de ultrassom para extração de compostos de microalgas.

| Espécie | Produtos | Tipo | Parâmetros | Resultados | Referências |
|---|---|---|---|---|---|
| *Chlorella vulgaris* | lipídeos | banho | 40 kHz; 2,68 W $m^2$ | 52,5 % de lipídeos (maior conteúdo lipídico comparado a outros métodos) | Araujo et al. (2013) |
| *Heterochlorella luteoviridis* | lipídeos e carotenoides | sonda | 20 kHz; 50 W $cm^2$ | 1,37 mg $g^{-1}$ de carotenoides e 129,3 mg $g^{-1}$ de lipídeos (80 e 71 % de rendimento comparado ao controle, respectivamente) | Jaeschke et al. (2017) |
| *Nannochloropsis oculata* | lipídeos | sonda | 20 kHz; 1000 W | 0,21 % de lipídeos (maior que outros métodos) | Adam et al. (2012) |
| *Nannochloropsis* spp. | compostos fenólicos | sonda | 24 kHz; 400 W | 0,33 de rendimento total de compostos fenólicos (5 vezes maior que o controle) | Parniakov et al. (2015) |
| *Chlorella vulgaris* | carotenoides | sonda | 35 kHz; 56,58 W $cm^2$ | 3,16 mg $g^{-1}$ de luteína (mais alta quantidade obtida comparado a outros métodos) | Deenu et al. (2013) |
| *Arthrospira platensis* | proteínas | sonda | 20 kHz; 1000 W | 28,42 g 100 $g^{-1}$ de proteínas (229 % maior que o controle) | Vernès et al. (2019) |

A extração com US não é resultado de um único mecanismo, mas sim de diferentes mecanismos independentes ou combinados decorrentes da cavitação, como fragmentação, erosão, capilaridade, destexturização e sonoporação (Chemat et al., 2017). Esses efeitos mecânicos podem aumentar a liberação de compostos intracelulares, rompendo a célula e facilitando a penetração do solvente (Martinez et al., 2020). Como essa tecnologia acelera a transferência de massa, ela pode impactar na redução do uso de solventes orgânicos levando à redução de resíduos e poluição (Chemat et al., 2017).

Recentemente, estudos com a combinação da tecnologia US e pressão têm sido avaliados para extração de compostos. O tratamento com US sob pressão, também denominado de manosonicação, pode aumentar o efeito derivado da cavitação (Martínez et al., 2020; Meullemiestre et al., 2017. No entanto, o efeito da combinação de US e pressão na extração de compostos em microrganismos ainda foi pouco investigado, sendo necessários mais estudos. Vernès et al. (2019) estudaram o uso da tecnologia de manotermosonicação para extração de proteínas da cianobactéria *Arthrospira platensis*. Neste estudo, a extração assistida por US resultou em um aumento de 229 % na concentração de proteínas quando comparada com o processo sem US.

Alguns autores, ainda, combinam a extração assistida por US com líquidos iônicos (ILs) como solventes (Rodrigues et al., 2018). Os ILs são sais compostos apenas por íons, com pontos de fusão abaixo de 100 °C, baixa pressão de vapor, alta solvabilidade e melhor estabilidade química e térmica quando comparados aos solventes orgânicos. Devido a essas características apresentam grande versatilidade e capacidade de regeneração e reutilização. Contudo, a maioria dos LIs apresenta desvantagens de alto custo e baixa biodegradabilidade (Rodrigues et al., 2018).

*3.5.2. Campo elétrico pulsado*

Campo elétrico pulsado (PEF, do inglês *Pulsed Electric Field*) pode ser descrito como um tratamento elétrico de curto período de tempo, de nanossegundos a milissegundos, e intensidade na ordem de kV $cm^{-1}$ (Vorobiev e Lebovka, 2008). Essa tecnologia pode ser classificada de acordo com o tempo de duração do tratamento, sendo: PEF convencional, denominado para a faixa de microssegundos a milissegundos; e campo elétrico pulsado de nanosegundos (nsPEF, do inglês *Nanoseconds Pulsed Electric Field*), denominado para 1 a 300 ns (Buchmann e Mathys, 2019).

O nsPEF induz efeitos intracelulares, enquanto PEF convencional atua principalmente sobre a membrana celular (Napotnik et al., 2016). Um dos benefícios do uso de nsPEF é o desencadeamento da proliferação celular, sem danificar componentes celulares valiosos devido aos efeitos não térmicos (Haberkorn et al., 2019).

Além disso, o uso do PEF em processos biotecnológicos é relevante devido à sua versatilidade, ao fácil escalonamento, a eficiência energética e aos efeitos de eletroporação. A eletroporação, ou eletropermeabilização, pode ser descrita como um aumento na permeabilidade da membrana quando exposta a pulsos elétricos externos. De acordo com o modelo de poro aquoso transitório, esses pulsos induzem a formação de poros hidrofílicos na bicamada lipídica da membrana celular. Esses poros são formados devido à diferença de potencial transmembrana, que fornece energia livre suficiente para reorganizar as estruturas fosfolipídicas da membrana (Vorobiev e Lebovka, 2008).

A eletroporação pode ser um processo reversível ou irreversível, dependendo dos parâmetros do pulso e do meio condutor aplicado. Um processo reversível é desejado em aplicações da biotecnologia e medicina, adequado quando se deseja a manutenção das

células vivas. O processo irreversível é indicado quando se deseja rompimento e, consequentemente, a morte da célula. Esse processo é aplicado na indústria alimentícia devido aos danos celulares que podem inativar as células de microrganismos (Vorobiev e Lebovka, 2008).

Segundo Teissie et al. (2005), o fenômeno de eletroporação reversível pode ser dividido nas seguintes etapas: gatilho, expansão, estabilização, nova vedação e memória. Durante o estágio de gatilho, o campo elétrico induz a polarização da membrana, formando os poros; essa etapa dura alguns microssegundos. Depois, ocorre a etapa de expansão do tamanho dos poros, que dura centenas de microssegundos a milissegundos. Então, ocorre a etapa de estabilização que consiste na recuperação da organização da membrana e, em seguida, a vedação, que consiste no fechamento dos poros, durando vários minutos. Finalmente, ocorre o estágio da memória, onde a viabilidade celular é preservada, mas a estrutura da membrana e as propriedades fisiológicas levam mais tempo para se recuperar.

Em relação aos fatores operacionais, a intensidade do campo elétrico depende da tensão elétrica aplicada e da distância entre os eletrodos (Buchmann e Mathys, 2019). Além disso, outros parâmetros podem influenciar os efeitos da eletroporação, como largura do pulso, duração do pulso, forma da onda, número de pulsos, taxa de repetição do pulso, tamanho da célula e condutividade elétrica extracelular (Buchmann et al., 2019; Napotnik et al. 2016).

Os efeitos do campo elétrico nas células são baseados no princípio da eletropermeabilização devido ao potencial transmembrana induzido. Essa tensão deve ser proporcional à força do campo e se sobrepor à tensão de repouso presente na membrana

em condições fisiológicas, normalmente de 70 mV (Kotnik e Miklavcic, 2006). Buchman e Mathys (2019) avaliaram as equações básicas do princípio do processamento de campo elétrico pulsado. Segundo os autores, a diferença de potencial transmembrana em função do tempo $\Psi_m(t)$ (V) pode ser derivada da Equação (1):

$$\Psi_m(t) = f \cdot \mathrm{E}(t) \cdot a_m \cdot \cos\theta \cdot (1 - e^{-t/\tau_m}) \quad (1)$$

onde $f$ é um fator de forma (1,5 para uma célula esférica), $E(t)$ (V m$^{-1}$) é a força do campo elétrico em função do tempo, $a_m$ (m) é o raio da célula, $\theta$ é o ângulo em relação na direção do campo elétrico, $t$ (s) é o tempo de tratamento e $\tau_m$ (s) é o tempo de carregamento da membrana.

O tempo de carregamento da membrana é determinado de acordo com a Equação (2):

$$\tau_m = a_m \cdot C_m \cdot (1/2\sigma_e + 1/\sigma_i) \quad (2)$$

onde $C_m$ ($F$) é a capacitância da membrana por unidade de área, $\sigma_e$ e $\sigma_i$ (S m$^{-1}$) são a condutividade elétrica extracelular e intracelular, respectivamente.

Com relação à condutividade elétrica extracelular, essa pode ser determinada pela Equação (3) e, para estimar a carga para o campo elétrico pulsado, a Equação (3) deve ser estendida na Equação (4):

$$R = 1/\sigma \cdot d/A \quad (3)$$

$$Z_{tot} = 1/(\sigma \cdot A/d + Y_c) \quad (4)$$

onde $R$ é a resistência (Ω), $\sigma$ é a condutividade elétrica do meio (S m$^{-1}$), $d$ é a distância entre os eletrodos (m), $A$ é a área de superfície do eletrodo (m$^2$), $Z_{tot}$ (Ω) é a impedância total igual a soma do inverso da resistência e do sistema de entrada $Y_c$ (S) (Buchmann et al., 2019).

A energia específica de entrada $W_s$ (J $kg^{-1}$) pode ser calculada de acordo com a Equação (5):

$$W_S = E^2 \cdot \tau_p \cdot \sigma \cdot n \quad (5)$$

onde $\tau_p$ (s) é a largura do pulso e *n* é o número de pulsos. O número de pulsos é derivado da Equação (6) em que $f$ (Hz) é a frequência e $t$ (s) é o tempo de permanência ou tratamento:

$$n = f \cdot t \quad (6)$$

Além de permitir a observação dos parâmetros envolvidos na diferença de potencial transmembrana, essa dedução também permite determinar a quantidade de energia necessária para o desenvolvimento do processo de PEF para diferentes escalas.

A exposição a um campo elétrico suficientemente forte pode causar um aumento significativo na condutividade elétrica e na permeabilidade da membrana plasmática da célula, sem causar danos permanentes à célula. No entanto, se o campo for muito alto, ou for aplicado por um longo período, a eletroporação pode causar danos, causando a morte celular (Kotnik e Miklavcic). Devido a possibilidade de se promover eletroporação nas células biológicas, o tratamento com PEF tem sido bastante estudado para assistir processos de extração de compostos de interesse (Postma et al., 2016; Martínez et al., 2017; Martínez et al., 2018; Goettel et al., 2013; Carullo et al. 2018; Coustets e Teissié, 2016; Lam et al., 2017; Parniakov et al., 2015; Sarkis et al., 2015).

*3.5.2.1. Efeito do campo elétrico pulsado na extração de compostos*

Devido à capacidade de aumentar a permeabilidade da membrana celular, o PEF vem sendo utilizado para a extração de diferentes compostos de microalgas, como

lipídeos, carotenoides, clorofilas e proteínas. Os principais trabalhos realizados que utilizam essa tecnologia estão descritos na Tabela 3. Além disso, essa tecnologia é considerada uma opção ambientalmente mais correta e seletiva (Goettel et al., 2013; Parniakov et al., 2015; Silve et al., 2018).

Em sua maioria, os estudos de aplicação de PEF como pré-tratamento extrativo têm demonstrado resultados promissores. Martínez et al. (2017) avaliaram a extração assistida por PEF de ficocianinas a partir de *A. platensis*; o maior conteúdo deste pigmento foi obtido a 110,1 J $g^{-1}$. Para mesma espécie de microalga, Jaeschke et al. (2019) observaram não só um bom desempenho extrativo com o uso do PEF (122 J $ml^{-1}$), como também identificaram que a capacidade antioxidante dos extratos obtidos com PEF foi maior do que aqueles obtidos por outros métodos convencionais.

Tabela 3 - Estudos envolvendo aplicação de campo elétrico pulsado (PEF) para extração de compostos de microalgas.

| Espécie | Tempo de tratamento (μs) | Pulsos | Intensidade (kV $cm^{-1}$) | Frequência (Hz) | Temperatura (°C) | Resultados | AUTORES |
|---|---|---|---|---|---|---|---|
| *Artrosphira platensis* | 150 | 50 pulsos quadrados de 3 μs | 25 | 0,5 | 40 | pureza de ficocianina com PEF 41% maior que outros tratamentos | Martínez et al. (2017) |
| *Chlorella vulgaris* | 150 | 50 pulsos quadrados de 3 μs | 25 | 0,5 | 40 | luteína com PEF 4,2 vezes maior que controle | Luengo et al. (2015) |
| *Porphyridium cruentum* | 150 | 50 pulsos quadrados de 3 μs | 10 | 0,5 | 22 | 32 mg $g^{-1}$ de ficoeritrina com pureza maior que outros métodos | Martínez et al. (2019) |
| *Haematococcus pluvialis* | 50000 | 10 pulsos de 5000 μs | 1 | 1 | - | 18,3 mg $g^{-1}$ de carotenoides totais (16 % mais extrato que outros métodos) | Martínez et al. (2019) |
| *Chlorella vulgaris* | 75 | 25 pulsos quadrados de 3 μs | 20 | 0,5 | 25 | ~1000 μg $g^{-1}$ de carotenoides (1,2 vezes maior que o controle) | Luengo et al. (2014) |
| *Arthrospira platensis* | - | Pulsos quadrados de 1 μs | 40 | 6 | 42 | 85,2 mg $g^{-1}$ de ficocianinas (com atividade antioxidante 50 % maior que de outros métodos) | Jaeschke et al. (2019) |
| *Auxenochlorella protothecoides* | 62 | Pulsos de 1 μs | 40 | 3 | 36 | 40 % de lipídeos (16 vezes maior que o controle) | Silve et al. (2018) |

Além da energia aplicada, outros fatores podem influenciar na extração mais eficiente de compostos intracelulares. A extração depende da permeabilização da membrana citoplasmática e também da etapa difusiva posterior. Esta etapa de difusão promove a liberação de compostos intracelulares através da membrana permeabilizada (Silve et al., 2018). Portanto, o tempo de espera é necessário para permitir a difusão suficiente após o tratamento com PEF, aumentando os rendimentos da extração. Na literatura são recomendados tempos de difusão de 4 h para levedura (Ganeva et al., 2003), 2 h para o tecido da casca da uva (Sack et al., 2010) e 1,5 h para as raízes de chicória (Loginova et al., 2010).

Diferentes autores detectaram diferentes compostos intracelulares de microalgas, como íons, carboidratos, proteínas, carotenoides e clorofilas após tempos de extração inferiores a 60 min (Goettel et al., 2013; Luengo et al., 2015; Postma et al., 2016). Para microalga *Auxenochlorella protothecoides*, Goettel et al. (2013) precisaram de 40 min de etapa difusiva para detecção de carboidratos e proteínas no extrato após aplicação de PEF.

Jaschke et al. (2019) avaliaram o tempo de difusão pós-tratamento com PEF para microalga *Arthrospira platensis*. Os autores observaram que foi possível extrair a mesma quantidade de ficocianinas e proteínas após 6 h de incubação com 56 e 112 J ml $^{-1}$, entretanto nos extratos com tratamento de PEF com maior intensidade (112 J $mL^{-1}$) foi possível detectar ficocianinas e proteínas em 15 min de etapa difusiva.

Para a mesma espécie de microalga, Martinez et al. (2017) observaram que as ficocianinas não foram liberadas imediatamente após a aplicação de PEF, independentemente da intensidade do tratamento. Os autores verificaram que pelo menos 150 min de etapa difusiva foram necessários para detecção do composto no extrato.

Segundo Martinez et al. (2019), este atraso foi atribuído ao peso molecular das ficocianinas (240 kDa) e ao tamanho dos poros formados pelos tratamentos com PEF. Os autores argumentam que compostos de baixo peso molecular podem atravessar a membrana citoplasmática imediatamente após sua eletroporação, enquanto a liberação de moléculas de maior peso molecular pode exigir que os poros criados pelo tratamento com PEF sejam alargados ao longo do tempo, até tamanho suficiente para permitir a liberação do composto.

Para extração de β-ficoeritrina (peso molecular 250 kDa) assistida por PEF produzida por *Porphyridium cruentum*, por exemplo, Martínez et al. (2019b) verificaram que foi necessário um intervalo de tempo de mais de 6 h até que o composto pudesse ser detectado no meio de extração, sendo que para intensidades menores de tratamento, até 12 h de incubação foram necessárias para detecção do composto no extrato.

Além da questão do alargamento dos poros, os autores explicam que esse comportamento indica que a extração do composto requer não apenas a difusão através da membrana celular, mas também a dissociação do composto das estruturas celulares. Ficobiliproteínas, como β-ficoeritrina, estão inseridas em uma estrutura celular organizada, o ficobilissomo, que, ao mesmo tempo, está localizado nos tilacoides do cloroplasto, em vez de flutuar livremente no citoplasma. Portanto, além da difusão do composto através das membranas celulares, também é necessária a dissociação do composto da estrutura celular.

Embora vários estudos tenham evidenciado o potencial dessa tecnologia para processos extrativos, ainda há múltiplos obstáculos a serem superados para que sua implementação seja difundida em grande escala. Os custos desses dispositivos

permanecem altos; entretanto, é esperado que, à medida que a tecnologia se desenvolva, esses equipamentos se tornem mais acessíveis, permitindo a incorporação do PEF em várias aplicações. Além disso, é fundamental aprofundar o conhecimento sobre seus efeitos na estrutura celular de diferentes microrganismos. Outro ponto importante está relacionado ao fato de que as tecnologias elétricas podem ser consideradas sustentáveis do ponto de vista ambiental, uma vez que apresentam potencial para reduzir a geração de resíduos de solventes tóxicos utilizados em processos convencionais.

# Capítulo 4 – Efeito do ultrassom no crescimento de microalgas

Esse capítulo apresenta o estudo da avaliação da influência do US no crescimento das microalgas *P. marina* e *C. zofingiensis*.

Primeiramente, é apresentada a metodologia empregada para o cultivo das microalgas, para realização dos tratamentos com ultrassom, bem como para as análises dos compostos extraídos. Os resultados desse estudo estão apresentados na forma de artigo já publicado na revista *Bioresource Technology*.

Os experimentos foram realizados no Laboratório de Tecnologia e Processamento de Alimentos (LATEPA), localizado no Departamento de Engenharia Química da UFRGS.

## 4.1. Materiais e métodos

### *4.1.1. Manutenção da cultura*

A microalga *Pseudoneochloris marina* foi obtida do Laboratório BioEng, do Instituto de Ciência e Tecnologia de Alimentos, da Universidade Federal do Rio Grande do Sul (UFRGS). A composição do meio f/2 (Guillard, 1975) modificado (Gonçalves et al., 2019) para manutenção da cepa consistiu em: 34 g $L^{-1}$ de sal marinho (Red Sea Salt), 17 g $L^{-1}$ de NaCl e 450 mg $L^{-1}$ de $NaNO_3$. As soluções estoque foram preparadas segundo Lourenço (2006) e foram adicionadas ao meio de cultura f/2 na concentração de1 mL $L^{-1}$: solução de fosfato contendo 5 g $L^{-1}$ de $NaH_2PO_4.H_2O$, solução de silicato contendo 30 g $L^{-1}$ de $Na_2SiO_3$, solução de metais traço contendo 9,8 mg $L^{-1}$ de $CuSO_4.5H_2O$, 22 mg $L^{-1}$ de $ZnSO_4.7H_2O$, 10 mg $L^{-1}$ de $CoCl_2.6H_2O$, 180 mg $L^{-1}$ de $MnCl_2.4H_2O$, 6,3 mg $L^{-1}$ de $Na_2MoO_4.H_2O$, 4,36 g $L^{-1}$ de $Na_2EDTA$ e 3,15 g $L^{-1}$ de $FeCl_3.6H_2O$,

solução de vitaminas contendo 100 mg $L^{-1}$ de tiamina ($C_{12}H_{17}N_4OS$), 0,5 mg $L^{-1}$ de cianocobolamina ($C_{63}H_{88}CoN_{14}O_{14}P$) e 0,5 mg $L^{-1}$ de biotina ($C_{10}H_{16}N_2O_3S$). A solução tampão foi composta por 50 g de tris ($C_4H_{11}NO_3$), aproximadamente 30 mL de HCl para ajuste de pH e água destilada para aferição de volume total de 200 mL. As soluções foram autoclavadas a 121 ºC por 15 min, exceto a solução de vitaminas que foi esterilizada por microfiltração. A renovação do banco da cepa foi realizada mensalmente.

Em relação a microalga *Chlorella zofingiensis*, esta foi fornecida pelo Departamento de Biologia Marina da Universidade Federal Fluminense (Niteroi, RJ, Brasil). A manutenção das cepas foi realizada mensalmente, sendo mantidas em meio WC (Guillard e Lorenzen, 1972) que consiste em: 36,76 mg $L^{-1}$ de $CaCl_2.2H_2O$, 36,97 mg $L^{-1}$ de $MgSO_4.7H_2O$, 12,60 mg $L^{-1}$ de $NaHCO_3$, 8,71 mg $L^{-1}$ de $K_2HPO_4$, 85,01 mg $L^{-1}$ de $NaNO_3$ e 28,42 mg $L^{-1}$ de $Na_2SiO_3$. As soluções estoque foram preparadas segundo Lourenço (2006) e foram adicionadas ao meio WC na concentração de 1 mL $L^{-1}$: solução de metais traço contendo 9,8 mg $L^{-1}$ de $CuSO_4.5H_2O$, 22 mg $L^{-1}$ de $ZnSO_4.7H_2O$, 10 mg $L^{-1}$ de $CoCl_2.6H_2O$, 180 mg $L^{-1}$ de $MnCl_2.4H_2O$, 6,3 mg $L^{-1}$ de $Na_2MoO_4.H_2O$, 100 mg $L^{-1}$ de $H_3BO_3$, 4,36 g $L^{-1}$ de $Na_2EDTA$ e 3,15 g de $FeCl_3.6H_2O$. A solução de vitaminas e tampão foram as mesmas que as utilizadas para *P. marina*.

### *4.1.2. Inóculo*

Os inóculos foram preparados com meio f/2 modificado para *P. marina* e meio WC para *C. zofingiensis*, os mesmos utilizados para manutenção das culturas. Em frascos do tipo Erlenmeyer (500 mL) foram adicionados de 180 mL de meio e 20 mL de cultura (10 % do volume de inóculo). Os inóculos foram mantidos por 7 dias em incubadora

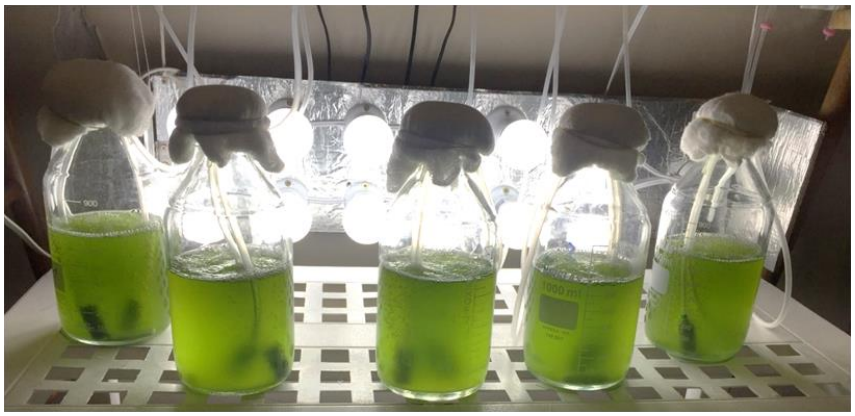
rotatória com agitação de 180 rpm, temperatura de 26 ºC e intensidade de luz de 3,0 klux, conforme Figura 6.

Figura 6 - Inóculos realizados previamente ao cultivo.

*4.1.3. Cultivos das microalgas*

Todos os cultivos foram realizados em frascos Duran de 1 L com adição de 70 ml de inóculo e 630 ml de meio (f/2 modificado ou WC), conforme Figura 7. Em 24 h de cultivo, para evitar a falta de nutrientes durante o experimento, foram adicionados 10 ml $L^{-1}$ de solução de fosfato contando 5 g $L^{-1}$ de $NaH_2PO4.H_2O$, e de solução de metais traço. Os frascos foram mantidos a 25 °C, 17 klux e aeração de 4 L $min^{-1}$ por 10 ou 13 dias. Foram coletadas amostras a cada 48 horas para realizar a análise da concentração de biomassa, carotenoides, clorofilas, lipídeos e nitratos. Ao final dos cultivos, todo o conteúdo dos biorreatores foi centrifugado (10.000 × g, 5 min, 4 °C), o sobrenadante foi descartado e a biomassa foi lavada com água destilada e centrifugada novamente. Após a centrifugação, a biomassa foi liofilizada (LIOTOP, Modelo L101, Brasil).

Figura 7 - Cultivos das microalgas em fotobiorreatores de 1L.



*4.1.4. Efeito da adição de nitrato no cultivo de C. zofingiensis*

Ao contrário da microalga *P.* marina, o meio utilizado para o crescimento da microalga *C. zofingiensis* não havia sido otimizado em estudos anteriores. Então, foi realizado um estudo avaliando a adição de $NaNO_3$ para favorecer o crescimento celular, a fim de se obter biomassa suficiente para as análises posteriores. Assim, foi avaliada a adição de 300, 450 ou 600 mg $L^{-1}$ de $NaNO_3$ no meio de cultivo da microalga, com o objetivo de se obter maior rendimento de biomassa e produção de compostos de interesse. As condições de cultivo foram as mesmas que as descritas no item 4.1.3.

*4.1.5. Aplicação de ultrassom durante o crescimento de P. marina*

A influência do US no crescimento da microalga e na produção de compostos foi avaliada de acordo com a fase de crescimento (exponencial e estacionária) e tempo de tratamento (10, 30 e 60 min), conforme apresentado na Tabela 4. O tratamento ultrassônico ocorreu por 5 dias consecutivos, iniciando-se em 72 h do início do cultivo durante a fase exponencial, e 192 h durante a fase estacionária. Um experimento controle

sem aplicação de US foi realizado em todas as etapas. Para o tratamento com US, os frascos de cultivo foram imersos em banho ultrassônico que opera em frequência de 38 kHz (Sanders do Brasil, UltraSonic Cleaner SW2000FI, Brasil) a 25 °C. A potência efetiva do equipamento de 200 W foi determinada por um método calorimétrico de acordo com Margulis e Margulis (2003). Todos os experimentos foram realizados em triplicata. A temperatura do banho ultrassônico foi monitorada durante os tratamentos, não excedendo 2 °C de variação (25 ± 2 °C), sendo, portanto, ser possível desconsiderar os efeitos térmicos.

Tabela 4 - Experimentos com a aplicação de ultrassom durante o crescimento da microalga *Pseudoneochloris marina*.

| **Ensaios** | **Descrição** |
|---|---|
| US EXP 10 | 5 dias de ultrassom por 10 min (72/96/120/144/168h) |
| US EXP 30 | 5 dias de ultrassom por 30 min (72/96/120/144/168h) |
| US EXP 60 | 5 dias de ultrassom por 60 min (72/96/120/144/168h) |
| US STA 10 | 5 dias de ultrassom por 10 min (192/216/240/264/288h) |
| US STA 30 | 5 dias de ultrassom por 30 min (192/216/240/264/288h) |
| US STA 60 | 5 dias de ultrassom por 60 min (192/216/240/264/288h) |

*4.1.6. Aplicação de ultrassom intermitente durante o crescimento de P. marina*

A partir dos resultados obtidos nos cultivos com aplicação de US contínuo, novas estratégias foram desenvolvidas com a aplicação de US de forma intermitente, intercalando tratamento ultrassônico e agitação magnética. Durante a fase exponencial (5 dias consecutivos, em 72h, 96 h, 120 h, 144 h e 168 h), foi aplicado tratamento

ultrassônico por 10 min (sendo 1 min de US e 9 min de agitação, até completar 10 min de tratamento – US INT 10) e 30 min (sendo 3 min de US e 7 min de agitação, até completar 30 min de tratamento – US INT 30). Um experimento controle sem aplicação de ultrassom foi realizado em paralelo. As mesmas condições utilizadas para o tratamento contínuo foram empregadas nessas estratégias investigadas.

*4.1.7. Aplicação de ultrassom durante o crescimento de C. zofingiensis*

Experimentos com aplicação de US contínuo e intermitente foram conduzidas durante o crescimento da microalga *C. zofingiensis*, conforme descrito na Tabela 5. Um experimento controle sem aplicação de ultrassom foi realizado em paralelo. As mesmas condições de tratamento ultrassônico foram empregadas nessas estratégias investigadas.

Tabela 5 - Experimentos com a aplicação de ultrassom durante o crescimento da microalga *C. zofingiensis*.

| **Ensaios** | **Descrição** |
|---|---|
| US CON 10 | 4 dias de ultrassom por 10 min (72/96/120/144h) |
| US CON 30 | 4 dias de ultrassom por 30 min (72/96/120/144h) |
| US INT 10 | 4 dias de ultrassom (72/96/120/144h) por 10 min de tratamento (1min US/ 9 min agitação) |

*4.1.8. Métodos analíticos*

*4.1.8.1. Análise de biomassa*

A concentração de biomassa foi acompanhada durante o cultivo por medição da densidade óptica a 750 nm em espectrofotômetro (PG Instruments Ltd., Modelo T80

UV-Vis, Reino Unido) e correlacionada com o peso das células secas, através de uma curva padrão, previamente determinada (Figuras 1A, 2A e 3A, Apêndice A).

*4.1.8.2. Análise de clorofila e carotenoides totais*

A quantificação de carotenoides totais e clorofila foi realizada por método colorimétrico segundo Lichtentlhaler & Buschmann (2001), utilizando metanol (90 %) como solvente.

Diariamente foram coletados 2 mL de amostra, em duplicata, adicionados em tubos eppendorff e centrifugados (10000 x g por 10 minutos). O sobrenadante foi reservado para análise de nitrato. No precipitado foi adicionado 2 mL de metanol 90 %, e armazenado em local escuro a 4 °C por 12 h (*overnight*). No dia seguinte, as amostras foram centrifugadas (10000 x g por 10 minutos), e o sobrenadante lido em espectrofotômetro nos comprimentos de onda de 470 nm, 652,4 nm e 665,2 nm.

A quantidade de clorofila *a* e *b* foi determinada pela aplicação das Equações (7) e (8), e a concentração de carotenoides totais (μg $mL^{-1}$) pela Equação (9):

$$C_a = 16{,}82\, A_{665{,}2\,nm} - 9{,}28\, A_{652{,}4nm} \quad (7)$$

$$C_b = 36{,}92\, A_{652{,}4\,nm} - 16{,}54\, A_{665{,}2nm} \quad (8)$$

$$C_{Ct} = \frac{1000\, A_{470\,nm} - 1{,}91\, C_a - 95{,}15\, C_b}{225} \quad (9)$$

onde $C_a$ corresponde a concentração de clorofila *a* (μg $mL^{-1}$), $C_b$ a concentração de clorofila *b* (μg $mL^{-1}$) e $C_{Ct}$ a concentração de carotenoides totais (μg $mL^{-1}$).

*4.1.8.3. Extração exaustiva de carotenoides*

A concentração de carotenoides foi determinada de acordo com Mandelli *et al.* (2011). Para realização da análise, foram pesados 0,1 g de biomassa liofilizada, e macerada em contato com os solventes acetato de etila e metanol, utilizando gral e pistilo. O extrato foi coletado e centrifugado por 7 min a 3500 rpm. Esse procedimento foi repetido até que o solvente não apresentar mais coloração. Após a obtenção do extrato, a suspensão foi filtrada através de membrana de 0,22 µm de polietileno, coletada em frasco âmbar e concentrada em rotaevaporador a 30°C. Então, o concentrado foi ressuspendido numa mistura de éter de petróleo e éter dietílico (1:1 v/v), e saponificado *overnight* (16 h) com solução de KOH em metanol (10%, m/v) a temperatura ambiente. Essa etapa é necessária para a eliminação de interferentes da análise, como os lipídeos, clorofilas e ésteres de carotenoides. Após a saponificação, foram realizadas lavagens das amostras até a obtenção de pH neutro.

A fase éter contendo os carotenoides foi coletada em Erlenmeyer em presença de sulfato de potássio. Nessa etapa, a presença do sal é importante para eliminar qualquer resquício de água que tenha permanecido na amostra. Após essa etapa, as amostras foram transferidas para balões de fundo chato e rota-evaporadas (*Fisatom*, Model 450-5, Brazil). Depois da eliminação completa do solvente (éter etílico e éter de petróleo), os pigmentos foram ressuspendidos em volume conhecido de etanol para posterior leitura em espectrofotômetro. Nessa etapa foram realizadas lavagens com etanol do balão de fundo chato até que o solvente não mais apresente coloração. Para garantir a solubilização de todo o conteúdo de pigmentos, foi utilizado um banho de ultrassom. Após a ressuspensão,

foram realizadas leituras em espectrofotômetro a 445 nm (*PG Instruments Ltd.*, Model T80 UV-Vis, United Kingdom).

A absortividade molar da luteína ($\varepsilon$ = 2,550) em etanol foi utilizada para o cálculo da concentração, e os resultados foram expressos em miligramas de luteína por grama de biomassa seca (mg $g^{-1}$). As extrações exaustivas de cada lote de biomassa foram realizadas em triplicata.

*4.1.8.4. Perfil de carotenoides*

A determinação do perfil de carotenoides das amostras coletadas foi de acordo com Rodrigues et al. (2014). Após a etapa de extração exaustiva, as amostras contendo os carotenoides foram analisadas por espectrofotômetro e cromatografia líquida de alta eficiência (Waters, Alliance e2695 Separations Module, USA) com detector UV-Vis e uma coluna C30 (YMC, 5 μm, 250 × 4.6 mm id., Waters, Wilmington – DE, USA). A fase móvel utilizada foi metanol e éter metil-terc-butil (MTBE) com gradiente linear de 95:5 a 70:30 nos primeiros 30 min, de 70:30 a 50:50 nos 20 min subsequentes, e 50:50 nos últimos 15 min. Os cromatogramas foram analisados a 451 nm, e a identificação foi realizada de acordo com a ordem de eluição dos compostos na coluna C30, levando em consideração as características espectrais de cada pico, em comparação com os dados do padrão (all-trans-β-carotene, Sigma Aldrich, USA). A faixa de concentrações de solução padrão avaliada foi de 0,125 a 15 μg $mL^{-1}$.

*4.1.8.5. Análise de nitrato*

O consumo de nitrato foi determinado por espectrofotometria, utilizando a metodologia de Cataldo et al. (1975). O sobrenadante reservado da análise de carotenoides foi utilizado para esta análise, sendo medidos 0,25 mL de extrato, transferidos para Erlenmeyer de 125 mL, e ainda adicionados 0,8 mL de solução de ácido salicílico ($C_7H_5O_3$) em $H_2SO_4$ concentrado. Após aguardar o tempo de reação (20 minutos), foram adicionadoa 19 mL de NaOH 2 M, e realizada a leitura da absorbância a 410 nm em espectrofotômetro. Os resultados foram convertidos em teores de $N\text{-}NO_3$ por peso seco, utilizando uma equação da curva de calibração previamente determinada (Figuras 4A, 5A, 6A, 7A e 8A, Apêndice A).

*4.1.8.6. Determinação de lipídeos*

O teor de lipídeos foi determinado pelo método sulfo-fosfo-vanilina (SPV) (Mishra et al., 2014). Amostras foram retiradas a cada 48 h durante o cultivo, centrifugadas e retirado o sobrenadante. Na biomassa resultante, foi adicionado 2 mL de ácido sulfúrico, e a mistura foi mantida sob aquecimento por 10 min. Posteriormente, as amostras foram resfriadas em banho de gelo por aproximadamente 5 min. Após o resfriamento, o reagente fosfo-vanilina, previamente preparado, foi adicionado as amostras. A reação ocorreu por 15 min no escuro, e posteriormente, as amostras foram analisadas em espectrofotômetro a 530 nm. A concentração de lipídeos nas amostras foi determinada por curva de calibração com padrão de óleo de oliva (Sigma Aldrich, USA) em clorofórmio em concentrações variando de 0,4 a 2 mg $L^{-1}$ (Figura 9A, Apêndice A).

*4.1.8.7. Análise de proteínas*

A determinação de proteínas foi realizada segundo a metodologia descrita por Lowry et al. (1951). Previamente, foram preparadas as soluções A, B, e diariamente a C: solução A contendo 2 g de carbonato de sódio anidro, 0,02 g de tartarato de sódio e potássio em 100 mL de NaOH 0,1N; solução B contendo 0,5 g de sulfato de cobre em 100 mL de água destilada; e solução C contendo 50 mL da solução A e 1 mL da solução B.

Uma quantidade de 0,025 g de biomassa liofilizada foi pesada em tubos de ensaio com tampa de rosca. Em seguida, foram adicionados 10 mL de água destilada, e a mistura foi armazenada em geladeira *overnight*. Após este período, as amostras foram centrifugadas a 3000 × g por 10 min, descartado o sobrenadante e adicionados 5 mL de NaOH 1 M. Na sequência, as amostras foram aquecidas e mantidas em ebulição a 100 °C por 30 min. Após resfriamento das amostras, estas foram centrifugadas a 10.000 × g por 10 min, e com auxílio de pipeta, 100 µL do sobrenadante foram transferidos para tubo de ensaio, adicionados de 2,5 mL de solução C, e mantido em banho termostático a 37 °C por 10 min. A solução de Folin 1 N foi adicionada, 300 µL em cada tubo, e imediatamente homogeneizado em agitador vórtex, mantendo no escuro por 30 min. Posteriormente, foi realizada a leitura de absorbância em espectrofotômetro a 750 nm.

*4.1.9. Análise Estatística*

Os resultados foram analisados por análise de variância (ANOVA) e teste Tukey a 95% de confiança ($p \leq 0,05$), utilizando software Statistica 12. Os cultivos foram realizados em triplicata e a análises em duplicata.

## 4.2. Artigo - Effect of ultrasound on *Pseudoneochloris marina* and *Chlorella zofingiensis* growth

Nesta seção são apresentados os resultados obtidos na forma de artigo científico publicado na revista *Bioresource Technology* (v. 373, 128741, 2023).

*Abstract*

The present work aims to evaluate the ultrasound (US) effects during *Pseudoneochloris marina* and *Chlorella zofingiensis* growth. For *P. marina*, US treatment did not result in an increase in cell proliferation and reduced cell density when used for 60 min (exponential phase, for 5 days), indicating a possible occurrence of cell damage. For *C. zofingiensis*, the application of discontinuous US for 10 min resulted in an increase of 65 % in the biomass concentration when compared to the control. These distinct behaviors indicate that microalgae species react differently to physical stimuli. After US treatment, a reduction of carotenoid, chlorophyll, lipid and protein concentrations was observed, which may be related to changes in the metabolic pathways to produce these compounds. Overall, the results of the present study show the potential of discontinuous US to enhance microalgae cell proliferation.

Keywords: sonication; lipids; carotenoids; proteins; biomass.

### *4.2.1. Introduction*

Microalgae have been attracted attention due to the sustainability of their cultivation process and to their synthesis capacity of biocompounds, such as proteins, lipids, carbohydrates, and pigments. If compared to other plant sources, microalgae production has the advantage of using non-arable lands and having higher productivity (Chisti, 2020). Despite their potential as a source of valuable compounds, only few microalgae species are cultivated for commercial purpose, such as *Chlorella vulgaris*, *Dunaliella salina*, *Arthrospira platensis* and *Haematococcus pluvialis* (Molina-Grima et al., 2022; Stirk & van Staden, 2022). However, other microalgae have been studied and are considered potential strains to be explored.

Among green microalgae, *Chlorella zofingiensis* is a promising strain that can be used to produce carotenoids, accumulating up to 4 mg $g^{-1}$ of astaxanthin (Nishshanka et al., 2021). Although *H. pluvialis* is mostly used for astaxanthin production, this microalga grows relatively slowly, and its cultivation results in low biomass yield, which contributes to the high astaxanthin prices in the market (2500-15000 USD/kg) (Patel et al., 2022; Rahman, 2020). Besides carotenoids, *C. zofingiensis* has the capacity to synthetize high amounts of lipids (up to 33 %) with a fatty acid profile suitable for biodiesel production (Gorgich et al., 2021; Nishshanka et al., 2021).

Other microalgae species are even less explored, such as *Pseudoneochloris marina*. To the best of our knowledge, only one study has investigated its capacity to synthetize valuable compounds, obtaining up to 13 % of lipids and 3.6 mg $g^{-1}$ of carotenoids under appropriate cultivation conditions (Gonçalves et al., 2019). Therefore, it is necessary to explore different cultivation conditions to improve biomass concentration and productivity of target compounds during its cultivation.

The main strategy employed to achieve this goal is the submission of microalgae cells to stress environments, as changes in luminosity, salt, nitrogen, and carbon dioxide concentrations. Under abiotic stress many species of algae may accumulate neutral lipids and secondary metabolites to protect cells against oxidative stress (Song et al., 2022). In addition, the use of chemical additives, as phytohormone and EDTA, and the application of innovative technologies, such as electrical technologies and ultrasound (US), have also been considered for this purpose (Haberkorn et al., 2021; Sivaramakrishnan & Incharoensakdi, 2019; Song et al., 2022).

Among these strategies, US application has shown an increase of biomass production and lipid concentration in the cells due to effects associated with cavitation (Han, Pei, Hu, Jiang, et al., 2016; Ren, Xiao, et al., 2019; Singh et al., 2019). These effects may be related with changes in the cell membrane permeability, changes in the metabolic pathways of the cells, changes in enzymatic activity, increase of the oxygen uptake by the cells, and release of cell clusters (Han, Pei, Hu, Jiang, et al., 2016; Ma et al., 2015; Ren, Xiao, et al., 2019; Ren, Zhu, et al., 2019). However, few researchers have explored US application so far during microalgae growth, and little is known about how this technology impacts the mechanisms of cell growth.

In this context, the present work evaluates the effects of US application during the cultivation of two microalgae species: *P. marina* and *C. zofingiensis*. As previously mentioned, both strains have potential to accumulate target compounds, as lipids and carotenoids, when cultivated under appropriate conditions. Therefore, the objective is to find the best cultivation conditions to improve biomass concentration and lipid and carotenoid productivity using US to promote environmental stress. In addition, this work also seeks to contribute for the elucidation of the defense behavior of the cells when exposed to ultrasonic treatment.

### *4.2.2. Materials and methods*

#### *4.2.2.1. Microalgae strains*

Two microalgae strains were used in the present work: *P. marina* and *C. zofingiensis*. *P. marina* (supplied by BioEng Laboratory, Institute of Food Science and Technology, Federal University of Rio Grande do Sul) was maintained in a modified f/2

medium with $NaNO_3$ (450 mg $L^{-1}$) and an additional amount of NaCl (17 g $L^{-1}$) to induce saline stress (Gonçalves et al., 2019; Guillard, 1975). *C. zofingiensis* was supplied by the Department of Marine Biology of the Federal University Fluminense (Niteroi, RJ, Brazil) and was maintained in WC medium (Guillard & Lorenzen, 1972). The pre-cultures were prepared in 500 mL Erlenmeyer flasks with 200 mL working volume using modified f/2 medium or WC and inoculated with 20 mL of stock culture. The flasks were incubated in a rotatory shaker at 26 °C and 180 rpm with continuous illumination of 40 μmol $m^{-2}$ $s^{-1}$ for 7 days.

*4.2.2.2. Cultivation conditions*

All cultivations were performed in 1-L Duran flasks with 70 mL of pre-culture and 630 mL of modified f/2 or WC medium. To avoid lack of nutrients, in the second day of cultivation, 10 ml $L^{-1}$ of phosphate (5 g $L^{-1}$ of $NaH_2PO_4.H_2O$) and metal traces solutions (solution A, for f/2 medium: 9,8 mg $L^{-1}$ of $CuSO_4.5H_2O$, 22 mg $L^{-1}$ of $ZnSO_4.7H_2O$, 10 mg $L^{-1}$ of $CoCl_2.6H_2O$, 180 mg $L^{-1}$ of $MnCl_2.4H_2O$, 6,3 mg $L^{-1}$ of $Na_2MoO_4.H_2O$, 4,36 g $L^{-1}$ of $Na_2EDTA$ and 3,15 g $L^{-1}$ of $FeCl_3.6H_2O$; solution B, for WC medium: 9,8 mg $L^{-1}$ of CuSO4.5H2O, 22 mg $L^{-1}$ of $ZnSO_4.7H_2O$, 10 mg $L^{-1}$ of $CoCl_2.6H_2O$, 180 mg $L^{-1}$ of $MnCl_2.4H_2O$, 6,3 mg $L^{-1}$ of $Na_2MoO_4.H_2O$, 100 mg $L^{-1}$ of $H_3BO_3$, 4,36 g $L^{-1}$ of $Na_2EDTA$ e 3,15 g $L^{-1}$ of $FeCl_3.6H_2O$) were added to the medium. In addition, the influence of $NaNO_3$ on *C. zofingiensis* growth was studied by the addition of different concentrations of this salt (300, 450 or 600 mg $L^{-1}$) in the WC medium. These experiments were performed before US application to obtain higher biomass yield and compounds production.

During all cultivations, the flasks were maintained at 25 °C, with luminosity of 220 μmol $m^{-2}$ $s^{-1}$, and aeration rate of 4 L $min^{-1}$ for 10 – 13 days. The flasks were immersed in the ultrasonic bath, as described in the next sections. Samples were withdrawn every 48 h to perform the following analyses: biomass and nitrate concentration in the medium, and carotenoids and lipid content in the biomass. At the end of the cultivation, the entire content of the bioreactors was centrifuged (10,000 × g, 5 min, 4 °C), the supernatant was discarded, and the biomass was washed with distilled water and centrifuged. After centrifugation, the biomass was freeze-dried (LIOTOP, Model L101, Brazil).

*4.2.2.3. Ultrasonic treatment during P. marina growth*

*Continuous ultrasonic treatment*

Table 1 shows the experiments performed to evaluate the US influence on *P. marina* growth and on production of internal compounds. US was applied during exponential or stationary growth phase, during 5 days, using different treatment times (10, 30 and 60 min). US treatment started after 72 h of the beginning of the cultivation. Control experiments (cultivations without US treatment) were performed for each condition. For US treatments, the flasks were immersed in an ultrasonic bath (Sanders, UltraSonic Cleaner SW2000FI, Brazil, 38 kHz) at 25 ± 2 °C. The temperature of the ultrasonic bath was monitored during the treatments to ensure that any effect observed on microalgae cells would be related only to US effects. The effective power of US equipment (200 W) was determined by a calorimetric method according to the methodology described by Margulis and Margulis (2003). All experiments were performed in triplicate.

Table 1 - Experiments with ultrasound application during the growth of the microalgae *P. marina* and *C. zofingiensis*.

| Microalgae | Essay | Description |
|---|---|---|
| | US EXP 10 | 5 days of continuous US for 10 min during exponential phase. Treatments were applied after 72, 96, 120, 144 and 168h of cultivation. |
| | US EXP 30 | 5 days of continuous US for 30 min during exponential phase. Treatments were applied after 72, 96, 120, 144 and 168h of cultivation. |
| | US EXP 60 | 5 days of continuous US for 60 min during exponential phase. Treatments were applied after 72, 96, 120, 144 and 168h of cultivation. |
| | US STA10 | 5 days of continuous US for 10 min during stationary phase. Treatments were applied after 192, 216, 240, 264 and 288h of cultivation. |
| | US STA 30 | 5 days of continuous US for 30 min during stationary phase. Treatments were applied after 192, 216, 240, 264 and 288h of cultivation. |
| | US STA 60 | 5 days of continuous US for 60 min during stationary phase. Treatments were applied after 192, 216, 240, 264 and 288h of cultivation. |
| | US INT 10 | 5 days of intermittent US for 10 min during exponential phase. Treatments were applied after 72, 96, 120, 144 and 168h of cultivation. |
| | US INT 30 | 5 days of intermittent US for 30 min during exponential phase. Treatments were applied after 72, 96, 120, 144 and 168h of cultivation. |
| *C. zofingiensis* | US CON 10 | 4 days of continuous US for 10 min during exponential phase. Treatments were applied after 72, 96, 120 and 144h of cultivation. |
| | US CON 30 | 4 days of continuous US for 30 min during exponential phase. Treatments were applied after 72, 96, 120 and 144h of cultivation. |
| | US INT 10 | 4 days of intermittent US for 10 min during exponential phase. Treatments were applied after 72, 96, 120 and 144h of cultivation. |

*Intermittent ultrasonic treatment*

Based on the results obtained with the experiments performed using continuous US treatment, new experiments were conducted applying US intermittently. The treatments were performed for 10 (US INT 10) and 30 min (US INT 30) during the exponential phase (5 consecutive days, after 72, 96, 120, 144, and 168 h of cultivation).

The experiment US INT 10 was performed applying US in a pulse regime of 1/9 min on/off until reaching 10 min of total US treatment. US INT 30 was carried out in a pulse regime of 3/7 min on/off until reaching 30 min of total US treatment. A control experiment without US application was performed in parallel (Control 4).

*4.2.2.4. Ultrasonic treatment during C. zofingiensis growth*

Table 1 describes the experiments conducted to evaluate the influence of continuous and intermittent US on *C. zofingiensis* growth. The methodology applied followed the procedure described in section 4.2.2.3. A control experiment without US application was performed in parallel.

*4.2.2.5. Analytical methods*

*Biomass concentration*

Biomass concentration during cultivation of *P. marina* and *C. zofingiensis* was estimated by the relationship between optical density. For that, a biomass calibration curve was previously performed, and each solution was measured in a spectrophotometer (PG Instruments Ltd., Model T80 UV–Vis, United Kingdom) at 750 or 680 nm, for *P. marina* and *C. zofingiensis*, respectively.

*Carotenoid and chlorophyll contents*

Total carotenoid and chlorophyll contents were determined by a colorimetric method (Lichtenthaler & Buschmann, 2001). For that, 2 mL of sample was withdrawn from the bioreactor and was centrifuged (10,000×g, 10 min); the supernatant was reserved for nitrate analysis. Two (2) ml of methanol (90 %v/v) was added to the precipitate, and the mixture was kept in the dark at 4 ° C for 12 h (overnight). After,

samples were centrifuged (10,000×g, 10 min), and the supernatant was measured at 470, 652.4, and 665.2 nm in a spectrophotometer (PG Instruments Ltd., Model T80 UV–Vis, United Kingdom).

The amounts of chlorophyll *a* and *b* and the concentration of total carotenoids were calculated by Equations (1), (2) and (3) (Lichtenthaler & Buschmann, 2001):

$$C_a = 16{,}82\ A_{665{,}2\ nm} - 9{,}28\ A_{652{,}4nm} \quad (1)$$

$$C_b = 36{,}92\ A_{652{,}4\ nm} - 16{,}54\ A_{665{,}2nm} \quad (2)$$

$$C_{Ct} = \frac{1000\ A_{470\ nm} - 1{,}91\ C_a - 95{,}15\ C_b}{225} \quad (3)$$

in which $C_a$ corresponds to chlorophyll *a* concentration (μg $mL^{-1}$), $C_b$ to chlorophyll *b* concentration (μg $mL^{-1}$), and $C_{Ct}$ to total carotenoid concentration (μg $mL^{-1}$).

Carotenoid profile was determined by high performance liquid chromatography (HPLC) according to Rodrigues et al. (2014). Initially, carotenoids were exhaustively extracted from the freeze-dried biomass (0.025 g), as described by Mandelli et al. (2012). For that, samples were hydrated overnight and then macerated using ethyl acetate and methanol. The extracts were dried and stored (-18 °C) until HPLC analyses. Right before the analysis, samples were dissolved in methanol/methyl tert-butyl ether (MeOH:MTBE 1:1, v $v^{-1}$). Carotenoids were then quantified by Waters HPLC 2695 series system (Wilmington, EUA), equipped with a diode array detector (Waters 2998 dual series), using YMC-C30 column (5 μm particle size, 250 mm × 4.6 mm). The flow rate was 0.9 mL $min^{-1}$, and the column temperature was 29 °C. The mobile phase (MeOH:MTBE) was eluted in a linear gradient from 95:5 to 70:30 in 30 min, followed by 50:50 in 20 min,

maintaining this proportion for 15 min. Carotenoid quantification were performed using the analytical curves of β-carotene (1–15 mg $L^{-1}$) and lutein (0.9–13 mg $L^{-1}$).

*Nitrate concentration*

Nitrate concentration was determined according to the methodology described by Cataldo et al. (1975), using the supernatant collected before total pigment analysis (as described in Section 4.2.6.2). An amount of 0.25 mL of this supernatant was mixed with a salicylic acid solution ($C_7H_5O_3$) in $H_2SO_4$ (50 g $L^{-1}$). After the reaction time (20 min), 19 mL of 2 M NaOH was added, and the absorbance was read at 410 nm in a spectrophotometer (PG Instruments Ltd., Model T80 UV–Vis, United Kingdom). The results were converted into N-$NO_3$ contents by dry weight, using the previously determined calibration curve equation.

*Lipid content*

The lipid content was measured according to the sulfo-phospho-vanillin (SPV) method (Mishra et al., 2014). Samples collected from the cultivation were centrifuged, and the supernatant removed. Two (2) mL of sulfuric acid was added to the biomass pellet, and the mixture was kept under heating for 10 min. Subsequently, the samples were cooled in an ice bath for approximately 5 min. After cooling, the previously prepared phospho-vanillin reagent was added to the samples. The reaction was carried out for 15 min in the dark, and the samples were analyzed in a spectrophotometer at 530 nm. A calibration curve of olive oil (Sigma Aldrich, USA) in chloroform (0.4 to 2 mg $L^{-1}$) was used to calculate the lipid concentration.

*Protein content*

The protein content was determined according to Lowry et al. (1951). The freeze-dried biomass (0.025 g) was hydrated overnight with 10 mL of distilled water and kept at 4 °C. Then, the hydrated biomass was centrifuged (10,000×*g*, 10 min), the supernatant was discarded, and 5 mL of NaOH (1 N) was added to the pellet. Samples were then mixed using a vortex, heated at the solution boiling point for 30 min, cooled to room temperature, and centrifuged (10,000×*g*, 10 min). The results were correlated to a calibration curve which was performed with bovine serum albumin as protein standard.

*4.2.2.6. Statistical data analyses*

All treatments were carried out in triplicate, and the analyses of each independent sample were performed in duplicate. The results were analyzed by ANOVA and Tukey test using the software Statistica® (12.0, Statsoft Inc., Tulsa, USA).

*4.2.3. Results and discussion*

*4.2.3.1. Influence of ultrasound on P. marina growth*

*Influence of continuous ultrasound on biomass concentration*

Figure 1 shows the normalized biomass concentration of *P. marina* over cultivation time for continuous US treatments applied at A) exponential and B) stationary growth phases, and for C) discontinuous US treatment. The results were normalized to their respective control to better evaluate the influence of the different variables. The growth curves obtained for US treatments at the exponential phase are very similar, except for the 60 min treatment (US EXP 60) that presented lower biomass concentration. Table 2 presents the biomass, carotenoid, chlorophyll, lipid and protein content of

*P.marina* with US application during exponential (EXP) and stationary phase (STA), and their respective control experiments at the end of the cultivation. The statistical analysis confirmed that biomass concentration for US EXP 60 was lower than the biomass obtained for the other treatments. At this condition, US treatment may have caused irreparable damage, preventing cell regeneration. Similar results were obtained by other authors (Han, Pei, Hu, Jiang, et al., 2016; Ren, Xiao, et al., 2019). Han et al. (2016) applied US bath to *Anabaena variabilis* cells at the end of the exponential phase and observed that longer treatments (more than 40 min) promoted cell damage, reducing the biomass concentration. Ren, Xiao, et al. (2019) also reported that excessive US application (probe, power higher than 30 W and time higher than 5s) inhibited *Scenedesmus* sp. cell growth.

From Table 2, it is also possible to observe that shorter US treatments during the exponential phase (US EXP 10 and US EXP 30) did not influence cell proliferation. Different results were reported by Ren, Xiao, et al. (2019), that observed an increase on biomass concentration of 27% when US probe (2s, 20 W) was applied to *Scenedesmus* sp. These distinct microorganism responses to US may be attributed to different application times, powers, and cell morphologies.

Regarding US application during the stationary phase, cell proliferation was not affected by the treatment (Figure 1B and Table 2). Similarly, Han, Pei, Hu, Jiang et al. (2016) reported that US had little effect on microalgae biomass concentration when applied during the stationary growth phase.

Figure 1 - Normalized biomass concentration ($C/C_{control}$) during cultivation of *P. marina* using US treatment: A) continuous US application at the exponential (EXP) phase during 10, 30, and 60 min; B) continuous US application at the stationary (STA) phase during 10, 30, and 60 min; and C) intermittent US treatment (INT) for 10 and 30 min.

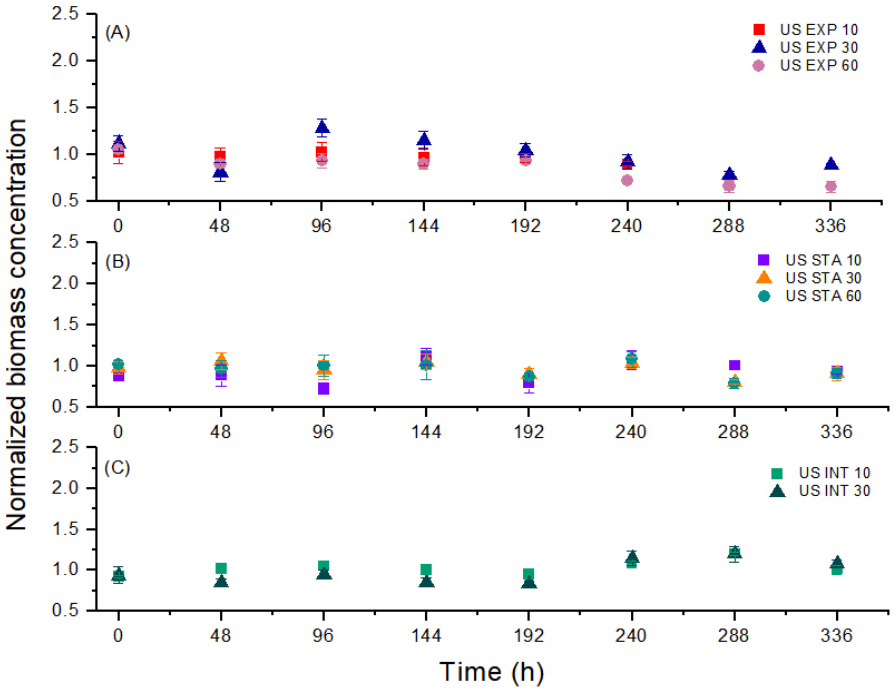


Table 2 - Biomass, carotenoid, chlorophyll, lipid and protein content of *P.marina* with US application, during exponential (EXP) and stationary phase (STA), and their respective control experiments at the end of the cultivation.

| Experiment | Biomass concentration ($g\ L^{-1}$) | Carotenoid content ($mg\ g^{-1}$) | Chlorophyll content ($mg\ g^{-1}$) | Lipid content ($g\ g^{-1}$) | Protein content ($mg\ g^{-1}$) |
|---|---|---|---|---|---|
| **Control 1** | 2.57± 0.07 $^{a}$ | 1.04 ± 0.05 $^{a}$ | 6.99 ± 0.30 $^{a}$ | 11.15 ± 1.37 $^{a}$ | 101.81 ± 9.18 |
| **US EXP 10** | 2.55 ± 0.01 $^{a}$ | 0.98 ± 0.08 $^{a}$ | 5.59 ± 0.68 $^{a}$ | 12.79 ± 1.25 $^{a}$ | 87.12 ± 4.24 $^{-}$ |
| **Control 2** | 2.29 ± 0.17 $^{A}$ | 2.39 ± 0.10 $^{A}$ | 8.35 ± 0.68 $^{B}$ | 21.54 ± 3.16 $^{A}$ | 109.43 ± 2.18 $^{A}$ |
| **US EXP 30** | 2.03 ± 0.06 $^{A}$ | 2.20 ± 0.26 $^{A}$ | 5.88 ± 0.24 $^{C}$ | 19.96 ± 1.22 $^{A}$ | 110.75 ± 4.98 $^{A}$ |
| **US EXP 60** | 1.50 ± 0.15 $^{B}$ | 1.39 ± 0.16 $^{B}$ | 10.93 ± 0.56 $^{A}$ | 22.65 ± 3.78 $^{A}$ | 82.68 ± 5.71 $^{B}$ |
| **Control 3** | 3.23 ± 0.17 $^{\alpha}$ | 0.89 ± 0.11 $^{\alpha}$ | 1.29 ± 0.08 $^{\alpha}$ | 20.94 ± 0.62 $^{\alpha}$ | 106.07± 6.78 $^{\alpha, \beta}$ |
| **US STA 10** | 3.02 ± 0.11 $^{\alpha}$ | 0.69 ± 0.09 $^{\alpha, \beta}$ | 1.07 ± 0.19 $^{\alpha}$ | 16.84 ± 0.60 $^{\beta}$ | 95.49 ± 8.50 $^{\beta}$ |
| **US STA 30** | 2.94 ± 0.26 $^{\alpha}$ | 0.52 ± 0.08 $^{\beta, \gamma}$ | 1.30 ± 0.07 $^{\alpha}$ | 17.16 ± 1.92 $^{\beta}$ | 103.39 ± 3.84 $^{\beta}$ |
| **US STA 60** | 2.96 ± 0.20 $^{\alpha}$ | 0.42 ± 0.06 $^{\gamma}$ | 1.16 ± 0.15 $^{\alpha}$ | 14.86 ± 2.01 $^{\beta}$ | 116.31 ± 5.51 $^{\alpha}$ |
| **Control 4** | 1.59 ± 0.17 $^{1}$ | 1.87 ± 0.29 $^{1}$ | 2.33 ± 0.18 $^{1}$ | 23.39 ± 1.45 $^{1}$ | 120.40 ± 9.16 $^{1}$ |
| **US INT 10** | 1.60 ± 0.09 $^{1}$ | 1.45 ± 0.07 $^{1}$ | 1.56 ± 0.07 $^{1}$ | 24.31 ± 2.30 $^{1}$ | 118.52 ± 7.09 $^{2}$ |
| **US INT 30** | 1.72 ± 0.06 $^{1}$ | 1.45 ± 0.19 $^{1}$ | 1.63 ± 0.32 $^{1}$ | 22.22 ± 1.30 $^{1}$ | 111.63 ± 10.50 $^{3}$ |

*Influence of continuous ultrasound on the synthesis of internal compounds*

Figure 2 presents the normalized carotenoid content over cultivation time for continuous US treatments applied at A) exponential and B) stationary growth phases, and for C) discontinuous US treatment. Overall, all experiments showed a similar trend, maintaining carotenoid concentration over time. From Table 2, it can be seen that US EXP 60 showed a reduction of the pigment concentration when compared to the control. Treatments applied at the stationary phase, for 30 and 60 min, also negatively affected the pigment concentration after 240 h of cultivation, reducing its concentration at the end of the cultivation (Table 2).

Figure 2 – Normalized carotenoid concentration *(*C/C$_{control}$) of *P. marina* biomass at different cultivation times: A) continuous US application at the exponential (EXP) phase during 10, 30, and 60 min; B) continuous US application at the stationary (STA) phase during 10, 30, and 60 min; and C) intermittent US treatment (INT) for 10 and 30 min. Different lowercase letters indicate significant difference between means ($p \leq 0.05$).

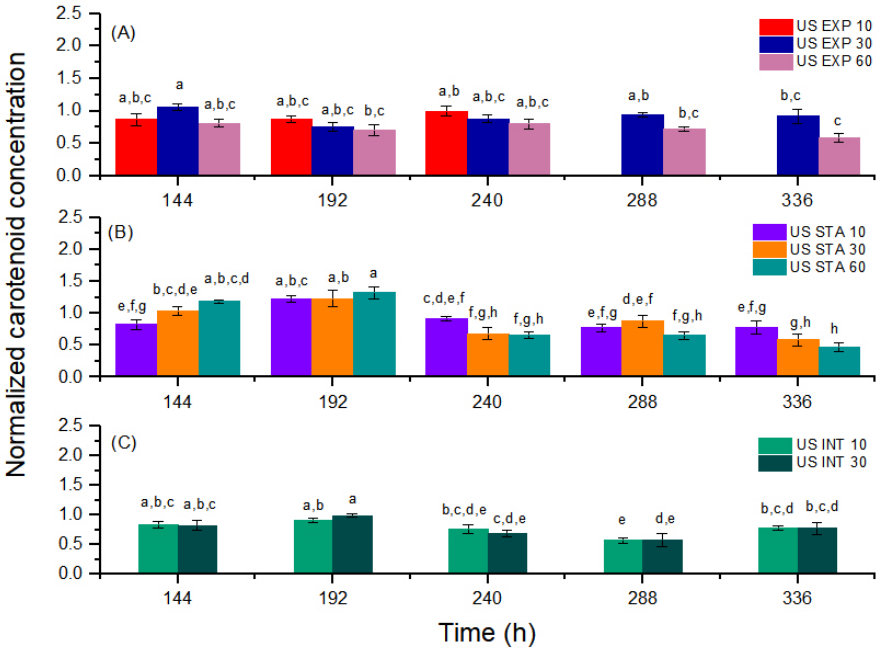



Figure 3 presents the normalized chlorophyll content for all treatments. All experiments showed a similar behavior, keeping chlorophyll concentration over time. Moreover, when compared to the control, US EXP 30 showed a reduction and US EXP 60 an increase in chlorophyll concentration at the end of the cultivation (Table 2). At the stationary phase, chlorophyll content was lower, which was expected since it is a primary pigment. Additionally, when US was applied at this growth phase, this technology did not influence chlorophyll concentration at the end of the cultivation (Figure 3 and Table 2).

Figure 3 - Normalized chlorophyll concentration *(*C/C$_{control}$) of *P. marina* biomass at different cultivation times: A) continuous US application at the exponential (EXP) phase during 10, 30, and 60 min; B) continuous US application at the stationary (STA) phase during 10, 30, and 60 min; C) intermittent US treatment (INT) for 10 and 30 min. Different lowercase letters indicate significant difference between means ($p \leq 0.05$).

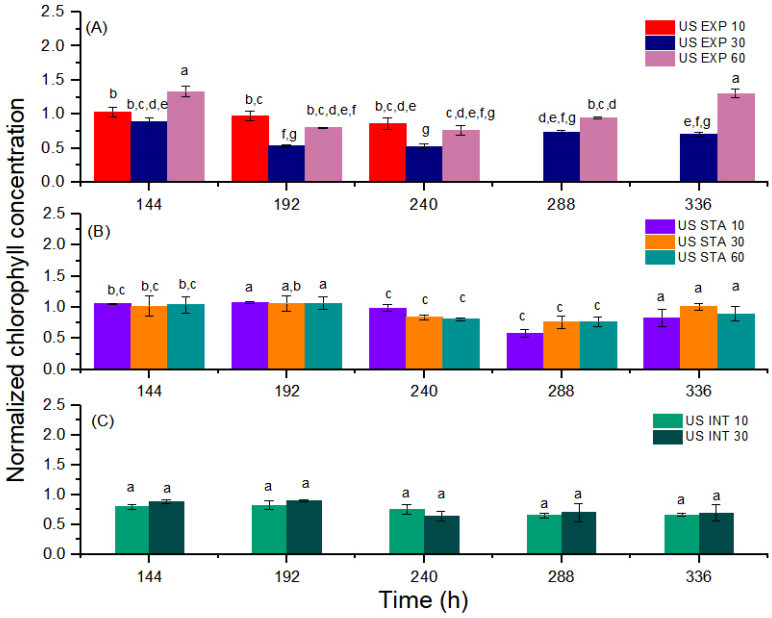
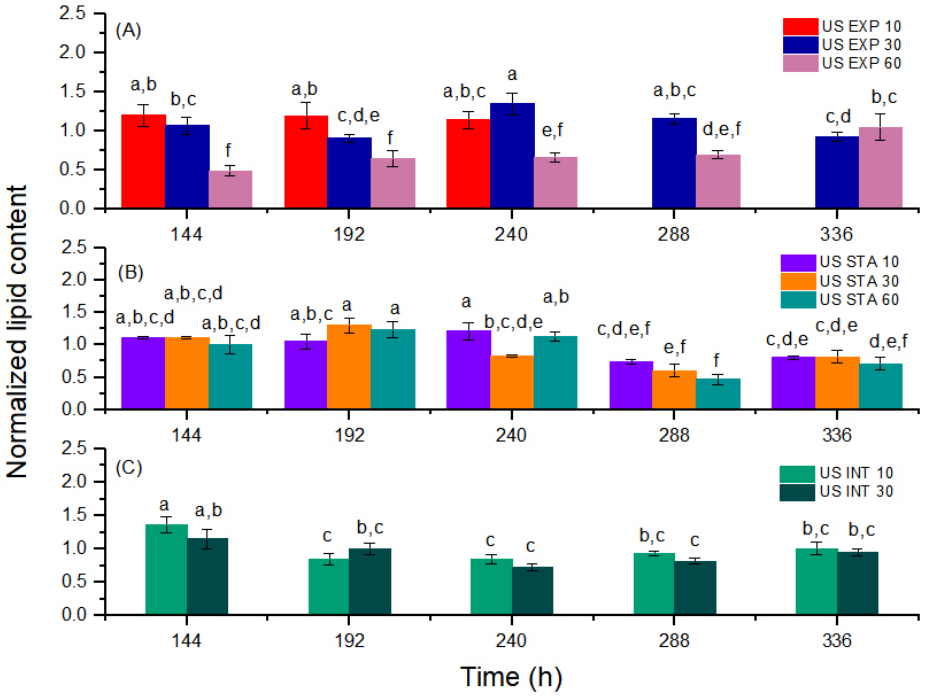



Figure 4 presents the normalized lipid concentration over cultivation time for continuous US treatments applied at A) exponential and B) stationary growth phases, and for C) discontinuous US. In general, lipid content remained constant over time for all treatments. Different results were reported by other authors that applied US at the exponential phase: Han, Pei, Hu, Zhang et al. (2016) reported an increase of 57 % in lipid concentration from *Scenedesmus quadriculata* using US bath (200W, 20 min); Ren, Zhu, et al. (2019) and Sivaramakrishnan & Incharoensakdi (2019) observed an increase of 37 and 72 % in lipid concentration from *Scenedesmus sp*. using US probe, respectively.

A different behavior was observed when US was applied at the stationary phase. US STA 30 and US STA 60 showed a decrease in lipid production at 288 h of cultivation. Furthermore, all experiments had a decrease at the end of the cultivation when compared

to the control (Table 2). These results are attributed to physical stress that may have triggered deviation of the metabolic route for lipid synthesis, decreasing its concentration in the cells (Singh et al., 2019). Singh et al. (2019) observed a similar behavior for *Tetradesmus obliquus*. After 13 days of cultivation, the authors noticed a slight reduction in the lipid content of cells treated with US.

Figure 4 – Normalized lipid content of *P. marina* biomass ($C/C_{control}$) at different cultivation times: A) continuous US application during exponential (EXP) phase during 10, 30, and 60 min; B) continuous US application during stationary (STA) phase during 10, 30, and 60 min; and C) intermittent US treatment (INT) for 10 and 30 min. Different lowercase letters indicate significant difference between means ($p \leq 0.05$).




The protein content of *P. marina* biomass was analyzed only at the end of the cultivation time. For US EXP 60, the use of the technology caused a decrease in protein content at the end of the cultivation (Table 2). This result was expected since protein synthesis is mostly affected by the nitrogen concentration in the medium, and stress

environments are rarely able to promote its accumulation. Therefore, this reduction can be attributed to the cell strategy to balance intracellular compounds to maintain its regular metabolic function (Pancha et al., 2014). Similar results were reported by Han, Pei, Hu, Jiang et al. (2016) and Kan et al. (2012). Han, Pei, Hu, Jiang et al. (2016) investigated the stress effects caused by US treatment on protein synthesis and found no increment in protein content. The same result was obtained by Kan et al. (2012), who studied the effects of salt stress on microalgae composition.

Regarding nitrate consumption, results showed that US did not promote significant changes in the consumption of this nutrient (data not shown); 450 mg $L^{-1}$ of nitrate was initially added to the medium, and, after 144 h of cultivation, this nutrient was no longer detected in the medium for all analyzed samples.

Based on all aforementioned results, it can be concluded that the use of US did not stimulate *P. marina* growth or internal compounds production. This may be attributed to the intensity of US treatment applied and to the characteristics and cell metabolism of *P. marina*. Furthermore, long US exposure (60 min) during the exponential phase has promoted a decrease in biomass, carotenoid, and protein concentration. These results indicate that US application for 60 min may have caused irreparable cellular damage probably due to cavitation effects associated with the increase of shear forces and free radical formation in the medium (Sillanpää, 2011).

Regarding the absence of positive US effects on *P. marina* metabolism, some aspects can be discussed. The optimized cultivation medium may have already imposed stress conditions to stimulate growth and synthesis of compounds; the growth medium used in the present work was chosen based on a previous study performed by Gonçalves

et al. (2019). These authors identified the optimal amount of nitrate and NaCl to promote cell proliferation and intracellular compounds production. This stress caused by the optimized medium, combined with high light intensity (17 klux or 238 μmol photons $m^2$ $s^{-1}$), may have been sufficient to reach the maximum growth capacity of the microalga. This fact is evidenced by the results obtained for the control experiment, which showed biomass concentration above 2 g $L^{-1}$. Han, Pei, Hu, Jiang et al. (2016) obtained a biomass concentration of 1.33 g $L^{-1}$ for *Anabaena variabilis* after application of US (200 W, 40 kHz) with 60 μmol photons $m^2$ $s^{-1}$. Singh et al. (2019) applied US (100 W, 33 kHz) with 7 μmol photons $m^2$ $s^{-1}$ during the growth of *Tetradesmus obliquus* and obtained an increase on biomass concentration of 22.8% (3.12 g $L^{-1}$).

The species of microalga used is also another important point to be considered. In the literature, divergent results were observed for different microalgae species when submitted to external stimuli. Particularly, *P. marina* has some characteristics that may not benefit cell stimulation by US; this microalga has relatively large cells (3 - 15 μm) and differs morphologically from many others by not having a cell wall. Joyce et al. (2014) investigated the effect of US on the viability of different microalgae species: *Dunaliella salina* (6 - 10 μm), *Chlamydomonas concordia* (6 - 10 μm), and *Nannochloropsis oculata* (2 - 4 μm). For the two species with larger cells, a reduction in cell density was observed. On the other hand, for *N. oculata* an increase on cell proliferation was noted. Furthermore, the authors found a reduction in cell concentration at different US application times: 4 minutes for *D. salina* and 16 minutes for *C. concordia*. These results were explained by the absence of a true cell wall in *D. salina* which allowed a faster cell disruption when compared to *C. concordia*.

Similar findings were observed by Greenly and Tester (2015) who evaluated cell disruption by US in various microalgae species with different cell wall sizes and compositions. These authors observed that *Chlamydomonas reinhardtii* (without cell wall) concentration halved after 2 s of treatment. In contrast, *Nannochloropsis* cell density (with robust cell wall and small size) decreased at the same level after 1 min.

Another issue to be considered is related to the US parameters (intensity and frequency) used during the experiments performed in the present study, which may not have been the most efficient for the process. Even though applying intensity and frequency (200W, 38 kHz) values similar to other authors, the application of continuous US may not have favored cell growth. According to Schläfer et al. (2000), under discontinuous treatments, cells receive the first stimulus, adapting themselves, and then receive a second stimulus, which is more effective for induction of cell growth. These authors also reported that discontinuous US (300 W $m^{-3}$ and 25 kHz) promoted an increase in ethanol production by *Saccharomyces cerevisiae* (the same effect was not observed when continuous US was applied). Therefore, to better evaluate US effects during *P. marina* growth, discontinuous US application was also evaluated.

*Influence of discontinuous ultrasound treatment*

Discontinuous US effects on *P. marina* growth were investigated using different application intervals. Regarding biomass concentration, US did not influence the results for all treatments (Table 2 and Figure 1C). Even with intermittent application, US effects were not observed, indicating that the cell characteristics of this microalga or the optimized cultivation media may be the reason for this result.

Similar to biomass concentration, intermittent US did not affect the synthesis of carotenoids at the end of the cultivation (Table 2). During the cultivation, on the other hand, at 288 h, carotenoid concentration decreased for US INT 10 and US INT 30 (Figure 2C). Regarding lipids, US INT 10 showed a reduction in the content from 192 h on; for US INT 30, a reduction was observed at 240 and 288 h (Figure 4C). At the end of the cultivation, however, no differences were detected for US treated and control samples (Table 2). The cavitation effects may have damaged intracellular enzymes involved in lipid and carotenoid metabolic pathways (Sivaramakrishnan & Incharoensakdi, 2019). The synthesis of lipids and carotenoids in microalgae occurs via the Calvin cycle; the carbon fixed during photosynthesis enters the plastidium and is converted to glyceraldehyde-3-phosphate (G3P). For lipid synthesis, G3P needs to be converted to pyruvate for subsequent formation of acetyl-CoA by the pyruvate dehydrogenase complex. For carotenoids, on the other hand, G3P is used to produce isopentenyl diphosphate (IPP) (Singh et al., 2019). Discontinuous US application may have negatively affected the conversion of G3P into pyruvate and IPP, affecting the synthesis of these compounds (Singh et al., 2019).

Different results were observed by other authors. Singh et al. (2019) used discontinuous US (1 min on/9 min off) to treat *Tetradesmus obliquus* and observed that US increased lipid and β-carotene yield by 34.5 and 31.5%, respectively. Sivaramakrishnan & Incharoensakdi (2019) also observed an increase of 72 % of lipid concentration from *Scenedesmus sp*. using intermittent US (2 s interval).

Concerning chlorophylls, intermittent US application did not affect their concentration (Figure 3C and Table 2). Protein content, on the other hand, showed a

decrease at the end of the cultivation for US INT 10 and US INT 30 (Table 2). This reduction may be the result of protein denaturation. According to Chia et al. (2019), higher ultrasonic power can produce greater cavitation effect that could result in protein denaturation, although greater mass transfer can be achieved.

Regarding nitrate concentration, it was observed that this compound was depleted within 144 h, similarly to the results obtained with continuous US treatment (data not shown). Therefore, discontinuous US did not stimulate *P. marina* growth. As previously mentioned, it is possible that *P. marina* cells characteristics did not favor US stimulation. In addition, this microalga does not synthesize secondary carotenoids, that are stimulated under stress environments.

*4.2.3.2. Influence of ultrasound on C. zofingiensis growth*

A study with continuous and discontinuous US treatment during the growth of *C. zofingiensis* was performed to evaluate the behavior of other species under US treatment. *C. zofingiensis* was chosen since this microalga presents cell wall and synthetize secondary carotenoids in higher amounts, mainly astaxanthin. Preliminary studies were carried out to evaluate different $NaNO_3$ concentrations in the growth medium to obtain the necessary biomass amount for further analyses. A $NaNO_3$ content of 450 mg $L^{-1}$ was chosen because higher concentrations did not promote an increase in biomass and protein concentration.

US was applied at the exponential phase since secondary carotenoids are synthetized at this stage. Figure 5 presents the normalized biomass concentration over cultivation time for continuous US treatments for 10 and 30 min (US CON 10 and US

CON 30) and for intermittent US treatments for 10 min (US INT 10). Results showed that discontinuous US promoted an increment on biomass production from 144 h on.

Figure 5 – Normalized biomass concentration ($C/C_{control}$) of *C. zofingiensis* at different cultivation times: continuous (CON) US treatment during 10 and 30 min, and intermittent (INT) US treatment for 10 min at the exponential phase.

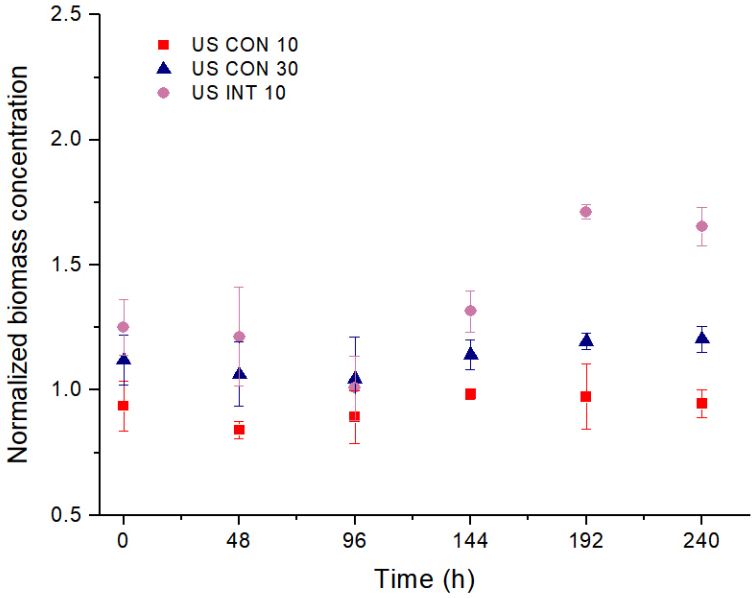



Table 3 presents the statistical analysis that compares the different treatments among themselves and among their respective controls at the end of the cultivation. The analysis of the biomass concentration results confirmed that discontinuous US was more beneficial than continuous treatment for growth: US INT 10 showed a 64% increase in biomass production when compared to the control. This finding corroborates the previously raised hypothesis that cells may need pauses between US exposure to better adapt to the stimulus (Schläfer et al., 2000). Moreover, it was also possible to infer that different microalgae species present different sensitivities to the US since *P. marina* and *C. zofingiensis* showed distinct responses to US treatment.

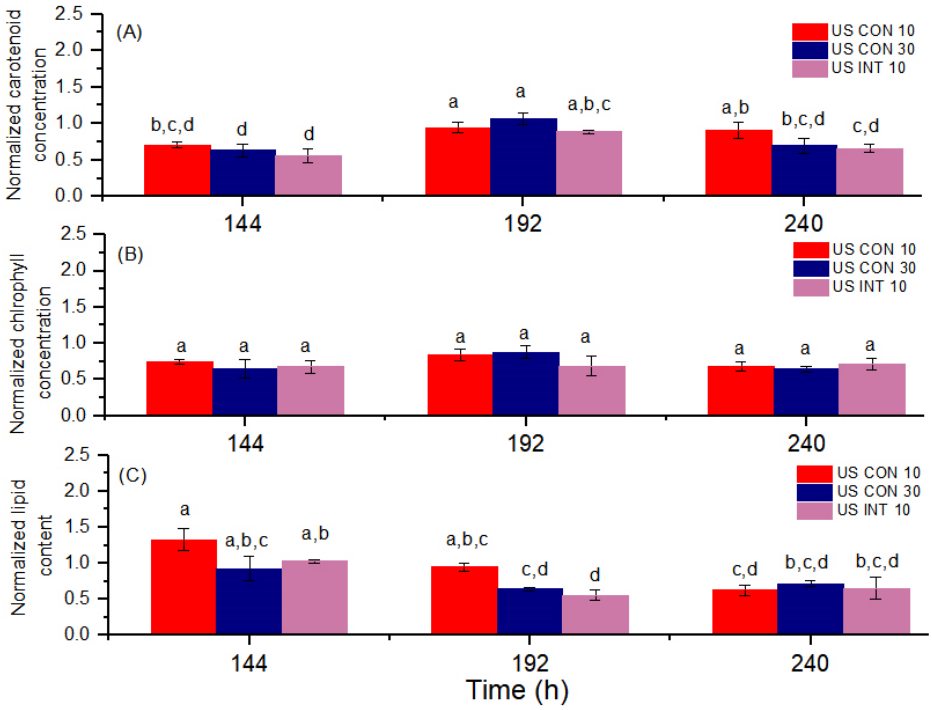
Table 3 - Biomass, carotenoid, chlorophyll, lipid and protein contents of *C. zofingiensis* with continuous (CON) US application for 10 and 30 min, and intermittent (INT) US treatment for 10 min at exponential phase. Different letters indicate significant difference between the means of the cultures compared to each other ($p \leq 0.05$).

| | **Biomass concentration ($g\ L^{-1}$)** | **Carotenoid content ($mg\ g^{-1}$)** | **Chlorophyll content ($mg\ g^{-1}$)** | **Lipid content ($g\ g^{-1}$)** | **Protein content ($mg\ g^{-1}$)** |
|---|---|---|---|---|---|
| Control | 1.33 ± 0.16 [b] | 1.63 ± 0.17 [a] | 20.48 ± 1.58 [a] | 11.31 ± 1.70 [a] | 109.41 ± 12.87 [a] |
| US CON10 | 1.25 ± 0.07 [b] | 1.48 ± 0.18 [a, b] | 13.93 ± 1.40 [b] | 7.16 ± 0.82 [b] | 134.37 ± 8.44 [a] |
| US CON 30 | 1.60 ± 0.07 [b] | 1.13 ± 0.16 [b] | 13.30 ± 0.80 [b] | 8.14 ± 0.41 [a, b] | 108.93 ± 12.78 [a] |
| US INT 10 | 2.19 ± 0.10 [a] | 1.07 ± 0.09 [b] | 14.61 ± 1.66 [b] | 7.39 ± 1.78 [b] | 104.31 ± 7.02 [a] |

Figure 6 presents the normalized carotenoid (A), chlorophyll (B), and lipid (C) concentrations over cultivation time for continuous and discontinuous US treatment. Regarding carotenoids, an increase in concentration was observed for all US treatments at 192 h. At the end of the cultivation (240 h), US CON 30 and US INT 10 showed a reduction in this pigment content compared to the control (Table 3). For chlorophylls, the concentration remained constant over cultivation time for all treatments. When compared to the control, all experiments showed a reduction in this concentration at the end of the cultivation (Table 3). Concerning lipids, a reduction in the concentration was observed for US CON 10 and US INT 10 at the end of the cultivation (Table 3). Since carotenoids and lipids showed a reduction in their contents at the end of the cultivation, a deviation in their metabolic route of production may have occurred. These results were similar to those observed for *P. marina*. As mentioned earlier, the application of US may have affected the conversion of G3P into its precursors, affecting the synthesis of lipids and carotenoids.

Therefore, considering the two microalgae species studied in the present work, it is possible to conclude that there was a distinct behavior concerning cell proliferation. At some conditions, *C. zofingiensis* reacted positively to US stimulation while *P. marina* reacted negatively. On the other hand, under other certain conditions, both were not affected. These results indicate that the presence or absence of a cell wall may be determinant to the sensitivity of different microalgae species to US.

Figure 6 – Normalized ($C/C_{control}$) carotenoid (A), chlorophyll (B), and lipid contents (C) of *C. zofingiensis* at different cultivation times: continuous (CON) US treatment during 10 and 30 min, and intermittent (INT) US treatment for 10 min at the exponential phase.




*4.2.3.3 Carotenoid profile*

Biomass obtained after the cultivation with US treatments at the best process condition were evaluated regarding changes on carotenoid profile: US INT 10 for *C. zofingiensis* and US INT 10 for *P. marina*.

Results showed that for both microalgae species, US treatment did not affect the carotenoid profile, that was similar to the control. The major carotenoids identified in *C. zofingiensis* were all-trans-lutein (72.0 %), all-trans-β-carotene (7.2 %), all-trans-zeaxanthin (3.4 %) and all-trans-α-carotene (2.8 %). For *P. marina*, the main carotenoids identified were all-trans-lutein (42.6 %), all-trans-β-carotene (16.2 %), all-trans-zeaxanthin (13.1 %) and all-trans-α-carotene (11.5 %). Ketocarotenoids astaxanthin and canthaxanthin, known to be produced by some microalgae as secondary carotenoids under stress conditions (Lee et al., 2016; Lemoine & Schoefs, 2010) were not detected in the analyzed biomass.

*4.2.4. Conclusions*

US application was evaluated during *P. marina* and *C. zofingiensis* growth. For *P. marina*, US did not stimulate biomass and internal compounds production when applied continuously or intermittently. Moreover, longer US exposures (60 min) in the exponential phase promoted a reduction in biomass, carotenoid and protein concentrations. In the stationary phase, a reduction in carotenoids and lipids were observed. For *C. zofingiensis*, on the other hand, discontinuous US treatment resulted in the increase of cell proliferation. This difference may be related to the absence of a true cell wall in *P. marina*. Lipids and carotenoids during *C. zofingiensi*s growth were also negatively affected when long treatments were applied. These effects may be linked to changes promoted by US cavitation on their metabolic pathway production. Overall, discontinuous US may be explored to stimulate biomass production depending on the microalga cell structure.

*4.2.5. Acknowledgements*

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# Capítulo 5 – Extração de carotenoides e clorofilas assistida por campo elétrico pulsado

Esse capítulo apresenta o estudo da aplicação de PEF na extração de carotenoides e clorofilas a partir da microalga *C. zofingiensis*. Primeiramente, é apresentada a metodologia empregada para o cultivo da microalga, para os tratamentos com PEF, bem como para as análises de carotenoides e clorofilas. Os resultados desse estudo estão apresentados na forma de artigo em processo de publicação em revista indexada.

Esse estudo foi desenvolvido durante o Doutorado Sanduíche no exterior na *Universidad de Zaragoza*, na Espanha, sob orientação do professor Javier Raso, pelo período de 10 meses.

## 5.1. Materiais e métodos

### *5.1.1. Inóculo*

A microalga *C. zofingiensis* foi adquirida no Banco Nacional de Algas (BEA 0468B, Ilhas Canárias, Espanha). As pré-culturas foram preparadas em frascos Erlenmeyer de 500 mL com um volume de trabalho de 200 mL, utilizando meio WC modificado (Guillard & Lorenzen, 1972) com 450 mg $L^{-1}$ de NaCl, inoculados com 20 mL de cultura. Os frascos foram incubados em um agitador rotatório a 25 °C e 180 rpm com iluminação contínua de 40 μmol $m^{-2}$ $s^{-1}$ por 7 dias.

*5.1.2. Cultivos da microalga*

Os cultivos foram realizados em frascos Roux de 1 L com meio WC e com a adição de um número inicial de células de $1\times10^5$ $mL^{-1}$. A densidade celular foi determinada por análise microscópica (microscópio L-Kc, Nikkon, Tóquio, Japão) em câmara de Thoma (ServiQuimia, Constantı, Espanha). Os frascos foram mantidos a 25 °C, 4 L $min^{-1}$ de aeração e 20 klux. No segundo dia de cultivo, 10 ml $L^{-1}$ de soluções de fosfato (5 g $L^{-1}$ de $NaH_2PO_4.H_2O$) e de metais traços (9,8 mg $L^{-1}$ de $CuSO_4.5H_2O$, 22 mg $L^{-1}$ de $ZnSO_4$. $7H_2O$, 10 mg $L^{-1}$ de $CoCl_2.6H_2O$, 180 mg $L^{-1}$ de $MnCl_2.4H_2O$, 6,3 mg $L^{-1}$ de $Na_2MoO_4.H_2O$, 100 mg $L^{-1}$ de $H_3BO_3$, 4,36 g $L^{-1}$ de $Na_2EDTA$ e 3,15 g $L^{-1}$ de $FeCl_3.6H_2O$) foram adicionadas ao meio para evitar a falta de nutrientes. Dois tipos de culturas foram avaliados, com e sem estresse celular, conforme Figura 8. Para a cultura sem estresse (cultura verde), as microalgas foram mantidas a 20 klux durante todo o crescimento; na cultura com estresse celular (cultura laranja), as microalgas foram submetidas a uma intensidade luminosa mais elevada (23 klux) a partir do 7º dia de crescimento (fim da fase exponencial).

Figura 8 - Cultivo da microalga *C. zofingiensis*, sem (cultura verde) e com (cultura laranja) estresse celular.

O crescimento das microalgas foi monitorado através da densidade ótica (DO), da massa seca das células e do número de células. A DO foi medida em espetrofotômetro (Genesys 10S UV-vis, Thermo Scientific) a 680 nm. O peso seco foi determinado por secagem a vácuo (GeneVac Ltd, UK) a 40 ºC utilizando 1 mL da suspensão de microalgas. Após 14 dias, quando a suspensão de microalgas atingiu aproximadamente 10 g $L^{-1}$ e 1 × $10^7$ células $mL^{-1}$, a biomassa foi recolhida para realização dos experimentos com PEF.

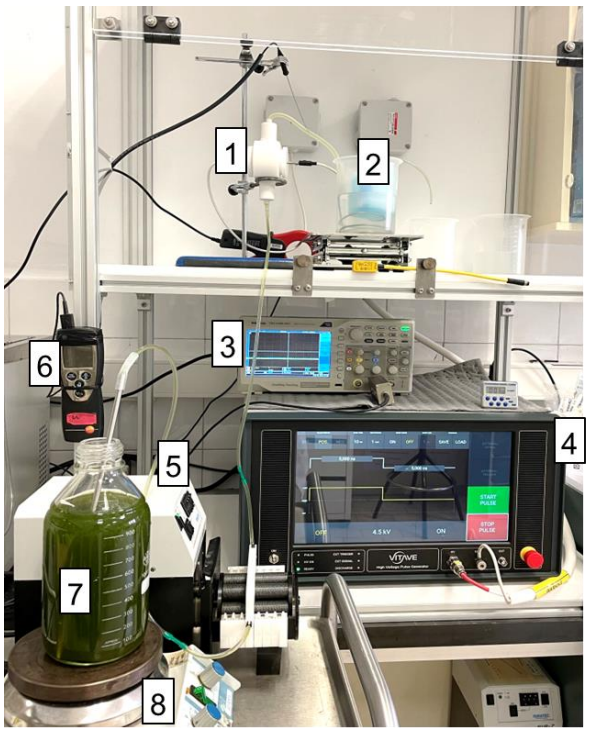
*5.1.3. Pré-tratamento com PEF*

Previamente aos tratamentos com PEF, a biomassa fresca foi centrifugada a 3.000 ×g durante 10 min a 25 ºC e ressuspendida em tampão citrato-fosfato McIlvaine (1 mS $cm^{-1}$; pH 7) até uma concentração final de $10^9$ CFU $ml^{-1}$. A biomassa foi tratada em uma câmara de fluxo contínuo utilizando um equipamento comercial de PEF (Vitave, Praga, República Checa). Foi utilizada uma bomba peristáltica (BVP, Ismatec, Wertheim, Alemanha) para bombear a biomassa na vazão de 5 L $h^{-1}$. A biomassa foi introduzida em uma câmara de eletrodos paralelos de titânio, com espaçamento de 0,4 cm, comprimento de 3,0 cm e largura de 0,5 cm, e tempo de residência de 0,43 s. A Figura 9 mostra o aparato experimental utilizado para o tratamento com PEF. Para induzir o tratamento elétrico, foram administrados pulsos monopolares em forma de onda quadrada com uma largura de 3,0 µs, com forças de campo elétrico de 15, 20 e 25 kV $cm^{-1}$ por um período de 150 µs (50 pulsos). Essas intensidades de campo elétrico correspondem a energias específicas totais de 34, 60 e 94 kJ $kg^{-1}$, respectivamente. Além disso, para o campo elétrico de 20 kV $cm^{-1}$, avaliou-se diferentes tempos de tratamento em 30, 75 e 150 µs (10, 25 e 50 pulsos), correspondendo a energias específicas totais de 12, 30 e 60 kJ $kg^{-1}$,

respetivamente. A tensão em tempo real foi monitorada por sonda de alta tensão (Tektronik, P6015A, Wilsonville, Oregon, Estados Unidos) ligada a um osciloscópio (Tektronik, TDS 220). A temperatura na saída foi medida por um termopar de tipo K (Ahlborn, Holzkirchen, Alemanha) incorporado ao circuito. Após os tratamentos, as suspensões foram rapidamente resfriadas a uma temperatura inferior a 20°C num espaço de tempo inferior a 5 segundos, utilizando um permutador de calor posicionado após a câmara de tratamento.

Após o pré-tratamento com PEF, foi avaliado 1) o efeito da incubação em tampão de pH 7 durante 24 h antes da extração; e 2) a influência da etapa difusiva com etanol 95% (1:1 v/v) realizadas por 0, 6 e 24 h após o tratamento com PEF.

Figura 9 - Aparato experimental de PEF.




1) câmara de tratamento; 2) banho de gelo; 3) osciloscópio; 4) gerador; 5) bomba; 6) termopar; 7) suspensão de microalga; 8) agitador magnético.

*5.1.4. Extração com moinho de bolas*

Para determinar o teor total de carotenoides e clorofilas produzidos por *C. zofingiensis*, foi realizada extração com moinho de bolas (Mini-Beadbeater-Plus; BioSpec, Bartlesville, Estados Unidos). Alíquotas de 1 ml de biomassa fresca foram centrifugadas (3.000 ×g, 5 min) e ressuspendidas em etanol a 95 % (1:1). A mistura foi transferida para um tubo de 2 ml com tampa de rosca e foram adicionadas esferas de vidro de 0,5 mm de diâmetro numa proporção de 1:5 (esferas de vidro/suspensão microalgal). A destruição das células foi verificada por observações microscópicas (Eclipse E400, Nikkon, Tóquio, Japão). Para alcançar a destruição de mais de 90% das células, foram efetuados três ciclos de 60 s, com 15 s de arrefecimento em banho de água gelada entre os ciclos. Em seguida, as suspensões foram centrifugadas (3.000 ×g, 5 min), e o sobrenadante foi avaliado quanto à concentração de carotenoides e clorofilas, conforme descrito na seção 5.1.5.

*5.1.5. Quantificação de pigmentos*

Para a extração de pigmentos, 100 µL da amostra (não tratada, tratada com PEF ou tratada com PEF e incubada) foram adicionados a 1 mL de etanol a 95 %. A mistura foi agitada em vortex, incubada no escuro à temperatura ambiente durante 20 min e centrifugada a 3.000 × g durante 5 min. A absorbância do sobrenadante foi medida a 470, 649 e 664 nm. As concentrações de carotenoides totais e clorofilas a e b foram calculadas de acordo com as equações 2 a 4 (Lichtenthaler & Buschmann, 2001):

$$C_a = 13.36\, A_{664\,nm} - 5.19\, A_{649\,nm} \quad (2)$$

$$C_b = 27.43\, A_{649\,nm} - 8{,}12\, A_{664\,nm} \quad (3)$$

$$C_{Ct} = \frac{1000\ A_{470\ nm} - 2.13\ C_a - 97.64\ C_b}{209} \quad (4)$$

em que $C_a$ corresponde à concentração de clorofila a (μg $mL^{-1}$), $C_b$ à concentração de clorofila b (μg/mL) e $C_{Ct}$ à concentração de carotenoides totais (μg $mL^{-1}$).

*5.1.6. Detecção de eletroporação*

A detecção de eletroporação das células de *C. zofingiensis* foi avaliada de acordo com a metodologia descrita por Berzosa et al. (2023). A libertação dos compostos (DNA e proteínas) foi monitorizada por medições espectrofotométricas em absorbâncias de 260 e 280 nm, respectivamente, após 0, 6 e 24 h do tratamento com PEF.

*5.1.7. Análise estatística*

Todos os tratamentos foram realizados em triplicata. Os resultados foram analisados por ANOVA e teste de Tukey utilizando o software Statistica® (12.0, Statsoft Inc., Tulsa, EUA).

## 5.2. Artigo - Pulsed electric field-assisted extraction of carotenoids from *Chlorella zofingiensis*

Nessa seção é apresentado o artigo ainda em processo de publicação.

*Abstract*

The present work aimed to evaluate the use of PEF as a pretreatment to carotenoid and chlorophyll extraction from two different *C. zofingiensis* biomasses, green (non-stressed) and orange (stressed). PEF treatment was performed varying the electric field strength (15, 20, 25 kV/cm) and the treatment time (30, 75, 150 μs). The effect of incubation in a pH 7 buffer for 24 h after PEF and before extraction was also evaluated. The application of an intermediate intensity combined with longer pulses (20 kv/cm with 50 pulses) was sufficient to promote the extraction, yielding the highest contents of carotenoids and chlorophylls. The incubation promoted higher extraction yields for treatments performed with shorter diffusion times and with mild electric field strengths. Overall, the findings

of this study suggest that the application of PEF combined with ethanol could represent a suitable approach for the extraction of carotenoids and chlorophylls from Chlorella zofingiensis biomass.

Keywords: pulsed electric fields, compounds, extraction, microalgae, pigments.

### *5.2.1. Introduction*

Due to the growing demand for natural products, there is a raising interest in substituting synthetic food additives, such as food colorants or antioxidants, for natural ones. As a result, the extraction of natural pigments, as carotenoids, has drawn the attention of many researchers. Microalgae are a potential source of carotenoids due to their fast growth rates, adaptability to diverse environments and various growth conditions (Daneshvar et al., 2021).

The green microalga *Chlorella zofingiensis* is known for being a rich source of carotenoids, particularly astaxanthin, a red pigment that also plays an antioxidant role. Astaxanthin is employed in industrial aquaculture as a feed additive to enhance the flesh coloration of salmons, trouts, shrimps, and crayfishes, improving the quality of the product and increasing its acceptance among consumers (Lim et al., 2018). In addition, the antioxidant properties of this carotenoid are also recognized in therapeutic applications due to their anti-inflammatory, immunostimulant, anti-tumor, anti-diabetic, cardioprotective, and neuroprotective qualities (Lu et al., 2019; Masoudi et al., 2021).

However, since carotenoids are located inside the cell, it is necessary to disrupt the cell structure to allow their extraction. Due to the lipophilic nature of these pigments, the commonly employed methods for their recovery involve drying the biomass and subsequent extraction with organic solvents. Frequently, the solvents used are harmful to the environment as acetone, petroleum ether, ethyl ether, and methanol (Mandelli et al.,

2012; Rodrigues et al., 2014). In addition, these conventional methods require lengthy periods and consume significant amounts of energy. To overcome these drawbacks, alternative technologies, such as pulsed electric field (PEF), ultrasound, and moderate electric field, have been applied to disrupt cell structures, allowing the use of more environmentally friendly solvents.

PEF is an electrical technology that consists of applying high-intensity electric field pulses of short duration (μs to ms) through a biological matrix. Due to the electroporation effects, this technology has been used to disrupt cell membranes and improve the extraction of metabolites from microalgae (Han et al., 2019; Kotnik & Miklavčič, 2006; Luengo & Raso, 2017; Martínez et al., 2019).

Wang et al. (2023) applied PEF (99 kJ/kg) to extract proteins, phenolic compounds, chlorophylls, and carotenoids from *Chlorella*, using water and dimethyl sulfoxide (DMSO, 50 % of concentration) as solvents. Control experiments (without PEF) were also carried out for comparison. The results showed that higher carotenoid extraction yields were obtained with DMSO after 120 min of PEF treatment (12 mg/g). Comparatively, experiments performed with water yielded 0.3 mg/g after the same 120 min of PEF treatment.

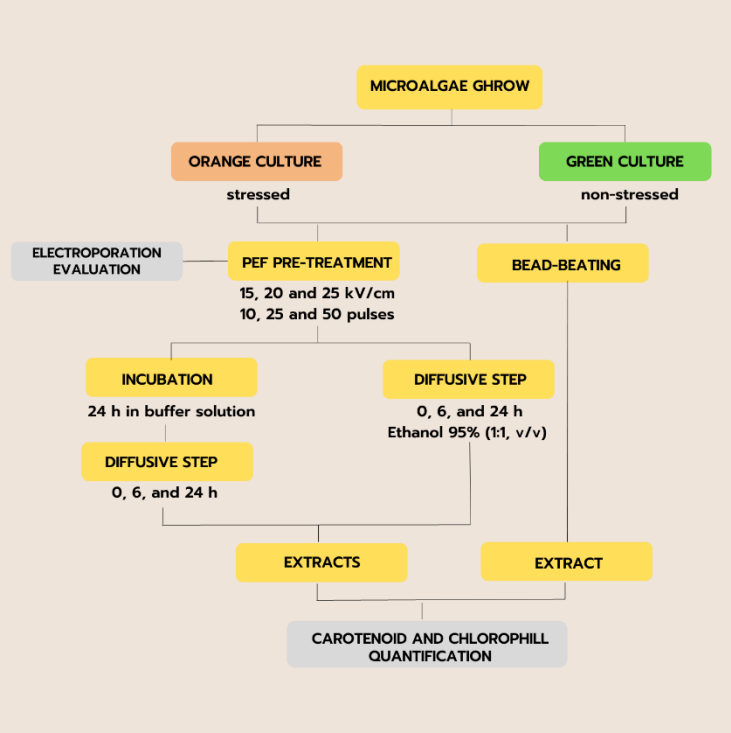
Besides the need for organic solvents to extract carotenoids from microalgae, another limitation for their industrial application is the low pigment concentration within the cells. Therefore, stress conditions, as nitrogen limitation and high luminosity and salinity, are applied to stimulate carotenoid production. The green microalga *C. zofingiensis*, for example, undergoes a color change to orange when stressed with high luminosity, resulting in an increased production of astaxanthin. Within this context, the

present study aimed to evaluate the use of PEF as a pretreatment to carotenoid and chlorophyl extraction from two different *C. zofingiensis* biomasses, green (non-stressed) and orange (stressed). The objective of this investigation was to better understand the microalgae behavior in response to PEF treatment by studying the influence of stress conditions on cell response.

### *5.2.2. Materials and methods*

The microalgae *C. zofingiensis* were cultivated, and fresh samples were subjected to PEF pretreatment, followed by a subsequent diffusive extraction step. Figure 1 illustrates a flowchart outlining the key steps involved in the process.

Figure 1 - Flowchart outlining the key steps involved in the different processes performed for carotenoid and chlorophyll extraction from *Chlorella zofingiensis*.



*5.2.2.1. Microalgae strain*

The microalgae strain *C. zofingiensis* was purchased from the National Bank of Algae (BEA 0468B, Canary Islands, Spain). The pre-cultures were prepared in 500 mL Erlenmeyer flasks with 200 mL working volume, using a modified WC medium (Guillard & Lorenzen, 1972) with 450 mg/L of NaCl, and were inoculated with 20 mL of stock culture. The flasks were incubated in a rotatory shaker at 25 °C and 180 rpm with continuous illumination of 40 μmol/$m^2$ s for 7 days.

*5.2.2.2. Cultivation conditions*

Microalgae cultures were grown in 1 L Roux flasks with WC medium and with the addition of an initial cell number of $1\times10^5$/mL. Cell density was determined by microscopic analysis (microscope L-Kc, Nikkon, Tokyo, Japan) in a Thoma cell chamber (ServiQuimia, Constantı, Spain). The flasks were maintained at 25°C, 4 L/min of aeration, and 20 klux. To avoid lack of nutrients, on the second day of cultivation, 10 ml/L of phosphate (5 g/L of $NaH_2PO_4.H_2O$) and metal traces solutions (9.8 mg/L of CuSO4.5H2O, 22 mg/L of $ZnSO_4.7H_2O$, 10 mg/L of $CoCl_2.6H_2O$, 180 mg/L of $MnCl_2.4H_2O$, 6.3 mg/L of $Na_2MoO_4.H_2O$, 100 mg/L of $H_3BO_3$, 4.36 g/L of $Na_2$EDTA e 3.15 g/L of $FeCl_3.6H_2O$) were added to the medium. Two types of growing cultures were performed, with and without cell stress. For the culture without stress (green culture), the microalgae were maintained at 20 klux during the whole growth; in the cell-stressed culture (orange culture), the microalgae were subjected to higher light intensity (23 klux) from the 7th day of growth (end of the exponential phase).

Microalgae growth was monitored by optical density (OD), cell dry weight, and the number of cells measured. OD was measured by a spectrophotometer (Genesys 10S UV−vis, Thermo Scientific) at 680 nm. The dry weight of microalgae was determined by vacuum drying (GeneVac Ltd, UK) at 40 ºC using 1 mL of the microalgal suspension. After 14 days, when microalgae suspension achieved approximately 10 g/L (dry base) and $1 \times 10^7$ cells/mL, the biomass was collected for further PEF experiments.

*5.2.2.3. PEF pre-treatments*

Before the treatments, fresh microalgal biomass was centrifuged at 3,000 ×g for 10 min at 25 ºC and re-suspended in a citrate phosphate McIlvaine buffer (1 mS/cm; pH 7) to a final concentration of $10^9$ CFU/ml. This biomass was PEF-treated in a continuous flow chamber using a commercial PEF equipment (Vitave, Prague, Czech Republic). A peristaltic pump (BVP, Ismatec, Wertheim, Germany) was utilized to propel microalgal biomass at a flow rate of 5 L/h. The biomass was pumped through a titanium parallel electrode chamber (3.0 cm x 0.5 cm), with a gap of 0.4 cm, and residence time of 0.43 s. To induce electrical treatment, square waveform monopolar pulses with a width of 3.0 μs were applied at electric field strengths of 15, 20, and 25 kV/cm at 150 μs, which correspond to total specific energies of 34, 60, and 94 kJ/kg, respectively. Also, at electric field strength of 20 kV/cm, three treatment times were evaluated: 30, 75, and 150 μs (corresponding to total specific energies of 12, 30, and 60 kJ/kg, respectively). The real-time voltage was monitored using a high voltage probe (Tektronik, P6015A, Wilsonville, Oregon, United States) connected to an oscilloscope (Tektronik, TDS 220). The temperatures at the outlets were measured by incorporating a type K thermocouple into

the circuit (Ahlborn, Holzkirchen, Germany). After treatments, the suspensions were rapidly cooled down to below 20°C within a time span of less than 5 seconds using a heat exchanger positioned after the treatment chamber.

The energy per pulse (W) was calculated using Equation 1:

$$W = \int_0^t k \cdot E(t)^2 dt \quad (1)$$

in which $k$ (S/m) is the electrical conductivity of the treatment medium, $E$ (V/m) is the electric field strength, and $t$ (s) is the duration of the pulse. The total energy (kJ) applied was calculated by multiplying the energy per pulse by the number of pulses. The total specific energy (kJ/kg) applied was determined by dividing the total energy by the mass of the treated medium.

After PEF pre-treatment, it was evaluated: 1) the effect of incubation in pH 7 buffer for 24 h before extraction; and 2) the influence of a diffusive step with ethanol 95% (1:1 v/v) performed during 0, 6, and 24 h of PEF treatment.

*5.2.2.4. Bead milling*

Extraction with bead milling (Mini-Beadbeater-Plus; BioSpec, Bartlesville, United States) was performed to determine the total content of carotenoid and chlorophyll produced by *C. zofingiensis*. Aliquots of 1 ml of fresh microalgal biomass was centrifugated (3,000 ×g, 5 min) and re-suspended in 95% ethanol. The mixture was transferred into a 2 ml screw cap tube, and 0.5 mm diameter glass beads were added at a weight ratio of 1:5 (glass beads/microalgal suspension). Cell disruption was checked by microscopic observations (Eclipse E400, Nikkon, Tokyo, Japan). To achieve the destruction of over 90% of the cells, three cycles of 60 s were performed, with 15 s of cooling in an ice-water bath between cycles. The suspensions were then centrifuged

(3,000 ×g, 5 min) and the supernatant was evaluated regarding carotenoid and chlorophyll concentration as detailed in Section 5.2.2.5.

*5.2.2.5. Pigment quantification*

For pigment extraction, 100 µL of the sample (non-treated, PEF-treated, or PEF-treated and incubated) was added to 1 mL of 95 % ethanol. The mixture was vortexed, incubated in the dark at room temperature for 20 min, and centrifuged at 3,000×g for 5 min. The absorbance of the supernatant was measured at 470, 649, and 664 nm against a 95 % ethanol blank. The concentrations of total carotenoids and chlorophylls a and b were calculated according to Equations 2 to 4 (Lichtenthaler & Buschmann, 2001):

$$C_a = 13.36\, A_{664\,nm} - 5.19\, A_{649\,nm} \quad (2)$$

$$C_b = 27.43\, A_{649\,nm} - 8{,}12\, A_{664\,nm} \quad (3)$$

$$C_{Ct} = \frac{1000\, A_{470\,nm} - 2.13\, C_a - 97.64\, C_b}{209} \quad (4)$$

in which $C_a$ corresponds to chlorophyll *a* concentration (µg/mL), $C_b$ to chlorophyll *b* concentration (µg/mL), and $C_{Ct}$ to total carotenoid concentration (µg/mL).

*5.2.2.6. Electroporation detection*

The detection of electroporation of *C. zofingiensis* microalgae cells was evaluated according to the methodology described by Berzosa et al. (2023). The release of the

compounds (DNA and proteins) was monitored by spectrophotometric measurements at absorbances of at 260 and 280 nm, respectively after PEF treatments at 0, 6 and 24 h.

#### *5.2.2.7. Statistical analysis*

All treatments were carried out in triplicate. The results were analyzed by ANOVA and Tukey's test using the software Statistica® (12.0, Statsoft Inc., Tulsa, USA).

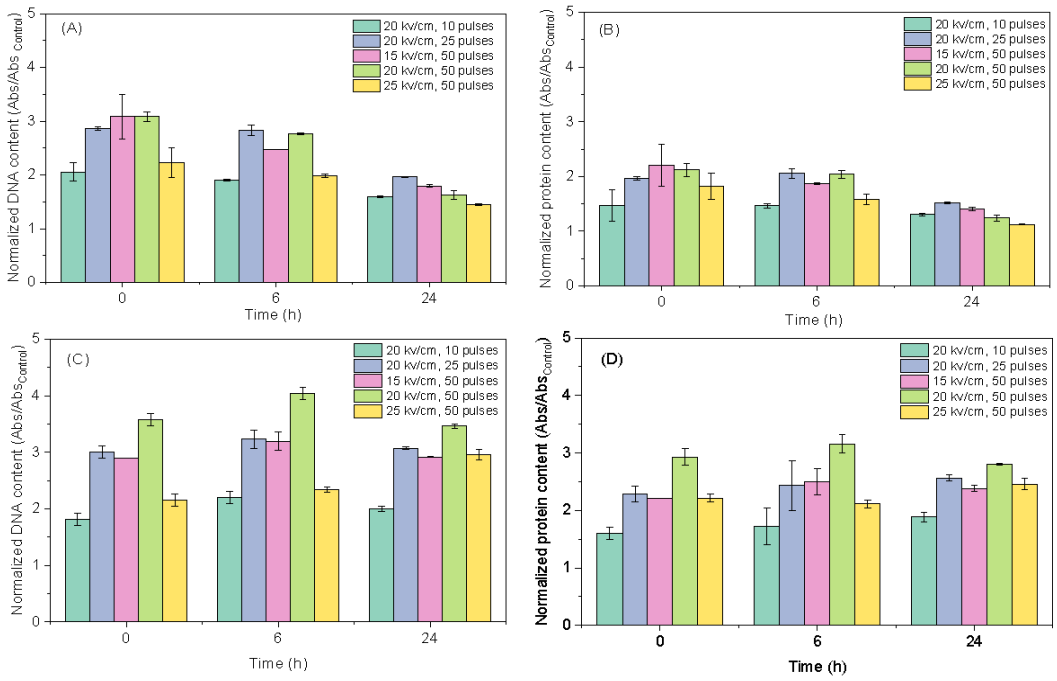
### *5.2.3. Results and discussion*

#### *5.2.3.1. Influence of PEF on electroporation*

The electroporation phenomenon was evaluated by DNA and protein release in the medium after PEF treatment, measuring the absorbance at 260 and 280 nm, respectively. The results were normalized against the control treatment and are shown in Figure 2 for orange (A and B) and green (C and D) cell cultures. It can be seen an increase in the detection of DNA and proteins in the medium since sample/control ratio values are greater than 1. This observation suggests that the application of PEF enhances cell permeability by inducing pore formation. This phenomenon is a result of the accumulation of charges on the membrane, promoting the rearrangement of phospholipids and electroporation (Gehl, 2003; Mahnič-Kalamiza & Miklavčič, 2022). Furthermore, it can be observed that electroporation occurred immediately after PEF application and, in most of cases, remained relatively constant over the following 24 hours. Since this analysis is solely used as an indicator of electroporation, no comparisons were made between the different treatments.

Berzosa et al. (2023) also observed an increase in absorbance right after PEF treatment of *Saccharomyces cerevisiae*. The researchers attributed this increment to the release of cytoplasmatic low molecular weight compounds, such as ATP, nucleosides, amino acids, and small peptides. According to the authors, the higher absorbance increment was observed in the first 15 min after the treatment. Aronsson (2005) performed a similar analysis on *Escherichia coli*, *Listeria innocua* and *Saccharomyces cerevisiae* biomasses after 6 to 42 h of PEF treatment and also reported a higher absorbance increment in the first hours after treatment. Yang et al. (2021), on the other hand, found different results after applying moderate pulsed electric field (3 to 7 kV/cm) to *Saccharomyces cerevisiae* biomass. The authors reported higher absorbance values when higher PEF intensities were applied, reaching maximum values after 30 h of treatment.

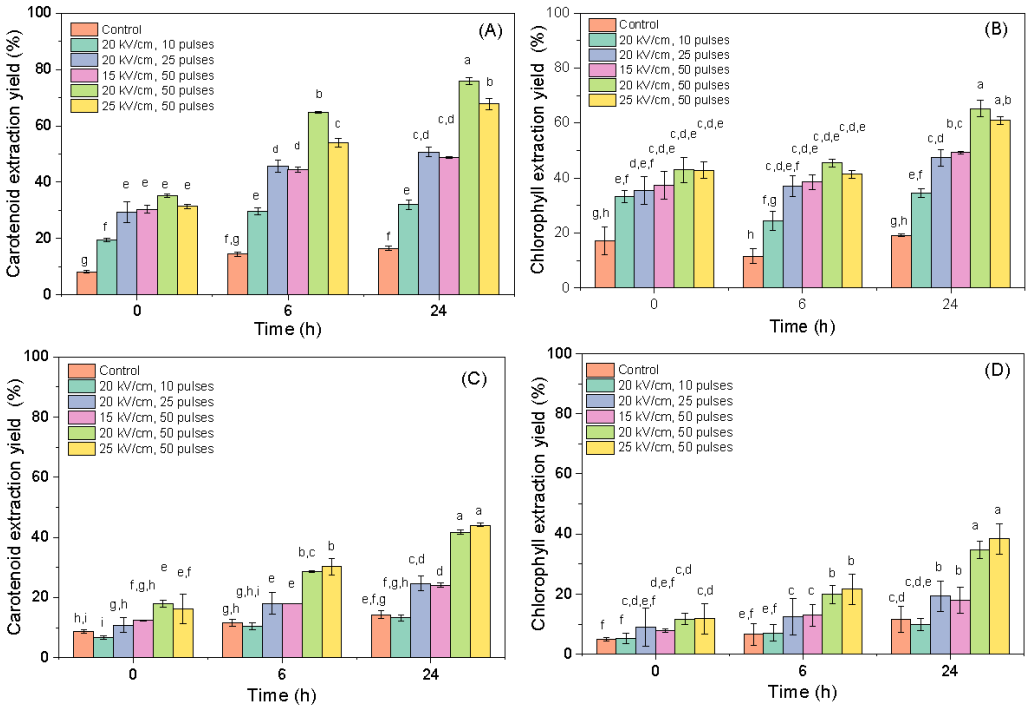
Figure 2 - DNA and protein measurement to evaluate electroporation of cells from orange (A, B) and green (C, D) cultures.



*5.2.3.2 Influence of PEF intensity and processing time on carotenoid and chlorophyll extraction*

Figure 3 shows the effect of electric field intensity (kV/cm) and treatment time (μs) on the extraction of carotenoids and chlorophylls from orange (A and B) and green (C and D) cultures of *C. zofingiensis*. The extraction yield was calculated considering the total content obtained by bead-milling extraction. As can be seen, PEF promoted cell permeabilization and electroporation just after treatment (time zero) since all treated samples showed higher amounts of carotenoids and chlorophylls than the control (except green microalga treated with 20 kV/cm and 10 pulses). These findings align with those found previously when analyzing DNA and protein release into the medium. To enhance this discussion, Table S1 displays the absolute concentration values of carotenoids and chlorophylls after PEF treatment, followed by a diffusive step with ethanol. The carotenoid content of the control at time zero of the diffusive step was 0.15 mg/g, while the other extracts (with PEF pretreatment) showed an average concentration of 0.57 mg/g. Regarding chlorophylls, at the beginning of the diffusive phase (time zero), the orange control sample had a chlorophyll content of 0.11 mg/g, while the other PEF pretreated extracts had an average concentration of 0.17 mg/g.

Figure 3 - Carotenoid and chlorophyll extraction yield (%) of the extracts after PEF treatments and a diffusive step with ethanol for orange (A and B) and green (C and D) cultures.




From Figure 3, it is also possible to observe that the extraction of these compounds increased as the number of pulses increases. Moreover, the extraction yields enhanced when the PEF intensity increased from 15 to 20 kV/cm (for the same number of pulses). On the other hand, when the PEF intensity augmented from 20 to 25 kV/cm, carotenoid and chlorophyll extraction yields were similar (except for carotenoids from orange culture at 6 and 24h). Martínez et al. (2017) found that the minimum electric field intensity for C-phycocyanin extraction was 15 kV/cm at 150 μs (50 pulses of 3 μs). Coustets et al. (2013) obtained a certain degree of electroporation on *Nannochloropsis salina* microalga cells when PEF treatment was applied at 6 kV/cm with 30 pulses of 2 ms. Zbinden et al. (2013) also reported that the use of intermediate electric field strength (45 kV/cm) promoted the highest lipid extraction from the microalga *Ankistrodesmus falcatus*.

The treatments with higher energies (20 and 25 kV/cm with 50 pulses) exhibited an increasing extraction yield over time for both cultures. This behavior was not observed in Figure 2 when evaluating the extend of electroporation, indicating a combined effect of PEF and ethanol on the extraction process. Luengo et al. (2014) also reported that ethanol was effective to extract carotenoids and chlorophylls from the fresh microalga *Chlorella vulgaris* PEF-treated. Parniakov et. al (2015) evaluated the extraction of carotenoids and chlorophylls from *Nannochlorpsis* spp. using dimethyl sulfoxide (DMSO), ethanol and water after 0 and 240 min of PEF treatment. The authors observed an increment on total carotenoid and chlorophyll content using PEF pre-treatment (20 kV $cm^{-1}$) followed by a diffusive step with ethanol and DMSO. Controversially, Martínez (2018) reported that ethanol was ineffective for carotenoid extraction from *Rhodotorula glutinis* yeast with PEF treatment at 15 kV/cm and 150 μs. The authors suggested that this could be due to the composition of the more robust plasma membrane.

It is important to mention that ethanol is included in the list of Generally Recognized as Safe (GRAS) solvents, as demonstrated by toxicological and medical studies that have shown no adverse effects on human health when used in food applications for an extended period (Food and Drug Administration, 2016). In terms of environmental, health, and safety hazards, ethanol is considered preferable compared to other solvents commonly used for carotenoid extraction, such as hexane and diethyl ether (Alfonsi et al., 2008).

Considering treatments carried out with 20 and 25 kV/cm at 50 pulses, the former can be regarded as the optimal condition. It promoted equivalent extraction yields of carotenoids and chlorophyll, while utilizing a lower amount of energy (kJ/kg). Table 1

shows the extraction yield of carotenoids and chlorophylls with PEF pretreatment at 20 kV/cm, 50 pulses in 24h (best condition) for orange and green cultures. In the case of the orange culture, this condition extracted 76 and 65% of carotenoids and chlorophylls, respectively, values approximately three to four times higher than that of the control (Table 1). Luengo et al. (2014), working with *C. vulgaris*, also reported an increase of 0.5, 0.7, and 0.8 times in extraction yields for carotenoids and chlorophylls a and b, respectively, right after PEF treatment (20 kV/cm and 75 μs) and extraction with ethanol as solvent. Luengo et al. (2015) applied PEF to extract lutein from *C. vulgaris* using ethanol as solvent (96 %). The authors observed that PEF treatment increased carotenoid extraction, if compared to control and best extraction conditions were 25 kV $cm^{-1}$, 75 μs and 40 °C. According to the authors, only ethanol was not enough to disrupt cell membrane and extract lutein.

Table 1 – Extraction yield of carotenoids and chlorophylls with PEF pretreatment at 20 kV/cm, 50 pulses in 24h (best condition) for orange and green cultures.

| **Culture** | **Carotenoid extraction yield (%)** | **Chlorophyll extraction yield (%)** |
|---|---|---|
| Orange | $76.30 \pm 1.44^{a}$ | $65.34 \pm 3.68^{a}$ |
| Green | $41.21 \pm 0.80^{b}$ | $33.90 \pm 1.16^{b}$ |

Table 2 displays the total contents of carotenoids and chlorophylls (obtained by bead-milling) for orange (stressed) and green (non-stressed) cultures. The orange culture exhibited greater amounts of carotenoids which can be attributed to potential alterations in cell structure resulting from distinct growth conditions. Specifically, the orange culture was subjected to light stress, which facilitated the production of the carotenoid astaxanthin. On the other hand, the green culture produced higher amounts of

chlorophylls but exhibited lower extraction yields, as depicted in Table 1. The green culture also showed lower extraction yields of carotenoids for all treatments. The contrasting growing conditions experienced by the green and orange cultures might have induced alterations in cell membrane structure, thereby influencing the process of electroporation.

Table 2 - Total contents of carotenoids and chlorophylls obtained from bead milling extraction for orange and green cultures.

| Culture | Carotenoid content (mg/g) | Chlorophyll content (mg/g) |
|---|---|---|
| Orange | $1.93 \pm 0.05^{a}$ | $2.03 \pm 0.02^{b}$ |
| Green | $1.31 \pm 0.01^{b}$ | $3.29 \pm 0.08^{a}$ |

*5.2.3.3. Influence of an incubation step before extraction*

An incubation step in buffer solution of 24h was evaluated after PEF treatment and before the diffusive step. Figures 4 and 5 depict carotenoid and chlorophyll extraction yields with and without the incubation step for orange and green cultures, respectively. In general, for both cultures, the extracts with incubation showed higher carotenoid extraction yields when mild PEF conditions were applied. In drastic conditions (high electric field strength and high number of pulses), there is no difference between incubated and non-incubated samples (except treatment performed with 20 kV/cm and 50 pulses). Martínez et al. (2018) found similar results, reporting a significant improvement of carotenoid extraction from fresh biomass of *R. glutinis* subjected to a PEF treatment (15 kV/cm, 150 μs) after adding an incubation step to the process (24 hours at 20°C in pH 7 buffer). The observed enhancement in extraction efficiency with incubation can be attributed to the presence of pigment-protein complexes. Carotenoids are predominantly

concentrated within the chloroplasts, where they are bound to proteins within the membrane thylakoids. Electroporation of the chloroplast membrane can facilitate the destabilization of the pigment-protein complex, thereby improving the extraction of carotenoids (Esser et al., 2010). Furthermore, incubating the PEF treated suspensions provided an extended period of interaction between the cells and the buffer solution, inducing the disruption of bonds between proteins and pigments due to their solubility in the medium. Consequently, this process enhanced extraction of carotenoids. Several studies have suggested that an incubation period following PEF treatment can enhance the release of water-soluble molecules, including proteins, into the surrounding medium (Coustets et al., 2013; Goettel et al., 2013). In drastic conditions, electroporation may have been sufficient to promote the release of carotenoids to the medium.

Luengo and Raso (2017) reported a different behavior when applying high electric field strengths, observing a higher extraction yield of pigments after 1 h of incubation in PEF-treated samples. The authors suggested that the increased electric field strength could induce localized defects in the membrane, leading to a pore size enlargement or an increase in the number of pores during the incubation period. This incubation in the aqueous media could also result in chloroplast plasmolysis due to osmotic imbalances within the cytoplasmatic space. Therefore, the chloroplast membrane, which houses the pigments, could become permeabilized, facilitating both the diffusion of the ethanol into the chloroplast and the diffusion of pigments towards the cytoplasm.

Regarding chlorophylls, the incubation step did not significantly impact extraction process for orange cultures. For green culture, on the other hand, incubation showed similar impact observed for carotenoids extraction.

Figure 4 – Carotenoid (A) and chlorophyll (B) extraction yields after PEF treatment with (blue) and without (yellow) incubation at time zero of the diffusive step for orange culture.

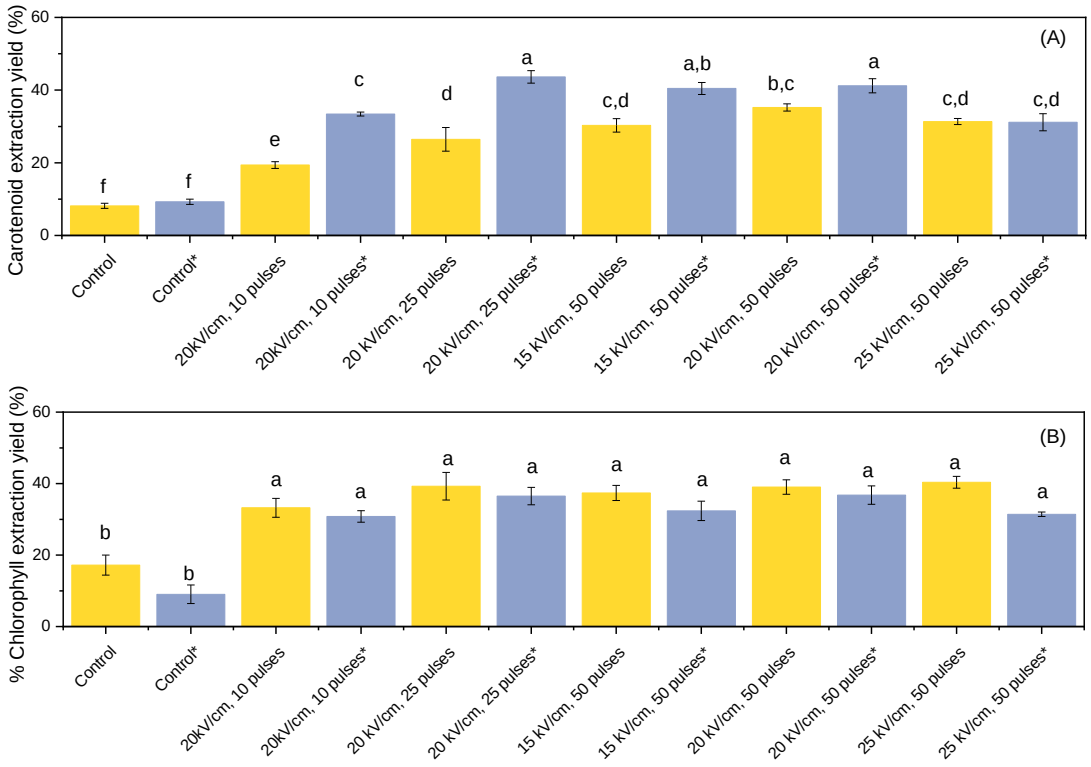
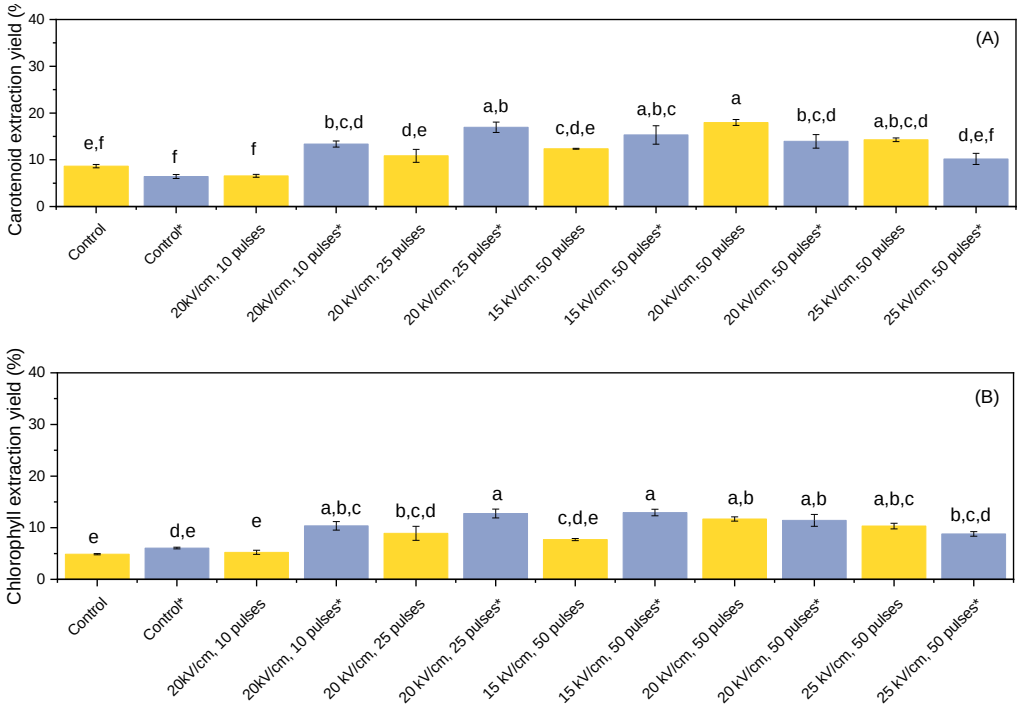


Figure 5 – Carotenoid (A) and chlorophyll (B) extraction yields after PEF treatment with (blue) and without (yellow) incubation at time zero of the diffusive step for green culture.




*Conclusions*

This study evaluated the potential of PEF as a pretreatment method for pigment extraction from fresh biomass of *C. zofingiensis*. The application of an intermediate intensity combined with high number pulses was sufficient to promote permeabilization and electroporation of the cell membrane, producing extracts with the highest yields of carotenoids and chlorophylls. The addition of an incubation step after PEF treatment and before the diffusive step further enhanced the extraction of carotenoids, leading to increased overall extraction yields. This study provided valuable insights into the specific parameters and conditions necessary for the successful application of PEF as a pretreatment method for pigment extraction. Understanding the optimal electric field

intensity and treatment time can significantly improve the efficiency and yield of the extraction process.

Overall, the used of PEF combined with ethanol as solvent represents an environmentally friendly alternative to conventional processes. By proposing an efficient method to extract valuable pigments from microalgae, this research can contribute to the sustainable development of various industries, including food, pharmaceuticals, and cosmetics.

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Table S1 - Carotenoid and chlorophyll content in the extracts after PEF treatment and a diffusive step with ethanol.

| | **Carotenoid concentration (mg/g)** | | | **Chlorophyll concentration (mg/g)** | | |
|---|---|---|---|---|---|---|
| | **0h** | **6h** | **24h** | **0h** | **6h** | **24h** |
| **Orange culture** | | | | | | |
| Control | 0.15 ± 0.03 | 0.28 ± 0.03 | 0.31 ± 0.03 | 0.36 ± 0.05 | 0.23 ± 0.05 | 0.38 ± 0.00 |
| 20 kV/cm, 10 pulses | 0.38 ± 0.03 | 0.56 ± 0.03 | 0.62 ± 0.05 | 0.67 ± 0.05 | 0.49 ± 0.08 | 0.69 ± 0.03 |
| 20 kV/cm, 25 pulses | 0.56 ± 0.10 | 0.87 ± 0.05 | 0.97 ± 0.05 | 0.72 ± 0.13 | 0.74 ± 0.10 | 0.95 ± 0.08 |
| 15 kV/cm, 50 pulses | 0.59 ± 0.03 | 0.87 ± 0.03 | 0.95 ± 0.00 | 0.77 ± 0.05 | 0.77 ± 0.00 | 1.00 ± 0.08 |
| 20 kV/cm, 50 pulses | 0.69 ± 0.03 | 1.26 ± 0.00 | 1.46 ± 0.03 | 0.87 ± 0.13 | 0.92 ± 0.03 | 1.33 ± 0.08 |
| 25 kV/cm, 50 pulses | 0.62 ± 0.03 | 1.05 ± 0.03 | 1.31 ± 0.05 | 0.87 ± 0.08 | 0.85 ± 0.03 | 1.23 ± 0.03 |
| **Green culture** | | | | | | |
| Control | 0.11 ± 0.01 | 0.15 ± 0.01 | 0.19 ± 0.01 | 0.16 ± 0.01 | 0.22 ± 0.03 | 0.38 ± 0.03 |
| 20 kV/cm, 10 pulses | 0.08 ± 0.01 | 0.13 ± 0.01 | 0.18 ± 0.01 | 0.17 ± 0.01 | 0.23 ± 0.02 | 0.32 ± 0.01 |
| 20 kV/cm, 25 pulses | 0.14 ± 0.02 | 0.23 ± 0.03 | 0.32 ± 0.01 | 0.3 ± 0.04 | 0.41 ± 0.04 | 0.63 ± 0.04 |
| 15 kV/cm, 50 pulses | 0.16 ± 0.00 | 0.23 ± 0.00 | 0.32 ± 0.01 | 0.26 ± 0.01 | 0.43 ± 0.03 | 0.59 ± 0.02 |
| 20 kV/cm, 50 pulses | 0.23 ± 0.01 | 0.37 ± 0.00 | 0.54 ± 0.01 | 0.39 ± 0.01 | 0.65 ± 0.02 | 1.14 ± 0.02 |
| 25 kV/cm, 50 pulses | 0.21 ± 0.04 | 0.39 ± 0.02 | 0.58 ± 0.01 | 0.39 ± 0.07 | 0.71 ± 0.07 | 1.26 ± 0.06 |

# Capítulo 6 – Considerações finais

No presente trabalho foi avaliada a influência da aplicação de tecnologias emergentes em microalgas: i) estudo do uso da tecnologia de US no crescimento das microalgas *P. marina* e *C. zofingiensis* e na síntese de compostos de interesse; ii) estudo do uso de PEF na extração de carotenoides e clorofilas a partir da microalga *C. zofingiensis*.

Na primeira etapa, o ultrassom foi aplicado nas etapas exponencial e estacionária do crescimento da microalga *P. marina* e na fase exponencial da microalga *C. zofingiensis* utilizando os modos contínuo e descontínuo da tecnologia. Os tratamentos ultrassônicos descontínuos se mostraram mais benéficos para a produção de biomassa do que o tratamento contínuo. Para *C. zofingiensis*, a aplicação de US descontínuo durante 10 min resultou num aumento de 65% na concentração de biomassa, 2,19 ± 0,10 g $L^{-1}$ em comparação com o controle que foi de 1,33 ± 0,16 g $L^{-1}$. Os tratamentos descontínuos permitem que o metabolismo celular se adapte ao primeiro estímulo, para, posteriormente, receber um novo estímulo de forma mais eficiente. Entretanto, as microalgas apresentaram comportamentos distintos entre as espécies reagindo de forma diferente aos estímulos físicos. Para *P. marina*, o tratamento com US, mesmo descontínuo, não aumentou a proliferação celular, e ainda reduziu a densidade celular quando utilizado por 60 min, indicando uma possível ocorrência de danos celulares.

Em relação à síntese de compostos de interesse, após o tratamento com US, observou-se uma redução das concentrações de carotenoides, clorofilas, lipídeos e proteínas. Esse comportamento pode estar relacionado a mudanças nas vias metabólicas para produção desses compostos.

No segundo estudo, o uso de PEF foi avaliado para extrair carotenoides e clorofilas da microalga *C. zofingiensis.* Essa tecnologia promove efeitos de eletroporação nas células devido à passagem de corrente elétrica através do material biológico que induz o aumento do potencial transmembrana, promovendo a formação de poros e o aumento das taxas de transferência de massa nos processos de extração.

A tecnologia de PEF usada como pré-tratamento de extração, juntamente com etanol como solvente, favoreceu a extração de carotenoides e clorofilas. O estudo avaliou a extração da biomassa fresca de *C. zofingiensis* em duas culturas distintas: verde (sem estresse celular) e laranja (com estresse celular). O melhor tratamento de PEF (20 kV $cm^{-1}$, 150 μs) extraiu 76 e 65% de carotenoides e clorofilas, respectivamente, valores de três a quatro vezes maior do que os observados no experimento controle.

A partir da avaliação das diferentes etapas de crescimento da microalga, foi possível observar que a cultura laranja apresentou maiores quantidades de carotenoides, devido ao estresse de luz a que foi submetida, o que promoveu a produção do carotenoide astaxantina. Por outro lado, a cultura verde produziu maiores quantidades de clorofilas, mas apresentou rendimentos de extração mais baixos. As diferentes condições de crescimento entre as culturas verde e laranja podem ter induzido alterações na estrutura da membrana celular, influenciando, assim, o processo de eletroporação.

Além disso, foi avaliada uma etapa de incubação em solução tampão de 24 horas após o tratamento com PEF e antes da etapa difusiva. Para ambas as culturas, os extratos com incubação apresentaram rendimentos mais altos de extração de carotenoides quando condições mais brandas de PEF foram aplicadas; em condições mais severas, não houve diferença entre as amostras incubadas e não incubadas (exceto no tratamento realizado

com 20 kV/cm e 50 pulsos). A incubação das suspensões tratadas com PEF proporcionou um período prolongado de interação entre as células e a solução tampão, podendo ter induzido o rompimento das ligações entre as proteínas e os pigmentos devido à sua solubilidade no meio. Consequentemente, esse processo aumentou a extração de carotenoides.

Diante do exposto, as s conclusões gerais do presente trabalho são apresentadas a seguir:

- a tecnologia de US descontínuo apresenta potencial para aumentar a proliferação de células de microalgas robustas. Dessa forma, sua utilização pode favorecer diferentes indústrias que utilizam os compostos sintetizados pelas microalgas (lipídieos, carotenoides, proteínas) como matéria-prima;
- a aplicação de PEF como método de pré-tratamento aumenta a eficiência de extração de carotenoides e clorofilas. Essa tecnologia possibilita a utilização de etanol como solvente, que é considerado um solvente menos agressivo ao meio ambiente.

A partir do trabalho realizado, algumas sugestões para trabalhos futuros são apresentadas a seguir:

- determinar a condição ótima de crescimento da microalga *C. zofingiensis* com aplicação de US, visto que outras intensidades, frequências, bem como fases de crescimento ainda podem ser avaliadas.
- desenvolver metodologias de análise para possibilitar o melhor entendimento dos efeitos de eletroporação promovidos pelo PEF, uma vez que a extração de compostos celulares é um indicativo de que as células foram rompidas; porém, os efeitos na

estrutura da membrana celular não são elucidados com base somente nesses resultados. Ainda, é necessário compreender o tempo de resposta das diferentes células ao campo elétrico, tempo de duração e tamanho dos poros.

- avaliar a pureza dos extratos obtidos com PEF, uma vez que essa tecnologia pode proporcionar a obtenção de extratos mais puros.

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# Apêndice - A

Figura 1A - Curva padrão de biomassa da microalga *P. marina* para os demais cultivos.

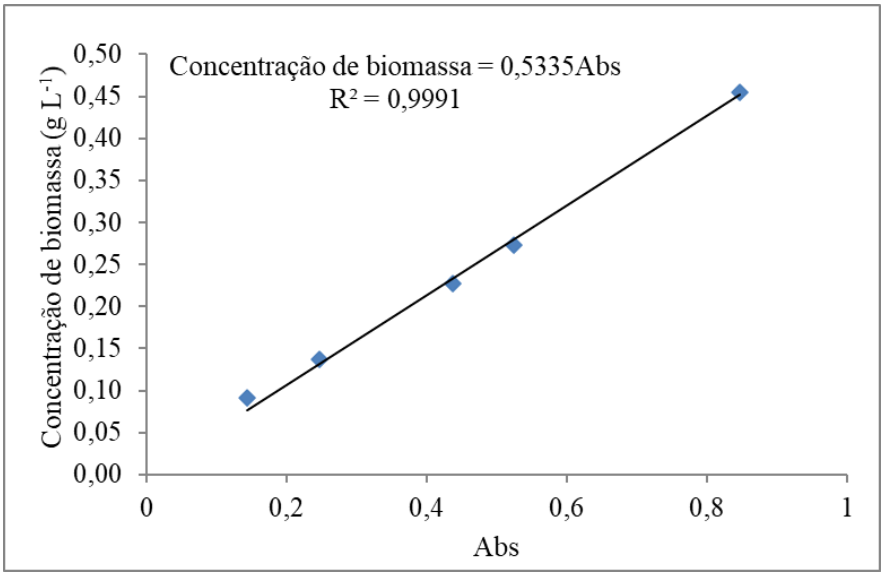
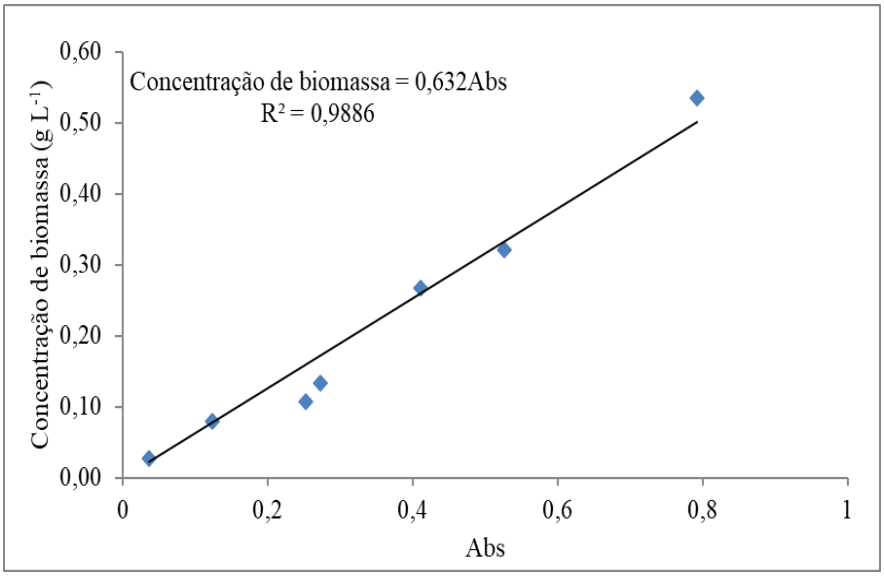



Figure 2A - Curva padrão de biomassa da microalga *P. marina* para os cultivos (Controle1/ U10exp(4)/ U10exp5).




Controle1: cultivos sem aplicação de ultrassom; U10exp(4): cultivos com aplicação de ultrassom por 4 dias durante a fase exponencial por 10 min; U10exp: cultivos com aplicação de ultrassom por 5 dias durante a fase exponencial por 10 min.

Figure 3A - Curva padrão de biomassa da microalga *C. zofingiensis*.

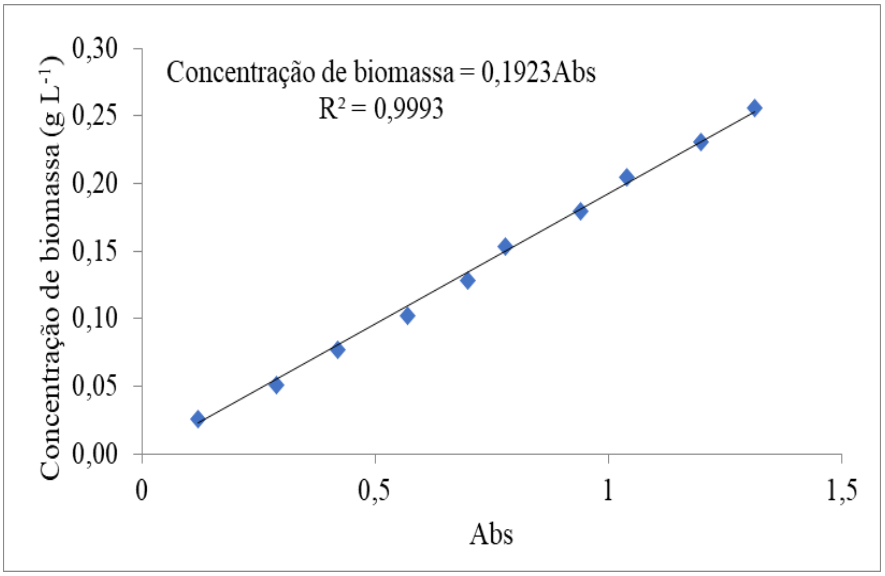
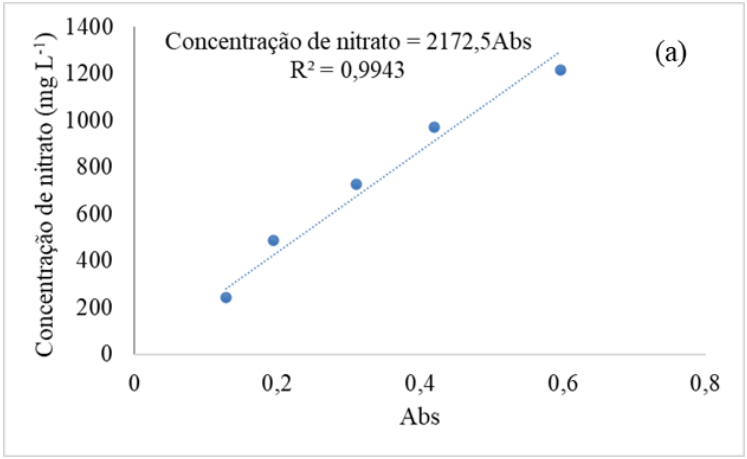
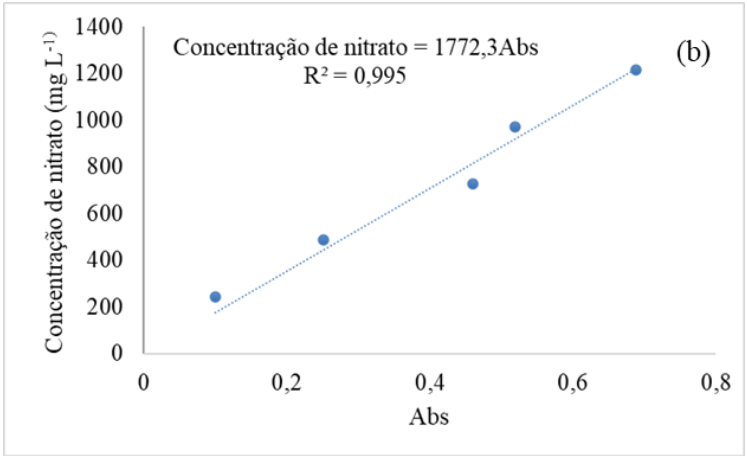


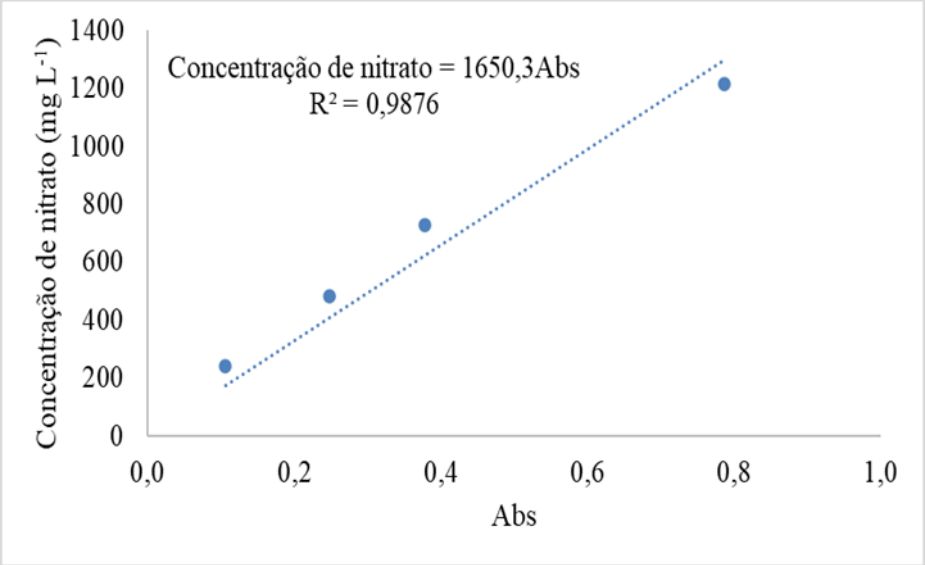
Figure 4A - Curvas padrão de nitrato para as análises dos cultivos Controle 1, U10exp(4), U10exp.



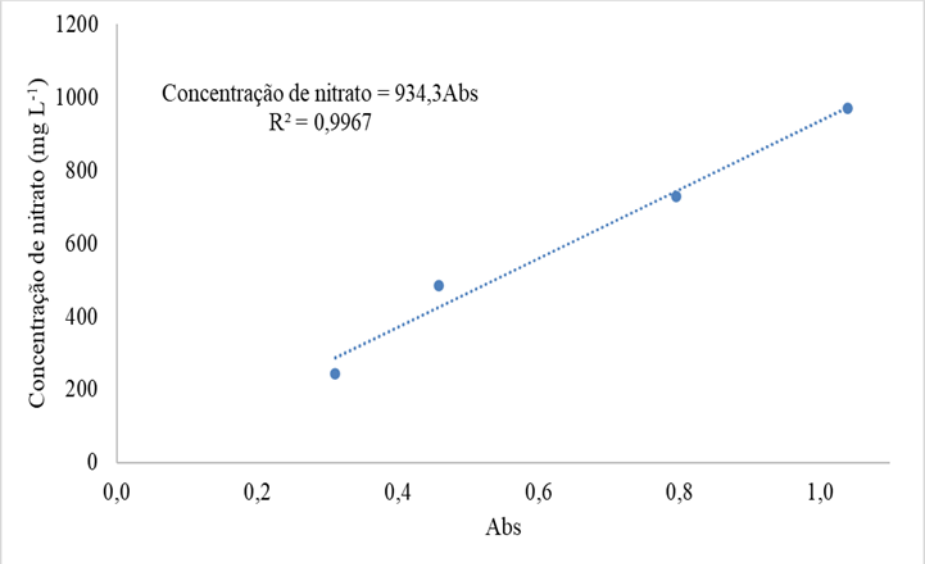

Controle1: cultivos sem aplicação de ultrassom; U10exp(4): cultivos com aplicação de ultrassom por 4 dias durante a fase exponencial por 10 min; U10exp:cultivos com aplicação de ultrassom por 5 dias durante a fase exponencial por 10 min.

Figure 5A- Curva padrão de nitrato para as análises dos cultivos Controle2, U30exp, U60exp.




Controle2: cultivos sem aplicação de ultrassom; U30exp: cultivos com aplicação de ultrassom por 5 dias durante a fase exponencial por 30 min; U60exp: cultivos com aplicação de ultrassom por 5 dias durante a fase exponencial por 60 min.

Figure 6A - Curva padrão de nitrato para as análises dos cultivos Controle3, U30est U60est.




Controle3: cultivos sem aplicação de ultrassom; U30est: cultivos com aplicação de ultrassom por 5 dias durante a fase estacionária por 30 min; U60est: cultivos com aplicação de ultrassom por 5 dias durante a fase estacionária por 60 min.

Figure 7A - Curva padrão de nitrato para as análises dos cultivos Controle4, U10int U30int.

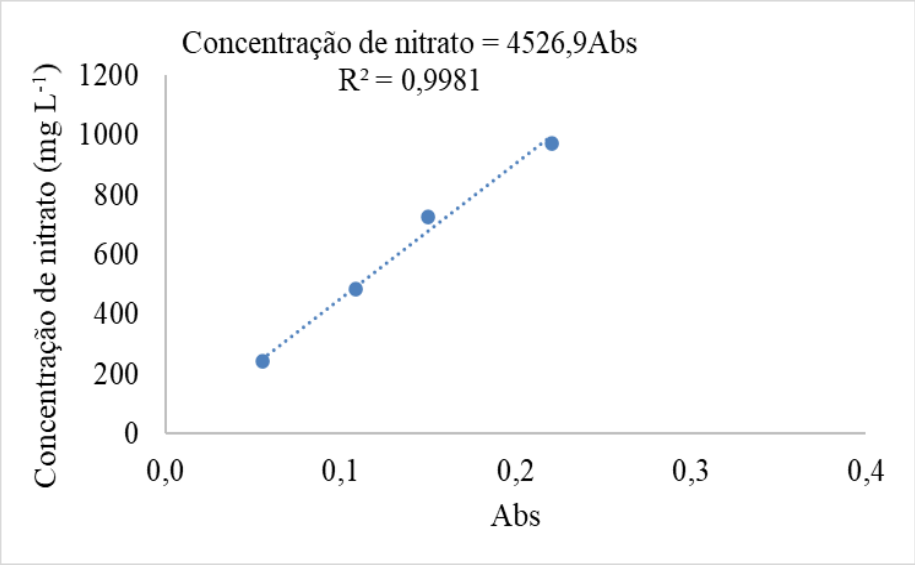



Figure 8A - Curva padrão de nitrato para as análises dos cultivos com *C. zofingiensis*.

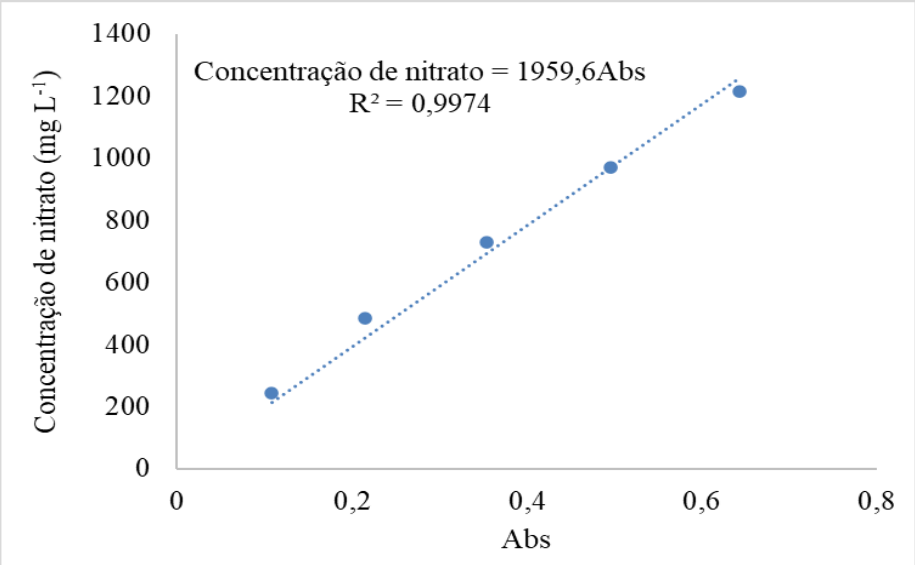
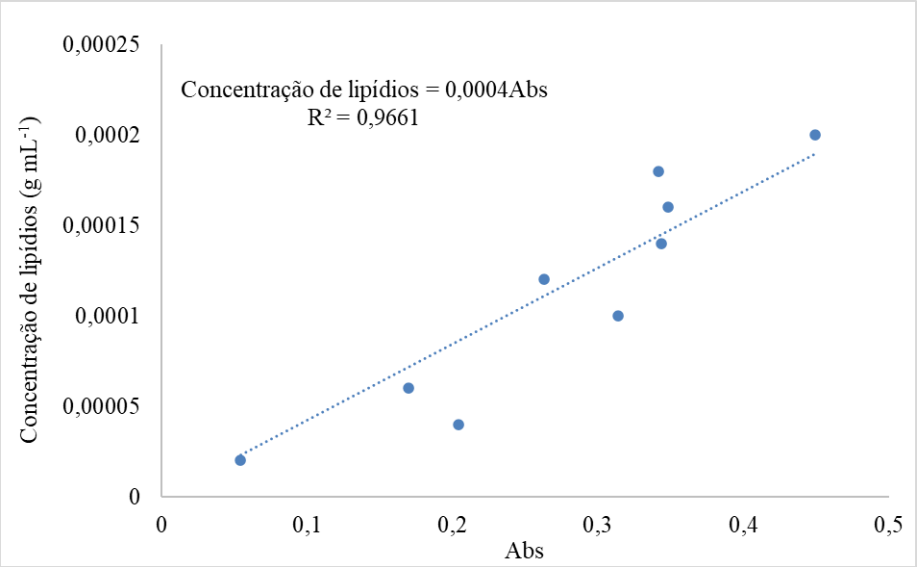


Figure 9A - Curva padrão de lipídeos.



# Anexo

Nesta seção é apresentado o artigo de revisão publicado na revista *Brazilian Journal of Chemical Engineering* (v. 40, p. 607–622, 2023).

## IMPACT OF ULTRASOUND AND ELECTRIC FIELDS ON MICROALGAE GROWTH: A COMPREHENSIVE REVIEW

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**Abstract**

Microalgae cultivation is still limited to small-scale systems with relatively high production costs. To overcome these limitations and to improve microalgae competitiveness, one alternative that has been explored is the submission of microalgae cells to stress conditions, promoting an increase of the biomass concentration and the production of internal compounds. In this context, the novel technologies of ultrasound (US), pulsed electric field (PEF), moderated electric field (MEF), and static electric field (SEF) may be used for this purpose. However, the application of these technologies to microalgae growth is still incipient, and their effects on cells are not yet fully understood. Therefore, it is still necessary to better understand and compile the influence of different process parameters on cell metabolism. The aim of this review is to contribute for elucidation of the mechanisms involved when novel technologies are applied to microalgae cells. Positive results obtained by different authors were correlated to process parameters, providing a concise comprehension of US and PEF applications for microalgae growth.

### Introduction

Meeting the demands for energy supply, food security, human health and biodiversity have been a constant challenge due to human population growth. The

implementation of innovative technologies and the increase of natural products usage, such as those produced by plants and microorganisms, are possible solutions to tackle these challenges (Haberkorn et al. 2021; Llamas et al. 2021).

Microalgae are an alternative source of biomass that show interesting advantages when compared to traditional crops, such as high growth rate, simple cultivation, low space occupation, and no need of arable land. Moreover, they promote CO2 fixation from the atmosphere, contributing to the reduction of the greenhouse effect (Daneshvar et al. 2021). In addition, microalgae contain high amounts of proteins, lipids, carbohydrates, and pigments, such as carotenoids and phycocyanin. Carotenoids can be used in pharmaceutical, chemical, food, and feed industries due to their coloring, antioxidant, and possible tumor-inhibiting activity (Liu et al. 2014). The antioxidant pigments astaxanthin, β-carotene, and lutein are the major carotenoids of commercial value found in microalgae (Gong and Bassi 2016). Lipids can be employed in the food industry, as ingredient and supplement, or even to obtain biofuels, such as biodiesel (Gong and Bassi 2016). Carbohydrates and proteins, on the other hand, can be employed as ingredients in the food and feed industries.

Microalgae are currently cultivated in relatively smallscale systems with production costs relatively high, affecting its economic viability and competitiveness on the market (Haberkorn et al. 2019). Therefore, it is important to explore alternatives to optimize its productive capacity. In this regard, studies have shown that submitting microalgae cells to environmental stress conditions (such as nutrient starvation, high temperature, intense light conditions, extreme pH, and high salinity) is an alternative to stimulate biomass growth and the biosynthesis of specific compounds (Han et al. 2016a).

Some studies have applied innovative technologies to promote cellular stress and other effects that can enhance cell proliferation (Loghavi et al. 2007; Han et al. 2016a; Buchmann et al. 2019; Haberkorn et al. 2019). Among them, ultrasound (US) and pulsed electric field (PEF) are technologies that have been studied for these purposes. Moderated electric field (MEF) and static electric field (SEF) may be also alternatives that have not been explored yet. US is a well-known technique for intensification of diverse physical and chemical processes, as it improves mass transfer process yields and modifies reaction pathways. The first investigations into US application on cell growth stimulation has shown that this technology can improve biomass production due to physical and chemical effects associated with the cavitation; however, little is known about how this phenomenon impacts the mechanisms of cell growth (Chisti et al. 2003; Han et al. 2016b; Singh et al. 2019).

Electrical technologies, such as PEF, MEF, and SEF are also alternatives to be applied for cell growth stimulation since they induce membrane damage, causing cellular stress. Few studies have addressed their utilization for this purpose (Loghavi et al. 2008; Mattar et al. 2014; Buchmann et al. 2019; Haberkorn et al. 2019; Al Daccache et al. 2020). Some authors suggested that electrical stimulation can influence not only growth but also the production of secondary metabolites. Thus, it is important to explore the effects of these technologies on cells to improve knowledge and operational development for future industrial applications.

In this framework, the present study provides an overview of US and PEF application for stimulation of microalgae growth, addressing their mechanisms of action on algae metabolic pathways to boost the production of compounds of interest for

pharmaceutical, chemical, and food industries. Regarding MEF and SEF, information about microalgae growth is scarce or inexistent from practical point of view. Thus, a revision of their application could not be accomplished, but they are considered potential alternatives throughout this review. Table 1 summarizes the advantages, disadvantages, the gap of knowledge that still exists to fully understand the mechanism of action, and the economic potential of these technologies.

Regarding the economic potential, few studies pointed out the viability of applying the technologies on an industrial scale. For electrical technologies, the main drawbacks are related to high equipment costs since special materials (as titanium) are required for the electrodes manufacture.

Moreover, especially for PEF, the pulse modulator is expensive, increasing capital costs. Operational costs of US and electrical technologies may also be high, but they depend on

the energy input required for the processes, which is still not well stablished. So far, studies regarding economic analysis are available mostly in the food technology area in applications that require higher energy inputs (Bocker and Silva 2022).

**Ultrasound**

US is based on the passage of ultrasonic waves (20 kHz – 10 MHz) through a medium. Depending on the frequency, this technology may be divided into two areas: low and high intensity waves. Low intensity US employs high frequencies (between 1 and 10 MHz) and low power levels, producing minimal physical and chemical changes in the material in which it is applied; it is used in non-destructive applications, as

diagnostics in the medical area or as a non-invasive analytical tool in various food materials during processing and storage to ensure quality and safety. High intensity US, on the other hand, utilizes low frequencies (from 20 to 100 kHz) and high-power levels that generate cavitation, inducing physical and chemical modifications in the materials (Sillanpää et al. 2011; Ojha et al. 2017). Thus, it is used in food and bioprocessing for cell destruction.

When high intensity ultrasonic waves propagate through a liquid medium, compression and rarefaction zones are induced along the wave cycle. Due to these pressure differences, cavitation bubbles are formed when the rarefaction cycles exceed the attractive forces in the medium. These small cavitation bubbles are formed because the liquid medium is incompressible and cannot readily accommodate the pressure changes in the system (Sillanpää et al. 2011). Once these bubbles reach a certain size, they collapse, often violently, generating shear forces within the fluid, producing intense local heating (~ 5000 K) and high pressures (~ 1000 atm) at a microscopic level. This phenomenon is called acoustic cavitation and may promote bond breakage and free radical formation. These radicals can react with each other to form new molecules or radicals, or can diffuse into the bulk liquid, reacting with other compounds (Sillanpää et al. 2011; Kentish and Feng 2014). Also, due to the formation and the collapse of cavitation bubbles, turbulence zones (associated with high-speed microjets) are formed, promoting agitation and shock waves. As a result of these effects, high intensity US has been studied as an alternative technology to assist extraction, homogenization, emulsification, dehydration, inactivation of enzymes and microorganisms, among other processes (Chisti 2003).

Depending on the medium in which US waves are propagated, different effects are produced: in solids, mechanical and thermal impacts are more important; in liquids, cavitation and streaming are the main effects; and in gases, streaming is the major phenomenon. The main applications of US in these mediums are summarized in Fig. 1a. Most part of the applications are in liquids, since in this media US effects are more intense due to cavitation. Power ultrasound has been used in distinct types of chemical reactions to promote several benefits: higher reaction rate, shorter reaction time, lower energy consumption, better catalyst effectiveness and less use of harmful reactants or solvents. US application promotes chemical or physical changes and facilitates transport processes within the reaction medium due to cavitation and formation of free radicals (Yao et al. 2020). This technology can also be applied in solids to assist metal processes, such as welding and wire bonding, and for de-icing of turbines and food dehydration. In gases, US waves effects are less intense, and the technology is useful mainly for defoaming of liquids (Yao et al. 2020).

**Microalgae growth assisted by ultrasound**

Recently, ultrasonic treatment has been suggested as an alternative to enhance microorganism growth and synthesis of compounds. Table 2 summarizes the main studies regarding US application for microalgae growth. As can be seen, US may influence either the production of internal compounds or cell growth, depending on the microorganism and operational conditions. According to these studies, the main effects of US application in microalgae cells are: changes in metabolic pathway; increase of membrane permeability; changes in enzymes structure; increase of aeration in the culture medium;

release of cells clusters formed during cultivation; and changes in cellular components and genetic functions. Figure 2 illustrates the effects of US application on microalgae growth. These effects are a consequence of the cavitation phenomenon, and they are explained in more details as follows.

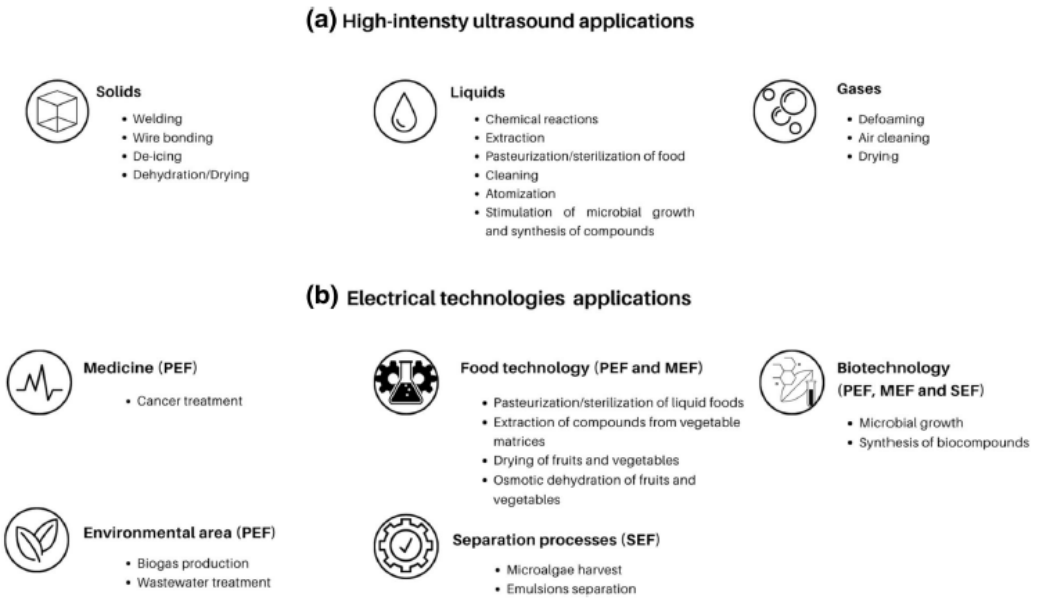


**Fig. 1** Applications of **a** high-intensity ultrasound (US) and **b** electrical technologies: pulsed electric field (PEF), moderate electric filed (MEF) and static electric field (SEF)

## Changes in metabolic pathways

When microalgae experience changes in the environment, the metabolism pathway of the cells may be changed to allow them to survive under the new conditions. In this context, lipid production is one tool used by cells that allows the microorganisms adapt to stress environments. Under environmental stress, caused by a deficiency of mineral nutrients or excessive irradiation, many species of algae continue to actively synthesize fatty acids while the growth rate slows (Sharma et al. 2012). Without a focus on growth, cells produce fatty acids (triglycerides) that serve as an ideal mechanism to

deflect large amounts of ATP and NADPH, which would be channeled into cell growth in other circumstances. These triglycerides can be used as an energy-rich carbon source when the growing conditions are favorable (Chakravarty and Mallick 2022).

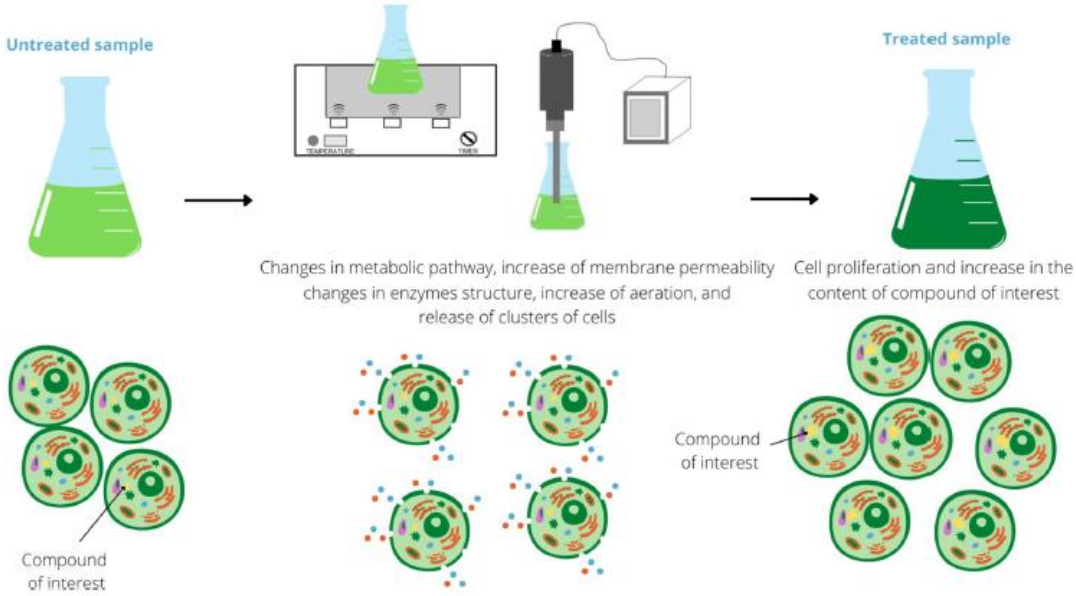


**Fig. 2** Main effects of ultrasound application on microalgae stimulation

In addition, some species of algae also use this reserve of carbon to reduce photosynthesis rates, directly absorbing part of the incoming light. Carotenoids, which normally act as accessory pigments, allow an increase in the absorption of light by the photosynthetic apparatus, acting as shields to absorb light that, otherwise, could cause photooxidative damage (Sharma et al. 2012). Therefore, the increase of the production of secondary metabolites, such as lipids and carotenoids, in a stressful situation can be explained by this change in the cellular metabolic pathway. Batghare et al. (2018) reported that US was able to induce an increase of 26% of astaxanthin production in batch

cultivation of *Phaffia rhodozyma*. Han et al. (2016a) revealed that ultrasonic treatment enhanced 23% of lipid production in *Anabaena variabilis*. Singh et al. (2019) reported results that corroborate these findings: US application increased lipid and β-carotene production in microalga *Tetradesmus obliquus* by 34 and 31%, respectively.

According to Han et al. (2016a), the influence of ultrasonic treatment on lipid accumulation in *Anabaena variabilis* is due to the harsh environment generated by the physical and chemical changes in the medium, as high temperature and pressure, and free radical production. The authors explained that, in adverse environments, cells change their metabolism, prioritizing lipid production. Moreover, Sivaramakrishnana and Incharoensakdia (2019) confirmed that an increase of lipid content in *Scenedesmus* sp. under optimized US conditions was caused by the environment created in the bulk medium: local points of high temperatures and pressures and reactive oxygen species (ROS) generation; according to these authors, ROS-derived electrons are used for lipid synthesis.

***Membrane permeability***

Increase of membrane permeability is another factor that may influence the synthesis of internal compounds, as well as cell proliferation. Depending on US intensity, pores can be created in the cell membrane. This phenomenon is called sonoporation. Low-intensity US normally leads to stable cavitation of microbubbles and usually generates small pores. The stable cavitation occurs when bubbles increase only to a certain size and do not disrupt. This phenomenon occurs because, along the wave cycle, there is a gas influx during the expansion, and a gas efflux during the compression. When the expansion

phase extends, the microbubble increases until a certain size and remains stable, with low amplitude oscillation. These oscillations create microstreams around the microbubble, and, if the bubble is close to a cell membrane, the membrane will experience shear stress and push-and-pull effects during expansion and compression, leading to the creation of pores. When high intensity US is applied, pore formation is due to the inertial cavitation of microbubbles. In this case, bubbles size oscillates until implosion, leading to shock waves and microjets that can perforate cell membranes. Therefore, it is believed that pores formed by stable cavitation are smaller than those promoted by high intensity US. These small pores may also be transient, once cell membrane may regenerate after US application (Lentacker et al. 2014).

Besides pore formation due to mechanical effects, there is evidence that the free radicals formed during cavitation could also play a role in the increase of cell membrane permeability, and some studies suggest that these effects are more pronounced at low power US. One possibility is that free radicals can stimulate $Ca^{2+}$ influx by modulating existing ion channels. The $Ca^{2+}$ influx is mediated by $K^{+}$, leading to hyperpolarization of the cell membrane; it is known that oxidants can regulate channels activity, which are formed by protein structures (affecting their expression), opening time and opening probability (Bogeski et al. 2011). Other possibility is that free radicals may react with lipids, resulting in the rearrangement of the cell membrane (Lentacker et al. 2014).

Ren et al. (2019b) suggested that ultrasonic treatment can improve the conversion efficiency of substrates into intracellular compounds due to a more efficient diffusion of substrates, nutrients, and metabolite products across the cell membrane. The authors observed that the lipid content and the biomass concentration from Scenedesmus sp.

treated by US was higher than the amount observed in control cells. Similar effects were observed in other microorganisms, as *Saccharomyces cerevisiae* and *Bacillus sphaericus*: Dai et al. (2017) observed an increase of the growth rate of *S. cerevisiae*, and Avhad and Rathod (2015) reported higher fibrinolytic enzyme yield from *B. sphaericus*.

***Other effects: enzyme activity, oxygen uptake and cluster dispersion***

Other phenomena may also be related to US application during microalgae growth, as changes in enzymatic activity, increase of the oxygen uptake by the cells and cluster dispersion. Some studies reported these effects in other microorganisms; however, they may also influence microalgae growth due to the similarity in cell structure.

Regarding changes in enzymatic activity, initial studies in this area were focused on enzymatic inactivation and showed that US is not able to inactivate all enzymes, mainly under mild conditions. The degree of enzyme sensitivity to US depends on its structure and US conditions: prolonged exposure times of high-power US are more effective due to free radical formation and strong shear forces that may destroy enzyme structure. On the other hand, low intensity and short duration US can be applied to increase enzymatic activity (Ma et al. 2015; Wang et al. 2018). In these conditions, US may promote disintegration of enzyme molecular aggregates, inducing changes in enzymatic structure, exposing more active sites and facilitating the contact between enzymes and substrates (Ma et al. 2015). Additionally, US intense mixing can eliminate concentration gradients in the culture medium, improving nutrient availability to microorganisms and reducing the inhibition effects caused by excess of substrates during cell growth.

Pawar and Rathod (2018) observed an increase of uricase and proteinase production along Bacillus licheniformis growth cycle assisted by US. Singh et al. (2015) studied US assisted ethanol synthesis by *S. cerevisiae* and observed that sonication increased enzyme-substrate affinity and reduced inhibition effects caused by excess of substrate and products formation.

Concerning the increase of oxygen uptake, there is evidence that shear forces, promoted by US in liquids, can break air bubbles dissolved in cultivation medium into smaller ones, increasing the interfacial area available for mass transfer between gas and liquid phases (Kumar et al. 2004; Batghare et al. 2018) reported that the enhance of gas liquid mass transfer and oxygen uptake in culture medium improved astaxanthin production by *Phaffia rhodozyma* cells.

On the other hand, US can also be used to degas liquids, removing oxygen and carbon dioxide, for example (Villamiel et al. 2021). Both processes are believed to occur simultaneously during US application: degassing and absorption (Kumar et al. 2004). The prevalence of one phenomenon over the other may be related to the applied US parameters, as time and frequency. However, there are only few theoretical studies of US application on gas-liquid mass transfer, and more studies are needed to better understand these effects (Akbari et al. 2017; Chen et al. 2021).

In addition, mixing promoted by US favors the dispersion of cell clusters. These aggregates often decrease the cell contact with light, carbon dioxide, and nutrients, reducing its growth rate. According to Han et al. (2016a), the vibration and cavitation effects of the ultrasonic treatment were important to separate clusters of microalgae, improving *Anabaena variabilis* growth. Ren et al. (2019a) reported similar results

indicating that appropriate ultrasonic treatment may help to separate clustered algal cells and benefit growing.

The intensity of all the aforementioned effects on cell metabolism and structure depends on US parameters, as well as on cells growth phase of US application. A deeper discussion about these topics is presented as follows.

## Recommendations for ultrasound practice on microalgae growth

US has been applied at different growth phases using several US parameters. Therefore, conclusions regarding the best operational conditions to be used are still missing in the literature. Analyzing data shown in Table 2, some inferences can be stated: US is applied mostly during the exponential growth phase; frequencies vary from 18 to 40 kHz and power intensity does not exceed 200 W. More details about US parameters and cell characteristics influence on growing rate are presented below. However, it is important to emphasize that the optimal conditions will depend on the microorganism, how it is cultivated, and the product to be extracted.

### *Growth phase*

The growth phase in which US is applied is one of the main factors that influences microalgae growth. Generally, US application at the beginning of the cultivation (lag or log phase) has been more effective to increase biomass production. On the other hand, US applied in the stationary phase has shown to be more promising for accumulation of secondary metabolites, such as carotenoids (Batghare et al. 2018; Sivaramakrishnan and Incharoensakdi 2019; Singh et al. 2019; Han et al. 2016a) applied US in the beginning of

stationary phase of *Anabaena variabilis* and observed not only an increase in the concentration of biomass, but also in the production of lipids. Singh et al. (2019) reported similar results when studying the application of US at the beginning of stationary phase during *Tetradesmus obliquus* growth: biomass, lipid and carotenoid concentration were higher if compared to control experiments. In contrast, Ren et al. (2019b) suggested that the ultrasonic treatment in the log and lag phases improved the cellular stimulus to produce compounds, although pigment concentration did not increase considerably using US. Thus, further research concerning the influence of US on growth phase is needed to validate all these statements.

***US power and frequency***

Concerning the power and frequency, the studies listed in Table 2 applied power varying from 15 to 200 W and frequencies from 18 to 37 kHz. These conditions resulted either in the increase of cell concentration or internal compounds production. Studies performed with microalgae and with the probe equipment showed that high power levels may have an inhibitory effect on cell growth. Sivaramakrishnan and Incharoensakdi (2019) applied power from 0 to 50 W during *Scenedesmus* sp. growth and observed the highest biomass and lipid accumulation when 20 W was used; a further increase in power resulted in the decrease of biomass and lipid concentration probably due to cell damage promoted by intense US application. Ren et al. (2019a) evaluated US application during lag, logarithmic and stationary phases of Scenedesmus sp. using frequencies from 18 to 30 kHz and power from 10 to 50 W. These authors also observed, for biomass and lipid

concentration, an increase when 20 W was used and a decrease when higher power was applied.

Other authors used the bath equipment to stimulate microalgae growth (Han et al. 2016a; Singh et al. 2019). If compared to the aforementioned studies, these works were performed with higher power (100–200 W) and frequency (33–40 kHz) and resulted in the increase of lipid and carotenoid concentration. As the bath equipment delivers less intense power to the sample (due to the wave dispersion in the water bath), US intensity was probably not enough to cause cell death.

***Treatment time***

Application time varies according to the US intensity, the microorganism that is being treated, and the equipment type (probe or bath). Usually, shorter treatments (seconds to minutes) are performed when the probe equipment is used, and longer processes are required when the bath equipment is applied (minutes). Han et al. (Han et al. 2016a) evaluated different treatment times using the bath equipment and observed that treatments longer than 40 min reduced *Anabaena variabilis* cells viability. Other studies conducted with the bath equipment, on the other hand, suggested times of application ranging from 5 to 10 min as higher exposure to US could cause irreversible cell damage (Avhad and Rathod 2014; Han et al. 2016a).

Rajasekhar et al. (2012) also observed that prolonged exposure to sonication at high power intensities (> 10 min, probe equipment) significantly reduced *Microcystis aeruginosa* cells concentration. This reduction may have occurred because irreparable damage was caused to the cells. The same authors compared the resistance of 3 different microorganisms (*Anabaena circinalis*, *M. aeruginosa* and *Chlorella* sp.) to US applied at

the same conditions. The results showed that Chlorella was more resistant than the other species. A possible explanation for this result is the presence of gas vacuoles in the cyanobacteria *A. circinalis* and *M. aeruginosa*, that makes the cell structure less resistant to US. These findings highlight that the specification of application time, as well as other parameters, strongly depends on the characteristics of each microorganism. However, it is possible to conclude that short times are recommended to avoid cell death.

***Microalgae species***

As already mentioned, cell sensitivity to US can vary depending on microalgae species. Joyce et al. (2014) applied US, using the same process conditions, to different microalgae species: *Dunaliella salina* (6–10 μm), *Chlamydomonas concordia* (6–10 μm) and Nannochloropsis oculata (2–4 μm). For the two largest species, US treatment reduced biomass concentration, whereas for *N. oculata*, an increase in cell concentration was observed. Additionally, reduction in cell concentration of *D. salina* occurred after 4 min of treatment, and the same effect was observed in *C. concordia* only after 16 min. The fast disruption of *D. salina* can be explained by the absence of a cell wall. *C. concordia*, on the other hand, has a thin cell wall, requiring longer sonication time. Greenly and Tester (2015) evaluated the resistance of different microalgae species to US, with various sizes and cell wall compositions. These researchers observed that *Chlamydomonas reinhardtii* (with deficient cell wall) cell concentration was halved after 2 s of treatment, while *Nannochloropsis* (with robust cell wall and small size) concentration was halved only after 1 min of US application.

As mentioned above, cyanobacteria with gas vacuoles may not be an ideal microorganism to be stimulated by US (Tang et al. 2004; Rajasekhar et al. 2012); US frequencies may be close to the vacuole resonance frequency, making them more susceptible to undergo acoustic cavitation and collapse (Rajasekhar et al. 2012; Tang et al. 2004) observed that cyanobacteria with and without gas vacuoles can respond differently to sonication. The authors worked with, *Microcystis aeruginosa* (which has gas vacuole) and *Synecocchus* (which does not have gas vacuole) and found that, after sonication, the former suffered a greater membrane permeability. Ahn et al. (2003) also reported that green algae were more resistant to US treatment than cyanobacteria; when applying the same US process conditions, green algae were unaffected, while cyanobacteria were affected by the treatment.

***US application mode***

US application mode (continuous or intermittent) may also affect cell growth. Sivaramakrishnan and Incharoensakdi (2019) evaluated different US intervals (0–5 s) on biomass and lipid content improvement from *Scenedesmus* sp. Best results were obtained at 2 s, and an increase of US application interval resulted in the decrease of biomass and lipid production; higher interval time resulted in the decrease of biomass and lipid content and did not influenced pigment production by the cells. Singh et al. (2019) applied intermittent US (1 min on/9 min off) to treat *Tetradesmus obliquus* and observed an enhancement of lipid and β-carotene yield by 34.5 and 31.5%, respectively. This effect was also observed for other microorganisms: Avhad and Rathod (2014) observed that increasing the ultrasonic duty cycle (increasing the US exposure time) confers a strong pressure on the *Bacillus sphaericus* cell for a longer time, which causes cell wall

breakdown, decreasing cell viability and productivity; Schlaäfer et al. (2000) reported that intermittent sonication (300 W/m3 and 25 kHz) more than doubled the yield of ethanol synthetized by *Saccharomyces cerevisiae*, but this effect did not occur when US was applied continuously.

Other authors, on the other hand, applied US continuously during microalgae growth and reported that US stimulated biomass and internal compounds synthesis (Han et al. 2016a, b). However, in these studies, authors did not explore different application modes or time intervals between US treatments. Therefore, more studies are needed to better understand the effect of application mode on cell growth and synthesis of compounds. Nevertheless, it is recommended to avoid long time exposure to US since it can promote cell death, as discussed in "Treatment time".

**Electric field technologies**

The major electrical technology applied for biotechnological purposes is PEF. PEF involves the application of electric fields higher than 1000 V $cm^{-1}$ and treatment times from nano to milliseconds (Vorobiev and Lebovka 2009; Barba et al. 2015). Moreover, MEF and SEF are alternatives that still need to be investigated. MEF consists of the passage of alternating electric field with intensities up to 1000 V $cm^{-1}$ (Sensoy and Sastry 2004). This process is usually applied for minutes and is based on the nonthermal effects of electricity; when the combined effects of electricity and temperature are present, this technology is called ohmic heating (Sastry 2008). In contrast, SEF consists of direct current application through two electrodes (one positively and the other negatively charged), leading to an unidirectional flow of charges and particles. However, SEF is

probably not so widely studied and applied, if compared to PEF, due to electrochemical reactions that occur because of the direct current used. These reactions involve the oxidation of electrodes and electrolysis of water. Moreover, other limitations are the excessive heat that may be generated in the medium and the low durability of the electrodes (Geada et al. 2018). Overall, these electrical technologies are attractive for food and biotechnological applications due to its versatility, ease of scaleup and energy efficiency (Vorobiev and Lebovka 2009).

When electrical technologies are applied to biological cells, electroporation of cell membrane may occur, increasing the membrane permeability. The most accepted model that describes this phenomenon is the transient aqueous pore model. According to this model, electric field induces the formation of hydrophilic pores in the lipid bilayer of the cell membrane. These pores are formed due to the difference in transmembrane potential, which provides sufficient free energy to rearrange the phospholipid structures of the membranes (Vorobiev and Lebovka 2009). Electroporation can be reversible or irreversible, depending mainly on the intensity of the applied electric field.

According to Teissie et al. (2005), reversible electroporation can be divided in the following steps: trigger, expansion, stabilization, resealing, and memory. During the trigger stage, electric field induces membrane polarization, forming pores that last just a few microseconds. Then, there is the pore expansion step, which lasts from hundreds of microseconds to milliseconds. After, in the stabilization step, the membrane recovers, followed by the resealing stage, which consists of pore sealing; this step can last several minutes. Finally, in the memory stage, cell viability is preserved, but the membrane structure and the physiological properties may take longer to recover. A reversible process

is desired for biotechnological and medical applications because it is suitable for living cells. When the irreversible process occurs, on the other hand, pores are created and expanded to a certain size that depends on the intensity of the treatment and on the cell characteristics. This process results on cell death because pores are not resealed. Therefore, irreversible electroporation is indicated for food processing since cell damage can inactivate microorganisms and enzymes, as well as increase mass transfer rates in extraction processes (Vorobiev and Lebovka 2009).

Current applications for PEF are mainly in the medical and food engineering area. More recently, this technology has also been applied to environmental and biotechnological processes. Regarding MEF, most part of the applications are in the food technology field and, lately, it has been considered to biotechnological processes. SEF is mainly applied to separate or concentrate particles from a liquid mixture. In the biotechnology area, this technology may be used for microalgae harvesting. A summary of the electrical technologies applications is shown in Fig. 1b.

***Microalgae growth assisted by electrical technologies***

Studies related to cellular stimulation with electric field have increased in the past years due to the promising results obtained by different authors. When restricting the research to microalgae, however, few studies were found, as can be seen in Table 3. Moreover, no studies were found regarding MEF and only one was found concerning SEF. Therefore, the following discussion is focused on PEF impacts on cell growth.

Literature evidence suggests that PEF treatment during the early to mid-exponential state of microalgae growth shows positive results either related to the increase

of biomass or concentration of compounds. Buchmann et al. (2019) evaluated the effect of nanosecond pulsed electric field (nsPEF) application on *Arthrospira platensis* and observed an increase of 13% on cell growth after treatment.

Similarly, Haberkorn et al. (2019) reported an increase of approximately 17% of biomass yield using *Chorella vulgaris*. Beside these results, there is a lack of understanding of the underlying treatment mechanisms. For nsPEF, however, some theories have been raised to explain the effects of this technology in the cells. The main theory assumes a transient intracellular and sublethal stress induction, formation of reactive oxygen and nitrogen species, and electroporation of the cell membrane as the principal effects to stimulate cell proliferation (Buchmann et al. 2019; Haberkorn et al. 2021). The sublethal stress induces cellular response pathways, such as increased concentration of intracellular calcium ions, that may promote cell stimulation. Figure 3 shows the main effects caused by PEF on microalgae cells. These effects will be discussed in more details as follows.

***Electroporation***

Spherical biological cells, such as microalgae, can be interpreted as an electric circuit model where the cell membrane exhibits dielectric properties, acting as a capacitor. An external electric field application promotes accumulation of charges at the membrane, changing its conductance (Fig. 3). In PEF applications with long pulses (micro to milliseconds), these accumulated charges promote reversible or irreversible electroporation (Haberkorn et al. 2021).

The rearrangement of phospholipids in the membrane leads to pore formation that increases the membrane permeability, enabling the exchange of internal and external compounds. This effect was observed specially for other types of microorganisms. For example, *Hanseniaspora* sp. treated by PEF consumed more sugar during fermentation to produce cider (Al Daccache et al. 2020; Mattar et al. 2014) reported higher consumption of carbon during *Saccharomyces cerevisiae* growth when the inoculum was treated with PEF. However, this effect may be extrapolated for microalgae due to the similarity in cells structure. According to Napotnik et al. (2016), longer pulses (mili and microseconds) affect mainly the cell membrane in eukaryotic cells, inducing electroporation. Short pulses (nanoseconds), on the other hand, induce the creation of small pores (also called nanopores) and affect intracellular organelles. Therefore, nsPEF treatments impact sub-cellular compartments.

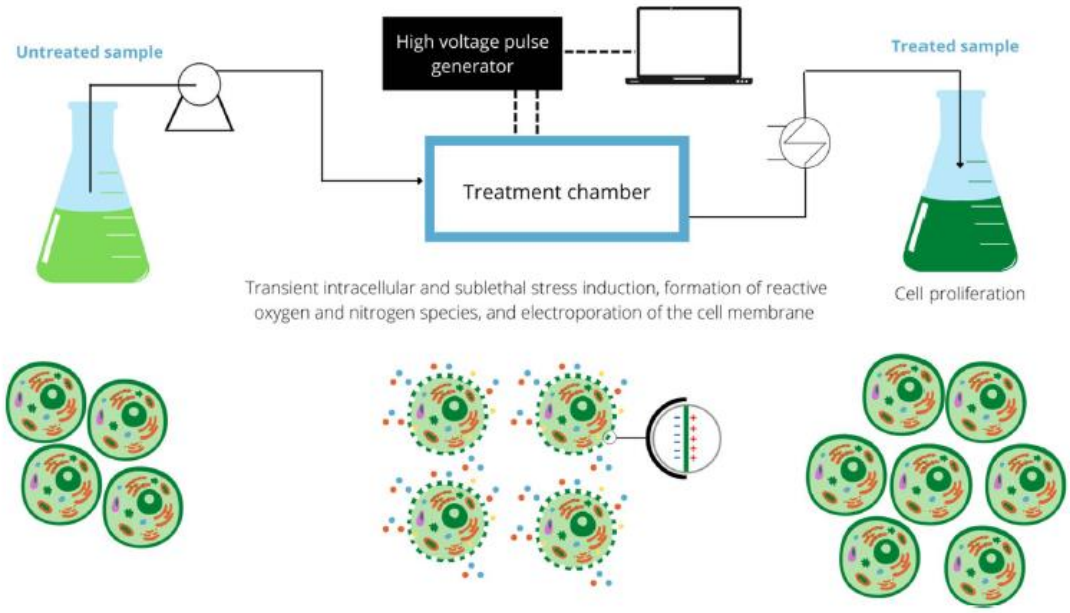


**Fig. 3** Main effects of electrical technologies application on microalgae stimulation

***Calcium release***

At mild electric field intensities, nsPEF treatments may affect organelles, changing $Ca^{2+}$ intracellular levels. In this case, electricity is conducted through the permeabilized cell membrane, allowing nonthermal interactions with subcellular structures (Schoenbach et al. 2007). At higher electric filed intensities, on the other hand, short pulses may induce apoptosis. Therefore, nsPEF at mild intensities has been recently used to induce cell growth as these conditions seem to affect cell metabolism and induce electroporation of internal organelles.

Concerning $Ca^{2+}$ release, several researchers have reported that nsPEF can increase their intracellular concentration. During nsPEF application, $Ca^{2+}$ can be released from intracellular storage compartments, such as endoplasmic reticulum, mitochondria, and vacuoles, due to nanopore formation in the membrane of these organelles. nsPEF may also trigger intracellular voltage-gated $Ca^{2+}$ channels, allowing its release from these organelles. $Ca^{2+}$ can also influx from the extracellular medium across the cell membrane, where different $Ca^{2+}$ channels and pumps enable its transport (Batista Napotnik et al. 2016; Haberkorn et al. 2021). Depending on the type of cell and its physiology, changes in $Ca^{2+}$ concentration can lead to several effects, such as cell division, cytoskeleton rearrangements, endo and exocytosis, apoptosis, and necrosis (Semenov et al. 2013). Buchmann et al. (2019) attributed the higher proliferation of *Arthospira platensis* to $Ca^{2+}$ concentration changes when this microorganism was nsPEF treated. They reported overexpression of two proteins involving with $Ca^{2+}$ regulation in the cytosol. These results suggest that nsPEF could act as a nonchemical trigger for intracellular $Ca^{2+}$

release, upregulating $Ca^{2+}$ signaling pathways, and, consequently, promoting cell proliferation (Haberkorn et al. 2021).

### *Formation of reactive oxygen and nitrogen species*

Concerning free radical formation, although PEF is considered a non-ionizing radiation technology, some researchers have pointed out that at high voltages PEF can induce water ionization and dissociation, causing the formation of free radicals. Intra and extracellular formation of reactive oxygen species (ROS) may cause damage in DNA or in other important proteins, influencing the cellular life cycle (Walker et al. 2006; Schoenbach et al. 2007). In plant and animal cells, ROS play an essential role in cell signaling, being involved in cell growth, development, responses to external stimuli, and programmed cell death (Haberkorn et al. 2021).

Some researchers also reported an increase in reactive nitrogen species (RNS) during nsPEF application (Su et al. 2015; Haberkorn et al. 2021). These free radicals are also key signaling molecules during plant growth (Bailey-Serres and Mittler 2006). Increase of intracellular RNS concentration may promote activation of mitotic processes, defense genes, stress response genes, or cell death. It may also lead to an enhance in intracellular $Ca^{2+}$ concentration, which induces $Ca^{2+}$-regulated cellular processes (Lamattina et al. 2003).

### *Other effects*

Cellular stress caused by an external electric field may increase the synthesis of metabolites of interest by cells. Different pathways can be activated in response to stress,

such as an increase in lipid and pigment production. Buchmann et al. (2019) reported an increase in C-phycocyanin and allophycocyanin contents of approximately 19% after nsPEF treatment. This effect has also been shown in other types of microorganisms. Vaessen et al. (2018) observed an increase of trehalose concentration in *Lactobacillus plantarum* after PEF treatment. Loghavi et al. (2008) and Ohba et al. (2016) also observed an increase of bacteriocin activity and exopolysaccharides concentration from *L. acidopholus* and *L. lactis* after MEF and PEF treatments, respectively.

***Recommendations for electrical technologies***

Practice on microalgae growth Table 3 shows the operational conditions used in the main studies performed so far using electrical technologies during microalgae growth. For PEF, pulse repetition rate, number of pulses, pulse width, electric field intensity, and waveform can be important variables to be taken into account. For SEF, which operates with a direct current, electric field intensity and time are essential parameters to be considered.

Concerning PEF application, the influence of pulse repetition frequency has been reported as an important parameter to stimulate microalgae growth. Many authors stated that a series of short pulses led to higher membrane permeabilization than one single long pulse, even if this pulse lasts the accumulated duration of the series of the short pulses (Silve et al. 2014; Buchmann and Mathys 2019). According to Silve et al. (2014), the higher efficiency of the low repetition rate may be related to the resealing rate of the membranes, since its conductance would be higher right after the pulse application, preventing the creation of additional pores; all the studies conducted so far with nsPEF

(Table 3) applied a three times repetition treatment with an intertreatment interval of 3 h to improve *Chlorella vulgaris* and *Arthrospira platensis* proliferation.

Regarding pulse width, short pulses (10–100 ns) may affect the transmembrane potential of organelles, which could induce abiotic and sublethal stress, increasing the biomass yield (Haberkorn et al. 2019). With respect to the number of pulses, long treatments are better than short ones, since cells already treated with PEF become more sensitive to the treatment. According to Pakhomova et al. (2011), this sensitization could be related to cell physiology modifications (as diameter increase), leading to a lower energy threshold for permeabilization. Similar findings were reported by Buchman et al. (2019), who suggested that neither a single treatment nor a single repetition affected *Arthrospira platensis* cellular proliferation; however, with a three-fold repetition, the researchers observed an increase on biomass concentration.

Additionally, permeabilization rates depend on the electric field intensity. The exposure to a sufficiently strong electric field can cause a significant increase in the natural transmembrane potential, leading to cell membrane permeabilization (Sillanpãã et al. 2011). However, if the exposure is too strong or too long, the electroporation may be irreversible, causing cell damage (Kotnik and Miklavčič 2006). Therefore, the electric field intensity must be carefully chosen. Common electric field intensities ranged from 7 to 20 kV $cm^{-1}$ for reversible electroporation. However, studies involving the influence of this parameter on microorganism growth assisted by PEF have not been assessed so far. Regarding the waveform, it is still unclear how this parameter affects microbial growth.

Considering SEF, only one study was found in the literature (Nezammahalleh et al. 2016). The researchers evaluated different application times (10–70 min) and observed

that all the treatment times resulted in higher biomass concentrations (when compared to the control), and the highest growth stimulation was obtained with 50 min treatment. According to the authors, longer treatments led to a decrease of biomass concentration, which was explained by the peroxide accumulation in the medium. The authors attributed growth stimulation by SEF to cell permeabilization, linking the positive effects to the enhancement of substrates and products transport through the cell. These assumptions were supported by electrical conductivity measurements of the medium along the treatments, as an increase of this parameter was observed with the increase of treatment time. The researchers also evaluated chlorophyll, carotenoid, carbohydrates, and lipid contents of the cells during the cultivation period and did not observe an influence of SEF on the synthesis of these compounds. Regarding electric field intensity, there are no information in the literature about the influence of this parameter on cell growing when SEF is applied. Overall, the electrical technologies have been little explored, as can be seen by the low number of studies shown in Table 3, and more research is needed to better understand the effects of operational conditions on microalgae growth.

## Conclusion

This review presented the state of the art about the application of US and PEF on microalgae growth. Regarding US application, there is evidence that cell metabolism can be altered due to different effects, such as changes in metabolic pathways, increase of membrane permeability, oxygen uptake, among others. US may be applied during the lag or exponential growth phase for cell proliferation and in the stationary phase for

accumulation of secondary metabolites. Low frequencies (18–40 kHz) and power lower than 200 W may be more beneficial for microalgae growth.

Concerning PEF, the main mechanisms involved are electroporation of the cell membrane, transient intracellular and sublethal stress induction, which induces increased concentration of intracellular calcium ions, and formation of reactive oxygen and nitrogen species. A series of short pulses (10–100 ns) of low-rate repetition was reported to better induce changes in cell metabolism. However, other parameters, such as electric field intensity and phase of application, should be better explored. The use MEF and SEF to promote cell proliferation is a relatively new topic, and research is needed to evaluate the benefits that these technologies may bring to the biotechnology industry. The consolidation of these technologies in the biotechnology industry depends on a better understanding of the underlying mechanisms and on the establishment of correlations between operational parameters and caused effects.

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## Declarations

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**Table 1** – Summary of the different approaches discussed in this review.

| Technology | Advantages | Disadvantages | Effects on microalgae growth | Lack of knowledge | Economic potential |
|---|---|---|---|---|---|
| **US** | Simple, rapid, and environmental-friendly technology, possibility of continuous operation. Easy scale-up. | Depending on the process parameters, it may cause increase of the temperature of the medium. High intensities may promote cell death. | Increase of membrane permeability; changes in enzymes structure; increase of aeration in the culture medium; release of clusters of cells formed during cultivation; and changes in cellular components and genetic functions. | Information regarding correlating operational conditions and caused effects are still missing in the literature. | Relatively low capital costs. Operational costs are mainly related to electricity expenses, which depends on the require energy input. |
| **PEF** | Flexibility, ease of scale-up, high energy efficiency, possibility of continuous operation. Environmental-friendly technology. | High electric field strength may cause irreversible electroporation. Safety precautions regarding equipment handling. | Transient intracellular and sublethal stress induction, formation of reactive oxygen and nitrogen species, and electroporation of the cell membrane. | Understanding of the underlying treatment mechanisms. Incomplete reporting and insufficient characterization and control of processing parameters. Few studies conducted so far regarding microalgae growth. | High capital costs. Operational costs are mainly related to electricity expenses, which depends on the require energy input, and are higher compared to other technologies. Safety precautions increase the operational costs. |
| **MEF** | Flexibility, ease of scale-up, high energy efficiency, possibility of continuous operation. Environmental-friendly technology. | Safety precautions regarding equipment handling. | Induction of sublethal stress and electroporation of the cell membrane. MEF can provide the optimum growing temperature at a short time. | Understanding of the underlying treatment mechanisms. So far, studies were conducted only in fermentation processes with *Lactobacillus*, *Streptococcus*, and *Saccharomyces*. | Relatively high capital costs. Operational costs are mainly related to electricity expenses, which depends on the require energy input. Safety precautions may also increase the operation costs. |
| **SEF** | Flexibility, ease of scale-up, high energy efficiency, possibility of continuous operation. Environmental-friendly technology. | Safety precautions regarding equipment handling. Electrochemical reactions promote water hydrolysis and low durability of the electrodes. Depending on the process parameters, it may cause increase of the temperature of the medium. | Increase of membrane permeability. | Understanding of the underlying treatment mechanisms. Incomplete reporting and insufficient characterization and control of processing parameters. Only one study conducted so far regarding microalgae growth. | Similar to MEF. |

**Table 2** – Applications of ultrasound for microalgae growth and the operating conditions.

| Species | Microorganism | Type | Frequency (kHz) | Power (W) | US time | Interval | Growth phase | Main results | | References |
|---|---|---|---|---|---|---|---|---|---|---|
| | | | | | | | | Biomass | Products | |
| *Anabaena variabilis* | Cyanobacterium | bath | 40 | 200 | 5 min | - | beginning of the stationary | 1.36 g $L^{-1}$ | 46.9 % of lipids (1.46-fold increase) | (Han et al. 2016a) |
| *Scenedesmus quadriculata* | Microalga | bath | 40 | 200 | 20 min | - | end of exponential | 1.17 g $L^{-1}$ | 38.9 % of lipids (57 % higher than control) | (Han et al. 2016b) |
| *Tetradesmus obliquus* | Microalga | bath | 33 | 100 | 10 min | 1 min on/9 min off ever12 h | beginning of the stationary | 3.12 g $L^{-1}$ (22.8 % higher than control) | 0.39 g $g^{-1}$of lipids and 1.17 ± 0.05 mg $g^{-1}$ of β-carotene (34.5 % and 31.5 % higher than control, respectively) | (Singh et al. 2019) |
| *Scenedesmus* sp. | Microalga | probe | 18 | 20 | 10 min | - | exponential | 1.56 g $L^{-1}$ | 240 mg $L^{-1}$ of lipids | (Ren et al. 2019a) |
| *Scenedesmus* sp. | Microalga | probe | 20 | 20 | 2s | - | exponential | 2.78 g $L^{-1}$ (26.9 % higher than control) | 28.5 % of lipids (37 % higher than control) | (Ren et al. 2019b) |
| *Scenedesmus* sp. | Microalga | probe | - | 20 | 4 min | 2s interval | exponential | 2.68 g $L^{-1}$ (1.4-fold increase) | 49 % of lipids (72.4 % higher than control) | (Sivaramakrishnan and Incharoensakdi 2019) |
| *Chlorella sorokiana* | Microalga | probe | - | 100 | 10 min | - | stationary | 0.63 g $L^{-1}$ (1.5 fold increase) | 64.4 % of lipids (39 % higher than the control) | (Xie et al. 2020) |

**Table 3** – Application of electric field treatments on microbial growth and synthesis of compounds.

| Species | Microorganisms | Technology | Growth phase | Wave shape | Frequency (Hz) | Treament parameters | Electric field strength ($V\ cm^{-1}$) | Main results | References |
|---|---|---|---|---|---|---|---|---|---|
| *Chlorella vulgaris* | Microalga | SEF | exponential | - | - | 50 min | 2770 | 51 % of biomass increase | (Nezammahalleh et al. 2016) |
| *Chlorella vulgaris* | Microalga | nsPEF | exponential | rectangular | 5 | pulse width of 100 ns, residence time of 0.61 s; 3 treatments, intertreatment interval of 3 h | 10000 | increased biomass yields by up to 17.53 % | (Haberkorn et al. 2019) |
| *Arthrospira platensis* | Cyanobacterium | nsPEF | exponential | rectangular | 9 | pulse width of 100 ns, residence time of 0.23 s; 3 treatments, intertreatment interval of 3 h | 10000 | 13.1 % of increase in dry matter; 19% of increase in C-phycocyanin and allophycocyanin contents | (Buchmann et al. 2019) |
| *Chlorella vulgaris* | Microalga | nsPEF | early exponential | rectangular | 7 | pulse width of 100 ns, residence time of 0.61 s; 3 treatments, intertreatment interval of 3 h | 10000 | Relative biomass increases of 50 % | (Haberkorn et al. 2021) |