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MILENA FERNANDES DA SILVA

POTENCIAL BIOTECNOLÓGICO DE MICRO-ORGANISMOS FOTOSSINTETIZANTES PRODUTORES DE COMPOSTOS BIOATIVOS DE INTERESSE NA INDÚSTRIA DE ALIMENTOS

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Tese apresentada ao Programa de Pós-Graduação em Ciências Biólogicas da Universidade Federal de Pernambuco como parte dos requisitos à obtenção do grau de Doutor em Ciências Biólogicas.

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“Combater o bom combate, completar a corrida e perseverar na fé; pois Deus nos deu um espírito de fortaleza, de amor e de sabedoria.”

(2 Tm 4,7; 1,7)

RESUMO

Nos últimos anos, microalgas e cianobactérias tem atraído grande atenção em todo o mundo devido à sua capacidade de produzir compostos bioativos com efeitos benéficos para a saúde humana, tais como os polifenóis. No entanto, sua recuperação rentável, a partir de abordagens de extração "verde" inovadoras, é atualmente um tópico de pesquisa quente que é reconhecida como um grande desafio. Neste contexto, o objetivo deste trabalho foi avaliar o potencial biotecnológico de micro-organismos fotossintetizantes para produzir substâncias bioativas naturais de interesse na indústria de alimentos. Extratos etanólicos e aquosos da biomassa liofilizada de *Chlorella vulgaris*, *Dunaliella salina*, *Scenedesmus* sp. e *Arthrospira (Spirulina) platensis*, obtidos por extração clássica sólido-líquido, foram submetidos a triagem quanto à sua capacidade antioxidante *in vitro*, bem como suas propriedades antibacterianas. Diferentes técnicas de extração verde, tais como as assistidas por ultrassons e micro-ondas e a de alta pressão e temperatura (HPTE), foram comparadas com o método clássico de extração sólido-líquido usando etanol como solvente, para selecionar o modo mais adequado para obter extratos com maior teor de compostos fenólicos e poder antirradicalar (ARP). Entre os extratos etanólicos estudados, o de *A. platensis* mostrou maior atividade antioxidante de acordo com ambos os métodos de radicais livres ABTS (66,09%±2,00) e DPPH (55,73%±0,18), bem como eficaz atividade antibacteriana. Uma vez que a HPTE foi a técnica de extração mais eficiente, foi utilizada em testes de otimização efetuados segundo um planejamento fatorial completo 3^2 e Metodologia de Superfície de Resposta (MSR). MSR revelou que as condições mais adequadas para extração tanto de polifenóis totais (26,00-28,04 $mg_{Ácido\ Gálico\ Equivalente}/g_{Biomassa\ Seca\ [BS]}$) como de flavonóides (10,25±0,34 $mg_{Catequina\ Equivalente}/g_{BS}^{-1}$) foram a temperatura mais elevada (T; 180°C) e concentração de etanol em água (Sc) entre 20-60%, enquanto o ARP foi maximizado (67,77-69,02 µmolTrolox g_{BS}^{-1}) a 90 ≤T≤ 135ºC e 20 ≤Sc≤ 60%. A CLAE permitiu identificar a catequina e os ácidos vanílico, gálico e siríngico como os principais compostos fenólicos do extrato (3,45-3,61, 1,06-2,02, 1,64-1,71 e 0,99-1,26 mg $100g_{BS}^{-1}$, respectivamente). Adicionalmente, foram realizados estudos de acordo com uma nova abordagem bioenergética e termodinâmica sobre o crescimento fotoautótrofo de *A. platensis* selecionada como micro-organismo modelo. Fotobiorreatores com diferentes relações superfície/volume (*S/V*) foram comparados a uma densidade de fluxo de fótons fotossintéticos (PPFD) de 70 µmol m^{-2} s^{-1}, e a influência deste parâmetro foi investigada no fotobiorreator horizontal (HoP; *S/V* 1,94 cm^{-1}) que mostrou o melhor desempenho. Um aumento da PPFD de 40 a 100 µmol m^{-2} s^{-1} no HoP acarretou uma progressiva melhora dos parâmetros cinéticos, bioenergéticos e termodinâmicos, provavelmente devido a uma distribuição mais favorável de luz. Este estudo demonstra o potencial da HPTE como uma técnica promissora para obter extratos ricos em fenólicos de *A. platensis* com alto ARP utilizando misturas binárias de solventes verdes (etanol/água). Além disso, HoP provou possuir a melhor configuração para produzir culturas de *A. platensis* de alto rendimento. Esta tese abre novos caminhos no uso desta tecnologia emergente para obtenção de extratos que podem ser utilizados como fonte natural de compostos para formular novos produtos alimentícios ou nutracêuticos.

Palavras-chave: Compostos bioativos. Micro-organismos fotossintetizantes. Extração verde. Fotobiorreator. Bioenergética e termodinâmica.

ABSTRACT

In recent years, microalgae and cyanobacteria have attracted great attention worldwide due to its ability to produce bioactive compounds with beneficial effects to the human health, such as polyphenols. However, their profitable recovery from innovating "green" extraction approaches is currently a hot investigation topic, which has been recognized as a big challenge. In this context, the objective of this study was to evaluate the biotechnological potential of photosynthetic microorganisms to produce natural bioactive substances of interest in the food industry. Aqueous and ethanolic extracts of freeze-dried biomass of the microalgae *Chlorella vulgaris*, *Dunaliella salina* and *Scenedesmus* sp. and the cyanobacterium *Arthrospira (Spirulina) platensis* obtained by classic solid-liquid extraction were first screened for their in vitro antioxidant capacity as well as their antibacterial properties. Different green extraction techniques, namely ultrasound-assisted, microwave-assisted and high pressure and temperature extractions (HPTE), were compared with classic solid–liquid extraction using ethanol as the solvent to select the most suitable way to get extracts with the highest content of phenolic compounds and antiradical power (ARP). Among the studied ethanolic extracts, the one of *A. platensis* exhibited the highest antioxidant activity by both ABTS (66.09%±2.00) and DPPH (55.73%±0.18) assays as well as effective antibacterial activity. Since HPTE was the most efficient extraction technique, it was utilized in optimization tests carried out according to a 3^2 full factorial design and Response Surface Methodology (RSM). RSM revealed that the most suitable conditions for extracting both total polyphenols (26.00-28.04 $mg_{Gallic\ Acid\ Equivalent}/g_{Dry\ Biomass\ [DB]}$) and flavonoids (10.25±0.34 $mg_{Catechin\ Equivalent}/g_{DB}^{-1}$) were the highest temperature (T; 180°C) and ethanol concentration in water (Sc) entre 20-60%, while ARP was maximized (67.77-69.02 µmolTrolox g_{DB}^{-1}) at 90 ≤T≤ 135ºC and 20 ≤Sc≤ 60%. HPLC allowed identifying catechin and vanillic, gallic and syringic acids as the main phenolic compounds of the extract (3.45-3.61, 1.06-2.02, 1.64-1.71 and 0.99-1.26 mg $100g_{DB}^{-1}$, respectively). In addition, studies were also performed according to a new bioenergetic and thermodynamic approach on the photoautotrophic growth of *A. platensis* selected as a model microorganism. Photobioreactors with different surface/volume ratios (*S/V*) were compared at photosynthetic photon flux density (PPFD) of 70 µmol m^{-2} s^{-1}, and the influence of this parameter was investigated in the horizontal photobioreactor (HoP; *S/V* 1,94 cm^{-1}), which showed the best performance. An increase in PPFD from 40 to 100 µmol m^{-2} s^{-1} revealed a progressive enhancement of the kinetic, bioenergetic and thermodynamic parameters probably due to the more favorable light distribution. This study demonstrates the potential of HPTE as a promising technique to obtain phenolic-rich extracts of *A. platensis* with remarkable ARP using binary mixtures of green solvents (ethanol/water). Furthermore, HoP proved to possess the best configuration to perform high-yield *A. platensis* cultures. This work opens new avenues in the use of this emerging technology to obtain extracts that can be potentially used as a natural source of compounds to formulate novel food or nutraceutical products.

Key-words: Bioactive compounds. Photosynthetic microorganisms. Green extraction. Photobioreactor. Bioenergetics and thermodynamics.

RIASSUNTO

Negli ultimi anni, microalghe e cianobatteri hanno attirato grande attenzione in tutto il mondo grazie alla sua capacità di produrre composti bioattivi con effetti benefici per la salute umana, come i polifenoli. Tuttavia, il loro recupero redditizio, dall'innovare approcci di estrazione "verde", è attualmente un argomento di ricerca calda, che è stata riconosciuta come una grande sfida. In tale contesto, ci si è prefissi in questo studio l'obiettivo di valutare il potenziale biotecnologico di microrganismi fotosintetici per produrre sostanze bioattive naturali di interesse per l'industria alimentare. Estratti acquosi ed etanolici di biomassa liofilizzata delle microalghe *Chlorella vulgaris*, *Dunaliella salina* e *Scenedesmus* sp. e del cianobatterio *Arthrospira (Spirulina) platensis*, ottenuti mediante estrazione solido-liquido classica, sono stati sottoposti a screening per la loro capacità antiossidante *in vitro* così come per le loro proprietà antibatteriche. Tecniche di estrazione verde, quali quelle assistite da ultrasuoni e microonde e quella ad alte pressione e temperatura (HPTE), sono state confrontate con il metodo classico di estrazione solido-liquido utilizzando l'etanolo come solvente, per selezionare il modo più adatto per ottenere gli estratti con il più alto contenuto di composti fenolici e il maggior potere antiradicalico (ARP). Tra gli estratti etanolici studiati, quello di *A. platensis* ha mostrato la più alta attività antiossidante secondo entrambi i saggi di neutralizzazione dei radicali liberi ABTS (66.09%±2.00) e DPPH (55.73%±0.18), nonché un'attività antibatterica efficace. Poiché l'HPTE è risultata la tecnica di estrazione più efficiente, è stata impiegata in prove di ottimizzazione eseguite secondo un disegno fattoriale completo 3^2 e Metodologia della Superficie di Risposta (MSR). MSR ha rivelato che le condizioni più adatte per l'estrazione tanto di polifenoli totali (26.00-28.04 $mg_{Acido\ Gallico\ Equivalenti}/g_{Biomassa\ Secca\ [DB]}$) quanto di flavonoidi (10.25±0.34 $mg_{Catechina\ Equivalente}/g_{DB}^{-1}$) sono la temperatura più elevata (T; 180°C) e la concentrazione di etanolo in acqua (Sc) tra il 20-60%, mentre l'ARP è stato massimizzato (67.77-69.02 µmolTrolox g_{DB}^{-1}) a 90 ≤T≤ 135 ºC e 20 ≤Sc≤ 60%. L'HPLC ha permesso di identificare la catechina, e gli acidi vanillico, gallico e siringico come i principali composti fenolici dell'estratto (3.45-3.61, 1.06-2.02, 1.64-1.71 and 0.99-1.26 mg $100g_{DB}^{-1}$, rispettivamente). Inoltre, sono stati eseguiti anche degli studi secondo un nuovo approccio bioenergetico e termodinamico sulla crescita fotoautotrofa di *A. platensis* selezionata come microorganismo modello. Fotobioreattori con differente rapporto superficie/volume (*S/V*) sono state confrontate a una densità di flusso fotonico fotosintetico (PPFD) di 70 µmol m^{-2} s^{-1}, e l'influenza di questo parametro è stata indagata nel fotobioreattore orizzontale (HoP; *S/V* 1,94 cm^{-1}), che ha mostrato la miglior performance. Un aumento della PPFD da 40 a 100 µmol m^{-2} s^{-1} ha rivelato un miglioramento progressivo dei parametri cinetici, bioenergetici e termodinamici probabilmente dovuto alla distribuzione della luce più favorevole. Questo studio dimostra il potenziale dell'HPTE come una tecnica promettente per ottenere estratti ricchi in composti fenolici di *A. platensis* con notevole ARP utilizzando miscele binarie di solventi verdi (etanolo/acqua). Oltretutto, HoP ha dimostrato di possedere la migliore configurazione per eseguire colture *A. platensis* ad alto rendimento. Questo lavoro apre nuove strade all'uso di questa tecnologia emergente per ottenere estratti che possono essere potenzialmente utilizzati come un'interessante fonte naturale di composti per formulare nuovi prodotti alimentari o nutraceutici.

Parole chiave: Composti bioattivi. Microrganismi fotosintetici. Estrazione verde. Fotobioreattore. Bioenergetica e termodinamica.

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Fig. 5. Fractions of the absorbed light energy stored as ATP (η_{ATP}) (empty symbols and solid lines) and used to increase the enthalpic content of the system (η_H) (filled symbols and dotted lines) during *A. platensis* cultivations performed: A) at PPFD of 70 μmol m^{-2} s^{-1} in: (◇) Open Pond, *S/V* = 0.25 cm^{-1}; (□) Shaken Flask, *S/V* = 0.48 cm^{-1}; (○) Horizontal Photobioreactor, *S/V* = 1.94 cm^{-1}; (△) Helicoidal Photobioreactor, *S/V* = 3.88 cm^{-1}. B) in the Horizontal Photobioreactor varying PPFD (μmol m^{-2} s^{-1}): (□) 40; (○)70;(△) 100.……..…

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LISTA DE ABREVIATURAS E SIGLAS

FDA - Food and Drug Administration

AESA - European Food Safety Authority

BHT - butil-hidroxitolueno

BHA - butil-hidroxianisol

GLA - ácido γ-linolénico

SPS - polissacarídeos sulfatados

BBM - Bold's basal medium

SLE - Extração sólido-líquido

UAE - Extração assistida por ultra-som

MAE - Extração assistida por microondas

HPTE - Extração alta pressão e temperatura

ARP - Poder antiradicar

RSA - radical scavenging activity

TP - Polifenóis totais

TF - Flavonóides totais

ABTS - Ácido 2,2'-azino-bis-(3-etilbenzotiazolina-6-sulfônico) sal diamônio

DPPH - 2,2-difenil-1-picrilhidrazill

Trolox - Ácido 6-hidroxi-2,5,7,8-tetrametil-cromano-2-carboxílico

TEAC - Capacidade antioxidante equivalente em Trolox

HPLC-DAD - Cromatografia líquida de alta eficiência com detector por arranjo de diodos

ANOVA - Análise de variância

R^2_{Adj} - Coeficiente de determinação ajustado

RSM - Metodologia de Superfície de Resposta

PPFD - Densidade de fluxo de fótons fotossintéticos.

S/V - Relação superfície/volume

DO - Densidade óptica

OP - Lagoa aberta

SF - Frasco agitado

HoP - Fotobioreator horizontal

HeP - Fotobioreator helicoidal

C - Carbono;

N - Nitrogênio

S - Enxofre

O - Oxigênio

H - Hidrogênio

X_{max} - Concentração celular máxima (g L^{-1})

$P_{X\,max}$ - Produtividade de biomassa máxima (g L^{-1} d^{-1})

μ_{max} - Taxa de crescimento específica máxima (d^{-1})

X_i e X_{i-1} - Concentrações de biomassa (g L^{-1})

t_i e t_{i-1} - Tempo (dias)

PSI - Fotosistema I

PSII - Fotosistema II

LISTA DE SÍMBOLOS

$1/Y_{GX}$ - Energia de Gibbs para o crescimento e manutenção celular

$1/Y_{GX}^{max}$ - Requerimento de energia Gibbs relacionado com o crescimento celular

μ - Velocidade específica de crescimento

m_G - Energia de Gibbs dissipada para a manutenção celular

Δg_{f° - Energia Molar de Gibbs de formação

h - Constante de Planck ($6.626 \cdot 10^{-34}$ J·s)

$\underline{c}$ - Velocidade da luz ($2.99626 \cdot 10^{8}$ m s^{-1})

N_A - Número de Avogadro ($6.022626 \cdot 10^{23}$ mol^{-1})

λ - Comprimento de onda

ΔG_a - Energia total de Gibbs absorvida pela fotossíntese

Y_{GX} – Rendimento de biomassa em energia de Gibbs

n_{Ph} – Número de fótons absorvidos para produzir 1 C-mol de biomassa (mol C-mol^{-1})

q_{O_2} - Produção molar de O_2

q_{H^+} - Consumo molar de H^+

η_{ATP} - Percentuais estimados de energia absorvida pela célula como ATP

η_H - Percentuais estimados de energia fixadas como conteúdo entálpico

Δg_{Ph} - Energia de Gibbs contida em 1 Einsten de fóton

ΔH - Variação de entalpia

ΔG_{ATP} - Energia de Gibbs transformada em ATP e utilizada para o crescimento e manutenção celular

Q - Energia de Gibbs liberada como calor

R - Constante dos gases (8.3145 J mol^{-1} K^{-1})

SUMÁRIO

INTRODUÇÃO

1 INTRODUÇÃO

Micro-organismos fotossintetizantes, como microalgas e cianobactérias, representam um importante reservatório de compostos biologicamente ativos (FREITAS et al., 2012). Esses organismos fotossintéticos microscópicos, capazes de converter a energia solar em biomassa, possuem diversos usos e aplicações sob diferentes áreas da ciência, principalmente, por serem uma das fontes mais interessantes de ingredientes de alimentos e produtos de alimentos funcionais, uma vez que, podem ser usados para melhorar o valor nutritivo dos alimentos devido à sua riqueza em compostos com atributos benéficos (STRANSKA-ZACHARIASOVA et al., 2016; PULZ; GROSS, 2004; BENELHADJ et al., 2016).

Atualmente, além da utilização de microalgas e cianobactérias para o tratamento de águas residuais, sustentabilidade da aquicultura ou para a produção de biocombustível, a procura de compostos bioativos de interesse alimentício têm atraído grande atenção em todo o mundo, principalmente no tocante à identificação de diversas substâncias sintetizadas por estes micro-organismos fotossintéticos. Com o objetivo de aumentar a disponibilidade e a diversidade química dos ingredientes bioativos nestes organismos, mais pesquisas têm sido desenvolvidas utilizando ferramentas biotecnológicas para descobrir e produzir novos compostos (CHEAH et al., 2015; VAZ et al., 2016; STRANSKA-ZACHARIASOVA et al., 2016).

Interesse tem surgido nesses micro-organismos, pois considerando a sua grande biodiversidade, e possibilidade de cultivo sob diferentes condições ambientais, as microalgas e cianobactérias podem se tornar fontes promissoras de produtos naturais de alto valor agregado, como por exemplo, os compostos bioativos estruturalmente diversos, incluindo ácidos graxos poliinsaturados (PUFA), polissacarídeos, minerais e vitaminas, enzimas e antioxidantes (BELAY, 2002; GUEDES et al., 2011; CUSTÓDIO et al., 2012; KIM; WIJESEKARA, 2010; DEWAPRIYA; KIM, 2014; BENELHADJ et al., 2016; RAPOSO; MORAIS, 2015). Além disso, apresentam ainda particular importância, pois possuem a capacidade de converter o CO_2 atmosférico em compostos orgânicos mais complexos e com valor comercial (GUEDES et al., 2013; GRIMI et al., 2014).

Uma das principais áreas de investigação da Ciência e Tecnologia de Alimentos, é a extração e caracterização de novos ingredientes naturais com atividades biológicas que podem contribuir para a saúde e o bem-estar da população como parte de novos alimentos funcionais. Estes ingredientes funcionais, são preferidos pelos consumidores por serem de origem natural,

sendo geralmente extraídos de fontes naturais, tais como plantas, microalgas e cianobactérias, etc (PLAZA et al., 2012; HERRERO et al., 2006). Pesquisadores têm relatado sobre o potencial das microalgas e cianobactérias como fonte de compostos bioativos (PLAZA et al., 2010; DE JESUS RAPOSO et al., 2013; GONÇALVES et al., 2016).

As microalgas e cianobactérias são uma importante fonte natural de grande número de substâncias bioativas que podem atuar como antioxidantes, antibacterianas, etc (PLAZA et al., 2012; OHNO et al., 2012; CATARINA GUEDES et al., 2013). Os antioxidantes apresentam um efeito positivo sobre a saúde humana, protegendo o corpo humano contra danos oxidativos causados por espécies reativas de oxigênio (ROS), que podem provocar diversos distúrbios à saúde, como câncer, diabete mellitus, doenças neurodegenerativas e inflamatória. Assim, o uso de antioxidantes sintéticos, tais como butil-hidroxitolueno (BHT) e butil-hidroxianisol (BHA), deve estar sob restrita regulamentação devido a potenciais riscos para a saúde (NGO et al., 2011).

As características antibacterianas das microalgas e cianobactérias, estas têm demonstrado atividade com potencial uso como aditivos alimentares antibacterianos para preservação de alimentos, uma vez que, a tendência atual adotada pela produção de alimentos, gabinetes legislativos e os consumidores, tem exigido um recuo progressivo da química de aditivos nos sistemas de conservação de alimentos, buscando substâncias alternativas, a fim de obter conservantes naturais contra a deterioração de alimentos e previnir doenças causadas por micro-organismos (CASTILHO et al., 2012; MENDIOLA et al., 2007).

Neste contexto, o presente trabalho tem como objetivo investigar o potencial de micro-organismos fotossintetizantes capazes de produzir compostos bioativos naturais de alto valor agregado visando sua aplicação na indústria de alimentos.

OBJETIVOS

2 OBJETIVOS

2.1 Objetivo Geral

Avaliar o potencial biotecnológico de micro-organismos fotossintetizantes na produção de compostos bioativos de interesse na indústria de alimentos.

2.2 Objetivos Específicos

- Avaliar as atividades biológicas antioxidante e antibacteriana dos extratos obtidos a partir da biomassa dos micro-organismos fotossintetizantes comerciais e isolado do Açude do Prada proveniente da cidade de Recife-PE utilizando extração por solvente (aquoso e orgânico);
- Selecionar o micro-organismo fotossintetizante que apresentar melhores atividades antioxidante e antibacteriana;
- Identificar o isolado selecionado através de método morfométrico;
- Determinar o método de extração mais eficiente para recuperar compostos funcionais a partir da biomasa do micro-organismo fotossintetizante selecionado através de tecnologias de extração verde alternativas (nomeadamente, assistidas por ultrassons e micro-ondas e a de alta pressão e temperatura);
- Avaliar a influência dos princiapis parâmetros bioenergéticos, rendimento de biomassa em energia de Gibbs (Y_{GX}), absorção de fótons para produzir 1 C-mol de biomassa (n_{Ph}), produções molares de O_2, consumo de H^+ e percentuais estimados de energia absorvida pela célula como ATP (η_{ATP}) e fixadas como conteúdo entálpico (η_H) no cultivo fotoautotrófico do micro-organismo fotossintetizante em diferentes fotobiorreatores e sobre diferentes condições de luz;
- Estudar em diferentes configurações de fotobiorreatores e sobre diferentes condições de luz no cultivo fotoautotrófico do micro-organismo fotossintetizante, os efeitos dos principais parâmetros termodinâmicos: variação de entalpia (ΔH), energia de Gibbs recuperada sobre a forma de ATP e utilizada para o crescimento e manutenção celular (ΔG_{ATP}) e energia liberada na forma de calor (Q).

REVISÃO DA LITERATURA

3 REVISÃO DA LITERATURA

3.1 Microalgas e cianobactérias: histórico e produção de biomassa

Os micro-organismos fotossintetizantes como microalgas e cianobactérias constituem um grupo de organismos muito heterogêneo, em geral microscópios, que são predominantemente aquáticos, com ampla distribuição no globo terrestre. (DERNER et al., 2006; LOURENÇO, 2006; KIM; WIJESEKARA, 2010).

As microalgas e cianobactérias são consideradas uma rica fonte natural de metabólitos secundários, incluindo nutrientes funcionais e peptídeos bioativos. Nessa linha, esses fatores associados às suas atividades biológicas, bem como seus efeitos na saúde, têm despertado o interesse de diversos pesquisadores acadêmicos e industriais (KIM; WIJESEKARA, 2010; KIM et al., 2016).

Os micro-organismos fotossintéticos possuem a habilidade de fixar dióxido de carbono utilizando a energia solar de forma 10 vezes mais eficiente do que as plantas terrestres, e requerem o mínimo de recursos para sobreviver sendo, portanto, considerados organismos com elevado potencial biotecnológico (MOSTAFA, 2012). Além disso, tais organismos apresentam-se em diversas formas de organização celular: unicelular, colonial ou filamentosa, e podem ser classificados em células eucarióticas, representadas pelas microalgas, pois possuem membrana nuclear, a qual contém a maior parte do genoma distribuído entre os cromossomos e nucléolo (Divisões Chlorophyta, Rhodophyta, Euglenophyta, Haptophyta (Prymnesiophyta), Heterokontophyta, Cryptophyta e Dinophyta); ou procarióticas, represenadas pelas cianobactérias que possuem DNA desorganizado, o qual fica livre no citoplasma com as membranas fotossintéticas, uma vez que não possuem membrana nuclear (Divisões Cyanophyta e Prochlorophyta) (TOMASELLI, 2004; LOURENÇO, 2006; DERNER et al., 2006).

Não está claro quantas espécies de microalgas existem, com estimativas em torno de 70.000 a um milhão. Apenas cerca de 44.000 têm sido descritas. Assim, novas espécies e gêneros estão sendo constantemente descobertos (NEOFOTIS et al., 2016). A classificação taxonômica dos micro-organismos fotossintetizantes é bastante vasta, pois apresenta uma ampla diversidade de espécies, com representantes de formas unicelulares que possuem desde micro a milímetros de diâmetro, células em colônia e filamentos. As técnicas modernas de biologia molecular têm sido usadas para confirmar ou alterar a classificação de microalgas e

cianobactérias, bem como tornaram uma ferramenta promissora para exploração de seus produtos (BEN-AMOTZ, 2009).

Ao longo do processo histórico, os micro-organismos fotossintetizantes vêm sendo empregados para várias finalidades, sendo a mais antiga, a partir da década de 1950 onde começaram a ser utilizadas como fonte para alimentação de humanos e animais, e de substâncias biologicamente ativas.

Ocorrência da presença e consumo de biomassa da cianobactéria procariota *Arthrospira* (*Spirulina*) *platensis* foram feitos por volta de 1950 nos lagos naturais: Chade (África) e o antigo Texcoco (México). A biomassa era usada como uma fonte alternativa de proteínas (teor de proteína > 50%) (BATISTA et al., 2011). Em 1960, seu uso foi levado a escala comercial com o gênero *Chlorella*, a qual surgiu com o estudo da ficologia aplicada, quando Beijerinck, em 1890, realizou cultivos desta microalga eucariota; e a partir de 1970, as microalgas foram destinadas a aquicultura, além de outros fins biotecnológicos (SPOLAORE et al., 2006).

Dentre as microalgas, a *Chlorella* teve sua produção comercial iniciada pela primeira vez em 1961 pela Nihon Chlorella Inc., usando as instalações do Instituto de Pesquisas de Microalgas localizada no Japão. Já, a empresa Dainippon Ink & Chemicals Inc. (DIC) foi a precursora na produção comercial de *A. platensis* em tanques por volta de 1978 em Bangkok, Tailândia (TOMASELLI, 2004).

A produção de biomassa algal se concentra, principalmente, na China, Japão, Taiwan, Estados Unidos e Índia (FRANCO et al., 2013). Um exemplo é a empresa Cyanotech Corporation (Hawai-USA) que é líder mundial na tecnologia de produção de astaxantina (BioAstin®), no que se refere, ao cultivo da microalga *Haematococcus*. A empresa também cultiva *A. platensis* (Figura 1), cuja biomassa produzida é usada como ingrediente alimentar de modo a aumentar a energia e a resposta imune (CYANOTECH, 2016).

Figura 1 – Cultivo comercial de *A. platensis* da empresa Cyanotech.

Fonte: Cyanotech (2016) (http://www.cyanotech.com/company/facility.html).

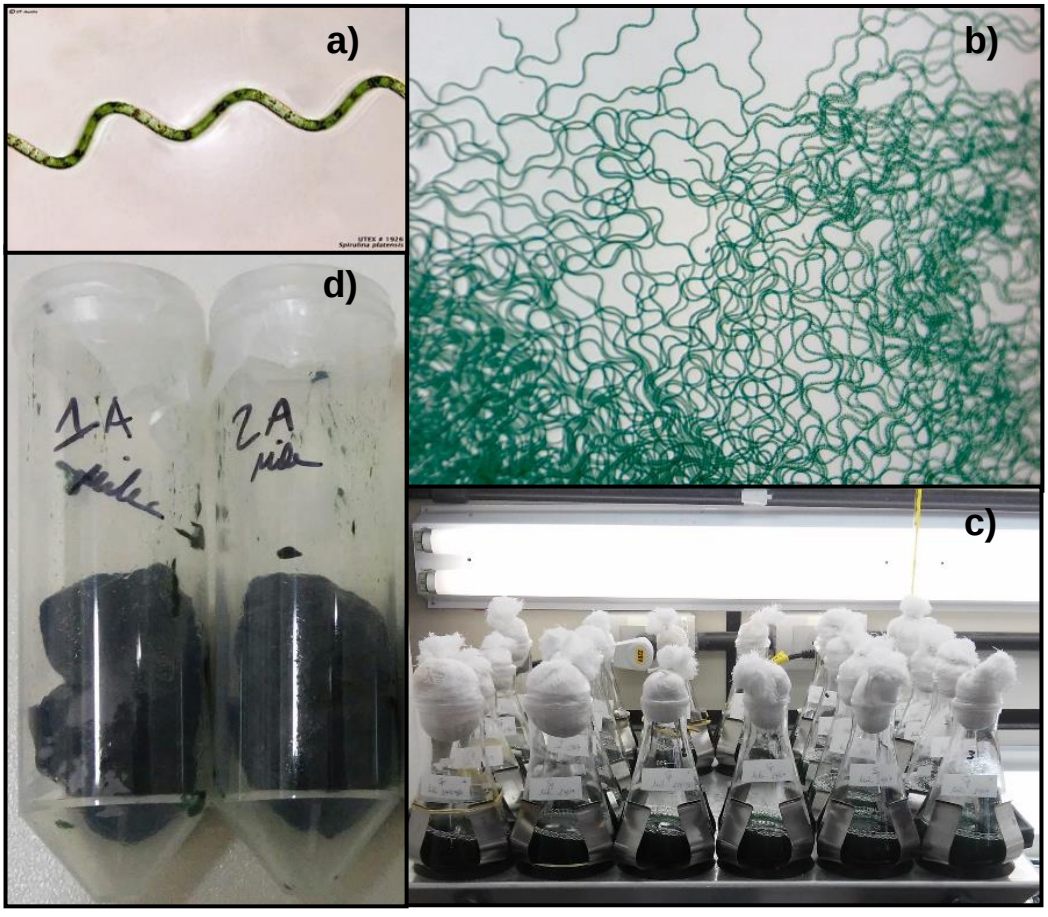
Baseado nesse contexto, este grupo diversificado de organismos merece destaque por apresentar elevada importância ecológica e econômica, com uso diversificado em vários países do mundo e numerosas aplicações comerciais (VIDOTTI; ROLLEMBERG, 2004).

3.1.1 Características das espécies de microalgas e cianobactérias

Uma ampla variedade de microalgas e cianobactérias são utilizadas para obtenção de diversos produtos e biomassa, dentre as quais podemos citar as espécies utilizadas no presente trabalho: *Arthrospira* (*Spirulina*) *platensis*, *Dunaliella tertiolecta*, *Chlorella e Scenedesmus* sp.

Arthrospira platensis (nome comum: *Spirulina*) é uma cianobactéria filamentosa e multicelular fotossintetizante (Figura 2), que pertence à classe *Cyanophyceae*, ordem *Oscillatoriales*, gênero *Arthrospira* e espécie *platensis.* São caracterizadas por possuirem células cilíndricas que formam ostricomas helicoidais, cujos seus filamentos apresentam mobilidade por deslizamento (VO et al., 2015).

Figura 2 – *Arthrospira (Spirulina) platensis* UTEX 1926: (a) e (b) Microscopia e Células da cianobactéria *A. platensis* com aumento de 10x, respectivamente. (c) Cultivo da *A. platensis* crescida em meio Schlösser (1982) em mesa agitadora. (d) Biomassa úmida da *A. platensis* obtida por centrifugação após processo de cultivo.

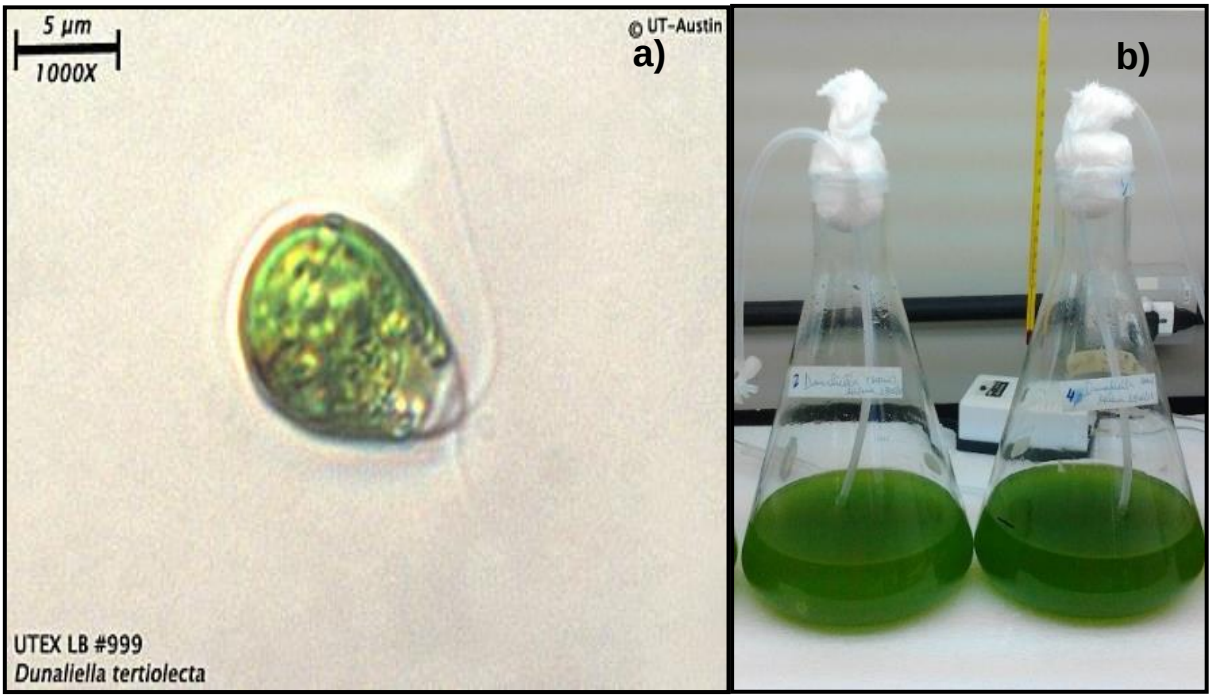


Fonte: (a, UTEX, 2016a; b, c e d, Autor do presente trabalho).

Em termos de aplicações comerciais, esta cianobactéria pode ser explorada pela indústria de alimentos para a produção de suplementos alimentares que promovem benefícios à saúde, uma vez que, estes organismos contêm, por exemplo, ficobiliproteínas que podem apresentar atividades antioxidante e anti-inflamatória, hipocolesterolémica, anti-câncer (BENELHADJ et al., 2016; HERNANDEZ et al., 2017).

A *Dunaliella tertiolecta* (Figura 3), por sua vez, é uma microalga pertencente ao filo *Chlorophyta.* As espécies do gênero *Dunaliella* apresentam características eucarióticas, fotossintéticas, unicelulares, biflageladas, com flagelos do tamanho aproximado ao da célula, bem como são desprovidas de parede celular rígida. O formato da célula da *Dunaliella* varia conforme a salinidade, o que favorece o acúmulo de metabólitos (TINOCO et al., 2015; DUFOSSÉ et al., 2005).

Figura 3 – *Dunaliella tertiolecta* UTEX 999: (a) Microscopia da microalga com aumento de 10x. (b) Cultivo da *Dunaliella tertiolecta* crescida em meio Guillard f/2 (GUILLARD; RYTHER, 1962).

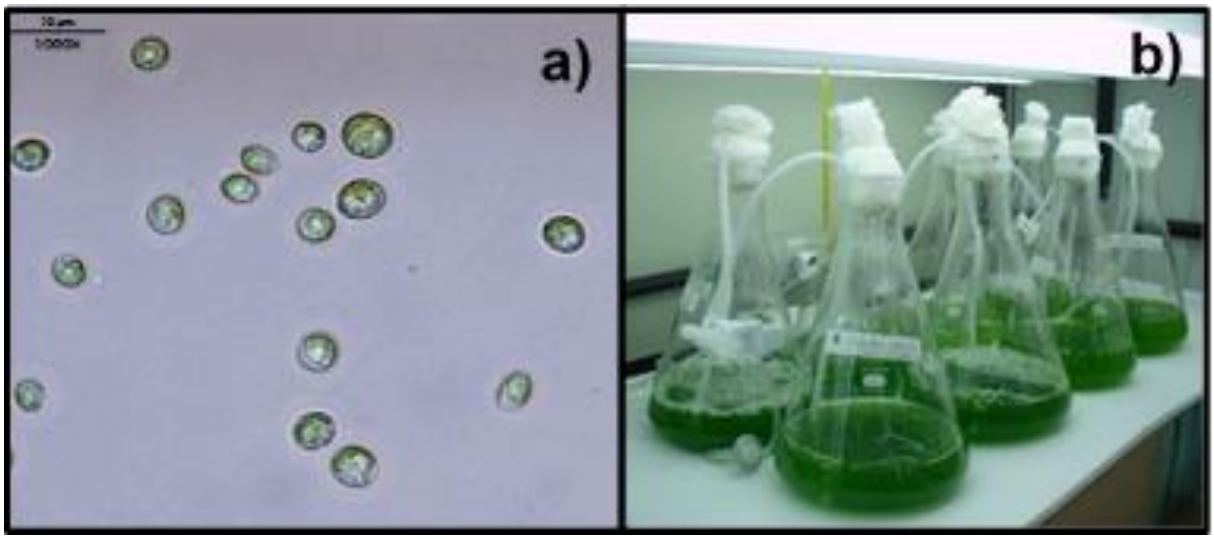


Fonte: (a, UTEX, 2016b; e b, Autor do presente trabalho).

O interesse nas aplicações comerciais desta microalga, em termos de exploração pela indústria alimentícia, tais como suplementos alimentares visando a promoção à saúde, encontra-se, particularmente, nas altas concentrações de carotenóides antioxidantes que este micro-organismo fotossintético apresenta (por exemplo, a luteína, astaxantina, zeaxantina, licopeno ou beta-caroteno) (TINOCO et al., 2015).

A *Chlorella vulgaris* é uma alga unicelular pertencente ao Filo *Chlorophyta*, Classe *Trebouxiophyceae*, Ordem *Chlorellales* e Família *Chlorellaceae* (Figura 4). Essas microalgas são cosmopolitas e apresentam forma de vida celular ou coloniale, possuindo forma esférica, com pequenas células globulares (3–8 μm em diâmetro). Além disso, é composta de diversas substâncias bioativas, tais como proteínas, vitaminas e minerais, polissacarídeos, clorofila e carotenóides alguns imunoestimuladores (CHA et al., 2010).

Figura 4 – *Chlorella vulgaris* UTEX 1803: (a) Microscopia da microalga com aumento de 10x. (b) Cultivo da *C. vulgaris* crescida em meio BBM (Bold's basal medium).

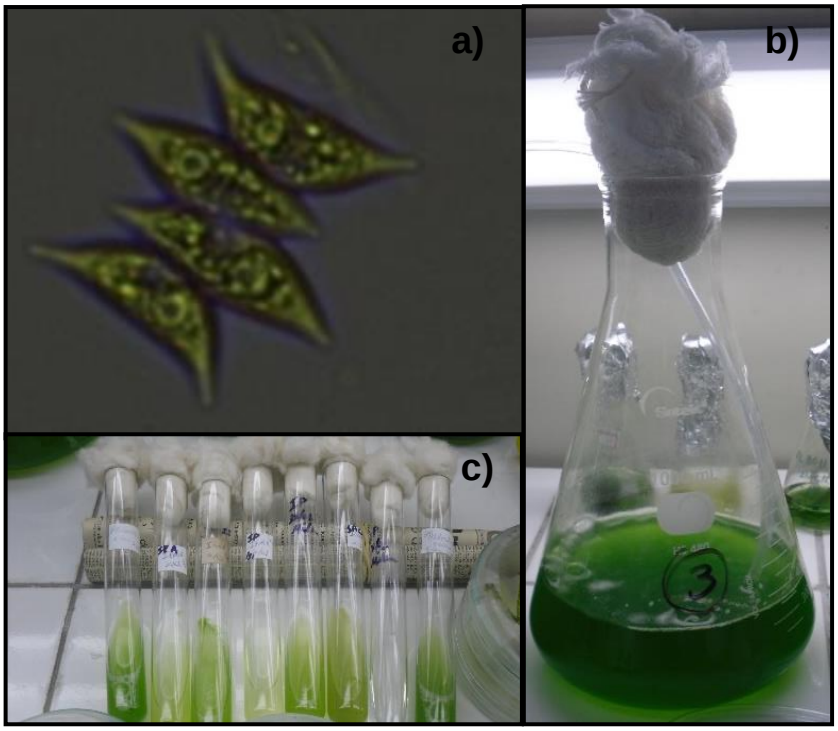


Fonte: (a, UTEX, 2016c; e b, Autor do presente trabalho).

A *C. vulg*aris, no que se refere as aplicações comerciais, é uma das microalgas mais utilizadas na indústria de alimentos funcionais, uma vez que, ela possui um amplo número de antioxidantes e proteínas (STRANSKA-ZACHARIASOVA et al., 2016).

Scenedesmus sp. pertence ao filo *Chlorophyta*, classe *Euchlorophyceae*, Ordem *Chlorococcales* e família *Scenedesmaceae* (Figura 5). É caracterizada como algas coloniais, não móveis, e apresentam altas taxas de crescimento. As espécies desse gênero, geralmente, consistem em 4, 8, 16 ou 32 células que se encontram dispostas de forma alinhada ou alternada. Adicionalmente, suas células são elipsoidais, com extremidades apresentando dois prolongamentos, nomeados de espinhos, que representam diferenciações da parede celular. As aplicações comerciais dessa microalga no campo da indústria alimentícia, despertam particular interesse, pois possui elevadas concentrações de carotenóides antioxidantes, tais como astaxantina e beta-caroteno (KIM et al., 2011).

Figura 5 – *Scenedesmus* sp.: (a) Célula de *Scenedesmus* sp. com aumento de 100x. (b) Cultivo da microalga crescida em meio líquido BG-11 (KIM et al., 2011). (c) Manutenção em tubos de ensaio da microalga *Scenedesmus* sp.



Fonte: (a, b e c, Autor do presente trabalho).

3.2 Cultivo de microalgas e cianobactérias

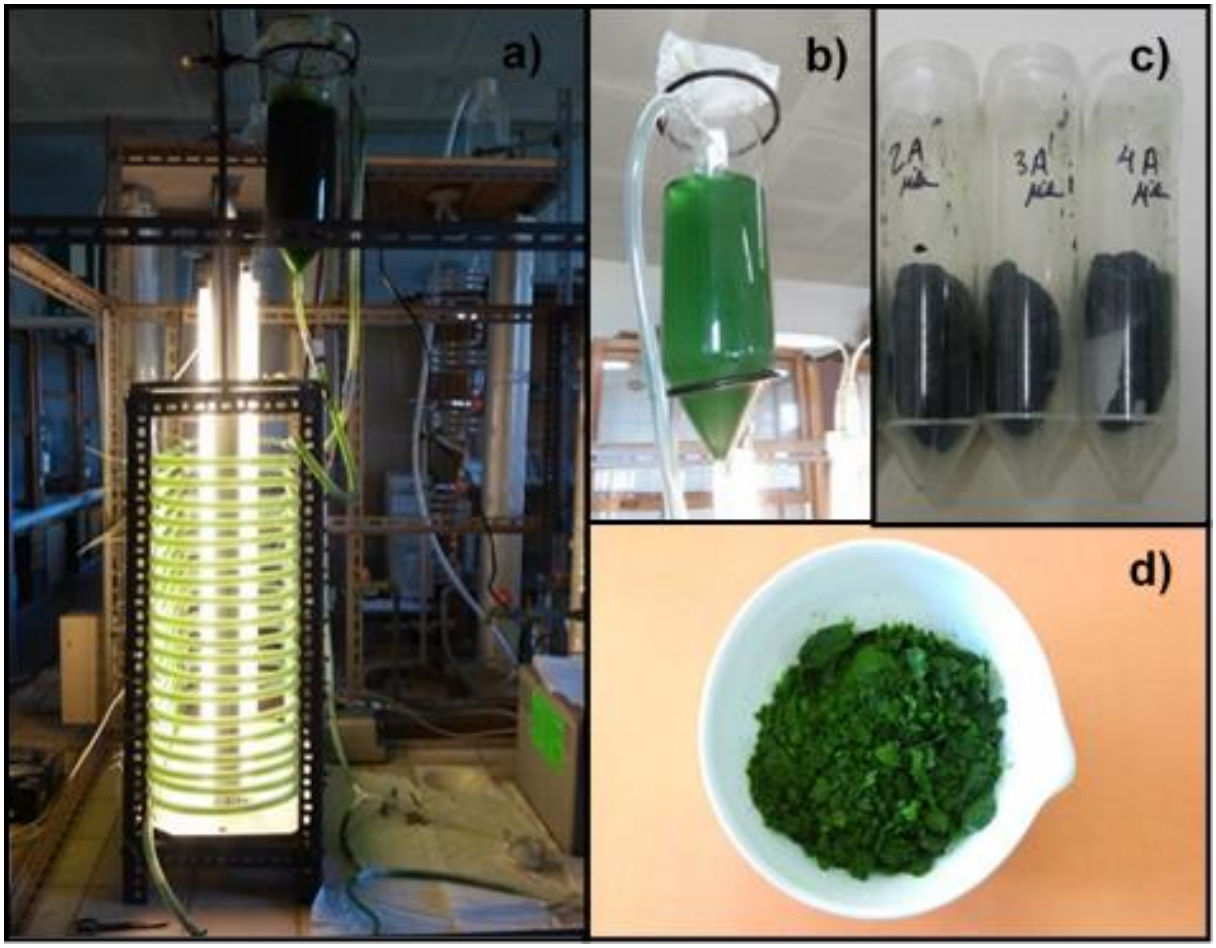
Diversos estudos têm sido desenvolvidos visando o desenvolvimento de tecnologias próprias para a obtenção de biomassa de microalgas e cianobactérias, sendo o cultivo destes micro-organismos fotossintetizantes a principal tecnologia para este fim (BELAY, 2002; PULZ; GROSS, 2004).

Chlorella, *Arthorospira*, *Dunaliela salina e Haematococcus pluvialis* são os principais micro-organismos fotossintetizantes cultivados comercialmente, com a produção para obtenção de biomassa e bioprodutos uma atividade já estabelecida (DERNER et al., 2006).

Particurlamente, os sistemas de cultivo de microalgas e cianobactérias podem ser classificados pelo seu desenho e construção, bem como pelo seu mecanismo de circulação, aeração e interação com o ambiente. Para suprir as necessidades comerciais, o cultivo desses organismos está crescendo progressivamente em todo o mundo, e tem sido realizado em larga escala, em sistemas, geralmente, classificados como fechados (fotobiorreatores) ou abertos (tanques) (LOURENÇO, 2006; UGWU; AOYAGI; UCHIYAMA, 2008; LANLAN et al., 2015).

Os fotobioreatores (PBRs) são sistemas fechados, os quais se classificam como do tipo coluna de bolhas, *air-lift* e arranjos tubulares espirais (Figura 6) e se diferenciam principalmente em função da fonte de luz (natural ou artificial), e de sua interação com o meio (sistemas abertos, fechados ou mistos) (ABOMOHRA et al., 2016).

Figura 6 – Cultivo de microalgas e cianobactérias: (a) Fotobioreator tubular com capacidade de 3,5 L, utilizado para o crescimento de *A. platensis*. (b) Primeiros dias de Cultivo em meio Schlösser (1982) da *A. platensis* em fotobiorreator. (c) Biomassa úmida da *A. platensis* obtida por centrifugação após fase estacionária de crescimento. (d) Biomassa seca após cultivo em fotobiorreator da cianobactéria *A. platensis*.



Fonte: Autor do presente trabalho.

Nos fotobioreatores, o controle das condições de cultivo é mais fácil e o potencial de contaminação é menor em comparação com os sistemas abertos, como resultado da menor exposição da cultura ao ambiente (WANG; LAN; HORSMAN, 2012; FRANCO et al., 2013). Em termos de produção em larga escala em sistemas fechados, apresentam grande potencial para gerar produtos de alto valor agregado (PULZ, 2001; RICHMOND, 2004). Nesse sentido, os sistemas fechados podem garantir aumento de produtividade volumétrica e maior

quantidade e qualidade de biomassa. Entretanto, a utilização desses sistemas implica em maior complexidade técnica, pessoal técnico qualificado e também maior gasto energético para resfriamento das culturas, resultando em maior custo operacional do que os sistemas abertos (RICHMOND, 2004; SPOLAORE et al., 2006)

Os sistemas abertos, por sua vez, são utilizados lagoas e tanques (Figura 7) que utilizam a luz solar e o CO_2 da atmosfera e podem ser construídos e explorados a baixos custos (FRANCO et al., 2013). Em larga escala os sistemas abertos mais utilizados são os tanques retangulares abertos, circulares com um misturador mecânico de cultura e o tipo raceway. Os tanques tipo raceway são responsáveis por mais de 90% da produção mundial de microalgas (OJAMAE, 2011).

Figura 7 – Cultivo de micro-organismos fotossintetizantes em tanques abertos.

Fonte: Savage (2011).

Vale ressaltar que, apesar de serem bastante utilizados, os sistemas de cultivo aberto em tanques do tipo raceways possuem alguns inconvenientes tais como: alta estratificação térmica entre dia e noite, especialmente em zonas temperadas; baixa relação superfície/volume e com isso menor produtividade volumétrica; baixa densidade celular e redução da eficiência de recuperação da biomassa; bem como são mais suscetíveis à

contaminação por espécies indesejáveis (FRANCO et al., 2013). Para Rawat et al. (2013) as lagoas são mais favoráveis em relação aos fotobioreatores pelo menor custo de produção.

A escolha do tipo de cultivo utilizado para a produção dos micro-organismos fotossintetizantes é um dos fatores fundamentais na viabilidade da obtenção de bioprodutos. Portanto, diversos esforços estão sendo realizados a fim de alcançar cultivos em larga escala com alta produtividade e de baixo custo.

3.3 Condições de cultivo

As condições para o cultivo de microalgas e cianobactérias são fatores importantes que influenciam o metabolismo destes organismos, de modo a potencializar a síntese de compostos específicos de interesse (MORAIS et al., 2015).

As diferentes funções desempenhadas pelas microalgas e cianobactérias podem variar substancialmente com as espécies e com as condições de cultivo, tais como intensidade da radiação de luz, temperatura, pH, nutrientes, agitação e fase de crescimento da cultura (VIDOTTI; ROLLEMBERG, 2004; LOURENCO, 2006).

Particularmente, a luz é a fonte de energia fotossintética, e é um dos fatores mais importantes que determina o crescimento de micro-organismos fotossintéticos (BEZERRA et al., 2011). A irradiância de luz deve ser fornecida com cuidado nos sistemas *indoor* utilizando luz artificial, uma vez que, o excesso de luz leva a um fenômeno nomeado "foto-oxidação "ou "fotoinibição". Ou seja, a concentração de células aumenta com a intensidade da luz até atingir um valor máximo, denominado “nível de saturação”. Um aumento adicional na intensidade de luz pode provocar danos no aparato fotossintético das células (BEZERRA et al., 2012).

A configuração do reactor, é um outro fator que pode impctar o crescimento celular. Fotobioreatores podem reduzir a área de cultivo por uma distribuição vertical do organismo fotossintético e ampliar a superfície exposta à luz, garantindo altas relações superfície/volume e aumentando a concentração de células. Assim, a luz é melhor capturada pelas células em fotobiorreatores tubulares, quando comparado com as lagoas abertas convencionais, que provavelmente devido à alta profundidade do meio de cultura, a luz tem que passar por espessas camadas para alcançar as células interiores (CONVERTI et al., 2006; RODRIGUES et al., 2010). Embora a lagoa aberta, é a mais utilizada configuração para a produção comercial de *A. platensis*, fotobioreatores tubulares vêm sendo intensamente estudados, não

apenas por causa de sua elevada produtividade celular, mas também devido a outras vantagens, tais como baixos níveis de contaminação, melhor distribuição de luz e, maior eficiência fotossintética (CONVERTI et al., 2006; ABOMOHRA et al., 2016).

O processo de produção de micro-organismos fotossintéticos envolve diversas áreas científicas, como biologia, metabolismo, engenharia de processos, parâmetros de operação, todos envolvidos com o desenvolvimento de fotobiorreatores e recuperação de células. O crescente aumento da produção mundial de microalgas e cianobactérias demonstra a relevância destes organismos, que foram e estão sendo cultivados com uso de novas tecnologias, visando a redução de custos e elevada produção.

3.4 Biotecnologia de microalgas e cianobactérias: aplicação alimentícia

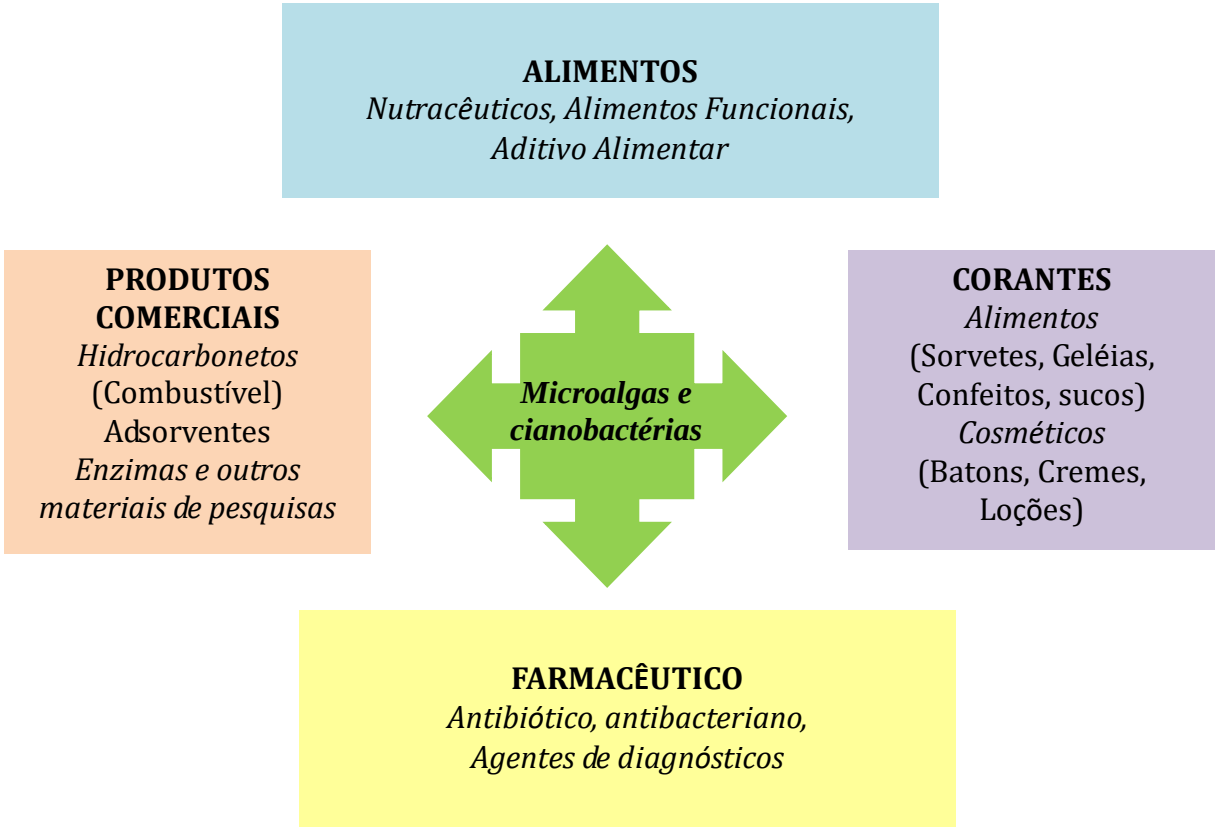
Ao longo dos últimos 30 anos, a biotecnologia de microalgas e cianobactérias tem desenvolvido e diversificado significativamente para diversas aplicações comerciais (VAZ et al., 2016). Microalgas e cianobactérias destacam-se por apresentar características de interesse para a indústria, por exemplo, na indústria farmacêutica, cosmética e alimentícia, devido alguns desses organismos produzirem compostos biologicamente ativos, como antioxidantes e antibióticos (HARUN et al., 2010; BARRA et al., 2014).

Dentre as vantagens no uso da biomassa microalgal, os micro-organismos fotossintéticos podem dobrar sua biomassa variando de 2 a 5 dias em média, podendo atingir elevados rendimentos sem aplicação de pesticidas, herbicidas ou fungicidas. Além disso, não necessitam de terras aráveis para o cultivo, e os requisitos nutricionais para o cultivo desses organismos podem ser encontrados em resíduos industriais, que podem transformar o que é considerado um problema, em matéria-prima para a produção de produtos com alto valor agregado (VAZ et al., 2016).

As microalgas e cianobactérias são eficientes na conversão de energia solar em metabólitos, tais como lipídios, proteínas, hidratos de carbono, pigmentos e vitaminas (BORGES, et al., 2011). Devido à sua enorme biodiversidade e variabilidade na composição bioquímica, bem como estratégias bioquímicas e moleculares para lidar com condições de estresse, aliada ao estabelecimento de tecnologias de cultivo em grande escala; vêm permitindo as microalgas e cianobactérias sintetizar vários produtos bioativos que podem ser utilizados em diversas aplicações (VAZ et al., 2016; DERNER et al., 2006) (Figura 8). Estes micro-organismos fotossintéticos possuem aplicações biotecnológicas em distintas áreas,

sendo sua biomassa microalgal, seca ou úmida, utilizada tanto para nutrição humana e animal, como suplementos alimentares, extração de compostos de alto valor, aquicultura e indústrias de biorremediação e biofertilização, assim como para a produção de biocombustíveis (PULZ; GROSS, 2004).

Figura 8 – Aplicações de microalgas e cianobactérias em diversos campos.



Fonte: Adaptada de Dufossé et al. (2005).

Apesar da aplicação mais comum desses micro-organismos fotossintetizantes está direcionada na aquicultura (alimentação de peixes, moluscos, etc.), diversas microalgas têm sido cultivadas por sua capacidade de sintetizar compostos nutracêuticos (por exemplo, ácidos graxos) e pigmentos carotenóides (astaxantina, betacaroteno, luteína, etc.) que apresentam propriedades terapêuticas, e elevado valor comercial (DERNER et al., 2006).

Na aplicação alimentícia, a comercialização de microalgas foi primeiro constatada por volta de 1960 no Japão, como fonte nutricional. Posteriormente, o elevado teor protéico da espécie *Spirulina* sp. (>60%), conduziu à sua introdução no mercado americano. O aumento da produção de microalgas no contexto de alimentos funcionais se verificou com a avaliação do seu potencial biotecnológico e nutricional visando o mercado nutracêutico (DERNER et

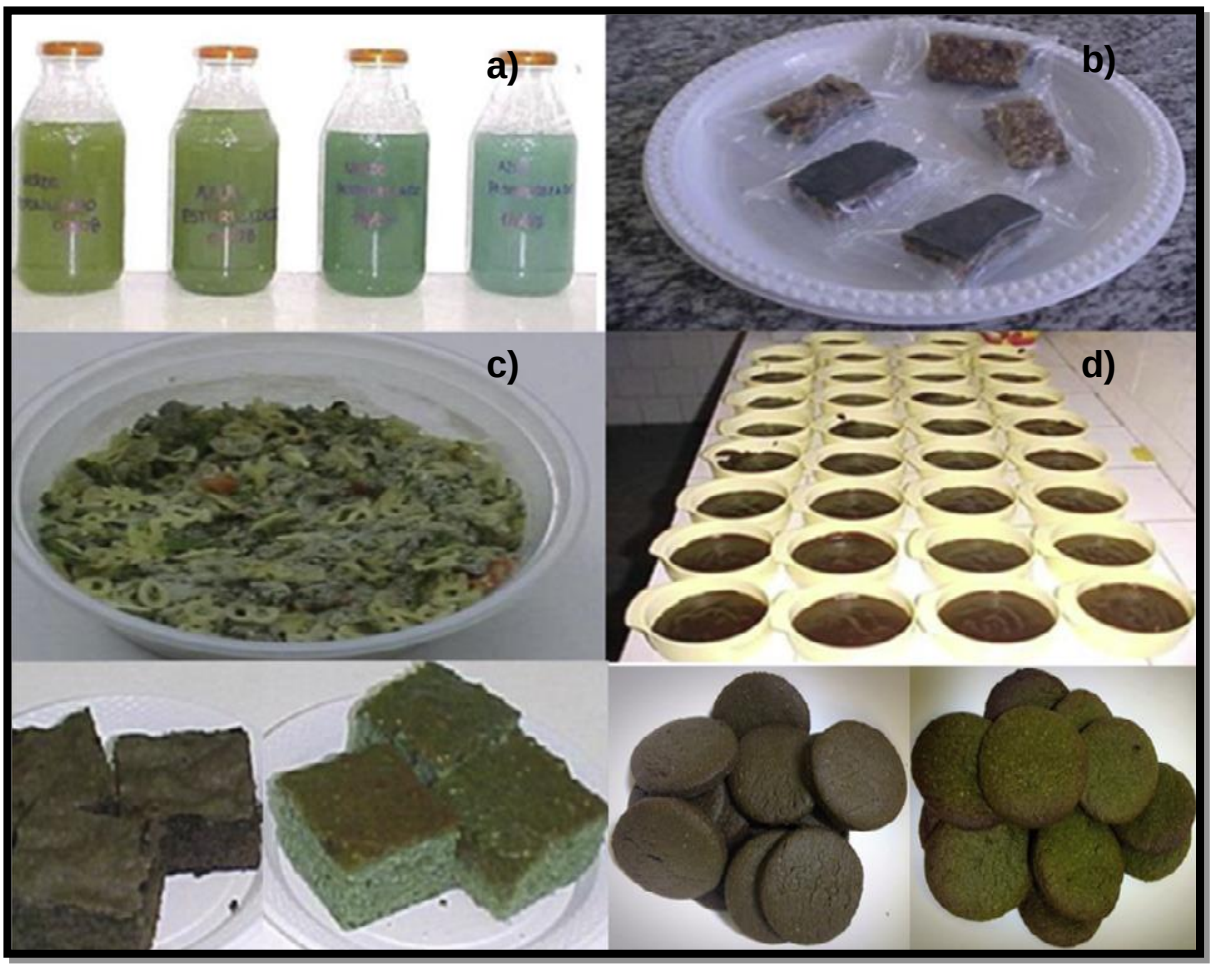
al., 2006; SPOLAORE et al., 2006). Há uma tendência crescente deste mercado, considerando a prosperidade econômica em todo o mundo, bem como o interesse cada vez maior no mundo ocidental por alimentos naturais (DERNER et al., 2006; SPOLAORE et al., 2006).

Nos últimos anos, as preocupações dos consumidores em matéria de saúde e questões de segurança sobre o consumo de alimentos processados têm aumentado. O órgão governamental americano - FDA (Food and Drug Administration), a agência Europeia para a Segurança Alimentar - EFSA (European Food Safety Authority) e outras autoridades mundiais, em termos de controle de alimentos, têm restringido, por exemplo, o uso de corantes sintéticos em alimentos, devido ao aumento no desenvolvimento de câncer ou reações alérgicas. Nesse sentido, a tendência na indústria de alimentos é a utilização de aditivos naturais, e neste cenário, as microalgas e cianobactérias são fontes naturais potenciais desses compotos (VAZ et al. 2016).

Microalgas e cianobactérias são uma das fontes mais interessantes de ingredientes de alimentos e alimentos funcionais, uma vez que, podem ser usadas para melhorar o valor nutricional dos alimentos, devido à sua riqueza em compostos com atributos benéficos (BENELHADJ et al., 2016). A sua composição química balanceada (proteínas de boa qualidade, perfil de ácidos graxos equilibrados, vitaminas, antioxidantes e sais minerais) e os seus atributos valiosos, podem ser aplicados na formulação de novos produtos alimentares (RODRÍGUEZ DE MARCO et al., 2014).

O uso de micro-organismos fotossintetizantes como fonte de alimentos funcionais é uma área prioritária na tecnologia de algas permitindo o estabelecimento de um sistema de produção eficaz com efeitos benéficos ambientais e relacionados à saúde. Diversas pesquisas têm sido realizadas em relação ao desenvolvimento de vários produtos alimentares saudáveis e mais atraentes, preparados a partir de microalgas e cianobactérias (BENELHADJ et al., 2016; RODRÍGUEZ DE MARCO et al., 2014). Produtos alimentares tradicionais, como biscoitos, massas, sobremesas geleificada, e sorvetes (VAZ et al., 2016; RODRÍGUEZ DE MARCO et al., 2014) têm sido desenvolvidos (Figura 9).

Figura 9 – Alimentos enriquecidos com *Spirulin*a sp.: (a) bebidas isotônicas, (b) barras de cereais, (c) sopas instantâneas, (d) pudim, (e) mistura de bolo em pó e (f) biscoitos.



Fonte: Adaptada de VAZ et al. (2016).

De acordo com Richmond et al. (2004), a biomassa das microalgas *Chlorella*, *Dunaliella* e *Scenedesmus*, bem como a cianobactéria *Spirulina*, quando processadas devidamente, apresentam um sabor atraente e podem ser incorporadas em diversos tipos de alimentos, ampliando a demanda no mercado. Adicionalmente, formulações em pó, tabletes, cápsulas ou extratos de *Spirulina* e *Chlorella* podem ser encontrados comercializados como alimento natural ou suplemento alimentar (DERNER et al., 2006).

Na alimentação humana e animal, as microalgas e cianobactérias representam uma fonte suplementar de proteínas, carboidratos, ácidos graxos de elevada importância, como os da família ômega 3 e ômega 6, pigmentos naturais, como os caroténoides, vitaminas, entre outras substâncias capazes de enriquecer o valor nutricional dos alimentos e produzir efeitos promotores à saúde, como melhor controle de peso, fertilidade e melhora nas respostas

imunes (SPOLAORE et al., 2006; DERNER et al., 2006). As microalgas apresentam ainda atividades probióticas e imunomodulatórias (PULZ; GROSS, 2004; SPOLAORE et al., 2006).

3.5 Compostos bioativos de microalgas e cianobactérias: vantagens na obtenção e atividades biológicas

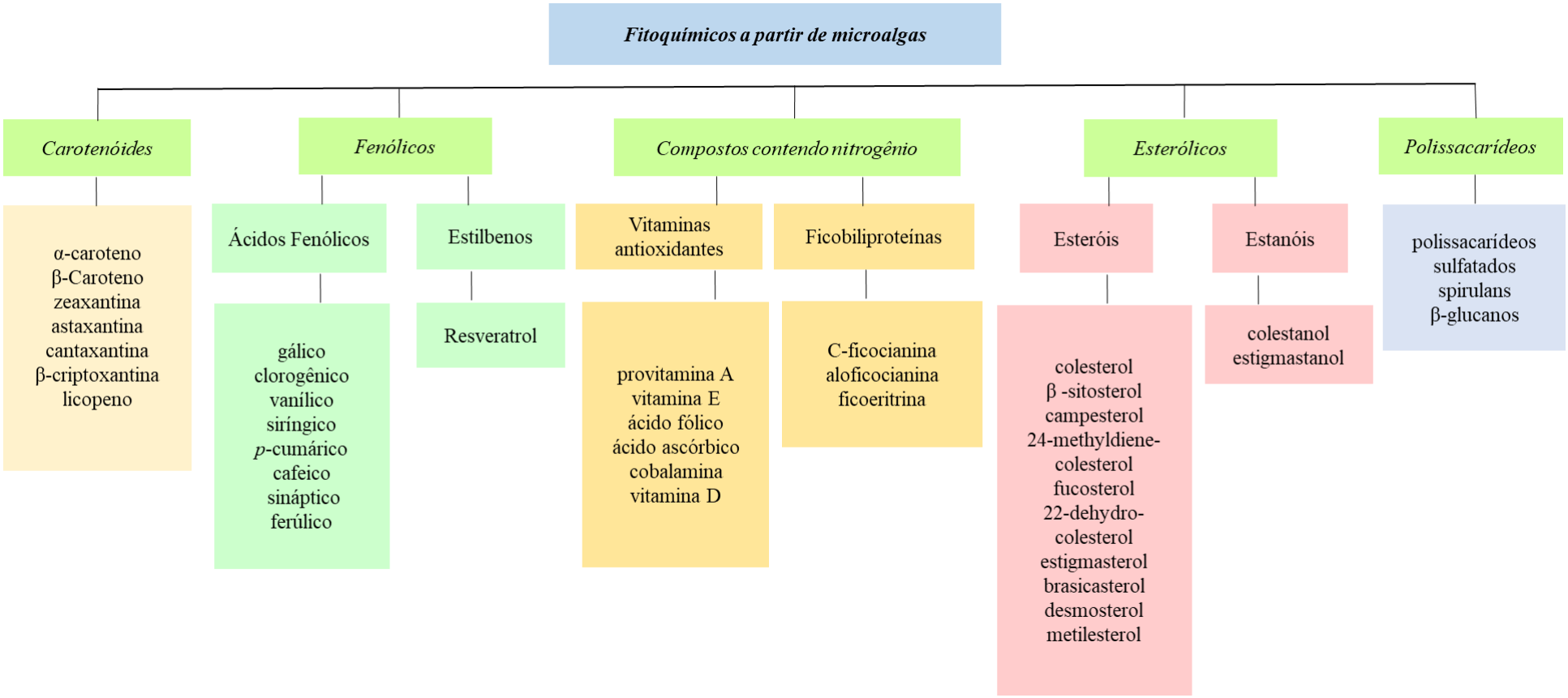
Compostos bioativos são substâncias fisiologicamente ativas com propriedades funcionais no corpo humano (DEL RIO et al., 2013). Nessa linha, os fitoquímicos são compostos bioativos encontrados em frutas, legumes e grãos integrais e não são considerados nutrientes essenciais, mas fornecem benefícios para a saúde (PRAKASH et al., 2012; LIU, 2004). No entanto, estes compostos não são específicos apenas para plantas, também podem ser encontrados em concentrações maiores em microalgas e cianobactérias (PLAZA et al., 2009; RAPOSO; MORAIS 2015).

Estudos epidemiológicos e em animais têm demonstrado previamente os benefícios para a saúde de fitoquímicos como resultado de seus múltiplos efeitos terapêuticos, especialmente, suas atividades anti-inflamatória, antioxidante, hipolipidémicos e efeitos hipoglicémicos e anti-hipertensivos, assim como, a sua capacidade para proteger danos contra o DNA e doenças cardiovasculares; todos os quais associados na redução do risco de doenças crônicas relacionadas ao estresse oxidativo, um grupo de doenças já considerado uma das causas mais comuns de mortalidade prematura em todo o mundo (PRAKASH et al., 2012; GINZBERG et al., 2000; RAPOSO; MORAIS 2015).

No entanto, apesar de sua riqueza em muitos fitoquímicos e fitonutrientes - carotenóides e polissacáridos, tais como beta-glucanos e polissacarídeos sulfatados (SPS); bem como vitaminas antioxidantes e enzimas, compostos fenólicos, esteróis e PUFAs - microalgas e cianobactérias ainda não receberam toda a atenção que merecem. Além disso, o rendimento, qualidade e diversificação de fitoquímicos nesses organismos podem ser facilmente mantidos ou alterados, controlando os seus meios de cultura e condições de crescimento, que podem ser melhorados a fim de elevar as concentrações de compostos específicos (DE JESUS RAPOSO et al., 2013).

Vários fitoquímicos (Figura 10) podem ser obtidos a partir de diferentes espécies ou diferentes linhagens da mesma espécie de microalgas e cianobactérias.

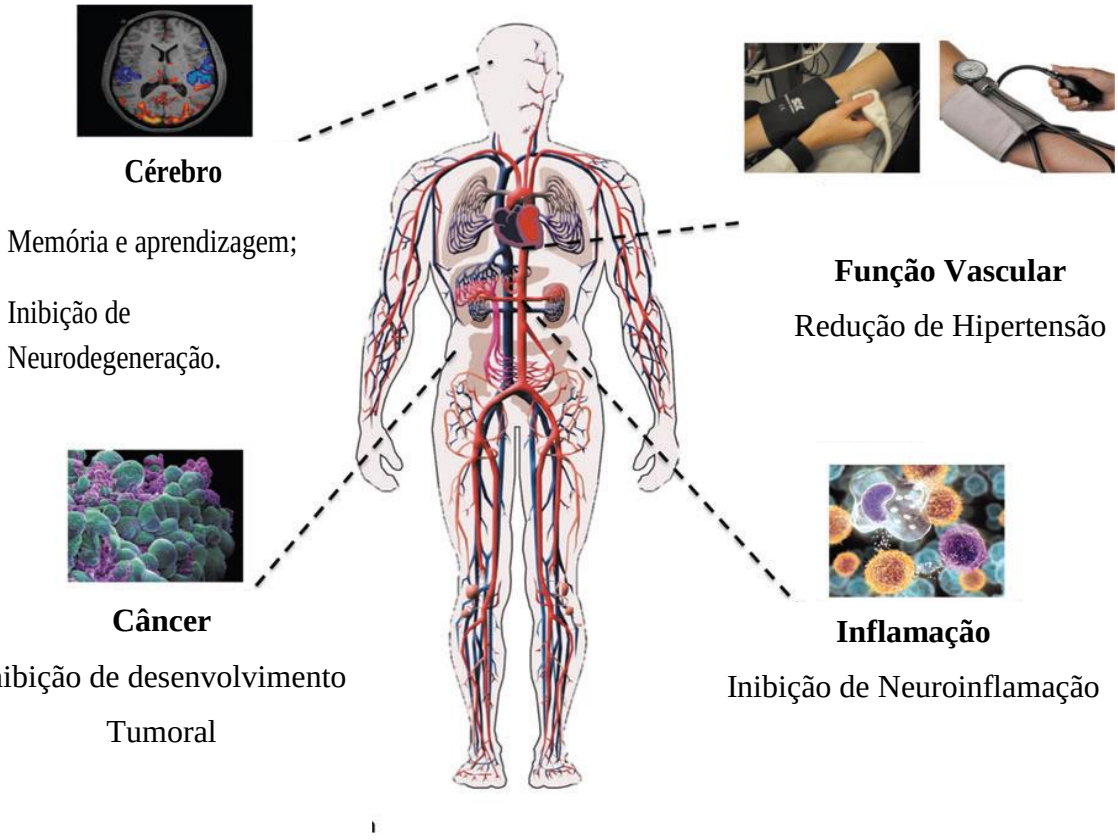
Figura 10 – Fitoquímicos mais conhecidos a partir de microalgas.



Fonte: Adaptado de RAPOSO; MORAIS (2015).

No campo alimentício um elevado entusiasmo para o desenvolvimento e fabricação de diversos biocompostos tem surgido, uma vez que, que podem potencialmente ser utilizados como ingredientes funcionais, tais como carotenóides, ácidos graxos, compostos poli-insaturados e polifenóis (MORAIS et al., 2015). Além disso, Li et al. (2014) têm relatado que estes últimos compostos podem ser usados também como conservantes de alimentos na indústria alimentícia para melhorar a estabilidade, qualidade, segurança e aceitação do consumidor. A figura 11 ilustra os efeitos potenciais da dieta de polifenóis em humanos (DEL RIO et al., 2013).

Figura 11 – Alguns dos potenciais benefícios de saúde da dieta de polifenóis em humanos.



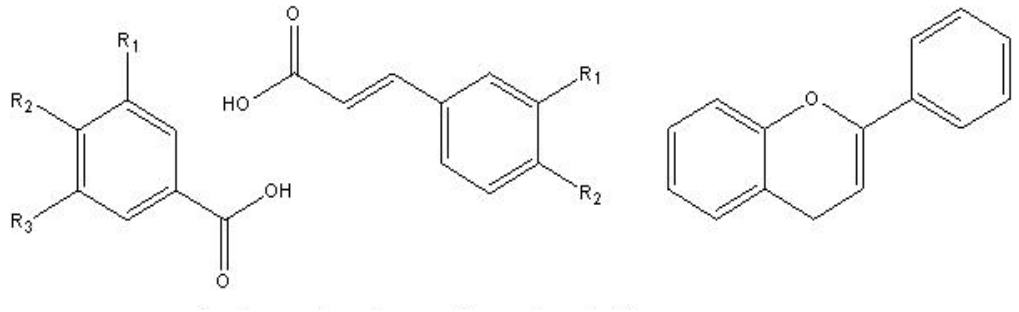
Fonte: Adaptada de Del Rio et al. (2013).

O estresse oxidativo imposto pelas espécies reativas de oxigénio (ROS) desempenham um papel importante em muitas doenças crônicas e degenerativas, tais como cardiovasculares, câncer, envelhecimento, doenças neurodegenerativas e diabetes.

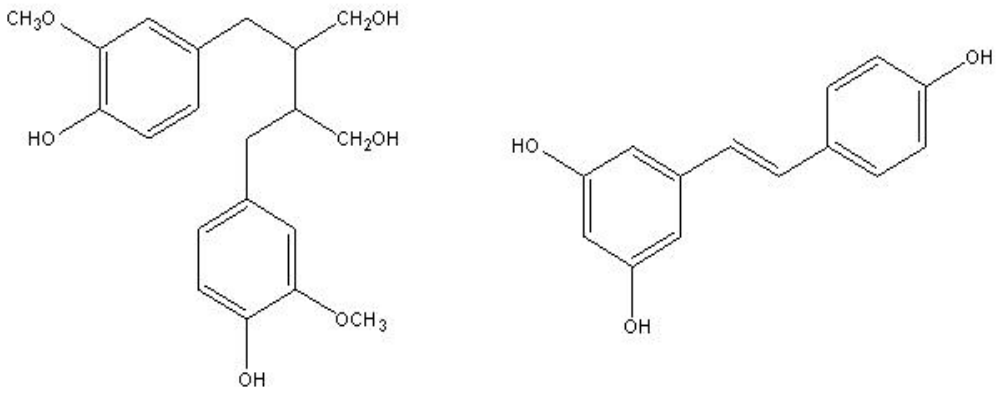
Polifenóis naturais são uma importante categoria de fitoquímicos que têm atraído cada vez mais atenção como potenciais agentes para a prevenção e tratamento de doenças

relacionadas ao estresse oxidativo. Essas substâncias bioativas são metabolitos secundários que existem em plantas, e têm sido consideradas como tendo elevada abilidade antioxidante e capacidade de sequestrar radicais livres, com o mecanismo de inibição das enzimas responsáveis pela produção das ROS. Adicionalmente, mais de 10.000 compostos polifenólicos foram identificados em várias plantas. Em termos de característica estrutural, os polifenóis ocorrem em formas conjugadas, com um ou mais resíduos de açúcar ligados a grupos hidroxilas, mas também existem ligações diretas do açúcar a um átomo de carbono aromático. Ligações com outros compostos, tais como aminas, ácidos orgânicos carboxílicos e, lípidos e, associação com outros fenóis são também comuns. Polifenóis (Figura 12) podem ser divididos em diferentes grupos pelo número de anéis de fenol que eles contêm e com base em elementos estruturais que se ligam a estes anéis, que foram classificados a partir de vários sub-classes, tais como os ácidos fenólicos, flavonóides, estilbenos e lignanas (LI et al., 2014; DEL RIO et al., 2013; NGO et al., 2011).

Figura 12 – Diferentes grupos de polifenóis e suas estruturas químicas.



Ácidos fenólicos (ácidos hidroxi-benzóico e cinâmico) **Flavonóides**

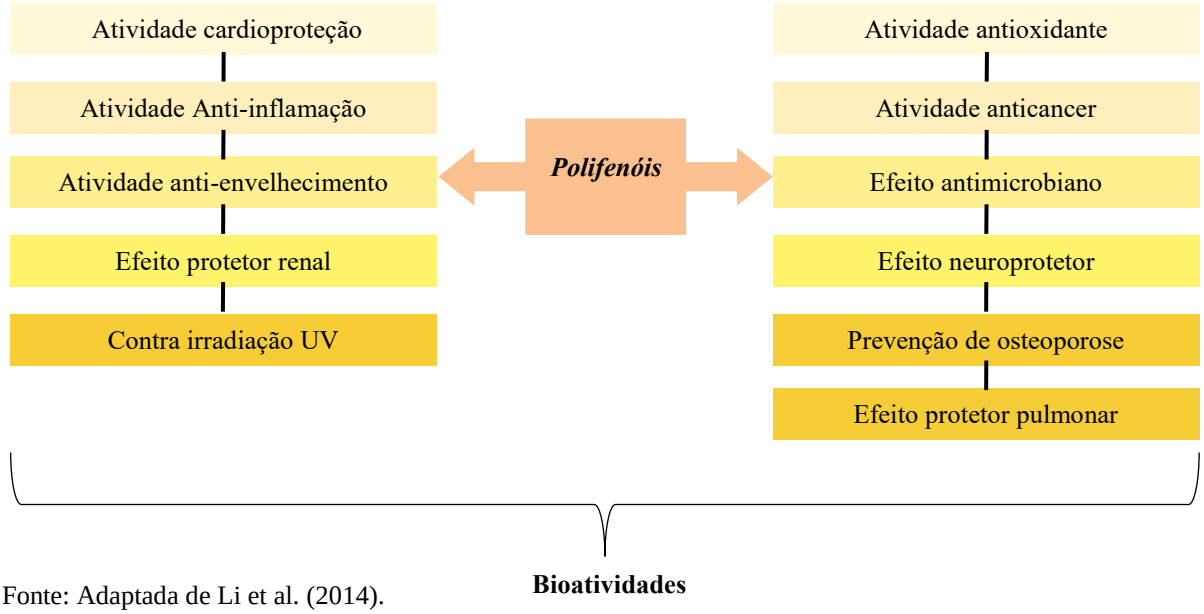


Lignanas **Estilbenos**

Fonte: Adaptada de Li et al. (2014)

Os polifenóis naturais têm demonstrado numerosas atividades biológicas e benéficas à saúde para prevenção e tratamento de doenças relacionadas a idade, câncer e doenças cardiovasculares. Polifenóis têm efeitos sobre adstringência, cor, sabor, odor e estabilidade oxidativa em alimentos. Estes compostos valiosos têm sido amplamente estudados, e verificou-se possuir muitas bioatividades importantes (Figura 13), como antioxidantes e antimicrobinas (LI et al., 2014).

Figura 13 – Algumas bioatividades de polifenóis naturais.



Fonte: Adaptada de Li et al. (2014).

3.5.1 Compostos com função antioxidante

Os antioxidantes são comumente usados para inibir, prevenir ou retardar a deterioração pela oxidação das espécies reativas de oxigénio (ROS), podendo atuar na redução dos radicais livres (antioxidante primário) ou por um mecanismo que não envolve a redução direta dos radicais livres (antioxidante secundário). Assim, o uso de antioxidantes sintéticos, tais como butil-hidroxitolueno (BHT) e butil-hidroxianisol (BHA), deve estar sob rigorosa regulamentação devido a potenciais riscos para a saúde (NGO et al., 2011).

A biomassa de microalgas é considerada uma rica fonte de antioxidantes naturais, tais como pigmentos, lípidos e polissacáridos, com potenciais aplicações em alimentos, cosméticos e medicina. Os antioxidantes primários, como os compostos fenólicos, são encontrados em extratos de microalgas e cianobactérias de diferentes grupos taxonômicos

(SCHOLZ; LIEBEZEIT, 2012). Assim, estes compostos são importantes no metabolismo das plantas e tem se tornado importante para a saúde humana devido as suas notáveis bioatividades, particularmente, atividades antioxidantes, as quais têm sido amplamente estudadas, incluindo o sequestro de radicais livres, inibição da oxidação lipídica, redução da formação de hidroperóxido, entre outras (PLAZA et al., 2012).

Os polifenóis podem também funcionar como antioxidantes por meio dos seus efeitos sobre o plasma, membranas, fatores de transcrição e as atividades de enzima *in vivo*. Por exemplo, fenólicos como a catequina, epicatequina e galato, induzem o efeito de dose-resposta na atividade antioxidante do plasma. Muitos polifenóis sequestram os radicais livres através do mecanismo de transferência de átomos de hidrogênio, devido a energias mais elevadas que estão envolvidas no processo de transferência de um único elétron. Polifenóis podem quelar metais de transição através de seus vários grupos OH e a unidade carbonila, quando presentes (LI et al., 2014).

3.5.2 Compostos com função antimicrobiana

As microalgas e cianobactérias têm demonstrado atividade antimicrobiana com potencial uso como aditivos alimentares antibacterianos para preservação de alimentos, uma vez que, a tendência atual adotada pela produção de alimentos, gabinetes legislativos e os consumidores, tem exigido um recuo progressivo da química de aditivos sintéticos nos sistemas de conservação de alimentos, buscando substâncias alternativas, a fim de obter conservantes naturais contra a deterioração de alimentos e previnir doenças causadas por micro-organismos (CASTILHO et al., 2012; MENDIOLA et al., 2007).

Estes micro-organismos fotossintéticos são fontes promissoras de antibióticos e com uma atividade antibacteriana eficaz. A atividade antimicrobiana destes organismos é devido à capacidade em sintetizar compostos, tais como ácidos graxos, compostos alifáticos halogenados, terpenóides, esteróis, compostos heterocíclicos contendo enxofre, hidratos de carbono, acetogeninas e polifenóis (MORAIS et al., 2015; PRAKASH et al., 2011; LI et al., 2014).

A atividade antimicrobiana de extratos a partir de microalgas e cianobactérias está envolvida com a sua composição lipídica. A ação antimicrobiana de microalgas também é notável por causa do potencial em produzir compostos tais como α- e β-ionona, β-ciclocitral, neofitadieno e fitol. A atividade antibacteriana destes micro-organismos fotossintéticos contra

patogénios humanos, tais como *Escherichia coli, Pseudomonas aeruginosa, Staphylococcus aureus e Staphylococcus epidermidis*, tem sido atribuída ao ácido γ-linolénico (GLA), ácido eicosapentaenóico, ácido hexadecatrienoico, docosahexaenóico, ácido palmitoléico, ácido láurico, ácido oléico, ácido láctico, e o ácido araquidônico (AMARO et al., 2011).

Baseado neste contexto, a produção de compostos bioativos naturais de interesse alimentício a partir de microalgas e cianobactérias visando aplicações funcionais com efeitos benéficos para a saúde humana, promove perspectivas futuras com potencial para o desenvolvimento sustentável (VAZ et al., 2016). Dessa forma, estas constatações motivaram o desenvolvimento deste trabalho de modo a investigar o potencial biotecnológico de micro-organismos fotossintetizantes na produção de compostos bioativos com propriedades antioxidantes e antibacterianas de interesse na indústria de alimentos.

CAPÍTULO I

4 ARTIGO - SCREENING OF PHOTOSYNTHETIC MICROORGANISMS PRODUCING OF COMPOUNDS WITH ANTIOXIDANT AND ANTIBACTERIAL ACTIVITIES OF INTEREST IN THE FOOD INDUSTRY

Este trabalho será submetido a revista *International Journal of Food Science & Technology* com fator de impacto igual a 1.64 (2016), o qual segue formatado conforme suas normas. Qualis Capes B2 (Biotecnologia). Anexo A.

Screening of photosynthetic microorganisms producing of compounds with antioxidant and antibacterial activities of interest in the food industry

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Summary

In order to promote natural sources of the bioactive molecules, this study aims to assess the potential of photosynthetic microorganisms producers of compounds with antioxidant and antibacterial activities. Aqueous and ethanolic extracts of freeze-dried biomass of the microalgae *Chlorella vulgaris, Dunaliella salina* and *Scenedesmus* sp. and the cyanobacterium *Arthrospira (Spirulina) platensis* obtained by classic solid-liquid extraction were screened for their in vitro antioxidant capacity by ABTS and DPPH free radical-scavenging assays as well as their antibacterial properties against *Escherichia coli* ATCC 25922, *Pseudomonas aeruginosa* ATCC 27853, *Enterococcus faecalis* ATCC 6057, *Bacillus subtilis* ATCC 6633 and *Staphylococcus aureus* ATCC 6538, which are the main spoiling agents in the food industry. The obtained results indicate that the ethanolic extracts of all the tested species exhibited higher antioxidant and antibacterial activities, when compared to aqueous extract. These results suggest that the better part of the bioactive compounds these extracts is hydrophobic and can better be extracted with organic solvente. Among the studied ethanolic extracts, the one of *A. platensis* exhibited the highest antioxidant activity by both ABTS (66.09%) and DPPH (55.73%) assays as well as effective antibacterial activity mainly against *Pseudomonas aeruginosa* ATCC 27853, *Enterococcus faecalis* ATCC 6057 and *Bacillus subtilis* ATCC 6633. These results demonstrated that the ethanolic crude extract of *A. platensis* could be used as a potential source of natural antioxidants and antibacterial, and therefore it may be considered as an accessible and safe alternative to synthetic compounds and a valuable tool for the biotechnology field.

Keywords: Microalgae, antioxidative and antibacterial activity, photosynthetic microorganisms, bioactive compounds.

Introduction

Photosynthetic microorganisms such microalgae and cyanobacteria constitute an important source for biomolecules with a wide range of applications that can be harnessed for commercial use (Maadane *et al*., 2015). They can produce vitamins, proteins, lipids, pigments and other molecules exploited for health, cosmetics and for energy production, food and feed additives (De Jesus Raposo *et al.,* 2013; Barra *et al.*, 2014).

Microalgae and cyanobacteria have gained an increasing interest due to the fact that they represent one of the most promising sources of compounds with biological activity that could be used as functional ingredients (Pulz & Gross, 2004; Rodríguez De Marco *et al*., 2014). Thus, functional foods are a priority area in algal technology permitting establishment of a cost effective microalgae production system with environmental and health-related beneficial effects (Benelhadj *et al*., 2016).

One of the main interests in Food Science and Technology is the extraction and characterization of new functional ingredients of natural origin. These biological active ingredients can be used not only as natural preservatives against food degradation, but also can be added to food as a functional ingredient able to promote our health (Rodríguez-Meizoso *et al*., 2010).

There is a growing worldwide interest toward finding new, safe and powerful antioxidants from natural sources in order to minimize oxidative damage to living cells and prevent oxidative deterioration in commercialized products such as food, pharma-ceuticals or cosmetics. Antioxidants compounds exert their action through different mechanisms including prevention of chain initiation, chelating of transition metal ion catalysts, decompositionof peroxidases, prevention of continued hydrogen abstraction andradical scavenging (Maadane *et al*., 2015).

Microalgae and cyanobacteria are a very interesting natural source of new compounds such as antioxidants and antimicrobials. In fact, some algae are organisms that live in complex habitats submitted to extreme conditions (for example, changes of salinity, temperature, nutrients, UV/vis irradiation), therefore, they must adapt rapidly to the new environmental conditions to survive, producing a great variety of secondary (biologically active) metabolites, which cannot be found in other organisms. Also, considering their great taxonomic diversity, investigations related to the search of new biologically active compounds from these organisms can be seen as an almost unlimited field (Rodríguez-Meizoso *et al*., 2010).

Based on this background, to promote natural sources of the bioactive molecules, this study aims to investigate the potential of photosynthetic microorganisms producers of compounds with antioxidant activity and antibacterial.

Materials and methods

Chemicals

All chemicals and reagents were of analytical grade and purchased from Sigma–Aldrich (St. Louis, MO, USA).

Microorganism and culture conditions

Three microalgae (*Chlorella vulgaris* UTEX 1803, *Dunaliella tertiolecta* UTEX 999 and *Scenedesmus* sp.) and the cyanobacterium *Arthrospira (Spirulina) platensis* UTEX 1926 were selected for this study. All microalgae and the cyanobacterium (execpt *Scenedesmus* sp. which was isolated from "Açude do Prada" in Recife-PE) were acquired by the Culture Collection of Algae at the University of Texas (Austin, TX, USA).

Microalgae were grown under constant aeration, except *A. platensis* with shaking frequency of 100 rpm, initial inoculum of 50 mg L^{-1}, temperature at 30 ± 1 °C, light 70± 1 µmol photons m^{-2} s^{-1} with white lamps and erlenmeyer flasks (1 L) containing 400 mL of Bold's basal medium (BBM) liquid proposed by Stein, J. (Ed.), Guillard f/2 medium (Guillard & Ryther, 1962), BG-11 liquid medium (Kim *et al*., 2011) and medium suggested by Schlösser (1982) per *Chlorella vulgaris* UTEX 1803, *Dunaliella tertiolecta* UTEX 999, *Scenedesmus* sp. and *A. platensis*, respectively.

The growth was monitored daily by measuring the optical density (OD) through a calibration curve relating OD to dry biomass weight at 685 nm, 680nm, 650 and 560nm per *Chlorella vulgaris* (Xu *et al*., 2008), *Dunaliella tertiolecta* (El Arroussi *et al*., 2015), *Scenedesmus* sp. (Xin *et al*., 2011) and *A. platensis* (Leduy & Therien, 1977), respectively. The biomass was harvested at the stationary phase by centrifugation at 10. 000 rpm for 10 min at 4 °C and then freeze-dried and stored at −20◦ until extraction.

Extraction procedure

Different extracts (aqueous, in distilled water and pure ethanolic) were obtained from dried cell biomass of the species mentioned in the section *2.2.*, with classic solid-liquid extraction.

For antioxidant activity was used a cell concentration of 10.00 mg mL^{-1} and for antibacterical activity was used 100 mg mL^{-1} of dried biomass. The samples were extracted by sonication (40 kHz, 400 W, Branson – P/S) with pulses of 60 s and 60 s off for 20 min on ice bath to prevent overheating, followed by centrifugation (15.000 rpm) for 10 min at 4 °C to obtain the supernatant liquid. The extraction was performed in the dark at room temperature (25 °C). After extraction, ethanolic extracts were concentrated under reduced pressure in a rotary evaporator, while aqueous extracts were then lyophilized. All extracts were stored at –20◦C until use.

Antioxidant activity using 2,2-azino-bis-(3-ethylbenzothiazoline)- 6-sulfonic acid (ABTS)

The antioxidant activity of the extracts was measured according to their ability to scavenge ABTS radical cation, as described by (Re *et al*., 1999). The mixture was incubated in the dark at room temperature (25°C) for 12–16 h (time required for radical formation) prior to use. The absorbance signal was measured at 734 nm. Trolox (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid) was used as the reference standard. The values were calculated and expressed as percentages of radical scavenging activity (RSA). The ABTS radical activity was calculated using the following equation:

Radical scavenging activity (%) = $[1-(A_{sample}-A_{sampleblank})/A_{control}] \times 100$.

A_{sample}: is the absorbance of ethanolic ABTS solution with the presence of all of the extract samples and standard. $A_{sampleblank}$: is the absorbance of extracts sample in ethanol (without ABTS in order to subtract the absorbance of colored extracts). $A_{control}$: is the absorbance of the ethanolic ABTS solution.

DPPH radical-scavenging activity

The DPPH (1,1-diphenyl-2-picryl-hydrazil) radical-scavenging activity was determined following the method developed by Brand-Williams *et al*. (1995) and modified by Fukumoto & Mazza (2000). In a 96-well microplate, 22 μL of each extract at various concentrations were mixed with 200 μL of a DPPH solution (25 mg L^{-1}) prepared fresh daily. Because of the turbidity of the extracts, the blanks were prepared after mixing 22 μL of extract at various concentrations with 200 μL of ethanol. The reaction was developed for 2 h in the dark at room temperature, and subsequently the absorbance was read at 517 nm using a Microplate Lector

LM-LGC (LGC Biotechnologies Ltda, São Paulo, Brazil). The DPPH concentration in the reaction medium was calculated from a calibration curve to further deduce the percentage of remaining DPPH (% DPPH). Ascorbic acid was used as a positive control. The DPPH radical activity was calculated using the following equation:

Radical scavenging activity (%) = $[1-(A_{sample}-A_{sampleblank})/A_{control}] \times 100$.

A_{sample}: is the absorbance of ethanolic DPPH solution with the presence of all of the extract samples and standard. $A_{sampleblank}$: is the absorbance of extracts sample in ethanol (without DPPH in order to subtract the absorbance of colored extracts). $A_{control}$: is the absorbance of the ethanolic DPPH solution.

Antibacterial activity

Microdilution method was used to determine antibacterial activity (NCCLS, 2003). A panel of microorganisms of importance for the food industry, including *Escherichia coli* ATCC 25922, *Pseudomonas aeruginosa* ATCC 27853, *Enterococcus faecalis* ATCC 6057, *Bacillus cereus* ATCC 33019 and *Staphylococcus aureus* ATCC 6538 were used.

The pre-inoculum for each standard strain was prepared in Müller Hinton broth. All strains were aerobically incubated at 37°C for 18 h. The 0.5 McFarland standard was prepared and used to approximate the concentration of the bacteria (1.5×10^8 CFU mL^{-1}). The negative controls contained no bacteria, and the positive controls contained bacteria and Müller Hinton broth. The samples contained 50 μL of the microalgae extracts. The microalgae extracts dilutions were ranging from 100 mg/mL to 5 mg/mL.

All experiments were performed in triplicate in a 96-well microplate (NUNC®), and the absorbance at 630 nm (Microplate Lector LM-LGC, LGC Biotecnologia Ltda, São Paulo, Brazil) was read after incubation for 24 h. The result was determined as the percentage inhibition.

Statistical analysis

Three replicates of each sample were used for statistical analysis and the values were reported as value ± the standard deviation. The Statistica v. 8.0 software (StatSoft, Tulsa, OK, USA) was used for data analysis. The results were considered statistically significant with p values ≤ 0.05.

Results and discussion

A screening of different extracts (aqueous and ethanolic) obtained from dried biomass of the microalgae *Chlorella vulgaris, Dunaliella salina* and *Scenedesmus* sp. and the cyanobacterium *Arthrospira (Spirulina) platensis* was carried out in order to examine their *in vitro* antioxidant activity, as well as antibacterial properties.

Two different methods were selected to determine the antioxidant activities of aqueous and ethanolic extracts from microalgae and cyanobacterium tested in this study. $ABTS^{\bullet+}$ radical decolorisation assay, one of the most commonly employed methods for measuring antioxidant capacity, which measures the ability of a compound to scavenge $ABTS^{\bullet+}$ radical. It is recommended for use in plant extracts because the long wavelength absorption maximum at 734 nm eliminates colour interference in plant extracts (Li *et al*., 2007). On the other hand, DPPH• is a purple stable radical that turned out yellowish when reacts with antioxidant analytes, and the degree of discoloration indicates the scavenging potentials of the antioxidant extract. The activity of the extracts is attributed to their hydrogen donating ability (Santoyo *et al*., 2006; Mendiola *et al*., 2007).

ABTS and DPPH radical scavenging percentages are presented in Table 1. According to the obtained results, all tested microalgae extracts exhibited various degrees of antioxidant activity whatever the assays at concentrations of 10 mg mL^{-1}. However, the tested ethanolic extracts displayed higher antioxidant activities than aqueous extracts.

The aqueous extracts of the microalgae and the cyanobacterium presented 7.2 to 25.7% and 5.4 to 16.7% of antioxidant activity according wiith ABTS and DPPH radical scavenging percentages, respectively. On the other hand, ethanolic extracts showed highest radical scavenging activity with 28.1 to 66.0% and 11.2 to 55.7% of antioxidant activity according with ABTS and DPPH methods, respectively.

Table 1 Antioxidant activity of the microalgae and cyanobacteria extracts (aquous and ethanolic) at concentration of 10 mg mL^{-1} performed by ABTS (Ácido 2,2'-azino-bis [3-etilbenzotiazolina-6-sulfônico] sal diamônio) and DPPH (1,1-diphenyl-2-picrylhydrazyl) radical scavenging assays. Ácido 6-hidroxi-2,5,7,8-tetrametil-cromano-2-carboxílico (Trolox) and ascorbic acid (Vit C) are used as positive control at concentration of 100 µg/ml.

Radical scavenging activity, RSA (%)		
Method ABTS	Aqueous extract	Ethanolic extract
Arthrospira platensis	25.73±0.18	66.09±2.00
Chlorella vulgaris	13.75±0.33	31.26±4.00
Dunaliella tertiolecta	7.23±0.49	28.16±0.01
Scenedesmus sp.	19.07±0.11	42.41±1.00
Trolox		90.20±0.15
Method DPPH	Aqueous extract	Ethanolic extract
Arthrospira platensis	16.73±0.12	55.73±0.18
Chlorella vulgaris	10.75±0.31	23.75±0.33
Dunaliella tertiolecta	5.43±0.24	11.23±0.49
Scenedesmus sp.	13.07±0.1	39.07±0.11
Vit. C		85.40±0.16

Means ± standard deviation of three experiments measurements.

The highest radical scavenging activity (RSA) was obtained with ethanolic extracts of *Arthrospira (Spirulina) platensis*, exhibiting the RSA value of 66.09% ± 2.00 (ABTS method) and 55.73% ± 0.18 (DPPH assays) at a concentration of 10 mg/mL. Trolox (Ácido 6-hidroxi-2,5,7,8-tetrametil-cromano-2-carboxílico) and ascorbic acid (Vit C) used as positive control showed an RSA of 90.2% and 85.4% at a concentration of 100 µg/mL, respectively. Our results corroborates to those of Pereira *et al*. (2015) that reported highest values of RSA (DPPH assay) in species of *Nannochloris* sp. SBL1 and *Desmochloris* sp. SBL3 with values of 60% and 61%, respectively, with the same concentration of this study (10 mg/mL). These results suggest that *A. platensis* species may be sources of compounds with anti-radical properties.

In addition, ethanolic extracts of *Scenedesmus* sp. and *Chlorella vulgaris* presented a considerable antioxidant activity with RSA values of 42.4% ± 1.00 and 31.2% ± 4.00 per ABTS methods and 39.0% ± 0.11 and 23.75% ± 0.33 with DPPH assay (Table 1).

Moreover, the results obtained in the present study are also in agreement with Maadane *et al*. (2015), who examined antioxidant activity using the DPPH radical from ethanolic extracts of *Dunalliela* sp., *Tetraselmi*s sp. and *Nannochloropsis gaditana* in which all presenting the ability of scavenging DPPH above of 80%. These authors suggested that the antioxidant compounds of microalgae could have different polarities, thus the antioxidant capacity of microalgae are strongly influenced by the extracting solvent.

In the literature, there are a number of reports on antioxidant capacity of some species belonging to the genera of *Chlorella* (Li *et al*., 2007), *Dunaliella* (Herrero *et al*., 2006), *Tetraselmis* (Custódio *et al*., 2014), *Phaeodactylum* (Guzmán *et al*., 2001) and *Navicula* (Hemalatha *et al*., 2013). Thus, when comparing the obtained data with other studies described in literature, should takes in consideration that antioxidant compounds compositions are remarkably influenced by microalgae growth conditions (nutrients availability, temperature, stress application) (Maadane *et al*., 2015). Furthermore, the extracting solvents used affect significantly antioxidant content and the ethanol might dissolve more radical scavenging active than other solvents (López *et al*., 2011; Goiris *et al*., 2012). Thus, Santoyo *et al*. (2006) reported that the ethanol turned out to be the most suitable solvent in extracting antioxidant components from *S. platensis* since ethanol extracts showed a high antioxidant activity together with a high extraction yield. Besides, ethanol is considered as safe (GRAS solvent).

As can be seen in Tables 2 and 3, the effects of different extracts (aqueous and ethanolic) on the antibacterial activity were also evaluated in this study. Thus, five different strains, including two gram negative bacteria (*Escherichia coli* ATCC 25922 and *Pseudomonas aeruginosa* ATCC 27853) and three gram positive bacteria (*Enterococcus faecalis* ATCC 6057, *Bacillus subtilis* ATCC 6633 and *Staphylococcus aureus* ATCC 6538) were used to screen the potential antibacterial activity of the aqueous and ethanolic extracts obtained from dried biomass of the microalgae *Chlorella vulgaris, Dunaliella salin*a and *Scenedesmus* sp. and the cyanobacterium *Arthrospira (Spirulina) platensis.*

Table 2 Inhibition percentage (%) of antibacterial activity of the aqueous extracts of microalgae and cyanobacteria.

		Arthrospira platensis		***Chlorella vulgaris***		***Dunaliella tertiolecta***		***Scenedesmus* sp.**	
		100	**50**	**100**	**50**	**100**	**50**	**100**	**50**
Strains	*Escherichia coli* ATCC 25922	ND	ND	ND	ND	ND	ND	ND	ND
	Pseudomonas aeruginosa ATCC 27853	40.0±0.01	ND	30.0±0.01	23.3±0.01	20.0±0.01	13.7±0.01	25.0±0.01	ND
	Enterococcus faecalis ATCC 6057	20.3±0.05	18.9±0.03	19.0±0.03	ND	ND	ND	17.6±0.05	13.9±0.05
	Bacillus subtilis ATCC 6633	34.8±0.14	ND	ND	ND	ND	ND	10.8±0.14	ND
	Staphylococcus aureus ATCC 6538	ND	ND	ND	ND	ND	ND	ND	ND

Cell extract concentration values given as mg/mL. ND = Not detected.

Table 3 Inhibition percentage (%) of antibacterial activity of the ethanolic extracts of microalgae and cyanobacteria.

		Arthrospira platensis		***Chlorella vulgaris***		***Dunaliella tertiolecta***		***Scenedesmus* sp.**	
		100	**50**	**100**	**50**	**100**	**50**	**100**	**50**
Strains	*Escherichia coli* ATCC 25922	40.0±0.01	ND	ND	ND	ND	ND	ND	ND
	Pseudomonas aeruginosa ATCC 27853	80.0±0.01	74.4±0.02	60.0±0.01	53.3±0.01	30.0±0.01	23.7±0.01	72.7±0.01	62.0±0.01
	Enterococcus faecalis ATCC 6057	78.3±0.03	59.9±0.04	70.0±0.03	ND	37.0±0.02	ND	71.1±0.02	60.1±0.05
	Bacillus subtilis ATCC 6633	64.8±0.14	ND	34.8±0.14	ND	24.8±0.14	ND	54.8±0.14	ND
	Staphylococcus aureus ATCC 6538	ND	ND	ND	ND	ND	ND	ND	ND

Cell extract concentration values given as mg/mL. ND = Not detected.

The results obtained showed that the ethanolic extracts (Table 3) of all the tested photosynthetic microorganisms exhibited highest percentage of inhibition of antibacterial activity against most of the bacterial species examined (*Pseudomonas aeruginosa* ATCC 27853, *Enterococcus faecalis* ATCC 6057 and *Bacillus subtilis* ATCC 6633). This effect was most evident at cell extract concentration at 100mg/ml, when compared to aqueous extracts (Table 2). This may occur probably due a better part of the molecules which have antibacterial properties in these extracts present a hydrophobic character and can better be extracted with organic solvents (Mudimu *et al*., 2014). Furthermore, the food-grade solvent - ethanol, had proven be one of the most appropriate solvents to extract antibacterial compounds from natural sources (Herrero *et al*., 2012); since it is low cost, with ease of preparation and good solvability (Guedes *et al*., 2011).

Among the studied ethanolic extracts (100mg/ml), *A. platensis*, followed by *Scenedesmus* sp. showed an inhibition above 50% of the growth of *Pseudomonas aeruginosa* ATCC 27853, *Enterococcus faecalis* ATCC 6057 and *Bacillus subtilis* ATCC 6633. In particular, *A. platensis* exhibited effective antibacterial activity against these same microorganisms aforementioned, with 80.0%, 79.9% and 64.8% of inhibition of the cell growth, respectively. In contrast, Shaieb *et al*. (2014), found that aqueous extract of *Spirulina platensis* has antibacterial activity against species tested, *E. coli, Serratia marcescens, Klebsiella pneumonia, Staphylococcus aureus* and *Micrococcus luteus*, except *B. cereus* and *P. aeruginosa* using agar plate diffusion test. The results also reveal that ethanolic extracts of *C. vulgaris* displays antibacterial activity with percentage of inhibition above 60% against *Pseudomonas aeruginosa* ATCC 27853 and *Enterococcus faecalis* ATCC 6057. Since that increasing at dilution of the cell extract to 50 mg/ml, the results also indicated that the ethanolic extracts of *A. platensis*, followed by *Scenedesmus* sp. exhibited highest percentage of inhibition of antibacterial activity against *Pseudomonas aeruginosa* ATCC 27853 and *Enterococcus faecalis* ATCC 6057 showed an inhibition above 60%.

In addition, for ethanolic extracts of all the tested photosynthetic microorganisms at both cell extract concentrations (100 to 50 mg/ml) no inhibition was observed against the growth of *Staphylococcus aureus* ATCC 6538. On the other hand, Kannan *et al*. (2014), reported that methanolic extract of *S. platensis* showed better antibacterial activity against *Staphylococcus aureus*, whe compared with the other organisms.

The other cell extract concentrations (25 to 5 mg/mL) showed no inhibition against the tested bacteria for both aqueous and ethanolic extracts investigated.

These results are in agreement with those of other studies, in which microalgae and cyanobacteria were screened to detect antibacterial (El-Baz *et al*., 2013; Mudimu *et al*., 2014). El-Baz *et al*. (2013), found antibacterial effect of ethanol extract of *Spirulina platensis* against different bacterial strains (*Escherichia coli, Staphylococcus aureus, Salmonella typhi*, and *Enterococcus faecalis*) using disc diffusion method. This result was similar to that found in the present work for antibacterial activity of the ethanolic extract of *A. plat*ensis with the highest percentage of inhibition against one Gram-negative bacteria (*Pseudomonas aeruginosa* ATCC 27853) and two Gram-positive bacteria (*Enterococcus faecalis* ATCC 6057 and *Bacillus subtilis*). Mudimu *et al*. 2014 reported that in methanol extracts of a large number of microalgal and cyanobacterial also exhibited antibacterial activities on both gram-positive and gram-negative bacteria.

In the literature, several studies reported that Cyanobacteria have been screened for potential antimicrobial activity, which have been attributed to different compounds belonging to a diverse range of chemical classes (Ozdemir *et al*., 2004; Benkendorff *et al*., 2005). In particular, the antimicrobial activity of a methanolic extract of the *S. platensis* was explained by the presence of c-linolenic acid, an antibiotically-active fatty acid present in high concentration in this alga (Demule *et al*., 1996). Besides, other fatty acids been also reported to have some antimicrobial activity, specifically lauric, palmitoleic and oleic acids (Benkendorff *et al*., 2005).

Rodríguez-Meizoso *et al*. (2010) previously described the potential antimicrobial activity of the *Haematococcus pluvialis* extracts using subcritical water extraction. The results showed antimicrobial activity against a panel of microorganisms of importance for the food industry, including *Staphylococcus aureus* ATCC 25923, *Escherichia coli* ATCC 11775, *Candida albicans* ATCC 60193 and *Aspergillus niger* ATCC 16404. Moreover, according to these authors the short chain fatty acids can be responsible by antimicrobial activity. Benkendorff *et al*. (2005), revealed that fatty acids have been reported as potential antimicrobial compounds

Conclusions

Data presented in this study illustrate that different extracts (aqueous and ethanolic) obtained from of the microalgae *Chlorella vulgaris*, *Dunaliella salina* and *Scenedesmus* sp. and the cyanobacterium *Arthrospira (Spirulina) platensis* were examined for their in vitro antioxidant activity using ABTS and DPPH free radical-scavenging assays, as well as antibacterial properties. The obtained results indicate that the ethanolic extracts of all the tested species exhibited higher antioxidant and antibacterial activities, when compared to aqueous extract. We conclude that the better part of the bioactive compounds these extracts is hydrophobic and can better be extracted with organic solvente. Results also demonstrated that ethanolic extract of *A. platensis* showed effective antibacterial activity mainly against *Pseudomonas aeruginosa* ATCC 27853, *Enterococcus faecalis* ATCC 6057, and *Bacillus subtilis* ATCC 6633. These results suggest that the ethanolic crude extract of *A. platensis* could be used as a potential source of natural antioxidants and antibacterial, and therefore it may be considered as an accessible and safe alternative to synthetic compounds.

Further studies are needed to investigate the increase of the production of specific bioactive components these extracts through process optimisation (e.g. growth conditions, extraction, downstream processing) and advanced biotechnology (e.g. genetic or metabolic engineering and metabolic flux modelling), besides possibility to isolate new compounds.

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CAPÍTULO II

5 ARTIGO - RECOVERY OF PHENOLIC COMPOUNDS OF FOOD CONCERN FROM *Arthrospira platensis* BY GREEN EXTRACTION TECHNIQUES

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Recovery of phenolic compounds of food concern from *Arthrospira platensis* by green extraction techniques

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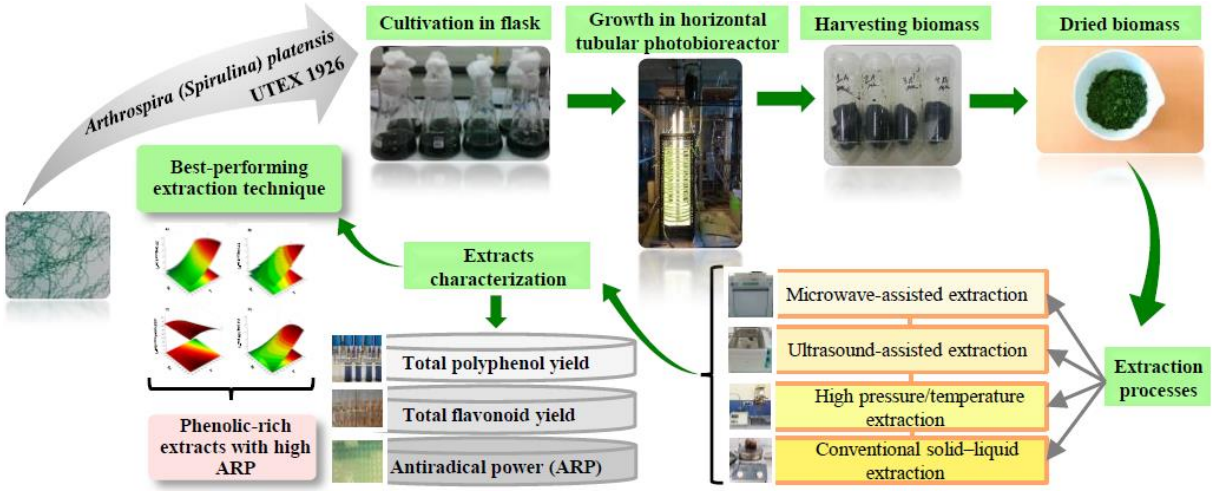
Graphical abstract



ABSTRACT

Arthrospira platensis UTEX 1926 has attracted attention for its ability to produce high-added value compounds such as polyphenols, but their profitable recovery is recognized as a big challenge. In this study, different green extraction techniques, namely ultrasound-assisted (UAE), microwave-assisted (MAE) and high pressure/temperature (HPTE) extractions were compared with classic solid–liquid extraction (SLE) in terms of phenolic compounds recovery from *A. platensis* using ethanol as solvent and antiradical power (ARP). HPTE proved the most efficient extraction method, allowing maximum total polyphenol yield (*TP)* of 3.32±0.08 mg of gallic acid equivalent [GAE] per gram of dry biomass [DB] and ARP of 58.30±0.12 $\mu mol_{Trolox}\ g_{DB}^{-1}$, whereas maximum total flavonoid yield (*TF)* was 2.80±0.18 mg of catechin equivalent [CE] g_{DB}^{-1} in MAE extract. Once HPTE had been selected as the best-performing extraction method, a 3^2-full factorial design was applied to evaluate the combined effects of temperature (90≤T≤180°C) and ethanol concentration in water (20≤Sc≤100% v/v) on *TP*, *TF* and ARP by Response Surface Methodology (RSM). RSM revealed that the most suitable conditions for *TP* (26.00-28.04 $mg_{GAE}\ g_{DB}^{-1}$) and *TF* (10.25±0.34 $mg_{CE}\ g_{DB}^{-1}$) were T=180°C and 20≤Sc≤60% (v/v), while ARP was maximized (67.77-69.02 $\mu mol_{Trolox}\ g_{DB}^{-1}$) at 90≤T≤135°C. HPLC analysis showed that catechin, vanillic, gallic and syringic acids were present in very low concentrations (up to 0.05±0.01 mg 100 g_{DB}^{-1}) in MAE, UAE and SLE extracts, while they were the most abundant phenolics in HPTE (3.45-3.61, 1.06-2.02, 1.64-1.71 and 0.99-1.26 mg 100 g_{DB}^{-1}, respectively). These compounds taken together effectively contributed to both *TP* (r = 0.928) and *TF* (r = 0.960), but not to ARP. This work opens new perspectives for HPTE use as emerging technique to obtain high-ARP phenolic-rich hydroalcoholic extracts of *A. platensis*, which may serve as natural source of compounds to

formulate functional foods or prepare dietary supplements.

Keywords: *Arthrospira platensis,* green extraction, phenolic compounds, antioxidants, response surface methodology, high pressure/temperature extraction.

1. Introduction

Cyanobacteria (photosynthetic prokaryotes), also known as blue–green algae, have a considerable potential not only for bioenergy production [1], but also for the production of nutraceuticals and pharmaceuticals, the former having a large and rapidly expanding market [2,3]. They can, in fact, be used to enhance the nutritional value of foods by addition of compounds with important beneficial attributes that confer health-promoting properties [4-6].

Many studies have also shown significant therapeutic applications of these photosynthetic microorganisms, which are recognized as natural sources of novel biologically active compounds with protective effects against cancer [7], viral, bacterial and fungal infections [8,9], allergies [10], inflammations [11], anemia [12], hyperlipidemia [13] and hyperglycemia [14].

Arthrospira platensis is an unbranched, helicoidal, and filamentous cyanobacterium [15] that stands out because of its high nutritional value related to high contents of several essential nutrients such as proteins [16], polyunsaturated fatty acids, vitamins (A, B_1, B_2, B_6, B_{12}, E and D), minerals (especially iron), and other high added-value phytochemicals including pigments (β-carotene, chlorophyll a, phycocyanin) and polyphenols [4,5,17-21].

The last compounds are an important group of secondary metabolites with antioxidant activity that play a key role in human health acting against free radicals, in particular the reactive oxygen and nitrogen species [22]. They also play an important role in algal cell defense against abiotic (UV irradiation) and biotic (pathogen interaction) stress. It is well documented that polyphenols are involved in the protection of cardiovascular system [23,24]. However, their profitable recovery is currently a hot investigation topic, which is recognized as a big challenge due to the inherent limitations and high costs of conventional extraction methods [25].

The presence of phenolic compounds in microalgae and cyanobacteria is well documented, but information about the presence of specific phenols as well as their bioactivity is still limited [26–28]. These compounds are mainly intracellular, which makes their recovery by conventional solvent extraction difficult [29], because of the need of lysing cells. Thus, it is necessary to develop new efficient cell disruption methods to recover, identify and purify these secondary metabolites in their active form for further application [21].

The extraction method can influence both the quality and quantity of target bioactive polyphenols as well as antiradical power of cyanobacteria extracts [30]. Innovating “green” extraction approaches are currently investigated by several research groups to improve the recovery of phenolic compounds contained in different biological raw materials [17,21,28-31]. Among them, supercritical-fluid (SFE), pressurized liquid (PLE), microwave-assisted (MAE) and ultrasound-assisted (UAE) extractions have been reported to enable faster heat and mass transfer, reduction in solvent consumption, savings in working time, yield increase and higher extract/product quality [32–36] compared to conventional extraction methods.

Since various factors influence the yield of phenolic compounds recovery such as the extraction temperature and solvent concentration, it is necessary to select the best-performing

extraction method. The extraction conditions can also be optimized with a high level of confidence by combining a suited experimental design with Response Surface Methodology (RSM), aiming to reduce costs and time as well as to increase efficiency of work [37,38].

High-pressure/temperature extraction (HPTE) in stirred reactor is an emerging technology, which has been investigated and compared in this study with conventional solid-liquid extraction (SLE), MAE and UAE. Recently, HPTE has been successfully applied to the extraction of bioactive phenolic compounds from different matrices such as grape and apple skins [39], barley grains [36], grape marc and olive pomace [40]. High temperature enhances the extraction efficiency by a decrease in both solvent viscosity and surface tension, hence promoting a better penetration into the matrix. Furthermore, the pressurized cell prevents solvent boiling at the extraction temperature, thus enhancing the intimate contact with the sample over the entire extraction period [38,40]. To the best of our knowledge, this is the first study where HPTE was used to recover phenolics with high antioxidant activity from *A. platensis* hydroalcoholic extracts.

Based on this, the main objective of this study was to select the most efficient extraction method to recover phenolic compounds from *A. platensis* biomass and to maximize, at the same time, the antiradical power (ARP) of the extracts. For this purpose, HPTE, UAE and MAE were compared with classic SLE, resulting HPTE the best-performing method. A 3^2-full factorial design combined with RSM was then applied to this method in order to assess the effects of extraction temperature and solvent concentration on total polyphenol yield, total flavonoid yield and ARP. Finally, possible correlations between phenolic compound contents, their individual constituents and ARP were also investigated.

2 Materials and methods

2.1 Chemicals

Salts to prepare the cultivation medium, ethanol (analytic grade), methanol (HPLC grade), acetonitrile, acetic acid, 6-hydroxy-2,5,7,8-tetramethylchromane-2-carboxylic acid (Trolox), 2,2′-Azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt (ABTS), potassium persulfate, Folin-Ciocalteu reagent, sodium carbonate and standards of phenolic compounds (gallic acid, catechin, vanillic acid, syringic acid, epicatechin and ferulic acid) were purchased from Sigma–Aldrich (St. Louis, MO, USA). CO_2 for cyanobacterial growth with purity of 99.8% was purchased from Siad (Milan, Italy).

2.2 Microorganism and culture conditions

Arthrospira platensis UTEX 1926 (University of Texas Culture Collection, Austin, TX, USA) was maintained and cultured in the culture medium suggested by Schlösser [41]. *A. platensis* was grown in a 3.5 L-horizontal tubular photobioreactor with initial biomass concentration of 0.40 g L^{-1}, exposed to light intensity of 100 ± 5 μmol photons m^{-2} s^{-1} at 30 ± 1 °C [42]. The pH was controlled at 9.5 ± 0.5 through the daily addition of pure CO_2 at a flow rate of 30 L/h. Both photobioreactor configuration and light intensity were chosen based on the best operating conditions previously established by our research group [42]. Biomass concentration (X) was determined daily by measuring the absorbance at 560 nm by a UV-Vis spectrophotometer, model Lambda 25 (Perkin Elmer, Milan, Italy), using the calibration curve $Abs_{560} = 0.0024\ X - 0.1129$ ($R^2 = 0.992$). All the measurements were done in triplicate. Once

the stationary phase had been reached, after about 9 days of cultivation, cells were separated from the culture medium by centrifugation (ALC 4226, Milan, Italy) for 10 min at 6000 ×*g*, dried at 105 °C for 24 h (moisture content < 1.0%), pulverized in a mortar and stored at –20 °C for further analyses.

2.3 Extraction processes

Dried biomass of *A. platensis* was extracted using ethanol as a solvent with a constant solid/liquid ratio of 1:10 (*w/v*) [22,24]. All extractions were performed using 2.0 g of dried biomass, except those at high pressure and temperature (3.0 ± 0.1 g), according to the schematic set-up of the overall process illustrated in Fig. 1.

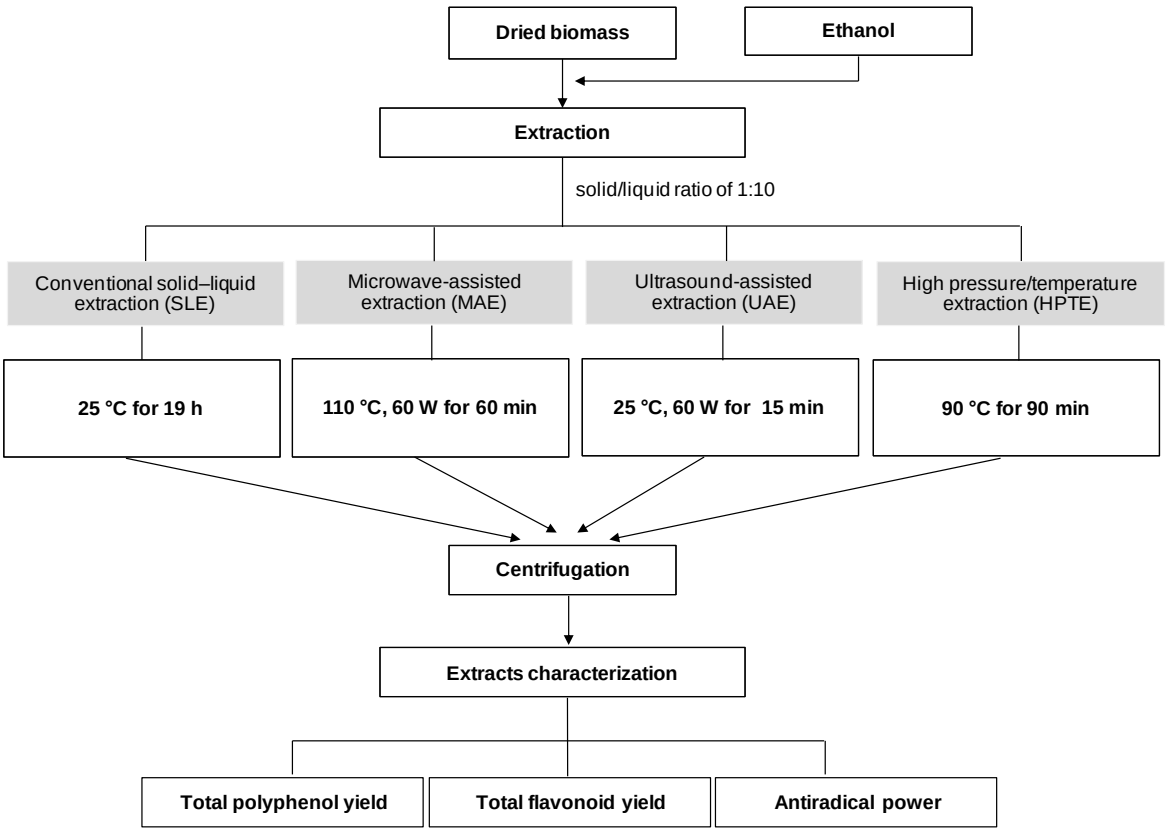


Fig. 1. Schematic set-up of the process for phenolic compounds recovery from *Arthrospira platensis* biomass by different extraction techniques.

Conventional solid–liquid extraction (SLE) was performed for 19 h in amber glass test tubes with caps, where the suspension was continuously mixed by a magnetic stirrer, model Mr. 3001 (Heidolph, Kelheim, Germany). The extraction was carried out in the dark at room temperature (25 ± 1 °C), and then the liquid was separated from solids by centrifugation under the same conditions as above and frozen at –20 °C [43].

Microwave-assisted extraction (MAE) was conducted at 110 °C under nitrogen atmosphere and microwave irradiation (60 W) for 60 min [44] in a professional multimode oven operating at 2.45 GHz (MicroSYNTH Milestone, Sirisole, Italy). Temperature was monitored by an optic-fiber thermometer directly inserted into the 100 mL-pressure-resistant reaction vessel (maximum pressure of 100 bar) made in polytetrafluoroethylene [44].

Ultrasound-assisted extraction (UAE) was carried out at 25 °C, 21.5 kHz and 60 W in an ultrasonic bath, model UTA 90 (FALC, Treviglio, Italy), following the methodology described by Cravotto et al. [45], but reducing the extraction time from 30 to 15 min according to preliminary experiments (data not shown).

High pressure/temperature extraction (HPTE) was performed in a high pressure–high temperature reactor, model 4560 (PARR Instrument, Moline, IL, USA), containing appropriate valves to allow introduction and removal of gases inside the reaction chamber and equipped with a mechanical stirrer [46,47]. The extraction was carried out at 90 °C and 2.5 bar for 90 min by flushing nitrogen through the reactor for 2 min in order to prevent oxidation of phenolics [48]. Since such equipment, able to operate up to 350 °C and 200 bar, did not have the ability to regulate pressure, high pressure was the result of temperature rise. After the extraction, the suspension was centrifuged as described above.

All the extractions were carried out in triplicate, and the extracts used for determinations of total polyphenol yield (*TP*), total flavonoid yield (*TF*) and antiradical power (ARP).

2.4 Analytical methods

2.4.1 Yield of total polyphenols

Total polyphenol content was quantified colorimetrically by the Folin–Ciocalteu assay [49]. Briefly, 4.80 mL of distilled water, 0.20 mL of sample and 0.50 mL of Folin–Ciocalteu reagent were placed into 15 mL-test tubes. After mixing and addition of 1.0 mL of 20% (*w/v*) sodium carbonate solution, distilled water was added to reach a final volume of 10 mL. All the solutions were mixed and allowed to stand at room temperature in the dark for 1 h. The concentration of total polyphenols was determined by absorbance measurements at 725 nm using the early-mentioned UV–Vis spectrophotometer. A calibration curve (Abs_{725} = 0.0017 *TP*, R^2 = 0.997) was made with standard solutions of gallic acid with concentration in the range 0.01–1.0 mg mL^{-1}. Total polyphenol yield (*TP*) was expressed in milligrams of gallic acid equivalent per gram of dry biomass ($mg_{GAE}\ g_{DB}^{-1}$).

2.4.2 Yield of total flavonoids

The yield of total flavonoids (*TF*) in the extract was determined by the colorimetric method described by Jia et al. [50] and modified by Yang et al. [51], which is specific for such an important class of phenolics. Briefly, 0.25 mL of diluted extract was mixed with 1.25 mL of deionized water and 0.075 mL of 5% sodium nitrite solution, and allowed to react for 5

min. Then, 0.15 mL of 10% aluminum chloride and, after 6 min, 0.5 mL of 1.0 M sodium hydroxide was added to the mixture. Distilled water was added to reach a final volume of 3.0 mL. Absorbance was read at 510 nm. A calibration curve (Abs_{510} = 0.0021 *TF*, R^2 = 0.991) was made with standard solutions of catechin with concentration in the range 0.01–0.50 mg mL^{-1}. *TF* was expressed in milligrams of catechin equivalent per gram of dry biomass (mg_{CE} g_{DB}^{-1}).

2.4.3 Antiradical power

The antiradical power (ARP) of extracts was measured according to their ability to scavenge $ABTS^{\bullet+}$ radical cation, as described by Re et al. [52] and applied by Gilbert-López et al. to microalgae extracts [38]. The mixture was incubated at room temperature in the dark for 12–16 h (time required for radical formation) prior to use. The absorbance was measured at 734 nm. ARP in the reaction medium was calculated from a calibration curve (Abs_{734} = -0.0013 Trolox conc. + 0.6239, R^2 = 0.997), and the results were expressed as micromoles of Trolox equivalent per gram of dry biomass (μmol_{Trolox} g_{DB}^{-1}).

2.4.4 Quantification of phenolic compounds by HPLC

The most abundant phenolic compounds of the extract were quantified by a high-performance liquid chromatograph, model 1100 (Agilent Technologies, Palo Alto, CA, USA), equipped with a C18 reverse-phase column, model 201TP54 (Vydac, Hesperia, CA, USA), coupled with a diode-array detector (DAD), model 1200 (Agilent), following the methodology described by De Faveri et al. [53]. The mobile phase was MilliQ water/acetic acid (99:1%, v/v) (solvent A) and methanol/acetonitrile (50:50%, v/v) (solvent B), while the

253 solvent gradient was varied according to the following conditions: from 5 to 30% B for 25
254 min, from 30 to 40% B for 10 min, from 40 to 48% B for 5 min, from 48 to 70% B for 10
255 min, from 70 to 100% B for 5 min, isocratic at 100% B for 5 min, followed by returning to
256 the initial conditions (10 min) and column equilibration (12 min). Runs were performed at 30
257 °C and flow rate of 1.0 mL min^{-1}, the injection volume was 20 µL, and peaks were monitored
258 at 280 nm. Gallic acid, catechin, vanillic acid, syringic acid, epicatechin and ferulic acid were
259 used as standards. Before analysis, samples were filtered through cellulose acetate filters with
260 0.22 µm-pore diameter. Phenolic compounds were identified by comparing the retention time
261 with that of the corresponding standard at 280 nm and comparing the spectrum obtained by
262 DAD with the standard one. The concentration of each phenolic compound was calculated
263 based on that of each standard solution and expressed in milligrams per 100 g of dry biomass
264 (mg 100 g_{DB}^{-1}). The sum of contents of all individual phenolic compounds quantified by
265 HPLC was referred to as SPC.
266
267 *2.5 Experimental design and statistical analysis*
268
269 A 3^2-full factorial experimental design combined with Response Surface Methodology
270 (RSM) was used to obtain and evaluate the results of HPTE at fixed time (90 min). To this
271 purpose, the extraction temperature ($90 \leq T \leq 180$ °C) and solvent (ethanol) concentration
272 (Sc) in the hydroalcoholic solution (20, 60 and 100% v/v) were selected as the independent
273 variables, whose coded values were −1 (lowest level), 0 (central point) and +1 (highest level),
274 while *TP* (mg_{GAE} g_{DB}^{-1}), *TF* (mg_{CE} g_{DB}^{-1}) and ARP (μmol_{Trolox} g_{DB}^{-1}) as the responses (Table
275 1).

The complete design consisted of 11 experiments with three replicates at the central point, which were added to estimate the experimental error as well as to check the reproducibility of results.

Table 1. Results and conditions of high pressure/temperature extraction tests performed on *A. platensis* biomass according to the 3^2-full factorial design.

Run	Independent variable		Response		
	T^A (°C)	$S_c{}^B$ (% v/v)	*TP*[C] ($mg_{GAE}\ g_{DB}^{-1}$)	*TF*[D] ($mg_{CE}\ g_{DB}^{-1}$)	ARP[E] ($\mu mol_{Trolox}\ g_{DB}^{-1}$)
1	90(-1)	20(-1)	6.19 ± 0.07^{b}	2.76 ± 0.05^{d}	69.02 ± 1.11^{b}
2	90(-1)	60(0)	$4.00 \pm 0.05^{a,b}$	2.00 ± 0.05^{a}	67.77 ± 0.20^{b}
3	90(-1)	100(1)	3.32 ± 0.08^{a}	1.45 ± 0.13^{c}	58.30 ± 0.12^{c}
4	180(1)	20(-1)	26.00 ± 0.02^{c}	10.25 ± 0.34^{g}	$60.95 \pm 0.05^{a,c}$
5	180(1)	60(0)	28.04 ± 2.76^{c}	8.83 ± 0.39^{f}	34.08 ± 1.00^{e}
6	180(1)	100(1)	14.81 ± 0.15^{f}	4.11 ± 0.17^{b}	25.47 ± 1.63^{d}
7	135(0)	20(-1)	11.93 ± 0.67^{e}	6.00 ± 0.04^{e}	68.11 ± 0.78^{b}
8	135(0)	100(1)	$4.00 \pm 0.02^{a,b}$	2.08 ± 0.04^{a}	62.16 ± 0.95^{a}
9[F]	135(0)	60(0)	9.33 ± 0.21^{d}	4.00 ± 0.04^{b}	63.12 ± 0.53^{a}
10[F]	135(0)	60(0)	9.07 ± 0.32^{d}	4.10 ± 0.06^{b}	61.18 ± 0.38^{a}
11[F]	135(0)	60(0)	9.20 ± 0.01^{d}	3.90 ± 0.01^{b}	60.65 ± 0.27^{a}

Different superscript lowercase letters in the same column show statistically significant differences among data at $p < 0.05$. Values are expressed as means ± standard deviations of three replicates.

[A] Extraction temperature. Coded values between brackets.

[B] Solvent (ethanol) concentration in the hydroalcoholic solution. Coded values between brackets.

[C] Total polyphenol yield; GAE: gallic acid equivalents; DB: dry biomass.

[D] Total flavonoid yield; CE: catechin equivalents.

[E] Antiradical power determined by the $ABTS^{\bullet+}$ radical scavenging method.

[F] Central point runs.

To describe the behaviour of the system, second-order polynomial models were used to correlate independent variables to responses according to the equation:

$$Y = \beta_0 + \sum \beta_i x_i + \sum \beta_{ii} x_i^2 + \sum \beta_{ij} x_i x_j \quad (1)$$

where Y is the response, β_0 is the interception coefficient, β_i are the linear terms, β_{ii} are the quadratic terms, β_{ij} are the interaction terms, and x_i and x_j are the coded levels of the independent variables. The quality of fit of the polynomial equations was expressed by the coefficient of determination (R^2).

The Student's *t*-test permitted us to check the statistical significance of the regression coefficients, while the Fisher's test for analysis of variance (ANOVA) was performed on experimental data to evaluate the statistical significance of models. The influences of the selected variables were assessed by ANOVA and Tukey's *post hoc* test for mean discrimination or mean comparison, depending on the data. The statistically significant differences were illustrated by different letters in tables and figures. All the analyses were carried out in triplicate (n = 3), and the results expressed as mean values ± standard deviations (SD).

To identify possible correlations among the contents of different groups of phenolic compounds (*TP* and *TF*), individual phenolic compounds detected by HPLC (catechin, epicatechin, gallic, vanillic, syringic and ferulic acids) and ARP, linear equations have been applied, and the values of the Pearson's coefficient (r) [54] compared.

315 The Statistica v. 10.0 software (StatSoft, Tulsa, OK, USA) was used for data analysis.
316 The results were considered statistically significant for p values ≤ 0.05.
317
318 **3. Results and discussion**
319
320 *3.1. Preliminary selection of the extraction method*
321
322 In the first part of this study, three green extraction techniques, namely ultrasound-
323 assisted (UAE), microwave-assisted (MAE) and high pressure/temperature (HPTE)
324 extractions, were compared with classic solid–liquid extraction (SLE) in terms of total
325 polyphenol yield (*TP*), total flavonoid yield (*TF*) and antiradical power (ARP).
326 As illustrated in Fig. 2A, *TP* in *Arthrospira platensis* ethanolic extracts was the
327 highest (3.32 ± 0.08 mg_{GAE} g_{DB}^{-1}) when using HPTE, followed by MAE (3.04 ± 0.08 mg_{GAE}
328 g_{DB}^{-1}) and UAE (2.07 ± 0.01 mg_{GAE} g_{DB}^{-1}). As suggested by Casazza et al. [47], the
329 effectiveness of HPTE to extract polyphenols may have been the result of optimal stirring
330 conditions and inert atmosphere. This extraction method was successful in terms of phenolic
331 compound recovery from other natural matrices such as grape and apple by-products
332 [39,44,47], olive oil solid waste [46], *Agave americana* (L.) leaves [48], barley grains [36],
333 agri-food waste from grape marc, olive pomace [40] and cyanobacteria [24]. On the other
334 hand, the conventional SLE showed the worst performance (1.61 ± 0.07 $mg_{GAE}g_{DB}^{-1}$) when
335 compared with all the selected emerging techniques, likely due to degradation caused by
336 exposition for prolonged time (19 h) at room temperature [47].
337 The type of extraction technique influenced differently and more markedly *TF* when
338 compared with *TP* (Fig. 2B). It is likely that the general structure of a 15-carbon skeleton of

TF, which are made of two phenyl rings and a heterocyclic ring, is responsible for the different thermosensitivity of these classes of compounds [55].

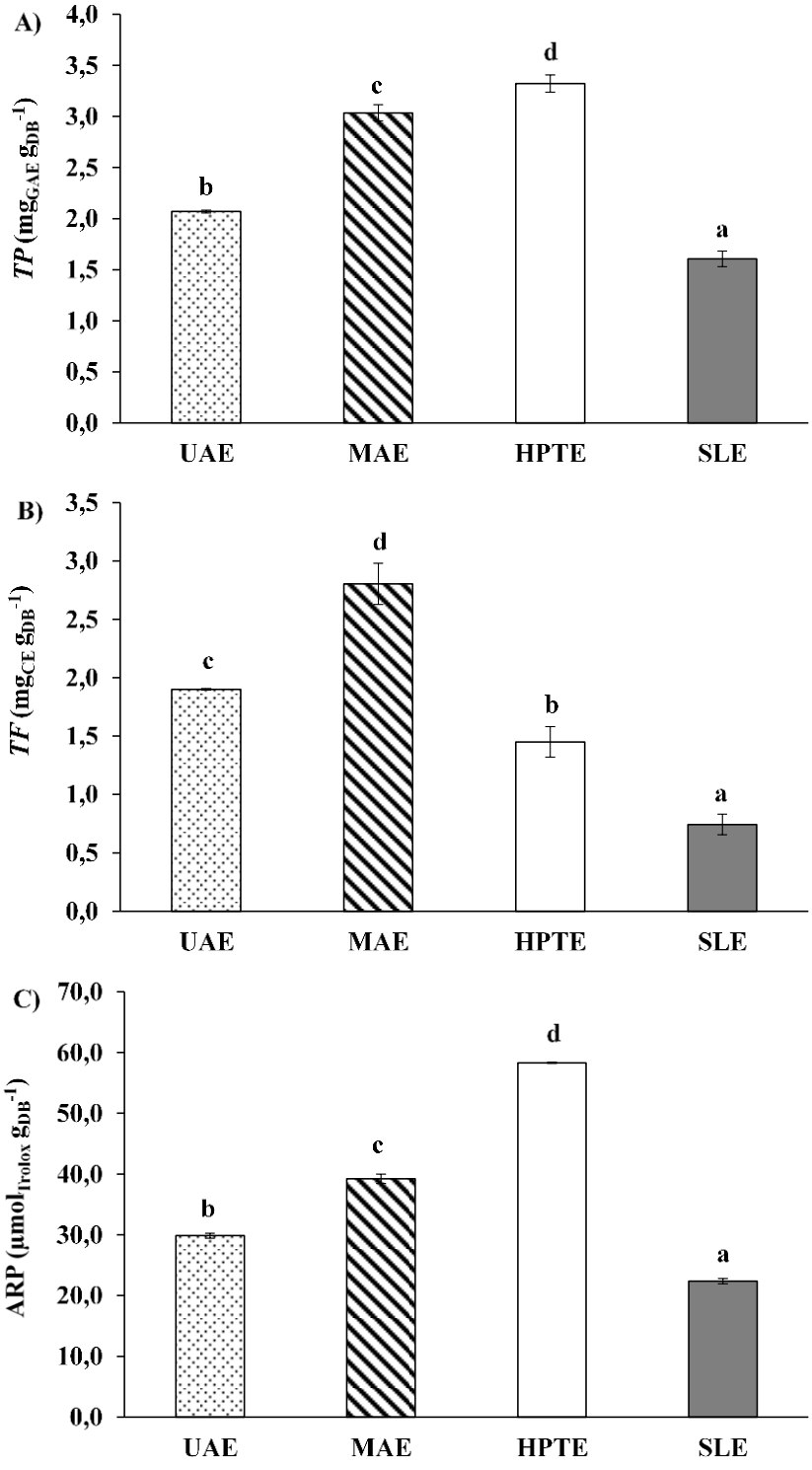


Fig. 2. Total polyphenol yield (*TP*) (A), total flavonoid yield (*TF*) (B), and antiradical power (ARP) (C) of ethanolic *Arthrospira platensis* extracts obtained by different extraction

techniques. Data are expressed as means of three replicates (n = 3). Different letters (from a to d) indicate statistically significant differences at $p < 0.05$. Error bars indicate means ± standard deviations. HPTE: high pressure/temperature extraction; MAE: microwave-assisted extraction; SLE: solid–liquid extraction; UAE: ultrasound-assisted extraction; GAE: gallic acid equivalents, CE: catechin equivalents.

The highest *TF* was in fact obtained when using MAE (2.80 ± 0.18 $mg_{CE}\ g_{DB}^{-1}$), followed by UAE (1.90 ± 0.01 $mg_{CE}\ g_{DB}^{-1}$) and HPTE (1.45 ± 0.13 $mg_{CE}\ g_{DB}^{-1}$), while SLE yielded only 0.74 ± 0.09 $mg_{CE}\ g_{DB}^{-1}$. These results taken together suggest that in MAE, which shatters cells using the shock of high-frequency waves [25], a temperature increase may have favored the release of flavonoids from the matrix, while a reduction of the extraction time compared with the SLE and HPTE may have enhanced their stability. On the other hand, the higher *TF* content of UAE compared with HPTE may have been the result of the well-known ability of ultrasound to break cell wall.

As expected by the well-known antioxidant activity of many polyphenols, ARP (Fig. 2C) showed a qualitatively similar trend as *TP* (Fig. 2A), in that the *A. platensis* extract obtained by HPTE exhibited the highest value of this response (58.30 ± 0.12 $\mu mol_{Trolox}\ g_{DB}^{-1}$), followed by MAE (39.22 ± 0.75 $\mu mol_{Trolox}\ g_{DB}^{-1}$), UAE (29.85 ± 0.39 $\mu mol_{Trolox}\ g_{DB}^{-1}$) and SLE (22.37 ± 0.42 $\mu mol_{Trolox}\ g_{DB}^{-1}$). Again, optimal stirring conditions and inert atmosphere [47] may have been the main reasons of the best HPTE performance compared with the other extraction methods.

To the best of our knowledge, there was no previous report on the production of phenolic-rich ethanolic extracts of *A. platensis* with high antioxidant activity by HPTE; therefore, the results of this study may be considered a useful starting basis for future advance in this field. In particular, HPTE extracts could be used as a potential source of easily-accessible natural antioxidants and a safe alternative to synthetic compounds.

389 *3.2 Full factorial design to select the best HPTE conditions*

390

391 Based on the results described in the previous section, HPTE was selected as the best-
392 performing extraction technique in terms of *TP* and ARP; therefore, an optimization study
393 was performed in the second part of this work to select the best extraction conditions for this
394 method.

395 As is well known, temperature and solvent concentration are the main key factors
396 influencing the extraction efficiency, because of their impact on the equilibrium (solubility),
397 mass transfer rate (diffusion coefficient), and stability of bioactive compounds [43]. Thus,
398 their effects on the extraction efficiency were investigated in order to maximize the yield in
399 phenolic compounds and antiradical power and to minimize costs [56].

400 The experimental results of HPTE tests performed according to the selected 3^2-full
401 factorial design demonstrate that both independent variables, namely the extraction
402 temperature (T, ºC) and solvent (ethanol) concentration in the hydroalcoholic solution (Sc, %
403 v/v), significantly influenced *TP*, *TF* and ARP, which varied within very wide ranges (Table
404 1).

405 One can see that the highest values of both *TP* (26.00 ± 0.02 mg_{GAE} g_{DB}^{-1} to 28.04 ±
406 2.76 mg_{GAE} g_{DB}^{-1}; $p > 0.05$) and *TF* (10.25 ± 0.34 mg_{CE} g_{DB}^{-1}) were achieved in *A. platensis*
407 extracts obtained at the highest temperature (180 °C), but while the former response was
408 maximized in the Sc range 20-60%, the latter was so at the lowest value (20%). In contrast
409 with these trends, ARP was favored by simultaneous decreases of both independent variables.

410 As previously suggested [46,57,58], these optimal results at high temperature may
411 have been due to a) decreases in solvent viscosity and surface tension, hence making
412 penetration into the matrix easier, b) increases in solvent diffusion rate and solutes mass

transfer, and c) enhancement of the disruption of the strong solute–matrix interactions (hydrogen bonds, dipole attractions and van der Waals forces).

It is noteworthy that, under all the conditions tested, the presence of water in the hydroalcoholic solution led to higher *TP* and *TF* values, when compared with pure ethanol as an extraction solvent (see section 3.1). Similarly, Paini et al. [40], who used hydroalcoholic solutions at 150 °C for 270 min to recover phenolic compounds from olive pomace by HPTE, found that TP and TF yields improved significantly when water was added to ethanol up to 25%. This behavior may be explained not only by the high solubility of some phenolic classes in polar solvents, due to their generally polar nature [59], but also by the high pressure in the reactor chamber that likely prevented solvent boiling at the extraction temperature, thus enhancing phenolic matrix disruption. The same reasoning was used by Herrero et al. [60] to explain the better yield of antioxidant recovery by pressurized liquid extraction from *A. platensis* using water and ethanol as solvents rather than hydrocarbons. Nonetheless, when Ferrari et al. [24] used HPTE at 180 °C for 90 min and 100% water as solvent to recover polyphenols from *A. platensis* biomass, *TP* was lower (13.43 mg_{GAE} g_{DB}^{-1}) than that obtained in this work at the same temperature and extraction time. These findings suggest that, although some water is necessary to perform highly effective HPTE, it is essential also the presence of a polar organic solvent to this purpose, which may be responsible for changes in both solubility of these compounds in water and water pressure-temperature diagram. Finally, the maximum ARP obtained in this study (69.02 ± 1.11 μmol_{Trolox} g_{DB}^{-1}) was almost the same as that (69.40 ±1.14 μmol_{Trolox} g_{DB}^{-1}) of hydroalcoholic extracts of *Tetraselmis* sp. [59], but remarkably higher than those of *Tetraselmis suecica*, *Chlorella vulgaris* and *Phaeodactylum tricornutum* (17-25 μmol_{Trolox} g_{DB}^{-1}) [27], all obtained by conventional SLE.

3.3 Quantification of phenolic compounds by HPLC

Single phenolic compounds belonging to flavonoid, phenolic acids and tannin groups are recognized as the dominant contributors to the antioxidant capacity, besides possessing diverse biological activities such as anti-inflammatory, anti-atherosclerotic and anti-carcinogenic ones [61]. Moreover, they have been associated with many aspects of food quality including color, flavor properties and nutritional value [62].

Contents of phenolic compounds that could be identified by HPLC in *A. platensis* extracts obtained by HPTE performed according to the 3^2-full factorial design are listed in Table 2. The major phenolics of the extracts and their maximum concentrations were catechin (3.45 ± 0.07 mg 100 g_{DB}^{-1} to 3.61 ± 0.01 mg 100 g_{DB}^{-1}; $p > 0.05$), vanillic (2.02 ± 0.01 mg 100 g_{DB}^{-1}), gallic (1.71 ± 0.04 mg 100 g_{DB}^{-1}) and syringic (1.26 ± 0.01 mg 100 g_{DB}^{-1}) acids. As can be observed, maximum contents of these compounds were detected in the extracts obtained under conditions (runs 4 and 5) able to maximize *TP* and *TF*, i.e., the highest temperature (180 ºC) and 20 ≤Sc≤ 60%, which suggests that they may be among the major contributors of both phenolics classes.

462 **Table 2.** Contents of phenolic compounds quantified by reverse phase-HPLC in *A. platensis* extracts obtained by high pressure/temperature
463 extraction (HPTE) runs performed according to the 3^2-full factorial design, ultrasound-assisted extraction (UAE), microwave-assisted extraction
464 (MAE) and conventional solid-liquid extraction (SLE).

Run	T[A] (°C)	S_c[B] (% v/v)	Phenolic compound content (mg 100 g_{DB}^{-1})*					
			GA[C]	*CA*[D]	*VA*[E]	*SA*[F]	*EP*[G]	*FA*[H]
1	90(-1)	20(-1)	0.09±0.01^{b,c,d}	0.41±0.03^{b,c}	0.22± 0.01^{c}	0.04± 0.00^{d,e}	0.07±0.00^{b}	-
2	90(-1)	60(0)	0.18±0.01^{e}	0.55±0.01^{b}	0.01±0.00^{a}	0.01±0.00^{a,b}	-	-
3	90(-1)	100(1)	0.05±0.00^{a,b,c}	0.18±0.01^{a,c}	0.02±0.00^{a}	-	-	-
4	180(1)	20(-1)	1.64±0.04^{h}	3.45±0.07^{d}	2.02±0.01^{h}	1.26±0.01^{h}	-	-
5	180(1)	60(0)	1.71±0.04^{i}	3.61±0.01^{d}	1.06±0.01^{f}	0.99±0.01^{g}	-	-
6	180(1)	100(1)	0.48±0.01^{f}	0.59±0.01^{b}	0.34±0.02^{d}	0.21±0.01^{f}	-	-
7	135(0)	20(-1)	0.12±0.01^{d,e}	1.30±0.10^{e}	1.20±0.10^{g}	0.02±0.01^{b,c,d}	-	0.03±0.00^{b,c}
8	135(0)	100(1)	0.12±0.01^{c,d,e}	0.26±0.07^{a,b,c}	0.12±0.01^{b}	0.03±0.01^{c,d,e}	0.17±0.01^{a}	0.04±0.00^{c}
9[I]	135(0)	60(0)	0.71±0.01^{g}	1.90±0.20^{f}	0.61±0.01^{e}	0.06±0.00^{e}	0.03±0.01^{c}	0.07±0.00^{d}
10[I]	135(0)	60(0)	0.78±0.01^{g}	1.70±0.10^{f}	0.68±0.00^{e}	0.05±0.00^{e}	-	0.09±0.01^{d}
11[I]	135(0)	60(0)	0.72±0.02^{g}	1.50±0.10^{f}	0.62±0.00^{e}	0.04±0.00^{e}	-	0.07±0.00^{d}
UAE[J]	25	100	-	-	0.01±0.00^{a}	-	-	0.01±0.00^{a,b}
MAE[K]	110	100	0.02±0.00^{a}	0.03±0.01^{a}	0.02±0.00^{a}	0.02±0.00^{a,b,c}	-	0.02±0.00^{a,b}
SLE[L]	25	100	0.03±0.01^{a,b}	-	0.05±0.01^{a,b}	0.04±0.00 ^{c,d,e}	-	0.02±0.00^{b,c}

Different superscript lowercase letters in the same column show statistically significant differences among data at $p < 0.05$. Values are expressed as means ± standard deviations of three replicates.

- = Not detected.

[A] Extraction temperature. Coded values between brackets.

[B] Solvent (ethanol) concentration in the hydroalcoholic solution. Coded values between brackets.

[C] Gallic acid.

[D] Catechin.

[E] Vanillic acid.

[F] Syringic acid.

[G] Epicatechin.

[H] Ferulic acid.

[I] Central point runs.

[J] Ultrasound-assisted extraction.

[K] Microwave-assisted extraction.

[L] Solid-liquid extraction.

* DB = dry biomass.

Fig. 3 illustrates, as an example, the reverse phase HPLC profile of these phenolic compounds in the *A. platensis* extract obtained by HPTE at 180 ºC and ethanol concentration in the hydroalcoholic solution of 60% (v/v) (run 5) (Fig. 3A) together with a micrograph of the microbial suspension before extraction (Fig. 3B).

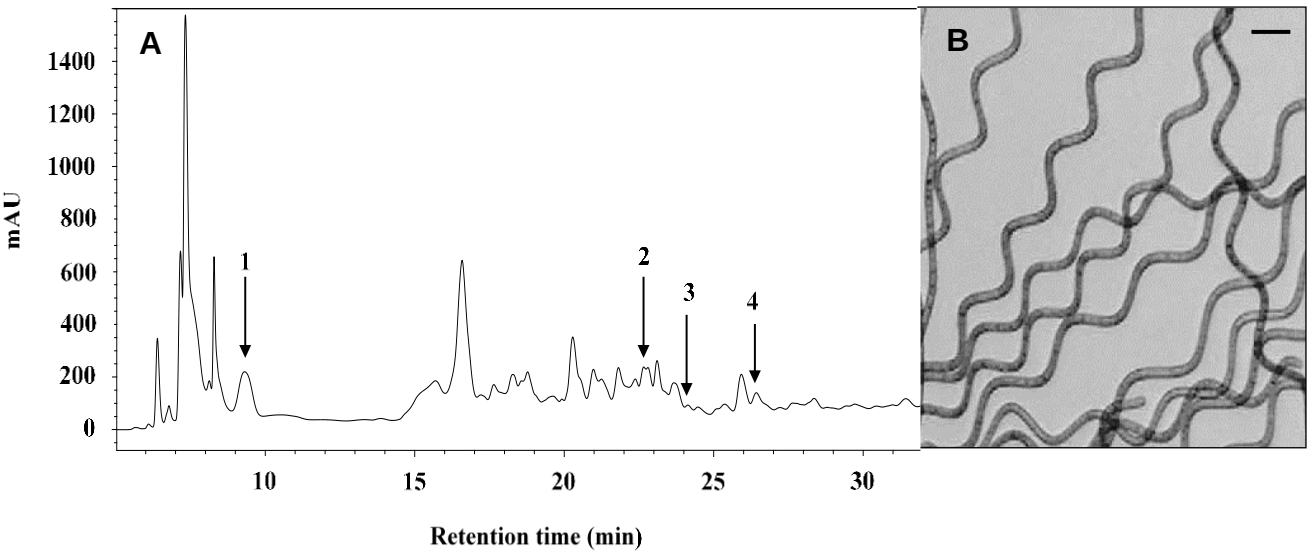


Fig. 3. A) Reverse phase HPLC chromatogram of phenolic compounds in the *Arthrospira platensis* extract obtained by high pressure/temperature extraction at 180 ºC and solvent (ethanol) concentration in the hydroalcoholic solution of 60% (v/v) (run 5). Phenolic compounds: (1) gallic acid; (2) catechin; (3) vanillic acid; (4) syringic acid. Elution time and maximum absorbance of individual phenolics were determined by HPLC-DAD with reverse phase C18 column. The results are expressed in milli absorption units (mAU). B) micrograph (bar 10 µm) of *A. platensis* suspension before extraction.

The overall content of phenolic compounds quantified by HPLC was much lower than the one obtained by the Folin–Ciocalteu colorimetric method. This finding was indeed expected not only from the weak selectivity of the Folin–Ciocalteu reagent, which can react also with some non-phenolic compounds [63,64], but also from the little fraction of identified phenolics.

The highest concentrations of vanillic and syringic acids were two and four orders of magnitude higher (2.02 ±0.01 mg 100 g_{DB}^{-1} and 1.26 ±0.01 mg 100 g_{DB}^{-1}, respectively) than those (25.5 $\mu g_{VA}100\ g_{DB}^{-1}$ and 0.34 $\mu g_{SA}100\ g_{DB}^{-1}$, respectively) detected in *A. platensis* extracts obtained by solid-phase/supercritical CO_2 extraction at 80 ºC for 60 min with 47.5:47.5:5.0 (v/v/v) methanol/water/NH_3 as a solvent mixture, and even higher than those reported for other cyanobacterial or microalgal extracts (5.29 $\mu g_{VA}100\ g_{DB}^{-1}$ and 5.48 $\mu g_{SA}100\ g_{DB}^{-1}$ for *Spongiochloris spongiosa*, 10.2 $\mu g_{VA}100\ g_{DB}^{-1}$ for *Cylindrospermum* sp., 12.0 $\mu g_{VA}100\ g_{DB}^{-1}$ for *Nostoc* sp., and 24.7 $\mu g_{VA}100\ g_{DB}^{-1}$ and 11.0 $\mu g_{SA}100\ g_{DB}^{-1}$ for *Anabaena doliolum*) [26], which suggests a crucial role of the extraction technique in the extract composition.

3.4 Response surface modeling

The Response Surface Methodology (RSM) was used to model *TP* (Y_1), *TF* (Y_2), ARP (Y_3) and the content of catechin (*CA*) (Y_4), i.e., the most abundant among the quantified phenolic compounds of the extract, by the second-order polynomial equations:

$$Y_1 = 9.66 + 9.22x_1 + 5.66{x_1}^2 - 3.66x_2 - 2.39{x_2}^2 - 2.08\ x_1 * x_2 \quad (2)$$

$$Y_2 = 4.17 + 2.83x_1 + 0.98{x_1}^2 - 1.89x_2 - 0.38{x_2}^2 - 1.21\ x_1 * x_2 \quad (3)$$

$$Y_3 = 63.04 - 12.43x_1 - 10.44{x_1}^2 - 8.69x_2 - 6.19\ x_1 * x_2 \quad (4)$$

$$Y_4 = 1.70 + 1.08x_1 - 0.68x_2 - 0.92{x_2}^2 - 0.65\ x_1 * x_2 \quad (5)$$

where x_1 and x_2 are the coded values of T and Sc. Eq. (4) and (5) were obtained after neglecting the statistically insignificant ($p > 0.05$) quadratic terms of Sc and temperature, respectively.

The analysis of variance (ANOVA) highlighted that the regression was statistically significant with high or satisfactory values of the adjusted determination coefficient (R^2_{Adj} = 0.925, 0.963, 0.849 and 0.895 for *TP*, *TF*, ARP and *CA*, respectively) and that all terms were statistically significant ($p < 0.05$) (see Supplemental Information, Table S1).

559 **Table S1.** Analysis of variance (ANOVA) of the effects of extraction temperature (T ºC,
560 coded values) and solvent (ethanol) concentration in the hydroalcoholic solution (Sc, % v/v,
561 coded values) on the total polyphenol (*TP*) and flavonoid (*TF*) yields, antiradical power
562 determined by the $ABTS^{\bullet+}$ radical scavenging method (ARP) as well as the content of
563 catechin (*CA*) in *A. platensis* extracts obtained by high pressure/temperature extraction
564 according to the 3^2-full factorial design.

	Source	SS[A]	df [B]	MS[C]	*F*-value	*p*-value
TP	T (L)[D*]	510.4193	1	510.4193	30202.32	0.000033
	T (Q)[E*]	81.1418	1	81.1418	4801.29	0.000208
	Sc (L)[D*]	80.5934	1	80.5934	4768.84	0.000210
	Sc (Q)[E*]	14.5377	1	14.5377	860.22	0.001160
	T (L)[D] x Sc (Q)[E*]	17.3056	1	17.3056	1024.00	0.000975
	Pure error	0.0338	2	0.0169	-	-
	Total SS[A]	718.4411	10			
TF	T (L)[D*]	48.05340	1	48.05340	4805.340	0.000208
	T (Q)[E*]	2.46974	1	2.46974	246.974	0.004025
	Sc (L)[D*]	21.54615	1	21.54615	2154.615	0.000464
	Sc (Q)[E*]	0.38065	1	0.38065	38.065	0.025279
	T (L)[D] x Sc (Q)[E*]	5.83223	1	5.83223	583.223	0.001710
	Pure error	0.02000	2	0.01000		
	Total SS[A]	79.41996	10			
ARP	T (L)[D*]	927.278	1	927.2780	548.3932	0.001819
	T (Q)[E*]	297.578	1	297.5781	175.9880	0.005634
	Sc (L)[D*]	453.270	1	453.2704	268.0646	0.003710
	Sc (Q)[E]	-	-	-	-	-
	T (L)[D] x Sc (Q)[E*]	153.264	1	153.2644	90.6407	0.010853
	Pure error	3.382	2	1.6909		
	Total SS[A]	2014.220	10			
CA	T (L)[D*]	7.06335	1	7.063350	176.5837	0.005615
	T (Q)[E]	-	-	-	-	-
	Sc (L)[D*]	2.84282	1	2.842817	71.0704	0.013780
	Sc (Q)[E*]	2.15035	1	2.150351	53.7588	0.018098
	T (L)[D] x Sc (Q)[E*]	1.72923	1	1.729225	43.2306	0.022359
	Pure error	0.08000	2	0.040000		
	Total SS[A]	14.59307	10			

565 [A] Sum of squares.

566 [B] Degree of freedom.

567 [C] Mean square.

568 [D] Linear effects.

569 [E] Quadratic effects.

570 [*]Statistically significant effects (*p*-value < 0.05).

571

572 To better visualize the combined effects of the two independent variables on *TP*, *TF*,
573 ARP and *CA*, Fig. 4 shows the three-dimensional graphs obtained by Eqs. (2-5). As confirmed
574 by significant linear and quadratic terms of the model equation, both T and Sc strongly
575 influenced *TP* in *A. platensis* extracts obtained by HPTE (Fig. 4A). In particular, the positive
576 linear regression coefficient of temperature was the most significant term of the model
577 (+9.22), followed by the positive quadratic one of the same variable (+5.66). Even though
578 they were responsible for an exponential increase in *TP*, especially evident at 180 ºC, the
579 negative linear (-3.66) and quadratic (-2.39) terms of Sc together with the negative interaction
580 between the two variables (-2.08) led to a maximization of this response (26.00 ± 0.02 mg_{GAE}
581 g_{DB}^{-1} to 28.04 ± 2.76 mg_{GAE} g_{DB}^{-1}; $p > 0.05$) at Sc values in the range 20-60%. The use of
582 higher Sc (100% v/v) led to a considerable decrease in *TP* (14.81 ± 0.15 $mg_{GAE}g_{DB}^{-1}$),
583 confirming the significance of a water fraction for efficient phenolic extraction from *A.*
584 *platensis* biomass. This result agrees with the observations of Esquivel-Hernández et al. [21],
585 who reported an enhancement of thiamine, riboflavin, C-phycocyanin and A-phycocyanin in
586 yields by microwave-assisted extraction from *A. platensis* when water/ethanol ratio was
587 increased from 0.25 to 0.81 (v/v) at 40 °C for 55 min.
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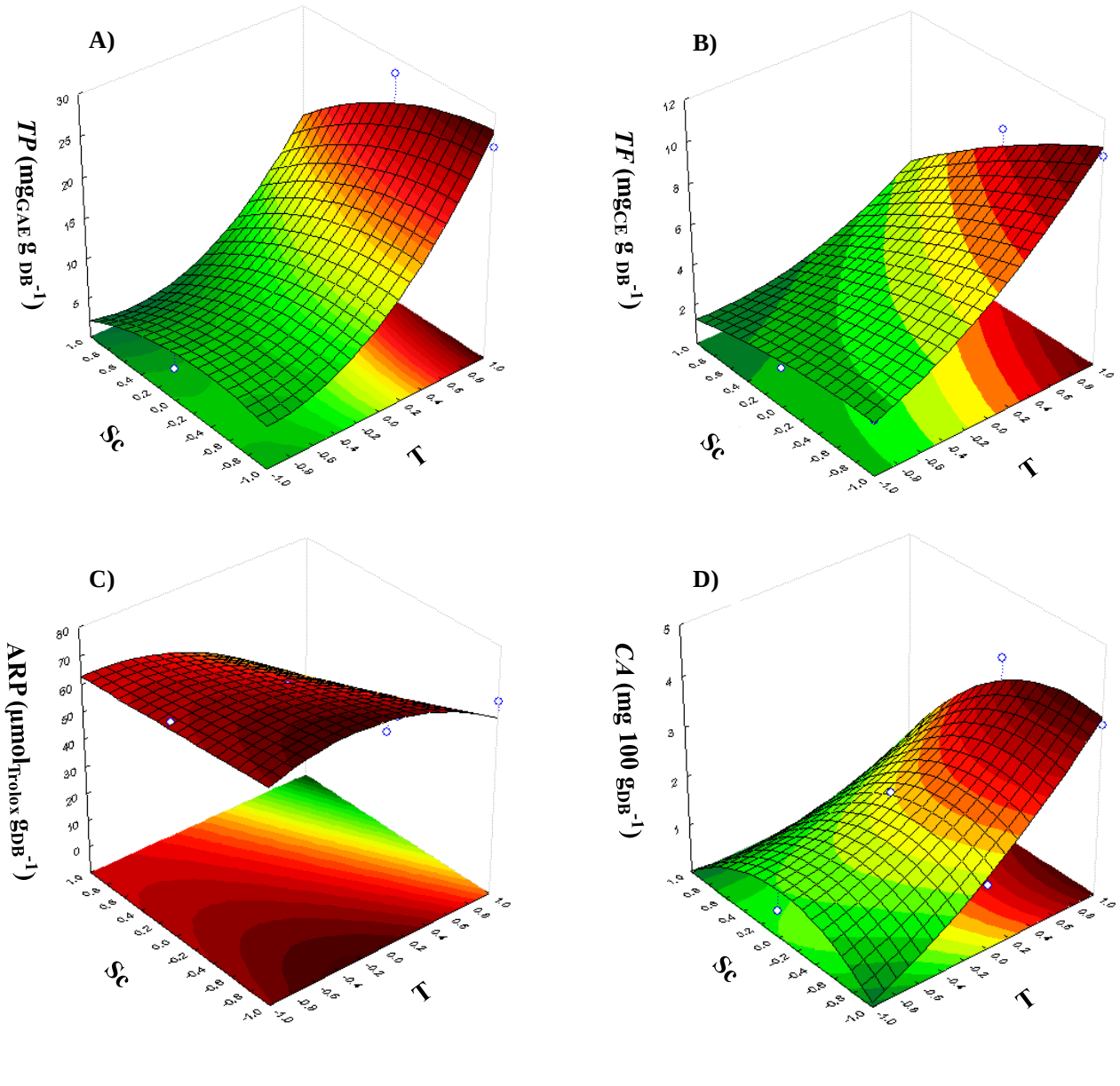

Fig. 4. Response surfaces of (A) total polyphenol yield (*TP*, mg_{GAE} g_{DB}^{-1}); (B) total flavonoid yield (*TF*, mg_{CE} g_{DB}^{-1}); (C) antiradical power determined by the $ABTS^{\bullet+}$ radical scavenging method (ARP, μmol_{Trolox} g_{DB}^{-1}) and (D) catechin content (*CA*, mg 100 g_{DB}^{-1}) as simultaneous functions of temperature (T, coded values) and ethanol concentration in the hydroalcoholic solution (Sc, coded values) in the *A. platensis* extracts obtained by high pressure/temperature extraction. GAE: gallic acid equivalents, CE: catechin equivalents.

Besides, the low temperature range between 90 and 135 ºC proved not to be efficient to recover polyphenols irrespectively of Sc, while a further T increase (180 ºC) for Sc up to 60% resulted in a remarkable rise in *TP*. For instance, at Sc of 20%, *TP* increased by no less than 93 and 118% when T was increased from 90 to 135 ºC and from 135 to 180 ºC, respectively. As early mentioned (see section 3.2), high temperature may have improved the extraction efficiency, because of enhanced diffusion rate and solubility of solutes [46,57,58], as well as the disintegration of cell wall and cell membrane [21].

As expected by the similarity of Eqs. (2) and (3), *TF* dependence on T and Sc (Fig. 4B) was very like that already discussed for *TP* (Fig. 4A). Temperature had a strong influence also on this response, in that the positive linear (+2.83) and quadratic (+0.98) terms of T were responsible for an exponential increase in *TF*, but the negative linear (-1.89) and quadratic (-0.38) terms of Sc together with the negative interaction term of both independent variables (-1.21) led to a maximization of this response (10.25 ± 0.34 $mg_{CE}\ g_{DB}^{-1}$) at the lowest Sc (20%) and the highest T (180 ºC) values. In particular, at this temperature, the *TF* increase was as higher as 115 and 149% when Sc was lowered from 100 to 60% and from 100 to 20%, respectively.

ARP of *A. platensis* extracts obtained by HPTE was influenced by T and Sc in a quite different way when compared with *TP* and *TF* (Fig. 4C). Being the negative linear regression coefficient of T the most significant term of the model (-12.43), followed by the negative quadratic term of the same variable (-10.44), the negative linear term of Sc (-8.69), and the interaction one between them (-6.19), ARP showed a parabolic trend and reached a maximum (67.77 ± 0.20 $\mu mol_{Trolox}\ g_{DB}^{-1}$ to 69.02 ± 1.11 $\mu mol_{Trolox}\ g_{DB}^{-1}$; $p > 0.05$) in runs 1, 2 and 7 carried out in the T and Sc ranges of 90-135°C and 20-60%, respectively (Table 1).

However, at the highest T (180 ºC), the negative influence of Sc became more marked, i.e., an ARP decrease by 44.1 and 58.2% took place when Sc was increased from 20 to 60%

and from 20 to 100%, respectively, which suggests that the antioxidant compounds mainly responsible for this activity in *A. platensis* extract may be more soluble in water than in ethanol. This Sc effect was opposite to that observed by Maadane et al. [65] for ARP determined by the DPPH assay for SLE extracts of other microalgae (*Nannochlorpsis gaditana*, *Dunaliella* sp., *Dunaliella salina*, *Phaedactylium tricornutum*, *Isochrysis* sp., *Navicula* sp., *Chaeotoceros* sp., *Chlorella* sp. and *Tetraselmis* sp.).

As shown in Fig. 4D, catechin content showed a trend similar to those of *TP* and *TF* as function of T and Sc. In particular, the positive linear term of temperature (+1.08) was responsible for a 112.3% increase in *CA* when temperature was raised from 135 to 180 ºC at Sc of 60%. But, the negative linear (-0.68) and quadratic (-0.92) terms of Sc, combined with the negative interaction between the independent variables (-0.65), led to a maximization also of this response (3.45 ± 0.07 mg 100 g_{DB}^{-1} to 3.61 ± 0.01 mg 100 g_{DB}^{-1}; $p > 0.05$) for $20 \leq Sc \leq 60\%$.

3.5. Statistical correlations among responses

Because of some qualitative similarities among the above results, the values of the Pearson's correlation coefficient (*r*) [54] of linear equations relating the contents of different groups of phenolic compounds (*TP* and *TF*), individual phenolic compounds detected by HPLC (catechin, epicatechin, gallic, vanillic, syringic and ferulic acids) and ARP have been used to identify possible relationships among them (Table 3).

Table 3. Correlation matrix with Pearson's correlation coefficients (*r*) for the responses investigated in *A. platensis* extracts obtained by high pressure/temperature extraction.

	TP[A]	*TF*[B]	ARP[C]	*GA*[D]	*CA*[E]	*VA*[F]	*SA*[G]	*EP*[H]	*FA*[I]
TP	1	0.958*	-0.538	0.903*	0.895*	0.816*	0.924*	-0.378	-0.271
TF		1	-0.297	0.870*	0.923*	0.942*	0.902*	-0.367	-0.167
ARP			1	-0.410	-0.245	-0.045	-0.376	0.240	0.301
GA				1	0.958*	0.751*	0.890*	-0.359	-0.006
CA					1	0.848*	0.852*	-0.389	0.045
VA						1	0.779*	-0.357	-0.000
SA							1	-0.242	-0.392
EP								1	0.083
FA									1

[A] Total polyphenol yield.

[B] Total flavonoid yield.

[C] Antiradical power determined by the $ABTS^{\bullet+}$ radical scavenging method.

[D] Gallic acid.

[E] Catechin.

[F] Vanillic acid.

[G] Syringic acid.

[H] Epicatechin.

[I] Ferulic acid.

* $p < 0.05$

One can see from this table a strong positive ($r = 0.958$) and statistically significant ($p < 0.05$) correlation between *TP* and *TF* and satisfactory positive correlations between *TP* and most of the individual phenolic compounds detected by HPLC, namely *GA* ($r = 0.903$), *CA* ($r = 0.895$), *VA* ($r = 0.816$) and *SA* ($r = 0.924$). The same applies to the positive correlations of

TF and these compounds (r = 0.870, 0.923, 0.942 and 0.902, respectively), whereas correlations between *TP* or *TF* with *EP*, *SA* and ARP were not statistically significant (p > 0.05).

To shed more light on these issues, either *TP* or *TF* determined by their respective colorimetric methods were plotted in Fig. 5 versus the sum of contents of individual phenolic compounds quantified by HPLC (SPC), with high Pearson's coefficients highlighting good linear correlation among the two assays.

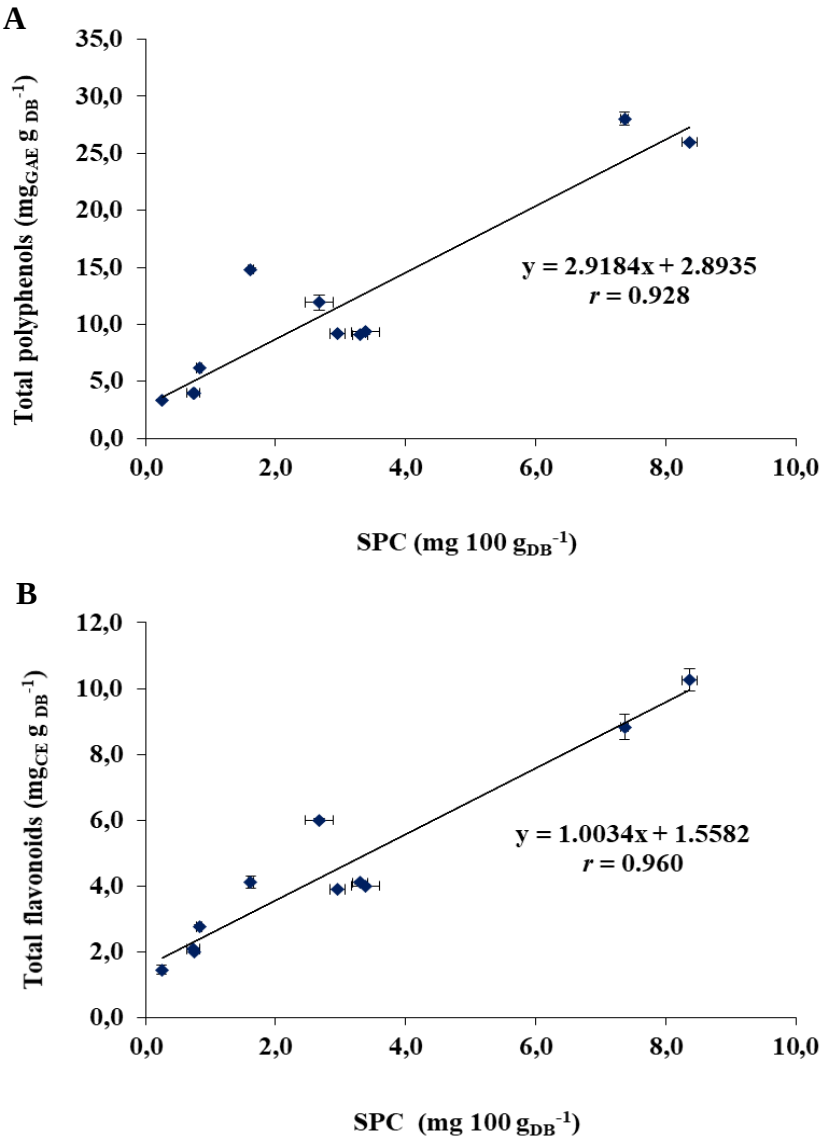

Fig. 5. Linear Pearson's correlations between the overall contents of total polyphenols and (A) total flavonoids, (B) the sum of contents of individual phenolic compounds quantified by

719 HPLC (SPC) in *Arthrospira platensis* extracts obtained by HPTE. GAE: gallic acid
720 equivalents, CE: catechin equivalents, HPTE: high pressure/temperature extraction. Data are
721 expressed as means of three replicates (n = 3). Error bars indicate means ± standard
722 deviations.
723
724 Statistically significant ($p < 0.05$) positive linear correlations of both *TP* (r = 0.928)
725 and *TF* (r = 0.960) and these compounds (Figs. 5A and B, respectively) indicate that the
726 phenolic compounds identified by HPLC were effective contributors to both groups of
727 phenolics.
728 On the other hand, the Pearson's coefficients of linear equations relating *TP* with ARP
729 and *TF* with ARP were not statistically significant ($p > 0.05$) for the extracts obtained under
730 the operating conditions investigated in this study (Table 3).
731 Since only polyphenols of a certain structure, particularly those carrying hydroxyl
732 groups are responsible for antioxidant properties, which depend on the ability to donate
733 hydrogen or electrons to free radicals [66], one can infer that the drastic HPTE conditions
734 might have somehow altered these structures. But a more likely reason is that photosynthetic
735 microorganisms are able to produce a wide range of alternative antioxidants to protect them,
736 including carotenoids, long-chain polyunsaturated fatty acids and polysaccharides [65,67].
737 For instance, Rodríguez-Meizoso et al. [68], who investigated the properties of
738 *Haematococcus pluvialis* extracts obtained by subcritical water extraction, observed that the
739 Trolox equivalent antioxidant capacity (TEAC) was correlated to vitamin E, together with
740 simple phenols, Maillard reaction products obtained during high temperature-extraction (200
741 ºC). Low correlations between antioxidant activity and phenolic contents (R^2 = 0.007-0.585)
742 were also found in a relatively large number of microalgae extracts obtained by three-step

743 sequential extraction with hexane, ethyl acetate and water [69], and the same occurred with
744 ethanolic extracts of nine microalgae strains ($R^2 = 0.154$) [65].

745 On the contrary, it was reported that the phenolic content significantly contributed to
746 the antioxidant capacity of *Isochrysis* sp., *Phaeodactylum* sp., *Chlorella* sp., *T. suecica*,
747 *Dunaliella salina*, *Fischerella ambigua*, *Nostoc muscorum*, *Oocystis pusilla* and *Scenedesmus*
748 *rubescens* [59,70]. These conflicting results may be explained with the fact that the
749 production of phenolics as well as other antioxidant compounds by microalgae and
750 cyanobacteria depends on growth conditions and nutritional and/or oxidative stress; therefore,
751 this aspect should be considered when the results are being compared with those of other
752 studies [71].

753

754 **4. Conclusions**

755

756 The high pressure/temperature extraction (HPTE) showed to be the most efficient
757 extraction method to recover total polyphenols and flavonoids from *Arthrospira platensis*
758 biomass as well as to obtain extracts with high antiradical power (ARP) using binary mixtures
759 of green solvents (ethanol/water). Results obtained according to a 3^2-full factorial design and
760 modeled by Response Surface Methodology clearly revealed that the extraction temperature
761 and solvent (ethanol) concentration in the hydroalcoholic solution (Sc) had a strong influence
762 on total polyphenol yield (*TP*), total flavonoid yield (*TF*) and ARP. The most suitable
763 conditions to efficiently recover *TP* and *TF* were shown to be the highest temperature (180
764 °C) and Sc in the range 20-60%, while a different trend was observed for ARP, which kept
765 always high in the whole tested temperature range ($90 \leq T \leq 135$ ºC) at 20-60%. HPLC
766 allowed identifying catechin and vanillic, gallic and syringic acids as the main phenolics in
767 the extract. Linear correlations between the overall content of these compounds taken together

and *TP* or *TF* suggested that they contributed significantly to either class of phenolics, whereas nothing similar was observed for ARP, suggesting that other cell constituents may play a major role in the antioxidant capacity of HPTE *A. platensis* extracts. This study demonstrates the potential of HPTE as a promising alternative technique to obtain high-ARP phenolic-rich hydroalcoholic extracts, and of *A. platensis* biomass as a potential natural source of compounds for functional food formulation or dietary supplement preparation. Furthermore, this work opens new avenues in the use of this emerging technology, for the recovery of other high-added value compounds from cyanobacterial biomass.

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CAPÍTULO III

6 ARTIGO - A NEW BIOENERGETIC AND THERMODYNAMIC APPROACH TO BATCH PHOTOAUTOTROPHIC GROWTH OF *Arthrospira (Spirulina) platensis* IN DIFFERENT PHOTOBIOREACTORS AND UNDER DIFFERENT LIGHT CONDITIONS

Este trabalho foi publicado na revista *Bioresource Technology* com fator de impacto igual a 5.651 (2016). Qualis Capes A1 (Biotecnologia). Anexo C.

A new bioenergetic and thermodynamic approach to batch photoautotrophic growth of *Arthrospira (Spirulina) platensis* in different photobioreactors and under different light conditions

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ABSTRACT

Photobioreactor configuration, mode of operation and light intensity are known to strongly impact on cyanobacteria growth. To shed light on these issues, kinetic, bioenergetic and thermodynamic parameters of batch *Arthrospira platensis* cultures were estimated along the time at photosynthetic photon flux density (PPFD) of 70 μmol m^{-2} s^{-1} in different photobioreactors with different surface/volume ratio (*S/V*), namely open pond (0.25 cm^{-1}), shaken flask (0.48 cm^{-1}), horizontal photobioreactor (HoP) (1.94 cm^{-1}) and helicoidal photobioreactor (HeP) (3.88 cm^{-1}). Maximum biomass concentration and productivity remarkably increased with *S/V* up to 1.94 cm^{-1}. HoP was shown to be the best-performing system throughout the whole runs, while HeP behaved better only at the start. Runs carried out in HoP increasing PPFD from 40 to 100 μmol m^{-2} s^{-1} revealed a progressive enhancement of bioenergetics and thermodynamics likely because of favorable light distribution. HoP appeared to be a promising configuration to perform high-yield indoor cyanobacterial cultures.

Keywords: Photobioreactor; bioenergetics; *Arthrospira platensis*; thermodynamics; light intensity.

1. Introduction

The energy management in a living cell is called bioenergetics. When combined with the fundamental principles of thermodynamics, it has proven to be a highly useful tool for the analysis of biosystems (Küçük et al., 2015). Photosynthetic microorganisms have recently gained huge attention worldwide (Cheah et al., 2015), since they are one of the most promising renewable and neutral energy sources, i.e., by consuming carbon dioxide, their cultivation has the additional benefit of combining valuable biomass production with CO_2 emissions reduction (Belay, 2002; Rodrigues et al., 2011). Among the photosynthetic microorganisms with commercial importance, the filamentous cyanobacterium (blue-green alga) *Arthrospira (Spirulina) platensis* has widely been studied, because its biomass has a large number of industrial applications besides being considered a high-value food (Belay, 2002; Benelhadj et al., 2016). *A. platensis* production is in fact increasing worldwide owing to its high contents of highly-valuable proteins, amino acids, essential fatty acids (i.e., γ-linolenic acid, GLA), polysaccharides, vitamins and pigments (β-carotene, chlorophyll a and phycocyanin) (Pulz and Gross, 2004); in addition, it contains other phytochemicals that find application in several industrial segments like those of health foods and therapeutics (Belay, 2002; Pulz and Gross, 2004; Raposo and Morais, 2015).

A lot of parameters can be tuned to obtain high biomass yield, among which are the mode of operation, light intensity and reactor configuration, which can strongly impact on the performance of *A. platensis* cultivation. Different types of processes have been developed to get an optimal compromise between high productivity and low production costs. Among these are: a) the fed-batch process that consists in periodically replacing a part of the exhaust medium with fresh medium to keep the culture volume constant; b) the batch culture systems, which are the most widely applied because of their simplicity and flexibility, even though not necessarily the most efficient ones; and c) large-scale continuous cultures, which have not

been applied extensively up to now, due to several difficulties in their control, among which are higher risk of contamination, use of feeding pumps, lower yields, etc.

Light irradiance should be provided with care to indoor systems performed using artificial light, since excess light leads to a phenomenon called “photooxidation” or “photoinhibition”. That is, cell concentration increases with light intensity until reaching a maximum threshold value at the so-called “saturation level”, beyond which a further increase in light intensity provokes damage of cell photosynthetic apparatus (Bezerra et al., 2012).

The reactor configuration is an additional factor greatly influencing cell growth. Photobioreactors can reduce the cultivation area by a vertical distribution of the photosynthetic organism and enlarge the surface exposed to light, thereby ensuring high surface/volume ratios and increasing cell concentration. Light is better captured by cells in tubular photobioreactors when compared to the conventional open ponds, where, owing to a relatively high depth of culture medium, it has to go through thick layers to reach the inner cells (Converti et al., 2006; Rodrigues et al., 2010). Although the open-channel raceway ponds is the most widely used configuration for *A. platensis* commercial production, tubular photobioreactors have been deeply studied, not only because of their high cell productivity, but also of many other advantages, such as low levels of contamination and better CO_2 solubilization in the medium, better light distribution and then higher photosynthetic efficiency (Converti et al., 2006).

Bioenergetic studies based on the Gibbs energy dissipation may be applied to describe or predict the microbial growth yield, the energy flow to ATP production, the increase in enthalpic content and the heat released by living organisms (Bezerra et al., 2012; Sassano et al., 2004; Torre et al., 2003). All of them are quite important to optimize any bioprocess and even to design the most suitable bioreactor to perform it; but, unfortunately, only a few studies dealt with the bioenergetic aspects of the growth of photosynthetic microorganisms

based on Gibbs energy balances. In particular, biomass yield constitutes one of the key parameters in any bioprocess or experiment involving microbial cultures, since it determines the final biomass concentration, which must be maximized to obtain high productivities (Von Stockar et al., 2006). In addition, the Gibbs energy dissipation per C-mol of biomass can be regarded as a simple thermodynamic measure of the amount of biochemical "work" required to convert the carbon source into biomass (Bezerra et al., 2012; Heijnen and Van Dijken, 1991, 1993; Liu et al., 2007).

Based on this background, if from one hand previous works demonstrated that the use of the fed-batch mode of operation is able to promote the growth of photosynthetic microorganisms minimizing a number of well-known adverse phenomena (shading, inhibitions related to excess salt level or osmotic pressure, and so on), from the other it masks their effects. Therefore, to shed light on these issues and because of a certain lack of detailed and recent theoretical studies on the mechanisms ruling *A. platensis* cultures, the main bioenergetic and thermodynamic parameters of the photoautotrophic batch growth of such a cyanobacterium were investigated in this study based on the model proposed by Torre et al. (2003). For this purpose, we investigated four different photobioreactor configurations with different surface/volume ratios, namely shaken flask, open pond, helicoidal photobioreactor and horizontal photobioreactor, and varying the light irradiance in the last, best-performing configuration.

2. Methods

2.1. Microorganism and culture conditions

A. (Spirulina) platensis UTEX 1926 was obtained from the Culture Collection of Algae of the University of Texas (Austin, TX, USA). To allow a large growth of biomass, the microorganism was maintained and cultivated in the culture medium suggested by Schlösser (1982) modified so as to have a nitrogen concentration equal to about 4-fold that of the original medium. The resulting medium had the following composition (per liter): 13.61 g $NaHCO_3$, 4.03 g Na_2CO_3, 0.50 g K_2HPO_4, 10.0 g $NaNO_3$, 1.00 g K_2SO_4, 1.00 g NaCl, 0.20 g $MgSO_4 \cdot 7\ H_2O$, 0.04 g $CaCl_2 \cdot 2\ H_2O$. All the nutrients were dissolved in distilled water containing (per liter): 6.0 mL of metal solution (97 mg L^{-1} $FeCl_3 \cdot 6\ H_2O$, 41 mg L^{-1} $MnCl_2 \cdot 4\ H_2O$, 5 mg L^{-1} $ZnCl_2$, 2 mg L^{-1} $CoCl_2 \cdot 6\ H_2O$, 4 mg L^{-1} $Na_2MoO_4 \cdot 2\ H_2O$, 750 mg L^{-1} $Na_2EDTA \cdot 2\ H_2O$), 1.0 mL of micronutrient solution (50.0 mg L^{-1} Na_2EDTA, 618 mg L^{-1} H_3BO_3, 19.6 mg L^{-1} $CuSO_4 \cdot 5\ H_2O$, 44.0 mg L^{-1} $ZnSO4 \cdot 7\ H_2O$, 20.0 mg L^{-1} $CoCl_2 \cdot 6\ H_2O$, 12.6 mg L^{-1} $MnCl_2 \cdot 4\ H_2O$, 12.6 mg L^{-1} $Na_2MoO_4 \cdot 2\ H_2O$) and 1.0 mL of B12 vitamin solution (0.15 mg L^{-1}).

Cells were grown batch-wise either at photosynthetic photon flux density (PPFD) of 70 μmol m^{-2} s^{-1} in different photobioreactor configurations with different surface/volume ratios (*S/V*), namely open pond (Sassano et al., 2004) (*S/V* = 0.25 cm^{-1}), shaken flask (Frumento et al., 2016) (*S*/V = 0.48 cm^{-1}), horizontal photobioreactor (Ferreira et al., 2010) (*S/V* = 1.94 cm^{-1}) and helicoidal photobioreactor (Bezerra et al., 2011; Frumento et al., 2016) (*S/V* = 3.88 cm^{-1}), or progressively increasing PPFD from 40 to 100 μmol m^{-2} s^{-1} in the horizontal photobioreactor that proved the best-performing configuration. For this purpose, fluorescent artificial light was ensured by a variable number of 36 W-lamps. Schematics of horizontal and helicoidal photobioreactors were illustrated in a previous study (Frumento et al., 2013).

Cultivations were carried out at temperature of 30 ± 2 °C, by incubating the equipment in a thermostated chamber, using an initial biomass concentration of 0.40 g L^{-1}. The pH was

controlled daily at 9.5 ± 0.2 through the addition of pure CO_2 from a cylinder. After growth, once the stationary phase had been reached after about 9 days of cultivation, biomass was separated from the culture medium by centrifugation at 7500 rpm for 10 min using a centrifuge, model 42426 (ALC, Milan, Italy). Recovered cells were washed twice with distilled water, dried at 105 °C for 24 h, pulverized in a mortar and stored at −20 °C for subsequent analysis of its elemental composition.

2.2. Analytical procedures

Cell concentration of *A. platensis* was determined daily by measuring the optical density (OD) at 560 nm by a UV-visible spectrophotometer, model Lambda 25 (PerkinElmer, Milan, Italy), and expressed in grams of dried biomass per liter of medium (g L^{-1}) through a calibration curve relating OD to dry biomass weight. All measurements were done in triplicate.

The elemental composition of dried biomass stored at the end of culture was determined by an elemental analyzer Flash EA1112 series (CE Instruments, Wigan, UK). Since biomass composition varied very little among the different photobioreactor configurations and light intensities, its average value obtained from all the runs ($CH_iO_jN_k$ where i = 1.59 ± 0.14, j = 0.50 ± 0.06, k = 0.10 ± 0.01) was used to estimate the bioenergetic and thermodynamic parameters of growth.

PPFD was measured in several points of the culture surface using a type sensor quantum/photometer/radiometer, model HD-9021 Delta OHM (Li-Cor Inc., Lincoln NE, USA).

3. Theory

3.1. Kinetic parameters of A. platensis *growth*

A. platensis cultures in different photobioreactor configurations under different light intensities has first been evaluated in terms of variations of the main growth parameters, namely the maximum cell concentration (X_{max}), the maximum specific growth rate (μ_{max}) and the maximum biomass productivity ($P_{X\,max}$).

To this purpose, μ_{max} was calculated by the equation:

$$\mu_{max} = \frac{1}{t_i - t_{i-1}} \ln\left(\frac{X_i}{X_{i-1}}\right) \quad (1)$$

where X_i and $X_{i\text{-}1}$ are biomass concentrations (g L^{-1}) at times t_i and $t_{i\text{-}1}$ (days), respectively.

Analogously, $P_{X\,max}$ was defined as:

$$P_{X\,max} = \frac{X_i - X_{i-1}}{t_i - t_{i-1}} \quad (2)$$

It should be noticed that t_i and $t_{i\text{-}1}$ are the cultivation times separately maximizing the functions μ_{max} and $P_{X\,max}$; therefore, they have not the same values in Eqs. (1) and (2).

3.2. Bioenergetic and thermodynamic parameters of A. platensis *growth*

To estimate the bioenergetics parameters of *A. platensis* growth under batch conditions, we applied the model proposed by Torre et al. (2003), according to whom, under ideal conditions, the energy carried by 8 photons at 580 nm is captured by photosynthetic

pigments held in the "reaction centers" of the photosystem II (PSII) and used to transfer 4 electrons from 2 water molecules to the photosystem I (PSI). In fact, according to Richmond (1983), the photosynthetic organisms can only use light with wavelength in the range 400-700 nm, and cyanobacteria have an energetic input (Li et al., 2001) equivalent to that of 580 nm monochromatic light (206.2 kJ mol^{-1} of photons). However, the yield of this process decreases as the conditions differ from the ideal ones. An equal number of photons with less energy is then released as heat, while the 4 electrons, through the PSI electron transport system, are used to reduce $NADP^+$ to NADPH:

$$2\ H_2O + E + 2\ NADP^+ \rightarrow 2\ NADPH + O_2 + 2\ H^+ \quad (3)$$

and finally transferred from NADPH to CO_2 through the Calvin cycle to produce biomass constituents.

The dissipation of Gibbs energy for cell growth and maintenance ($1/Y_{GX}$) was estimated, according to Heijnen (2001), as:

$$\frac{1}{Y_{GX}} = \frac{1}{Y_{GX}^{\max}} + \frac{m_G}{\mu} \quad (4)$$

where $1/Y_{GX}^{\max}$ is the growth-related Gibbs energy requirement, equivalent to 986 kJ per C-mol of dry biomass for photoautotrophic cultures, μ the specific growth rate and m_G the specific rate of Gibbs energy dissipation for maintenance, equivalent to 7.12 kJ $C\text{-}mol^{-1}$ h^{-1} at 30°C.

The photoautotrophic growth can be described by an overall mass balance equation, where the nitrogen (NO_3^-) and carbon (HCO_3^-) sources, H_2O, O_2, and H^+ are involved in the formation of one C-mol of dry biomass:

$$a\ HCO_3^- + b\ NO_3^- + c\ H_2O + d\ O_2 + e\ H^+ + CH_{1.59}O_{0.50}N_{0.10} = 0 \quad (5)$$

where *a*, *b*, *c*, *d* and *e* are the stoichiometric coefficients of the chemical species involved in biomass formation expressed in mol C-mol^{-1} and $CH_{1.59}O_{0.50}N_{0.10}$ is the average elemental composition of biomass experimentally determined in this work.

The stoichiometric coefficients estimated through material balances of carbon, nitrogen, oxygen, charge, and reduction degree are listed in Table 1.

Table 1. Molar Gibbs energy of formation (Δg_f) and stoichiometric coefficients (a–e)[a] of the chemical species involved in *A. platensis* biomass formation on modified Schlösser medium.

Species	HCO_3^-	NO_3^-	H_2O	O_2	H^+	Biomass
Δg_f (kJ mol^{-1})	-587.2	-111.4	-237.3	0	-54.4[b]	-67.0[c]
Stoichiometric coefficients						
Symbol	*a*	*b*	*c*	*d*	*e*	γ_X [d]
Value (mol C-mol^{-1})	-1.000	-0.100	0.255	1.272	-1.100	5.09

[a] Stoichiometric coefficients estimated through material balances of carbon, nitrogen, oxygen, charge, and reduction degree, according to the bioenergetic model of Eq. (5).

[b] Value calculated at pH 9.5 through the Gibbs equation (Eq. (7)) using $\Delta g°'_{fH^+}$ = -39.87 kJ mol^{-1} at pH 7.0.

[c] Value expressed in kJ C- mol^{-1} instead of kJ mol^{-1}.

[d] Reduction degree of biomass (dimensionless) referred to the average elemental composition of biomass ($CH_{1.59}O_{0.50}N_{0.10}$) experimentally determined in this study.

The following Gibbs energy balance should have been employed to estimate the moles of photons (Einsteins), n_{Ph}, to sustain the autotrophic growth of one C-mol of *A. platensis* biomass under biological standard conditions (298 K, pH = 7, 1 bar, concentrations of 1 mol L^{-1}):

$$n_{Ph} = -\frac{a\,\Delta g^{o\prime}_{f_{HCO_3^-}} + b\,\Delta g^{o\prime}_{f_{NO_3^-}} + c\,\Delta g^{o\prime}_{f_{H_2O}} + d\,\Delta g^{o\prime}_{f_{O_2}} + e\,\Delta g^{o\prime}_{f_{H^+}} + \Delta g^{o\prime}_{f_X} + 1/Y_{GX}}{\Delta g^{o\prime}_{Ph}} \quad (6)$$

where $\Delta g°'_{f_{HCO_3^-}} = -587.2$ kJ mol^{-1}, $\Delta g°'_{f_{NO_3^-}} = -111.4$ kJ mol^{-1}, $\Delta g°'_{f_{H_2O}} = -237.3$ kJ mol^{-1}, $\Delta g°'_{f_{O_2}} = 0$ and $\Delta g°'_{f_{H^+}} = -39.87$ kJ mol^{-1} are the molar Gibbs energies of formation of the chemical species involved in the photosynthetic event, while $\Delta g°'_{f_X} = -67.0$ kJ C-mol^{-1} and $\Delta g°'_{Ph} = h\underline{c}N_A/\lambda = 206.2$ kJ mol^{-1} are those associated with the formation of biomass and the absorption of 1 mol of photons, respectively, being $h = 6.626 \cdot 10^{-34}$ J·s the Planck constant, $\underline{c} = 2.99626 \cdot 10^{8}$ m s^{-1} the light velocity, $N_A = 6.022626 \cdot 10^{23}$ mol^{-1} the Avogadro number and $\lambda = 580$ nm the early-mentioned reference wavelength.

However, culture conditions were quite different from the biological standard ones, and the actual Δg_f values should have been used for this calculation. Nonetheless, as stressed by Torre et al. (2003), the actual Gibbs energies of formation do not differ at all or differ by no more than 3% from those under standard biological conditions, with exception of $\Delta g_{f_{H^+}}$, which is by definition a function of pH; therefore, this parameter was recalculated ($\Delta g_{f_{H^+}} = -54.4$ kJ mol^{-1}) for the actual conditions (pH 9.5; 30°C) by the well-known equation of Gibbs:

$$\Delta g_{f_{H^+}} = \Delta g^{o\prime}_{f_{H^+}} + RT \ln \frac{10^{-9.5}}{10^{-7.0}} \quad (7)$$

where T is the absolute temperature (K) and R the ideal gas constant (8.3145 J mol^{-1} K^{-1}).

Once n_{Ph} had been estimated, the molar Gibbs energy associated with the photosynthetic growth of one C-mol of biomass (ΔG_a) was calculated as:

$$\Delta G_a = n_{Ph}\,\Delta g_{Ph} = \Delta H + \Delta G_{ATP} + Q \quad (8)$$

and assumed to be equivalent to the sum of the energy fixed by the system to increase its own enthalpic content (ΔH), the Gibbs energy transformed into ATP and used for growth and maintenance (ΔG_{ATP}) and that released as heat (Q).

ΔG_{ATP} and ΔH were calculated from n_{Ph}, the Gibbs energy associated to one mol of ATP (286.2 kJ mol^{-1}) and the sum (E^0 = 1.236 V) of the average potential variations between PSI and PSII (E^0 = 0.580 V) and between PSI and NADPH (E^0 = 0.656 V) reported in the literature for most of synthetic organisms (Torre et al., 2003).

The rates of O_2 production (q_{O_2}) and H^+ consumption (q_{H^+}) were calculated by multiplying their respective stoichiometric coefficients by the cell concentration expressed in C-mol L^{-1}, by using the biomass elemental composition experimentally determined in this study ($CH_{1.59}O_{0.50}N_{0.10}$), and by dividing by the cultivation time.

4. Results and discussion

Previous works demonstrated that the mode of operation, the photobioreactor configuration and the light intensity strongly influence the performance of *A. platensis* cultivation. In particular, Ferreira et al. (2010) found that fed-batch addition of nitrate as

nitrogen source in a horizontal photobioreactor working at PPFD of 60, 120 and 240 μmol m^{-2} s^{-1} led to photo-limitation, photosaturation and photoinhibition, respectively, hence confirming the leading role of light intensity. A subsequent study performed under comparable conditions but comparing different photobioreactor configurations under fed-batch addition of two different nitrogen sources (nitrate and urea), highlighted that also these factors, besides the light intensity, exert a strong influence on the growth of such a cyanobacterium acting on its bioenergetic parameters (Bezerra et al., 2012). However, if from one hand the use of the fed-batch mode of operation is able to promote the growth minimizing a number of adverse phenomena (shading, inhibitions related to excess salt level or osmotic pressure, and so on), from the other it join them masking their separate effects.

To shed better light on these effects, the main growth, bioenergetic and thermodynamic parameters have been calculated in this study from batch cultures of *A. platensis* carried out either at PPFD of 70 μmol m^{-2} s^{-1} in four photobioreactor configurations, namely open pond (OP), shaken flask (SF), horizontal photobioreactor (HoP), helicoidal photobioreactor (HeP), or progressively increasing PPFD from 40 to 100 μmol m^{-2} s^{-1} in HoP that proved to be the best-performing configuration.

4.1. Kinetic parameters of A. platensis *growth*

It can be seen in Fig. 1 that biomass concentration was dramatically influenced by the photobioreactor configuration, in that its maximum value (X_{max}) strongly raised from 2.14 ± 0.11 g L^{-1} to 3.56 ± 0.25 g L^{-1} and 7.11 ± 0.53 g L^{-1} when the surface to volume (*S/V*) was increased from 0.25 cm^{-1} (OP) to 0.48 cm^{-1} (SF) and 1.94 cm^{-1} (HoP), respectively (Table 2). Such a behavior may be ascribed to a better light distribution in HoP along with the occurrence of the well-known shading effect of outer cell layers on the inner ones in systems with insufficient exposition to light (SF and OP), which resulted in reduced light availably to

cells (Danesi et al., 2004). A separate reasoning should be applied to HeP, which, despite a *S/V* ratio that was twice that of HoP, offered unexpectedly a sigmoidal growth curve, with better performance during the log phase but a X_{max} value about 15% lower (6.02 ± 0.27 g L^{-1}) (Table 2). Such an observation could be explained with a combination of different positive and negative factors in HeP compared with HoP, among which the most important may have been the higher *S/V* ratio, resulting in better growth up to 4 days, and a more intense agitation, leading to excess mechanical stress to cells responsible for a lower X_{max} value.

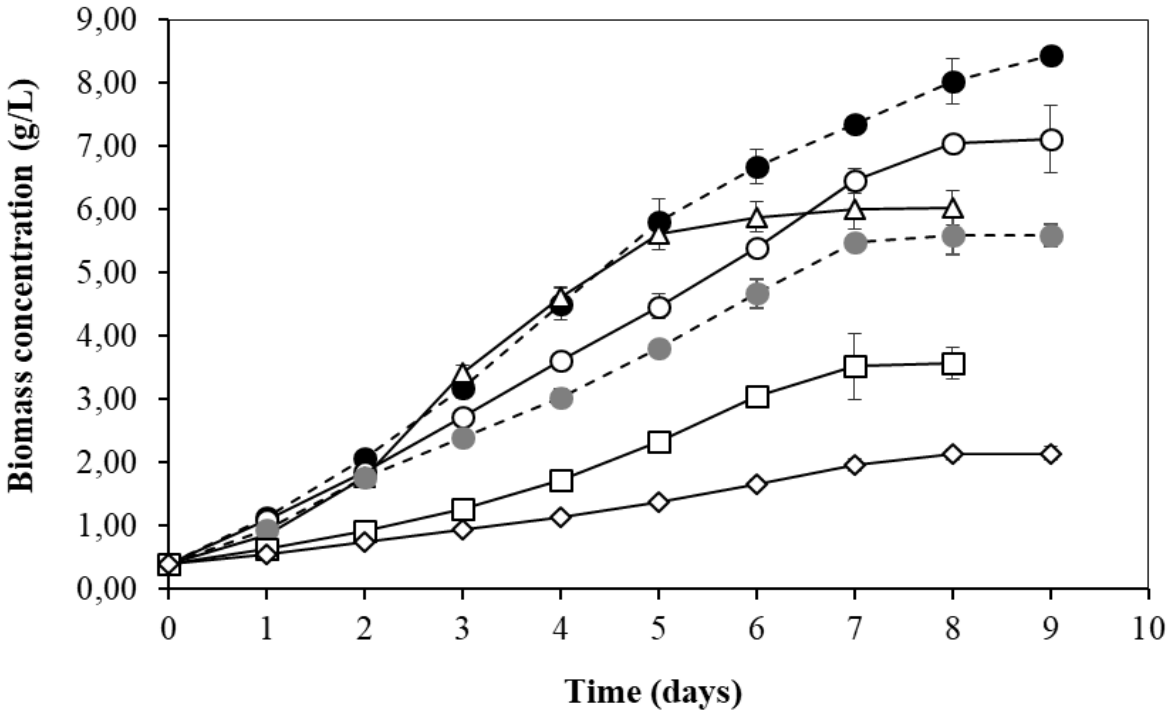

Fig. 1. Biomass concentration during *A. platensis* cultivations performed at PPFD of 70 μmol m^{-2} s^{-1} in different photobioreactor configurations with different *S/V* ratios: (◇) Open Pond, *S/V* = 0.25 cm^{-1}; (□) Shaken Flask, *S/V* = 0.48 cm^{-1}; (○) Horizontal Photobioreactor, *S/V* = 1.94 cm^{-1}; (△) Helicoidal Photobioreactor, *S/V* = 3.88 cm^{-1}. Cultivations carried out in the Horizontal Photobioreactor at different PPFD (μmol m^{-2} s^{-1}): (···●···) 40; (···●···) 100.

The main growth parameters of these cultures summarized in Table 2 clearly show that HoP was by far the best photobioreactor configuration in terms not only of maximum cell concentration but also of maximum specific growth rate (μ_{max} = 0.988 ± 0.012 day^{-1}), while HeP ensured the highest maximum cell productivity ($P_{X\ max}$ = 1.12 ± 0.04 g L^{-1} d^{-1}). These results confirm that, even though HeP was the best configuration at the beginning of runs, its performance worsened along the time; thus, such conditions may not be the best option for a slow-growth system like this, especially when used in long-term (continuous or fed-batch) operations.

As far as the effect of light intensity is concerned, the results of Table 2 also shows that *A. platensis* growth in the best photobioreactor configuration (HoP) was favored by a progressive increase in PPFD up to 100 μmol m^{-2} s^{-1}, reaching X_{max} = 8.44 ± 0.13 g L^{-1}, $P_{X\ max}$ = 1.08 ± 0.07 g L^{-1} d^{-1} and μ_{max} = 1.047 ± 0.006 day^{-1}. Lower X_{max} values were reported for a two-plane tubular photobioreactor for outdoor culture (4.2 g L^{-1}), elevated panels (5.0 g L^{-1}) and mainly open ponds (0.8 g L^{-1}), while a similar combined airlift-tubular photobioreactor system allowed reaching comparable X_{max} values (6.5-10.6 g L^{-1}) but lower productivity (0.29-0.62 g L^{-1} d^{-1}) under similar irradiation conditions (55 ≤ PPFD ≤ 120 μmol m^{-2} s^{-1}) (Converti et al., 2006). These results demonstrate that light intensity was the growth-limiting factor under the selected conditions, in agreement with previous findings that demonstrated the occurrence of photoinhibition in the same photobioreactor at PPFD as high as 240 μmol m^{-2} s^{-1} (Bezerra et al., 2012). The following bioenergetic study allowed shed light on these observations.

Table 2. Kinetic parameters of batch *A. platensis* cultures on modified Schlösser medium performed (a) in different photobioreactor configurations at photosynthetic photon flux density (PPFD) of 70 μmol m^{-2} s^{-1} and (b) in horizontal photobioreactor under different PPFD values.

	X_{max} (g L^{-1})[a]	$P_{X\,max}$ (g L^{-1} d^{-1})[b]	μ_{max} (d^{-1})[c]
a) Photobioreactor			
HoP	7.11 ± 0.53	0.86 ± 0.03	0.988 ± 0.012
HeP	6.02 ± 0.27	1.12 ± 0.04	0.750 ± 0.011
SF	3.56 ± 0.25	0.45 ± 0.07	0.466 ± 0.006
OP	2.14 ± 0.11	0.22 ± 0.01	0.322 ± 0.005
b) PPFD (μmol m^{-2} s^{-1})			
40	5.59 ± 0.18	0.73 ± 0.02	0.869 ± 0.052
70	7.11 ± 0.53	0.86 ± 0.03	0.988 ± 0.012
100	8.44 ± 0.13	1.08 ± 0.07	1.047 ± 0.006

HoP = Horizontal Photobioreactor; HeP = Helicoidal Photobioreactor; SF = Shaken Flask; OP = Open Pond.

[a]X_{max} = maximum cell concentration.

[b]$P_{X\,max}$ = maximum cell productivity. Values obtained after 7 days, except for runs carried out in HeP at PPFD of 70 μmol m^{-2} s^{-1} (5 days) and HoP at PPFD of 100 μmol m^{-2} s^{-1} (5 days).

[c]μ_{max} = maximum specific growth rate. All values obtained after 1 day.

4.2. Bioenergetic parameters of A. platensis *growth*

Consistently with Westerhoff and Van Dam (1987), according to whom a high rate of Gibbs energy dissipation is related to high metabolic rates but low biomass yield, Bezerra et al. (2012) observed that the Gibbs energy dissipation for cell growth and maintenance ($1/Y_{GX}$) progressively increased in fed-batch *A. platensis* cultures as the likely result of the increased biomass level. The results of Fig. 2, which illustrates the decrease along the time of the reciprocal of this parameter (Y_{GX}), i.e. the biomass yield on Gibbs energy, confirm the

suspicion of those authors that the increase in the energy requirements observed when the system approached the stationary growth phase may have been related to a dramatic decrease in the specific growth rate, owing to the difficulty in synthesizing biomass from given carbon and energy sources (Heijnen, 1994), rather than to biomass decay.

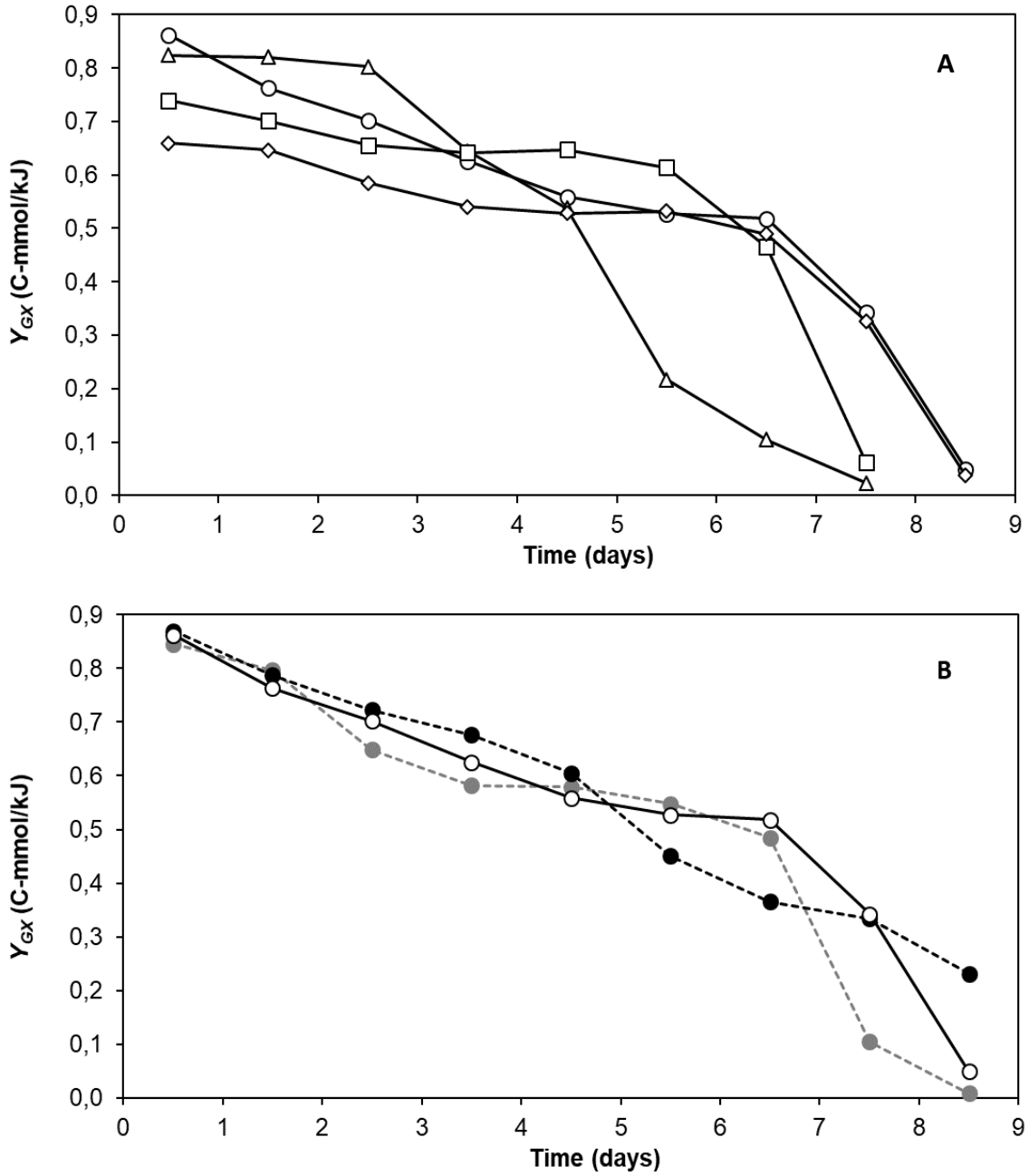

Fig. 2. Time behavior of biomass yield on Gibbs energy (Y_{GX}) during *A. platensis* cultivations performed: A) at PPFD of 70 μmol m^{-2} s^{-1} in: (◇) Open Pond, S/V = 0.25 cm^{-1}; (□) Shaken

Flask, *S/V* = 0.48 cm^{-1}; (○) Horizontal Photobioreactor, *S/V* = 1.94 cm^{-1}; (△) Helicoidal Photobioreactor, *S/V* = 3.88 cm^{-1}. B) in the Horizontal Photobioreactor varying PPFD (μmol m^{-2} s^{-1}): (···●···) 40; (—○—) 70; (···●···) 100.

A comparison among the different photobioreactor configurations (Fig. 2A) reveals that the best-performing one (HoP) allowed delaying the quick fall of Y_{GX} almost to zero by several days with respect to SF and HeP. It is also noteworthy that this occurrence always took place when μ was less than 0.01 day^{-1} (results not shown). As suggested by Bezerra et al. (2012), under these stress conditions the growth is so strongly affected that most of the Gibbs energy is addressed to cell maintenance. In addition, it should be noticed that, in OP and SF, Y_{GX} was at the beginning of the runs about 23 and 14% lower than in HoP (0.863 C-mmol kJ^{-1}), which means that biomass production required more energy, thus resulting in poorer performances. On the other hand, the helicoidal configuration, which at the beginning showed comparable performance to HoP because of its higher *S/V* ratio (3.88 cm^{-1}), after about 4 days suffered a quick worsening likely due to excess mechanical stress. As earlier mentioned, a progressive increase in light intensity in HoP favored cell growth because of photolimitation in the tested PPFD range; as a result, Y_{GX} fell sharply only after 6.5 days in runs carried out at PPFD of 40 and 70 μmol m^{-2} s^{-1}, while it decreased slightly even after 8.5 days at 100 μmol m^{-2} s^{-1} (Fig. 2B).

As shown in Fig. 3A, the moles of photons absorbed to produce one C-mol of biomass (n_{Ph}) progressively increased (in absolute value) throughout all the cultivations, just reflecting the decrease in Y_{GX} illustrated in Fig. 2.

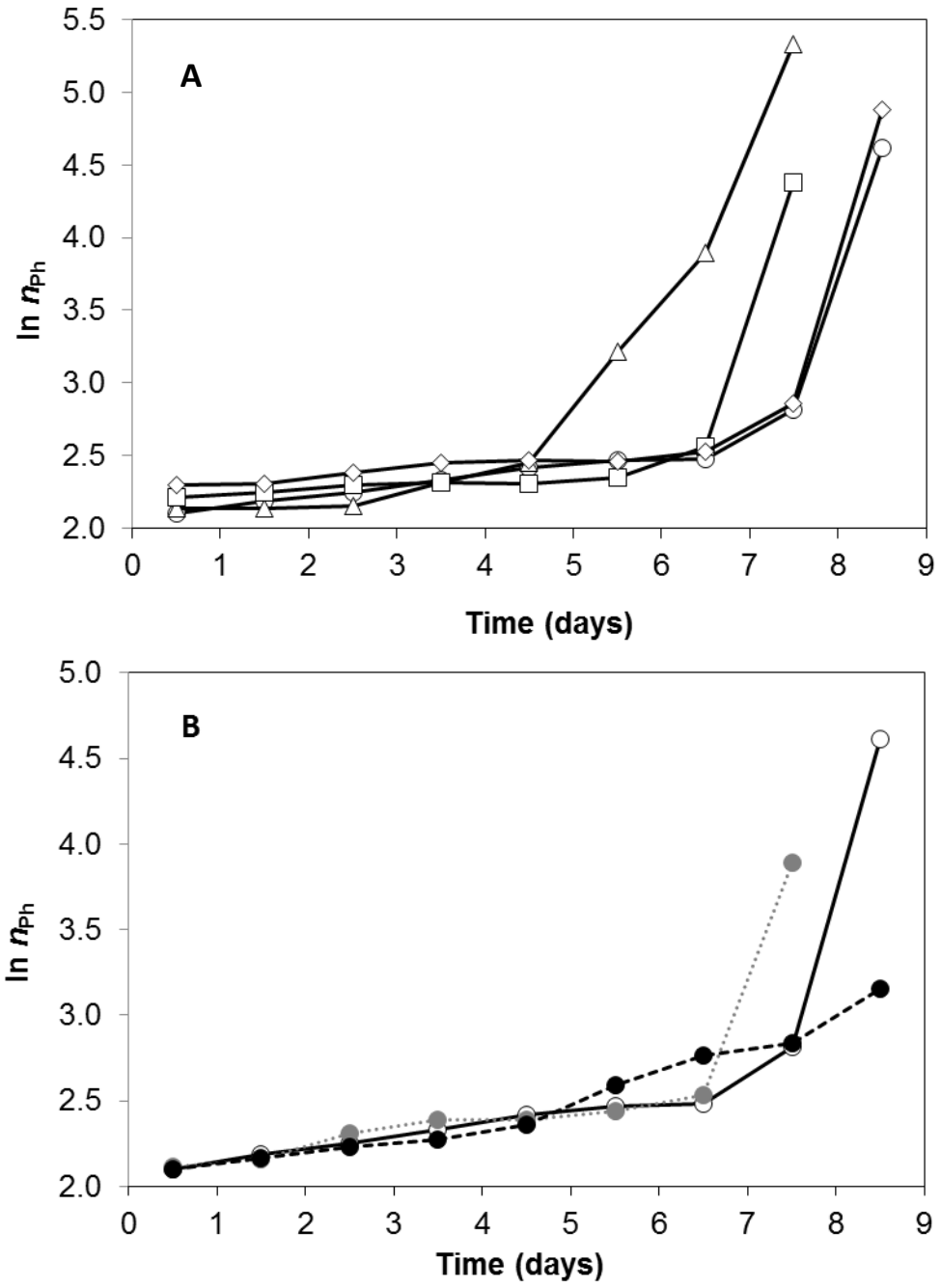

Fig. 3. Semi-log plot of the moles of photons required for the synthesis of 1 C-mol of *A. platensis* biomass (n_{Ph}, expressed in mol C-mol^{-1}) versus time during cultivations performed: A) at PPFD of 70 μmol m^{-2} s^{-1} in: (◇) Open Pond, *S/V* = 0.25 cm^{-1}; (□) Shaken Flask, *S/V* = 0.48 cm^{-1}; (○) Horizontal Photobioreactor, S/V = 1.94 cm^{-1}; (△) Helicoidal Photobioreactor, *S/V* = 3.88 cm^{-1}. B) in the Horizontal Photobioreactor varying PPFD (μmol m^{-2} s^{-1}): (···●···) 40; (—○—) 70; (···●···) 100.

As expected, at the beginning of runs where the microorganism grew at the highest μ, this bioenergetic parameter approached values very close to that reported to sustain growth under ideal conditions, i.e. 8 mol of photons C-mol^{-1} ($\ln n_{Ph}$ = 2.08) of biomass (Richmond, 1983); the release of 1 mol of O_2 by the photosynthesis does in fact imply the absorption of at least 8 mol of photons and the fixation of 1 mol of CO_2 to produce 1 C-mol of carbohydrate (Osborne and Geider, 1987). Also in terms of such a bioenergetic parameter, HoP was shown to be by far the best photobioreactor configuration, requiring only 8.2-11.9 mol of photons ($\ln n_{Ph}$ = 2.10-2.48) for the growth of 1 C-mol of biomass up to 6.5 days. As already observed in previous works carried out under fed-batch conditions (Bezerra et al., 2012; Sassano et al., 2004), the additional energy requirement with respect to the above ideal situation was negligible at the beginning, but considerably increased only during the stationary phase (about 100 mol of photons C-mol^{-1} after 7.5 days; $\ln n_{Ph}$ = 4.60) indicating that most of the energy was lost. On the contrary, OP and SF were the less performing configurations within the first 6.5 days, requiring 9.1-13.0 mol of photons C-mol^{-1} ($\ln n_{Ph}$ = 2.21-2.56), which suggests that, under stress conditions like these, biomass used the energy of absorbed photons preferably for maintenance rather than for growth. Disappointingly, HeP, after 4 days during which exhibited the best performance, started to have the highest photon requirement.

Confirming the observations made for Y_{GX}, a progressive increase in light intensity did not exert any appreciable influence on n_{Ph} up to 6.5 days, but had a remarkable effect on the time at which this parameter suddenly increased (7, 8 and 9 days for PPFD of 40, 70 and 100 μmol m^{-2} s^{-1}, respectively) (Fig. 3B). However, it is noteworthy that, irrespective of light conditions and configurations, the additional photons with respect to the theoretical requirement (8 mol of photons C-mol^{-1}) were partly utilized to reduce nitrate to ammonia (Hatori and Myers, 1966), which is the preferential form of nitrogen assimilated by photosynthetic microorganisms (Boussiba et al., 1984; Torre et al., 2003), and that such a

reduction was accompanied by the oxidation of a portion of NADPH produced by the photosynthesis (Bruinenberg et al., 1983).

Fig. 4 shows the time behaviors of the rates of O_2 development and H^+ consumption during cultivations performed at PPFD of 70 μmol m^{-2} s^{-1} in the four photobioreactor configurations tested in this study (panel A), or progressively increasing PPFD from 40 to 100 μmol m^{-2} s^{-1} in the sole HoP (panel B). These trends clearly show that, as biomass is the main product of photosynthetic metabolism, both rates faithfully followed cell growth, reaching maximum values in the late log phase, with exception of HeP where the maxima took place in the early log phase owing to the above-mentioned performance worsening along the time. Thus, consistently with the growth curves already illustrated in Fig. 1, the poorest O_2 release and H^+ consumption occurred in OP, while the highest rates were observed in HeP at the start or in HoP at the end of runs (Fig. 4A). As expected, both rates progressively increased with PPFD in HoP (Fig. 4B), hence confirming that the growth was light-limited and the system never suffered photoinhibition under the conditions tested in this work. However, an effect masked up to now was the speed up of both activities, which reached their maximum rates at PPFD of 100 μmol m^{-2} s^{-1} about 2.5 days before than at 40-70 μmol m^{-2} s^{-1}.

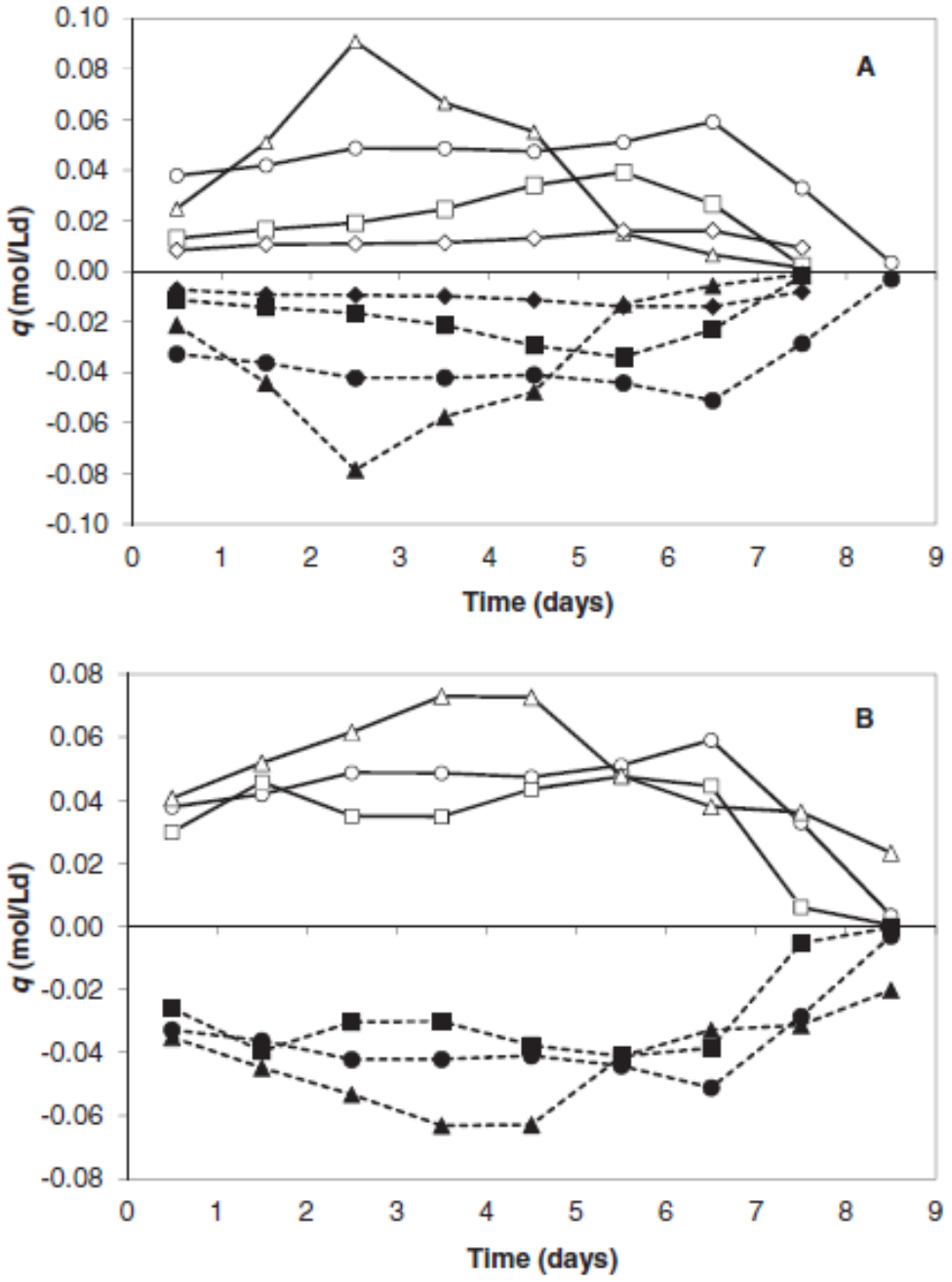

Fig. 4. Time behaviors of the rates of O_2 development (empty symbols and solid lines) and H^+ consumption (filled symbols and dotted lines) during *A. platensis* cultivations performed: A) at PPFD of 70 μmol m^{-2} s^{-1} in: (◇) Open Pond, *S/V* = 0.25 cm^{-1}; (□) Shaken Flask, *S/V* = 0.48 cm^{-1}; (○) Horizontal Photobioreactor, *S/V* = 1.94 cm^{-1}; (△) Helicoidal Photobioreactor, *S/V* = 3.88 cm^{-1}. B) in the Horizontal Photobioreactor varying PPFD (μmol m^{-2} s^{-1}): (□) 40; (○)70;(△) 100.

4.3. Thermodynamic parameters of A. platensis *growth*

As known, a portion of the Gibbs energy carried by the photons absorbed in PSII is used to transfer electrons to PSI and to create a proton-motive force across the plasma membrane, thus providing energy for ATP synthesis. In all the photobioreactor configurations, the highest values of the thermodynamic parameters taken into account in this estimation, i.e. the enthalpic content (ΔH), the energy recovered as ATP (ΔG_{ATP}) and that released as heat (Q), whose sum equals the total Gibbs energy effectively fixed by the photosynthesis (ΔG_a), were obtained in the stationary phase of growth (Table 3).

Table 3. Main thermodynamic parameters of *A. platensis* growth in different photobioreactor configurations and under different PPFD values.

	ΔG_{ATP} (kJ C-mol^{-1})			ΔH (kJ C-mol^{-1})			Q (kJ C-mol^{-1})		
Time (d)	0.5	3.5	7.5	0.5	3.5	7.5	0.5	3.5	7.5
a) Cultures in different photobioreactors at PPFD of 70 µmol m^{-2} s^{-1}									
HoP	228	287	466	244	308	499	1218	1534	2488
HeP	235	281	5749	252	301	6161	1257	1500	30703
SF	254	282	2219	272	302	2378	1357	1505	11852
OP	276	321	485	296	344	519	1475	1716	2588
b) Cultures in HoP at different light intensities (PPFD, µmol m^{-2} s^{-1})									
40	231	303	1358	248	325	1455	1235	1621	7251
70	228	287	466	244	308	499	1218	1534	2488
100	227	271	475	243	291	509	1210	1448	2538

HoP = Horizontal Photobioreactor; HeP = Helicoidal Photobioreactor; SF = Shaken Flask; OP = Open Pond; ΔG_{ATP} = Gibbs energy recovered as ATP; ΔH = enthalpy variation; Q = energy released as heat. Times 0.5, 3.5 and 7.5 days were selected as representative of the mid lag phase, mid log phase and early stationary phase of growth, respectively.

This means that, under these conditions, cell growth was so impaired that the synthesis of one C-mol of biomass required much more Gibbs energy than in the other growth phases,

and this occurrence was less marked in HoP, likely because of its favorable configuration and less turbulent conditions. Reynolds numbers (N_{Re}) in the ranges 600-2500 and 10-600 were in fact estimated in a similar air-lift tubular photobioreactor at biomass concentrations around 0.05 and 10 g L^{-1}, respectively (Converti et al., 2006), while Carlozzi and Torzillo (1996) warned that a turbulence excess with respect to optimal turbulence regime ($2680 < N_{Re} < 4000$) is deleterious for the culture.

To make comparisons possible, times 0.5, 3.5 and 7.5 days were selected as representative of the mid lag phase, mid log phase and early stationary phase of growth, respectively. As in the medium of Schlösser (1982) used in this study all the nutrients were present in large excess, the stress conditions suffered by the cyanobacterium in the stationary growth phase were the likely result of a shading effect depending on light availability to the system rather than of shortage of some nutrient, as it happens in batch heterotrophic cultures.

As previously suggested (Bezerra et al., 2012), exposition of the system to excess light intensity means that a progressively more significant portion of ΔG_a is dissipated via a mechanism called Non-Photochemical Quenching (NPQ) (Mozzo et al., 2008) to protect the photosynthetic apparatus against photodestruction (Heber et al., 2006; Karapetyan, 2008). However, such a situation was likely not to take place in HoP, where an increase in PPFD from 40 to 70 μmol m^{-2} s^{-1} reduced the energy lost as heat, while a further PPDF increase did not lead to any significant variation in the thermodynamic parameters (Table 3). The system exhibited practically the same behavior in HoP up to the achievement of the stationary growth phase, which was fully established after 7 and 8 days at PPFD of 40 and 70 μmol m^{-2} s^{-1}, respectively, but no less than 9 days at 100 μmol m^{-2} s^{-1} (Fig. 1). These results as a whole confirm that in the PPFD range tested in this study, despite of a variation in the overall Gibb energy availability that influenced the duration of cultures, the system kept under photolimited conditions. Such a situation is consistent with that observed in fed-batch *A.*

platensis cultures, where the highest values of the same thermodynamic parameters were observed at PPFD of 120 μmol m^{-2} s^{-1} and kept almost unvaried up to 240 μmol m^{-2} s^{-1}, because of light saturation occurrence (Bezerra et al., 2012).

Fig. 5A shows the time behavior of the percentages of absorbed light stored by the system as ATP (η_{ATP}) and fixed as enthalpy (η_H) in the different photobioreactor configurations at PPFD of 70 μmol m^{-2} s^{-1}, while Fig. 5B that of the same percentages in HoP at increasing PPFD. One can see that, in all the runs, both η_{ATP} and η_H decreased along the time, confirming the increasing difficulty of biomass to grow, and fell down after the achievement of the stationary growth phase. Whereas such decreases were almost regular for HoP, SF and OP, with higher values of both parameters in HoP ($6.4 \leq \eta_{ATP} \leq 13.2\%$; $13.8 \leq \eta_H \leq 28.2\%$), once more HeP exhibited the best performance at the beginning of the run up to 3.5 days ($10.7 \leq \eta_{ATP} \leq 12.8\%$; $22.9 \leq \eta_H \leq 27.3\%$), but behaved unsatisfactorily ($\eta_{ATP} = 0.52\%$ and $\eta_H = 1.12\%$) at the early stationary phase.

On the other hand, a progressive increase in light intensity did not imply in HoP any significant variation in these percentages up to the achievement of the early stationary phase (Fig. 5B), but, as earlier mentioned, it led to a simple delay, after which they fell down. As a matter of fact, whereas at PPFD of 40 μmol m^{-2} s^{-1} η_{ATP} fell to 2.2% after only 7.5 days, at higher PPFD this fall was less marked and was likely to occur after longer time (results not shown). These results taken together are consistent with photolimitation before the early stationary growth phase and a light-modulated shading effect after its appearance. Based on the proposed model, the percentage of absorbed Gibbs energy released as heat (η_Q) (results not shown) is the complement of η_{ATP} and η_H to 100%, which means that, consistently with the well-known low photosynthetic efficiency of every photosynthetic organisms, during all the cultivations most of the absorbed energy was released as heat, being this fraction much

higher at the early stationary phase ($79.7 \leq \eta_Q \leq 93.0\%$) than at the mid lag phase ($58.4 \leq \eta_Q \leq 59.2\%$).

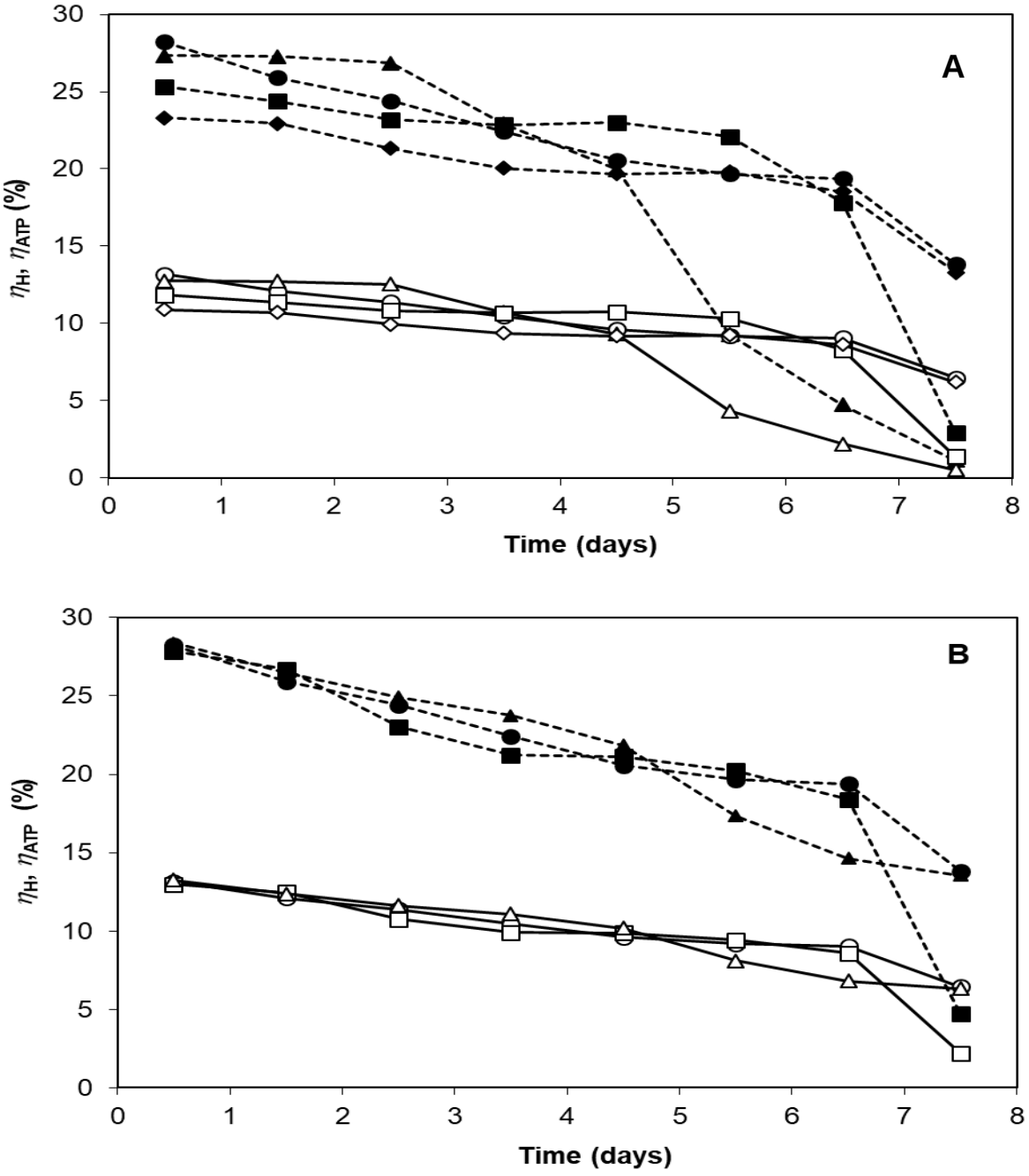

Fig. 5. Fractions of the absorbed light energy stored as ATP (η_{ATP}) (empty symbols and solid lines) and used to increase the enthalpic content of the system (η_H) (filled symbols and dotted lines) during *A. platensis* cultivations performed: A) at PPFD of 70 μmol m^{-2} s^{-1} in: (◇) Open Pond, S/V = 0.25 cm^{-1}; (□) Shaken Flask, S/V = 0.48 cm^{-1}; (○) Horizontal Photobioreactor,

S/V = 1.94 cm^{-1}; (△) Helicoidal Photobioreactor, *S/V* = 3.88 cm^{-1}. B) in the Horizontal Photobioreactor varying PPFD (μmol m^{-2} s^{-1}): (□) 40; (○)70;(△) 100.

5. Conclusions

Kinetic, bioenergetic and thermodynamic parameters of batch *A. platensis* cultures were influenced by photobioreactor configuration, surface/volume ratio (*S/V*) and light intensity. The photobioreactor configuration had a strong impact on biomass concentration, in that its maximum value remarkably increased when *S/V* was raised. However, the helicoidal photobioreactor showed the best performance only at the beginning of runs because of its highest *S/V* ratio, but subsequently it behaved poorly owing to a time-depending mechanical stress. The horizontal photobioreactor, where the growth was favored by a progressive irradiance increase, proved to possess the best configuration and showed the best bioenergetic and thermodynamic parameters.

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CONSIDERAÇÕES FINAIS

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Micro-organismos fotossintetizantes testados no presente estudo foram capazes de produzir compostos bioativos. Extratos etanólicos e aquosos da biomassa liofilizada das microalgas *Chlorella vulgaris, Dunaliella salina* e *Scenedesmus* sp. e da cianobactéria *Arthrospira (Spirulina) platensis*, obtidos por extração clássica sólido-líquido, foram submetidos a triagem quanto a sua capacidade antioxidante *in vitro* bem como a suas propriedades antibacterianas frente à *Escherichia coli* ATCC 25922, *Pseudomonas aeruginosa* ATCC 27853, *Enterococcus faecalis* ATCC 6057, *Bacillus subtilis* ATCC 6633 e *Staphylococcus aureus* ATCC 6538, principais deteriorantes na industria alimentícia. Os resultados permitem concluir que entre os extratos etanólicos estudados, o de *A. platensis* demonstrou potencial por apresentar maior capacidade antioxidante e eficaz atividade antibacteriana.

Posteriormente, estudos foram realizados a partir de uma comparação entre diferentes técnicas de extração verde, tais como as assistidas por ultrassons e micro-ondas e a de alta pressão e temperatura (HPTE) com o método clássico de extração sólido-líquido para selecionar o modo mais adequado para obter extratos hidroalcoólicos de *A. platensis* com o maior teor de compostos fenólicos (polifenóis totais, TP e flavonoides totais, TF) e poder antirradicalar (ARP). A HPTE demostrou ser a técnica mais eficaz para a recuperação destes compostos valiosos.

A metodologia de superfície de resposta revelou que para HPTE, a temperatura de extração (T) e concentração de etanol em água (Sc) apresentam forte influência sobre a extração de TP e TF, bem como ARP, em que seus conteúdos máximos notavelmente aumentam quando os efeitos combinados de T e Sc trabalham em conjunto. As condições mais adequadas em termos de rendimento de extração, mostraram ser a T mais elevada (180 °C) e Sc entre 20-60% (v/v) para a recuperação eficiente de TP e TF, enquanto que uma tendência diferente foi observada para ARP, que se manteve sempre elevado em toda a faixa de temperatura testada ($90 \leq T \leq 135$ °C) a 20-60% v/v. A CLAE permitiu identificar a catequina e os ácidos vanílico, gálico e siríngico como os principais fenólicos no extrato. Correlações lineares entre o conteúdo geral dos fenólicos acima mencionados e TP ou TF, sugeriram que eles contribuem significativamente para qualquer classe de fenólicos, enquanto que nenhuma semelhança foi observada para ARP, sugerindo que outros constituintes celulares podem desempenhar um papel importante na capacidade antioxidante dos extratos de *A. platensis* usando HPTE.

Adicionalmente, devido a certa falta de estudos teóricos detalhados e recentes sobre os mecanismos que regulam o crescimento de micro-organismos fotossintetizantes, foram

também realizados estudos de acordo com uma nova abordagem bioenergética e termodinâmica sobre o crescimento fotoautótrofo de *A. platensis* selecionada como micro-organismo modelo.

Os parâmetros cinéticos, bioenergéticos e termodinâmicos foram influenciados pela configuração do fotobiorreator, relação superfície/volume (*S/V*) e intensidade de luz. A configuração do fotobiorreator teve um forte impacto sobre a concentração de biomassa e a produtividade celular de *A. platensis* na medida em que seus valores máximos aumentaram notavelmente quando *S/V* foi aumentado. No entanto, o fotobiorreator helicoidal apresentou melhor desempenho apenas no começo, por causa da sua maior relação *S/V*, mas subsequentemente mostrou um pior desempenho, o qual pode ser atribuído ao estresse mecânico. O fotobiorreator horizontal, onde o crescimento foi favorecido por um aumento progressivo da irradiância devido a uma distribuição favorável da luz, provou possuir a melhor configuração, bem como foi o mais eficiente ao longo de todas as culturas mostrando os melhores parâmetros bioenergéticos e termodinâmicos.

Dessa forma, por apresentar alto ARP, bem como eficiente recuperação de compostos fenólicos quando extraídos pela técnica de HPTE, este estudo demonstra que o extrato hidroalcoólico de *A. platensis* possui potencial de aplicação na indústria alimentícia para formulação de alimentos funcionais ou preparação de suplementos dietéticos, e, pode ser considerado como uma alternativa segura e acessível aos compostos sintéticos. Assim, para o melhor de nosso conhecimento, não houve nenhum estudo prévio a usar HPTE para a recuperação de fenólicos e antioxidantes a partir de *Arthrospira (Spirulina) platensis* usando misturas binárias de solventes verdes (etanol/água), e, esta investigação fornece dados preliminares valiosos, através da demonstração de sua elevada capacidade antioxidante. Adicionalmente, HoP provou possuir a melhor configuração para produzir culturas de *A. platensis* de alto rendimento. Portanto, esta tese abre novos caminhos no uso desta tecnologia emergente, e pode ser considerada como uma ferramenta valiosa para o campo da biotecnologia alimentar.

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ANEXOS

ANEXO A – Normas de submissão para revista *International Journal of Food Science & Technology* correspondente ao capítulo I desta tese

ANEXO B – Artigo publicado apresentado no capítulo II desta tese

ANEXO C – Artigo publicado apresentado no capítulo III desta tese

ANEXO D – Publicações desenvolvidas durante o doutorado

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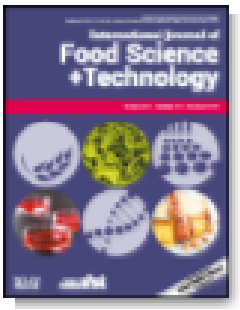

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ANEXO B – Artigo publicado apresentado no capítulo II desta tese

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Recovery of phenolic compounds of food concern from *Arthrospira platensis* by green extraction techniques

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ABSTRACT

Arthrospira platensis UTEX 1926 has attracted attention because of its ability to produce high value-added compounds such as polyphenols, but their profitable recovery is recognized as a big challenge. In this study, different green extraction techniques, namely ultrasound-assisted (UAE), microwave-assisted (MAE) and high pressure/temperature (HPTE) extractions were compared with classic solid–liquid extraction (SLE) in terms of phenolic compounds recovery from *A. platensis* using ethanol as solvent and antiradical power (ARP). HPTE proved the most efficient extraction method, allowing maximum total polyphenol yield (*TP*) of 3.32 ± 0.08 mg of gallic acid equivalent [GAE] per gram of dry biomass [DB] and ARP of 58.30 ± 0.12 $\mu mol_{Trolox}\ g_{DB}^{-1}$, whereas maximum total flavonoid yield (*TF*) was 2.80 ± 0.18 mg of catechin equivalent [CE] g_{DB}^{-1} in MAE extract. Once HPTE had been selected as the best-performing extraction method, a 3^2-full factorial design was applied to evaluate the combined effects of temperature ($90 \le T \le 180$ °C) and ethanol concentration in water ($20 \le Sc \le 100\%$ v/v) on *TP*, *TF* and ARP by Response Surface Methodology (RSM). RSM revealed that the most suitable conditions for *TP* (26.00–28.04 $mg_{GAE}\ g_{DB}^{-1}$) and *TF* (10.25 ± 0.34 $mg_{CE}\ g_{DB}^{-1}$) were $T = 180$ °C and $20 \le Sc \le 60\%$ (v/v), while ARP was maximized (67.77–69.02 $\mu mol_{Trolox}\ g_{DB}^{-1}$) at $90 \le T \le 135$ °C. HPLC analysis showed that catechin, vanillic, gallic and syringic acids were present in very low concentrations (up to 0.05 ± 0.01 mg 100 g_{DB}^{-1}) in MAE, UAE and SLE extracts, while they were the most abundant phenolics in HPTE (3.45–3.61, 1.06–2.02, 1.64–1.71 and 0.99–1.26 mg 100 g_{DB}^{-1}, respectively). These compounds taken together effectively contributed to both *TP* ($r = 0.928$) and *TF* ($r = 0.960$), but not to ARP. This work opens new perspectives for HPTE use as emerging technique to obtain high-ARP phenolic-rich hydroalcoholic extracts of *A. platensis*, which may serve as a natural source of compounds to formulate functional foods or prepare dietary supplements.

1. Introduction

Cyanobacteria (photosynthetic prokaryotes), also known as blue–green algae, have a considerable potential not only for bioenergy production [1], but also for the production of nutraceuticals and pharmaceuticals, the former having a large and rapidly expanding market [2,3]. They can, in fact, be used to enhance the nutritional value of foods by addition of compounds with important beneficial attributes that confer health-promoting properties [4–6].

Many studies have also shown significant therapeutic applications of these photosynthetic microorganisms, which are recognized as natural sources of novel biologically active compounds with protective effects against cancer [7], viral, bacterial and fungal infections [8,9], allergies [10], inflammations [11], anemia [12], hyperlipidemia [13] and hyperglycemia [14].

Arthrospira platensis is an unbranched, helicoidal, and filamentous cyanobacterium [15] that stands out because of its high nutritional value related to high contents of several essential nutrients such as proteins [16], polyunsaturated fatty acids, vitamins (A, B_1, B_2, B_6, B_{12}, E and D), minerals (especially iron), and other high added-value phytochemicals including pigments (β-carotene, chlorophyll *a*, phycocyanin) and polyphenols [4,5,17–21].

The last compounds are an important group of secondary metabolites with antioxidant activity that play a key role in human health

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acting against free radicals, in particular the reactive oxygen and nitrogen species [22]. They also play an important role in algal cell defense against abiotic (UV irradiation) and biotic (pathogen interaction) stress. It is well documented that polyphenols are involved in the protection of cardiovascular system [23,24]. However, their profitable recovery is currently a hot investigation topic, which is recognized as a big challenge due to the inherent limitations and high costs of conventional extraction methods [25].

The presence of phenolic compounds in microalgae and cyanobacteria is well documented, but information about the presence of specific phenols as well as their bioactivity is still limited [26–28]. These compounds are mainly intracellular, which makes their recovery by conventional solvent extraction difficult [29], because of the need of lysing cells. Thus, it is necessary to develop new efficient cell disruption methods to recover, identify and purify these secondary metabolites in their active form for further application [21].

The extraction method can influence both the quality and quantity of target bioactive polyphenols as well as antiradical power of cyanobacteria extracts [30]. Innovating "green" extraction approaches are currently investigated by several research groups to improve the recovery of phenolic compounds contained in different biological raw materials [17,21,28–31]. Among them, supercritical-fluid (SFE), pressurized liquid (PLE), microwave-assisted (MAE) and ultrasound-assisted (UAE) extractions have been reported to enable faster heat and mass transfer, reduction in solvent consumption, savings in working time, yield increase and higher extract/product quality [32–36] compared to conventional extraction methods.

Since various factors influence the yield of phenolic compounds recovery such as the extraction temperature and solvent concentration, it is necessary to select the best-performing extraction method. The extraction conditions can also be optimized with a high level of confidence by combining a suited experimental design with Response Surface Methodology (RSM), aiming to reduce costs and time as well as to increase efficiency of work [37,38].

High-pressure/temperature extraction (HPTE) in a stirred reactor is an emerging technology, which has been investigated and compared in this study with conventional solid-liquid extraction (SLE), MAE and UAE. Recently, HPTE has been successfully applied to the extraction of bioactive phenolic compounds from different matrices, such as grape and apple skins [39], barley grains [36], grape marc and olive pomace [40]. High temperature enhances the extraction efficiency by a decrease in both solvent viscosity and surface tension, hence promoting better penetration into the matrix. Furthermore, the pressurized cell prevents solvent boiling at the extraction temperature, thus enhancing the intimate contact with the sample over the entire extraction period [38,40]. To the best of our knowledge, this is the first study where HPTE was used to recover phenolics with high antioxidant activity from *A. platensis* hydroalcoholic extracts.

Based on this, the objective of this study was to select the most efficient extraction method to recover phenolic compounds from *A. platensis* biomass and to maximize, at the same time, the antiradical power (ARP) of the extracts. For this purpose, HPTE, UAE and MAE were compared with classic SLE, resulting HPTE the best-performing method. A 3^2-full factorial design combined with RSM was then applied to this method in order to assess the effects of extraction temperature and solvent concentration on total polyphenol yield, total flavonoid yield and ARP. Finally, possible correlations between phenolic compound contents, their individual constituents and ARP were also investigated.

2. Materials and methods

2.1. Chemicals

Salts to prepare the cultivation medium, ethanol (analytic grade), methanol (HPLC grade), acetonitrile, acetic acid, 6-hydroxy-2,5,7,8-tetramethylchromane-2-carboxylic acid (Trolox), 2,2′-Azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt (ABTS), potassium persulfate, Folin-Ciocalteu reagent, sodium carbonate and standards of phenolic compounds (gallic acid, catechin, vanillic acid, syringic acid, epicatechin and ferulic acid) were purchased from Sigma–Aldrich (St. Louis, MO, USA). CO_2 for cyanobacterial growth with purity of 99.8% was purchased from Siad (Milan, Italy).

2.2. Microorganism and culture conditions

Arthrospira platensis UTEX 1926 (University of Texas Culture Collection, Austin, TX, USA) was maintained and cultured in the culture medium suggested by Schlösser [41]. *A. platensis* was grown in a 3.5 L-horizontal tubular photobioreactor with initial biomass concentration of 0.40 g L^{-1}, exposed to light intensity of 100 ± 5 μmol photons $m^{-2} s^{-1}$ at 30 ± 1 °C [42]. The pH was controlled at 9.5 ± 0.5 through the daily addition of pure CO_2 at a flow rate of 30 L/h. Both photobioreactor configuration and light intensity were chosen based on the best operating conditions previously established by our research group [42]. Biomass concentration (X) was determined daily by measuring the absorbance at 560 nm by a UV–Vis spectrophotometer, model Lambda 25 (Perkin Elmer, Milan, Italy), using the calibration curve $Abs_{560} = 0.0024\,X - 0.1129$ ($R^2 = 0.992$). All the measurements were done in triplicate. Once the stationary phase had been reached, after about 9 days of cultivation, cells were separated from the culture medium by centrifugation (ALC 4226, Milan, Italy) for 10 min at 6000 × *g*, dried at 105 °C for 24 h (moisture content < 1.0%), pulverized in a mortar and stored at − 20 °C for further analyses.

2.3. Extraction processes

Dried biomass of *A. platensis* was extracted using ethanol as a solvent with a constant solid/liquid ratio of 1:10 (*w/v*) [22,24]. All extractions were performed using 2.0 g of dried biomass, except those at high pressure and temperature (3.0 ± 0.1 g), according to the schematic set-up of the overall process illustrated in Fig. 1.

Conventional solid–liquid extraction (SLE) was performed for 19 h in amber glass test tubes with caps, where the suspension was continuously mixed by a magnetic stirrer, model Mr. 3001 (Heidolph, Kelheim, Germany). The extraction was carried out in the dark at room temperature (25 ± 1 °C), and then the liquid was separated from solids by centrifugation under the same conditions as above and frozen at − 20 °C [43].

Microwave-assisted extraction (MAE) was conducted at 110 °C under nitrogen atmosphere and microwave irradiation (60 W) for 60 min [44] in a professional multimode oven operating at 2.45 GHz (MicroSYNTH Milestone, Sirisole, Italy). Temperature was monitored by an optic-fiber thermometer directly inserted into the 100 mL-pressure-resistant reaction vessel (maximum pressure of 100 bar) made in polytetrafluoroethylene [44].

Ultrasound-assisted extraction (UAE) was carried out at 25 °C, 21.5 kHz and 60 W in an ultrasonic bath, model UTA 90 (FALC, Treviglio, Italy), following the methodology described by Cravotto et al. [45], but reducing the extraction time from 30 to 15 min according to preliminary experiments (data not shown).

High pressure/temperature extraction (HPTE) was performed in a high pressure–high temperature reactor, model 4560 (PARR Instrument, Moline, IL, USA), containing appropriate valves to allow introduction and removal of gases inside the reaction chamber and equipped with a mechanical stirrer [46,47]. The extraction was carried out at 90 °C and 2.5 bar for 90 min by flushing nitrogen through the reactor for 2 min in order to prevent oxidation of phenolics [48]. Since this equipment, able to operate up to 350 °C and 200 bar, did not have the ability to regulate pressure, high pressure was the result of temperature rise. After the extraction, the suspension was centrifuged as described above.

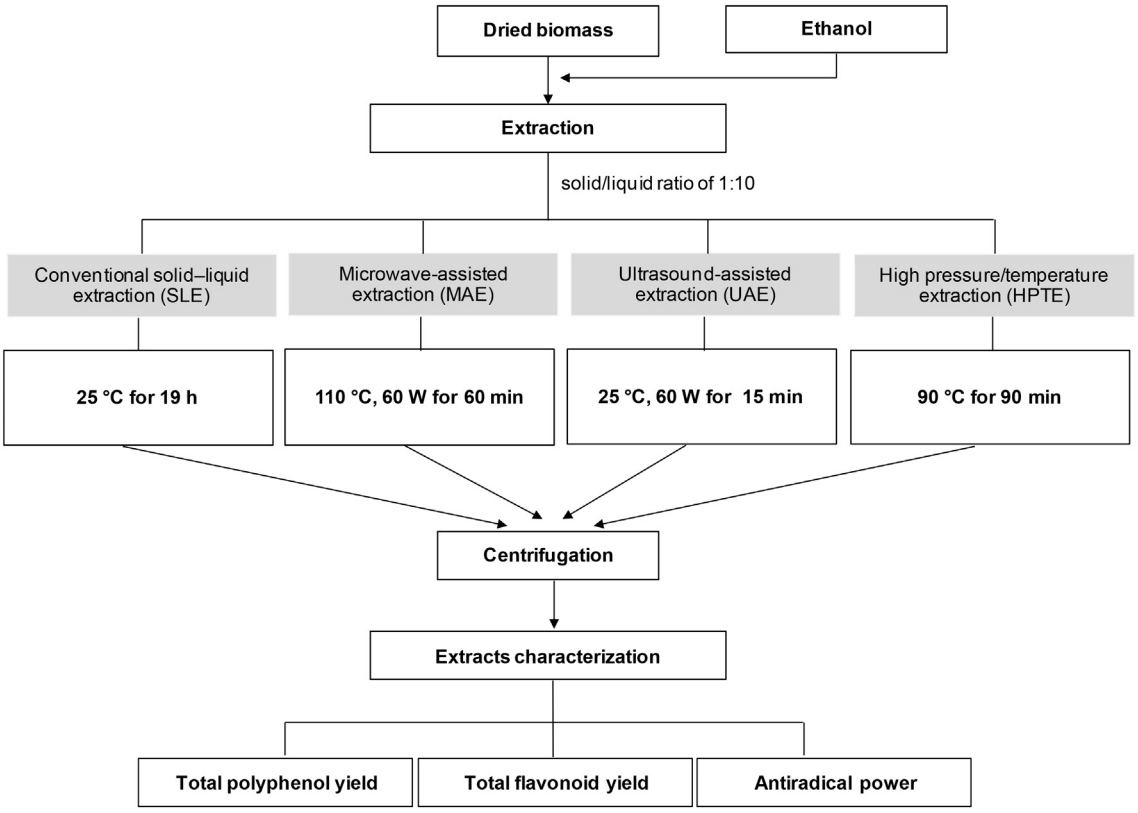

Fig. 1. Schematic set-up of the process for phenolic compounds recovery from *Arthrospira platensis* biomass by different extraction techniques.

All the extractions were carried out in triplicate, and the extracts used for determinations of total polyphenol yield (*TP*), total flavonoid yield (*TF*) and antiradical power (ARP).

2.4. Analytical methods

2.4.1. Yield of total polyphenols

Total polyphenol content was quantified colorimetrically by the Folin–Ciocalteu assay [49]. Briefly, 4.80 mL of distilled water, 0.20 mL of sample and 0.50 mL of Folin–Ciocalteu reagent were placed into 15 mL-test tubes. After mixing and addition of 1.0 mL of 20% (*w/v*) sodium carbonate solution, distilled water was added to reach a final volume of 10 mL. All the solutions were mixed and allowed to stand at room temperature in the dark for 1 h. The concentration of total polyphenols was determined by absorbance measurements at 725 nm using the early-mentioned UV–Vis spectrophotometer. A calibration curve (Abs_{725} = 0.0017 *TP*, R^2 = 0.997) was made with standard solutions of gallic acid with concentration in the range 0.01–1.0 mg mL^{-1}. Total polyphenol yield (*TP*) was expressed in milligrams of gallic acid equivalent per gram of dry biomass ($mg_{GAE}\ g_{DB}^{-1}$).

2.4.2. Yield of total flavonoids

The yield of total flavonoids (*TF*) in the extract was determined by the colorimetric method described by Jia et al. [50] and modified by Yang et al. [51], which is specific for such an important class of phenolics. Briefly, 0.25 mL of diluted extract was mixed with 1.25 mL of deionized water and 0.075 mL of 5% sodium nitrite solution, and allowed to react for 5 min. Then, 0.15 mL of 10% aluminum chloride and, after 6 min, 0.5 mL of 1.0 M sodium hydroxide were added to the mixture. Distilled water was added to reach a final volume of 3.0 mL. Absorbance was read at 510 nm. A calibration curve (Abs_{510} = 0.0021 *TF*, R^2 = 0.991) was made with standard solutions of catechin with concentration in the range 0.01–0.50 mg mL^{-1}. *TF* was expressed in milligrams of catechin equivalent per gram of dry biomass ($mg_{CE}\ g_{DB}^{-1}$).

2.4.3. Antiradical power

The antiradical power (ARP) of extracts was measured according to their ability to scavenge $ABTS^{\cdot+}$ radical cation, as described by Re et al. [52] and applied by Gilbert-López et al. to microalgae extracts [38]. The mixture was incubated at room temperature in the dark for 12–16 h (time required for radical formation) prior to use. The absorbance was measured at 734 nm. ARP in the reaction medium was calculated from a calibration curve (Abs_{734} = −0.0013 Trolox conc. + 0.6239, R^2 = 0.997), and the results were expressed as micromoles of Trolox equivalent per gram of dry biomass ($\mu mol_{Trolox}\ g_{DB}^{-1}$).

2.4.4. Quantification of phenolic compounds by HPLC

The most abundant phenolic compounds of the extract were quantified by a high-performance liquid chromatograph, model 1100 (Agilent Technologies, Palo Alto, CA, USA), equipped with a C18 reverse-phase column, model 201TP54 (Vydac, Hesperia, CA, USA), coupled with a diode-array detector (DAD), model 1200 (Agilent), following the methodology described by De Faveri et al. [53]. The mobile phase was MilliQ water/acetic acid (99:1%, v/v) (solvent A) and methanol/acetonitrile (50:50%, v/v) (solvent B), while the solvent gradient was varied according to the following conditions: from 5 to 30% B for 25 min, from 30 to 40% B for 10 min, from 40 to 48% B for 5 min, from 48 to 70% B for 10 min, from 70 to 100% B for 5 min, isocratic at 100% B for 5 min, followed by returning to the initial conditions (10 min) and column equilibration (12 min). Runs were performed at 30 °C and flow rate of 1.0 mL min^{-1}, the injection volume was 20 μL, and peaks were monitored at 280 nm. Gallic acid, catechin, vanillic acid, syringic acid, epicatechin and ferulic acid were used as standards. Before analysis, samples were filtered through cellulose acetate filters with 0.22 μm-pore diameter. Phenolic compounds were identified by comparing the retention time with that of the corresponding standard at 280 nm and comparing the spectrum obtained by DAD with the standard one. The concentration of each phenolic compound was calculated based on that of each standard solution and

Table 1
Results and conditions of high pressure/temperature extraction tests performed on *A. platensis* biomass according to the 3^2-full factorial design.

Run	Independent variable		Response		
	T[A] (°C)	S_c[B] (% v/v)	*TP*[C] ($mg_{GAE}\ g_{DB}^{-1}$)	*TF*[D] ($mg_{CE}\ g_{DB}^{-1}$)	ARP[E] ($\mu mol_{Trolox}\ g_{DB}^{-1}$)
1	90(−1)	20(−1)	6.19 ± 0.07[b]	2.76 ± 0.05[d]	69.02 ± 1.11[b]
2	90(−1)	60(0)	4.00 ± 0.05[a,b]	2.00 ± 0.05[a]	67.77 ± 0.20[b]
3	90(−1)	100(1)	3.32 ± 0.08[a]	1.45 ± 0.13[c]	58.30 ± 0.12[c]
4	180(1)	20(−1)	26.00 ± 0.02[c]	10.25 ± 0.34[g]	60.95 ± 0.05[a,c]
5	180(1)	60(0)	28.04 ± 2.76[c]	8.83 ± 0.39[f]	34.08 ± 1.00[e]
6	180(1)	100(1)	14.81 ± 0.15[f]	4.11 ± 0.17[b]	25.47 ± 1.63[d]
7	135(0)	20(−1)	11.93 ± 0.67[e]	6.00 ± 0.04[e]	68.11 ± 0.78[b]
8	135(0)	100(1)	4.00 ± 0.02 [a,b]	2.08 ± 0.04[a]	62.16 ± 0.95[a]
9[F]	135(0)	60(0)	9.33 ± 0.21[d]	4.00 ± 0.04[b]	63.12 ± 0.53[a]
10[F]	135(0)	60(0)	9.07 ± 0.32[d]	4.10 ± 0.06[b]	61.18 ± 0.38[a]
11[F]	135(0)	60(0)	9.20 ± 0.01[d]	3.90 ± 0.01[b]	60.65 ± 0.27[a]

Different superscript lowercase letters in the same column show statistically significant differences among data at $p < 0.05$. Values are expressed as means ± standard deviations of three replicates.
[A] Extraction temperature. Coded values between brackets.
[B] Solvent (ethanol) concentration in the hydroalcoholic solution. Coded values between brackets.
[C] Total polyphenol yield; GAE: gallic acid equivalents; DB: dry biomass.
[D] Total flavonoid yield; CE: catechin equivalents.
[E] Antiradical power determined by the $ABTS^{\cdot+}$ radical scavenging method.
[F] Central point runs.

expressed in milligrams per 100 g of dry biomass (mg 100 g_{DB}^{-1}). The sum of contents of all individual phenolic compounds quantified by HPLC was referred to as SPC.

2.5. Experimental design and statistical analysis

A 3^2-full factorial experimental design combined with Response Surface Methodology (RSM) was used to obtain and evaluate the results of HPTE at fixed time (90 min). To this purpose, the extraction temperature ($90 \le T \le 180$ °C) and solvent (ethanol) concentration (Sc) in the hydroalcoholic solution (20, 60 and 100% v/v) were selected as the independent variables, whose coded values were −1 (lowest level), 0 (central point) and +1 (highest level), while *TP* ($mg_{GAE}\ g_{DB}^{-1}$), *TF* ($mg_{CE}\ g_{DB}^{-1}$) and ARP ($\mu mol_{Trolox}\ g_{DB}^{-1}$) as the responses (Table 1).

The complete design consisted of 11 experiments with three replicates at the central point, which were added to estimate the experimental error as well as to check the reproducibility of results.

To describe the behavior of the system, second-order polynomial models were used to correlate independent variables to responses according to the equation:

$$Y = \beta_0 + \sum \beta_i x_i + \sum \beta_{ii} x_i^2 + \sum \beta_{ij} x_i x_j \quad (1)$$

where Y is the response, β_0 is the interception coefficient, β_i are the linear terms, β_{ii} are the quadratic terms, β_{ij} are the interaction terms, and x_i and x_j are the coded levels of the independent variables. The quality of fit of the polynomial equations was expressed by the coefficient of determination (R^2).

The Student's *t*-test permitted us to check the statistical significance of the regression coefficients, while the Fisher's test for analysis of variance (ANOVA) was performed on experimental data to evaluate the statistical significance of models. The influence of the selected variables was assessed by ANOVA and Tukey's post hoc test for mean discrimination or mean comparison, depending on the data. The statistically significant differences were illustrated by different letters in tables and figures. All the analyses were carried out in triplicate ($n = 3$), and the results expressed as mean values ± standard deviations (SD).

To identify possible correlations among the contents of different groups of phenolic compounds (*TP* and *TF*), individual phenolic compounds detected by HPLC (catechin, epicatechin, gallic, vanillic, syringic and ferulic acids) and ARP, linear equations have been applied, and the values of the Pearson's coefficient (*r*) [54] compared.

The Statistica v. 10.0 software (StatSoft, Tulsa, OK, USA) was used for data analysis. The results were considered statistically significant for p values ≤ 0.05.

3. Results and discussion

3.1. Preliminary selection of the extraction method

In the first part of this study, three green extraction techniques, namely ultrasound-assisted (UAE), microwave-assisted (MAE) and high pressure/temperature (HPTE) extractions, were compared with classic solid–liquid extraction (SLE) in terms of total polyphenol yield (*TP*), total flavonoid yield (*TF*) and antiradical power (ARP).

As illustrated in Fig. 2A, *TP* in *Arthrospira platensis* ethanolic extracts was the highest (3.32 ± 0.08 $mg_{GAE}\ g_{DB}^{-1}$) when using HPTE, followed by MAE (3.04 ± 0.08 $mg_{GAE}\ g_{DB}^{-1}$) and UAE (2.07 ± 0.01 $mg_{GAE}\ g_{DB}^{-1}$). As suggested by Casazza et al. [47], the effectiveness of HPTE to extract polyphenols may have been the result of optimal stirring conditions and inert atmosphere. This extraction method was successful in terms of phenolic compound recovery from other natural matrices such as grape and apple by-products [39,44,47], olive oil solid waste [46], *Agave americana* (L.) leaves [48], barley grains [36], agri-food waste from grape marc, olive pomace [40] and cyanobacteria [24]. On the other hand, the conventional SLE showed the worst performance (1.61 ± 0.07 $mg_{GAE}\ g_{DB}^{-1}$) when compared with all the selected emerging techniques, likely due to degradation caused by exposure for prolonged time (19 h) at room temperature [47].

The type of extraction technique influenced differently and more markedly *TF* when compared with *TP* (Fig. 2B). It is likely that the general structure of a 15-carbon skeleton of *TF*, which are made of two phenyl rings and a heterocyclic ring, is responsible for the different thermosensitivity of these classes of compounds [55]. The highest *TF* was in fact obtained when using MAE (2.80 ± 0.18 $mg_{CE}\ g_{DB}^{-1}$), followed by UAE (1.90 ± 0.01 $mg_{CE}\ g_{DB}^{-1}$) and HPTE (1.45 ± 0.13 $mg_{CE}\ g_{DB}^{-1}$), while SLE yielded only 0.74 ± 0.09 $mg_{CE}\ g_{DB}^{-1}$. These results taken together suggest that in MAE, which shatters cells using the shock of high-frequency waves [25], a temperature increase may have favored the release of flavonoids from the matrix, while a reduction of the extraction time compared with the SLE and HPTE may have enhanced their stability. On the other hand, the higher *TF* content of UAE compared with HPTE may have been the result of the well-known ability of ultrasound to break cell wall.

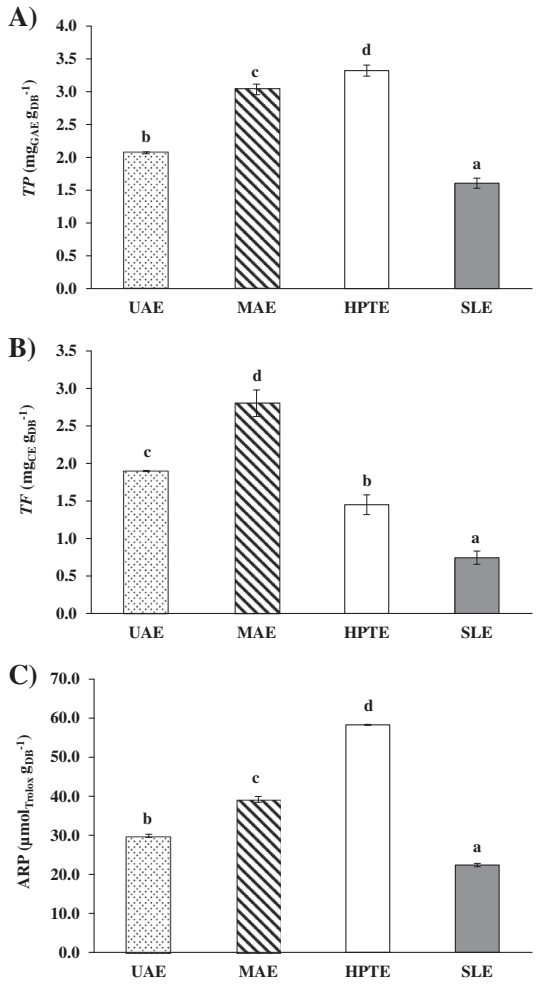

Fig. 2. Total polyphenol yield (*TP*) (A), total flavonoid yield (*TF*) (B), and antiradical power (ARP) (C) of ethanolic *Arthrospira platensis* extracts obtained by different extraction techniques. Data are expressed as means of three replicates ($n = 3$). Different letters (from a to d) indicate statistically significant differences at $p < 0.05$. Error bars indicate means ± standard deviations. UAE: ultrasound-assisted extraction; MAE: microwave-assisted extraction; HPTE: high pressure/temperature extraction; SLE: solid–liquid extraction; GAE: gallic acid equivalents, CE: catechin equivalents.

As expected by the well-known antioxidant activity of many polyphenols, ARP (Fig. 2C) showed a qualitatively similar trend as *TP* (Fig. 2A), in that the *A. platensis* extract obtained by HPTE exhibited the highest value of this response (58.30 ± 0.12 $\mu mol_{Trolox}\ g_{DB}^{-1}$), followed by MAE (39.22 ± 0.75 $\mu mol_{Trolox}\ g_{DB}^{-1}$), UAE (29.85 ± 0.39 $\mu mol_{Trolox}\ g_{DB}^{-1}$) and SLE (22.37 ± 0.42 $\mu mol_{Trolox}\ g_{DB}^{-1}$). Again, optimal stirring conditions and inert atmosphere [47] may have been the main reasons of the best HPTE performance compared with the other extraction methods.

To the best of our knowledge, there was no previous report on the production of phenolic-rich ethanolic extracts of *A. platensis* with high antioxidant activity by HPTE; therefore, the results of this study may be considered a useful starting basis for future advance in this field. In particular, HPTE extracts could be used as a potential source of easily-accessible natural antioxidants and a safe alternative to synthetic compounds.

3.2. Full factorial design to select the best HPTE conditions

Based on the results described in the previous section, HPTE was selected as the best-performing extraction technique in terms of *TP* and ARP; therefore, an optimization study was performed in the second part of this work to select the best extraction conditions for this method.

As is well known, temperature and solvent concentration are the main key factors influencing the extraction efficiency, because of their impact on the equilibrium (solubility), mass transfer rate (diffusion coefficient), and stability of bioactive compounds [43]. Thus, their effects on the extraction efficiency were investigated in order to maximize the yield in phenolic compounds and antiradical power and to minimize costs [56].

The experimental results of HPTE tests performed according to the selected 3^2-full factorial design demonstrate that both independent variables, namely the extraction temperature (T, °C) and solvent (ethanol) concentration in the hydroalcoholic solution (Sc, % v/v), significantly influenced *TP*, *TF* and ARP, which varied within very wide ranges (Table 1).

One can see that the highest values of both *TP* (26.00 ± 0.02 $mg_{GAE}\ g_{DB}^{-1}$ to 28.04 ± 2.76 $mg_{GAE}\ g_{DB}^{-1}$; $p > 0.05$) and *TF* (10.25 ± 0.34 $mg_{CE}\ g_{DB}^{-1}$) were achieved in *A. platensis* extracts obtained at the highest temperature (180 °C), but while the former response was maximized in the Sc range 20–60%, the latter was so at the lowest value (20%). In contrast with these trends, ARP was favored by simultaneous decreases of both independent variables.

As previously suggested [46,57,58], these optimal results at high temperature may have been due to a) decreases in solvent viscosity and surface tension, hence making penetration into the matrix easier, b) increases in solvent diffusion rate and solutes mass transfer, and c) enhancement of the disruption of the strong solute–matrix interactions (hydrogen bonds, dipole attractions and van der Waals forces).

It is noteworthy that, under all the conditions tested, the presence of water in the hydroalcoholic solution led to higher *TP* and *TF* values, when compared with pure ethanol as an extraction solvent (see Section 3.1). Similarly, Paini et al. [40], who used hydroalcoholic solutions at 150 °C for 270 min to recover phenolic compounds from olive pomace by HPTE, found that TP and TF yields improved significantly when water was added to ethanol up to 25%. This behavior may be explained not only by the high solubility of some phenolic classes in polar solvents, due to their generally polar nature [59], but also by the high pressure in the reactor chamber that likely prevented solvent boiling at the extraction temperature, thus enhancing phenolic matrix disruption. The same reasoning was used by Herrero et al. [60] to explain the better yield of antioxidant recovery by pressurized liquid extraction from *A. platensis* using water and ethanol as solvents rather than hydrocarbons. Nonetheless, when Ferrari et al. [24] used HPTE at 180 °C for 90 min and 100% water as solvent to recover polyphenols from *A. platensis* biomass, *TP* was lower (13.43 $mg_{GAE}\ g_{DB}^{-1}$) than that obtained in this work at the same temperature and extraction time. These findings suggest that, although some water is necessary to perform highly effective HPTE, it is essential also the presence of a polar organic solvent to this purpose, which may be responsible for changes in both solubility of these compounds in water and water pressure-temperature diagram. Finally, the maximum ARP obtained in this study (69.02 ± 1.11 $\mu mol_{Trolox}\ g_{DB}^{-1}$) was almost the same as that (69.40 ± 1.14 $\mu mol_{Trolox}\ g_{DB}^{-1}$) of hydroalcoholic extracts of *Tetraselmis* sp. [59], but remarkably higher than those of *Tetraselmis suecica*, *Chlorella vulgaris* and *Phaeodactylum tricornutum* (17–25 $\mu mol_{Trolox}\ g_{DB}^{-1}$) [27], all obtained by conventional SLE.

3.3. Quantification of phenolic compounds by HPLC

Single phenolic compounds belonging to flavonoid, phenolic acids and tannin groups are recognized as the dominant contributors to the antioxidant capacity, besides possessing diverse biological activities such as anti-inflammatory, anti-atherosclerotic and anti-carcinogenic ones [61]. Moreover, they have been associated with many aspects of food quality including color, flavor properties and nutritional value [62].Contents of phenolic compounds that could be identified by HPLC in *A. platensis* extracts obtained by HPTE performed according to the 3^2-full factorial design are listed in Table 2. The major phenolics of theextracts and their maximum concentrations were catechin (3.45 ± 0.07 mg 100 g_{DB}^{-1} to 3.61 ± 0.01 mg 100 g_{DB}^{-1}; $p > 0.05$), vanillic (2.02 ± 0.01 mg 100 g_{DB}^{-1}), gallic (1.71 ± 0.04 mg 100 g_{DB}^{-1}) and

Table 2
Contents of phenolic compounds quantified by reverse phase-HPLC in *A. platensis* extracts obtained by high pressure/temperature extraction (HPTE) runs performed according to the 3^2-full factorial design, ultrasound-assisted extraction (UAE), microwave-assisted extraction (MAE) and conventional solid-liquid extraction (SLE).

Run	T[A] (°C)	S_c[B] (% v/v)	Phenolic compound content (mg 100 g_{DB}^{-1})*					
			GA[C]	*CA*[D]	*VA*[E]	*SA*[F]	*EP*[G]	*FA*[H]
1	90(−1)	20(−1)	0.09 ± 0.01^{b,c,d}	0.41 ± 0.03^{b,c}	0.22 ± 0.01^{c}	0.04 ± 0.00^{d,e}	0.07 ± 0.00^{b}	–
2	90(−1)	60(0)	0.18 ± 0.01^{e}	0.55 ± 0.01^{b}	0.01 ± 0.00^{a}	0.01 ± 0.00^{a,b}	–	–
3	90(−1)	100(1)	0.05 ± 0.00^{a,b,c}	0.18 ± 0.01^{a,c}	0.02 ± 0.00^{a}	–	–	–
4	180(1)	20(−1)	1.64 ± 0.04^{h}	3.45 ± 0.07^{d}	2.02 ± 0.01^{h}	1.26 ± 0.01^{h}	–	–
5	180(1)	60(0)	1.71 ± 0.04^{i}	3.61 ± 0.01^{d}	1.06 ± 0.01^{f}	0.99 ± 0.01^{g}	–	–
6	180(1)	100(1)	0.48 ± 0.01^{f}	0.59 ± 0.01^{b}	0.34 ± 0.02^{d}	0.21 ± 0.01^{f}	–	–
7	135(0)	20(−1)	0.12 ± 0.01^{d,e}	1.30 ± 0.10^{e}	1.20 ± 0.10^{g}	0.02 ± 0.01^{b,c,d}	–	0.03 ± 0.00^{b,c}
8	135(0)	100(1)	0.12 ± 0.01^{c,d,e}	0.26 ± 0.07^{a,b,c}	0.12 ± 0.01^{b}	0.03 ± 0.01^{c,d,e}	0.17 ± 0.01^{a}	0.04 ± 0.00^{c}
9[I]	135(0)	60(0)	0.71 ± 0.01^{g}	1.90 ± 0.20^{f}	0.61 ± 0.01^{e}	0.06 ± 0.00^{e}	0.03 ± 0.01^{c}	0.07 ± 0.00^{d}
10[I]	135(0)	60(0)	0.78 ± 0.01^{g}	1.70 ± 0.10^{f}	0.68 ± 0.00^{e}	0.05 ± 0.00^{e}	–	0.09 ± 0.01^{d}
11[I]	135(0)	60(0)	0.72 ± 0.02^{g}	1.50 ± 0.10^{f}	0.62 ± 0.00^{e}	0.04 ± 0.00^{e}	–	0.07 ± 0.00^{d}
UAE[J]	25	100	–	–	0.01 ± 0.00^{a}	–	–	0.01 ± 0.00^{a,b}
MAE[K]	110	100	0.02 ± 0.00^{a}	0.03 ± 0.01^{a}	0.02 ± 0.00^{a}	0.02 ± 0.00^{a,b,c}	–	0.02 ± 0.00^{a,b}
SLE[L]	25	100	0.03 ± 0.01^{a,b}	–	0.05 ± 0.01^{a,b}	0.04 ± 0.00 ^{c,d,e}	–	0.02 ± 0.00^{b,c}

Different superscript lowercase letters in the same column show statistically significant differences among data at $p < 0.05$. Values are expressed as means ± standard deviations of three replicates.
– = Not detected.
[A] Extraction temperature. Coded values between brackets.
[B] Solvent (ethanol) concentration in the hydroalcoholic solution. Coded values between brackets.
[C] Gallic acid.
[D] Catechin.
[E] Vanillic acid.
[F] Syringic acid.
[G] Epicatechin.
[H] Ferulic acid.
[I] Central point runs.
[J] Ultrasound-assisted extraction.
[K] Microwave-assisted extraction.
[L] Solid-liquid extraction.
* DB = dry biomass.

syringic (1.26 ± 0.01 mg 100 g_{DB}^{-1}) acids. As can be observed, maximum contents of these compounds were detected in the extracts obtained under conditions (runs 4 and 5) able to maximize *TP* and *TF*, i.e., the highest temperature (180 °C) and 20 ≤ Sc ≤ 60%, which suggests that they may be among the major contributors of both phenolics classes.

Fig. 3 illustrates, as an example, the reverse phase HPLC profile of these phenolic compounds in the *A. platensis* extract obtained by HPTE at 180 °C and ethanol concentration in the hydroalcoholic solution of 60% (v/v) (run 5) (Fig. 3A) together with a micrograph of the microbial suspension before extraction (Fig. 3B).

The overall content of phenolic compounds quantified by HPLC was much lower than the one obtained by the Folin–Ciocalteu colorimetric method. This finding was indeed expected not only from the weak selectivity of the Folin–Ciocalteu reagent, which can react also with some non-phenolic compounds [63,64], but also from the little fraction of identified phenolics.

The highest concentrations of vanillic and syringic acids were two and four orders of magnitude higher (2.02 ± 0.01 mg 100 g_{DB}^{-1} and 1.26 ± 0.01 mg 100 g_{DB}^{-1}, respectively) than those (25.5 μg_{VA}100 g_{DB}^{-1} and 0.34 μg_{SA}100 g_{DB}^{-1}, respectively) detected in *A. platensis* extracts obtained by solid-phase/supercritical CO_2 extraction at 80 °C for 60 min with 47.5:47.5:5.0 (v/v/v) methanol/water/NH_3 as a solvent mixture, and even higher than those reported for other cyanobacterial or microalgal extracts (5.29 μg_{VA}100 g_{DB}^{-1} and 5.48 μg_{SA}100 g_{DB}^{-1} for *Spongiochloris spongiosa*, 10.2 μg_{VA}100 g_{DB}^{-1} for *Cylindrospermum* sp., 12.0 μg_{VA}100 g_{DB}^{-1} for *Nostoc* sp., and 24.7 μg_{VA}100 g_{DB}^{-1} and 11.0 μg_{SA}100 g_{DB}^{-1} for *Anabaena doliolum*) [26], which suggests a crucial role of the extraction technique in the extract composition.

3.4. Response surface modeling

The Response Surface Methodology (RSM) was used to model *TP* (Y_1), *TF* (Y_2), ARP (Y_3) and the content of catechin (*CA*) (Y_4), i.e., the most abundant among the quantified phenolic compounds of the extract, by the second-order polynomial equations:

$$Y_1 = 9.66 + 9.22x_1 + 5.66x_1^2 - 3.66x_2 - 2.39x_2^2 - 2.08\, x_1{}^*x_2 \quad (2)$$

$$Y_2 = 4.17 + 2.83x_1 + 0.98x_1^2 - 1.89x_2 - 0.38x_2^2 - 1.21\, x_1{}^*x_2 \quad (3)$$

$$Y_3 = 63.04 - 12.43x_1 - 10.44x_1^2 - 8.69x_2 - 6.19\, x_1{}^*x_2 \quad (4)$$

$$Y_4 = 1.70 + 1.08x_1 - 0.68x_2 - 0.92x_2^2 - 0.65\, x_1{}^*x_2 \quad (5)$$

where x_1 and x_2 are the coded values of T and Sc. Eqs. (4) and (5) were obtained after neglecting the statistically insignificant ($p > 0.05$) quadratic terms of Sc and temperature, respectively.The analysis of variance (ANOVA) highlighted that the regression was statistically significant with high or satisfactory values of the adjusted determination coefficient (R^2_{Adj} = 0.925, 0.963, 0.849 and 0.895 for *TP*, *TF*, ARP and *CA*, respectively) and that all terms were statistically significant ($p < 0.05$) (see Supplemental Information, Table S1).

To better visualize the combined effects of the two independent variables on *TP*, *TF*, ARP and *CA*, Fig. 4 shows the three-dimensional graphs obtained by Eqs. (2)–(5). As confirmed by significant linear and quadratic terms of the model equation, both T and Sc strongly influenced *TP* in *A. platensis* extracts obtained by HPTE (Fig. 4A). In particular, the positive linear regression coefficient of temperature was the most significant term of the model (+9.22), followed by the positive quadratic one of the same variable (+5.66). Even though they were responsible for an exponential increase in *TP*, especially evident at 180 °C, the negative linear (−3.66) and quadratic (−2.39) terms of Sc together with the negative interaction between the two variables (−2.08) led to a maximization of this response (26.00 ± 0.02 mg_{GAE} g_{DB}^{-1} to 28.04 ± 2.76 mg_{GAE} g_{DB}^{-1}; $p > 0.05$) at Sc values in the range 20–60%. The use of higher Sc (100% v/v) led to a considerable decrease in *TP* (14.81 ± 0.15 mg_{GAE} g_{DB}^{-1}), confirming the significance of a water fraction for efficient phenolic extraction from *A.*

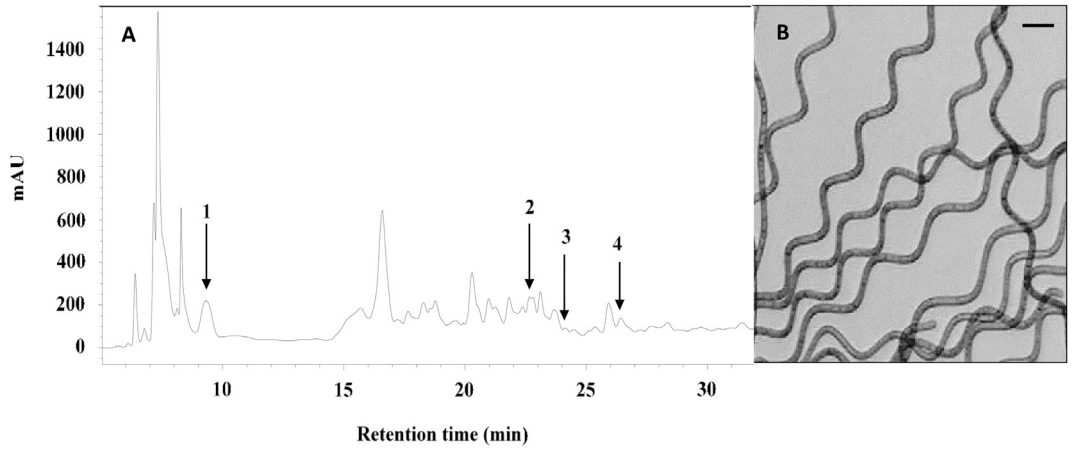

Fig. 3. A) Reverse phase HPLC chromatogram of phenolic compounds in the *Arthrospira platensis* extract obtained by high pressure/temperature extraction at 180 °C and solvent (ethanol) concentration in the hydroalcoholic solution of 60% (v/v) (run 5). Phenolic compounds: (1) gallic acid; (2) catechin; (3) vanillic acid; (4) syringic acid. Elution time and maximum absorbance of individual phenolics were determined by HPLC-DAD with reverse phase C18 column. The results are expressed in milli absorption units (mAU). B) micrograph (bar 10 μm) of *A. platensis* suspension before extraction.

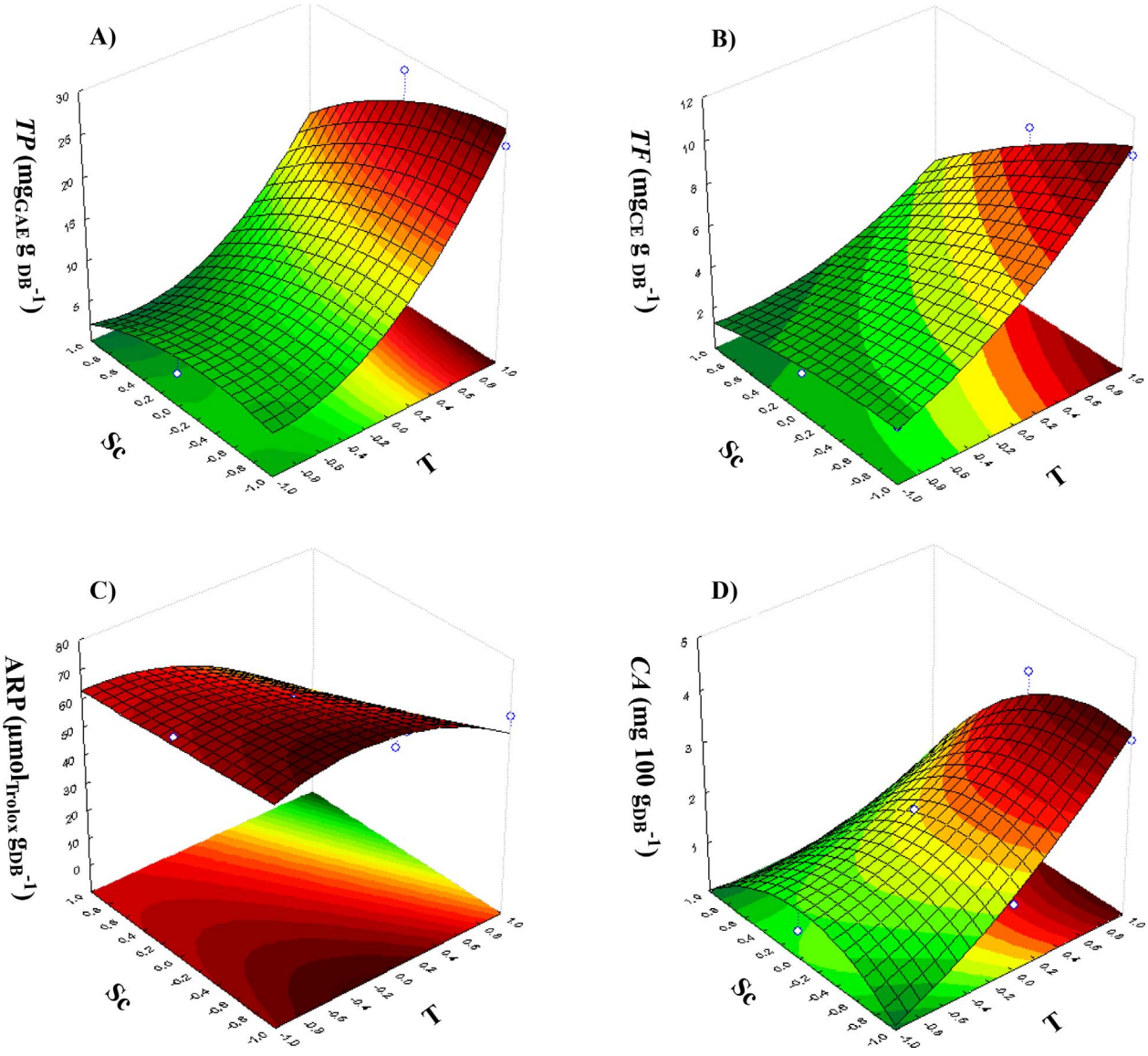

Fig. 4. Response surfaces of (A) total polyphenol yield (*TP*, $mg_{GAE}\ g_{DB}^{-1}$); (B) total flavonoid yield (*TF*, $mg_{CE}\ g_{DB}^{-1}$); (C) antiradical power determined by the $ABTS^{\cdot+}$ radical scavenging method (ARP, $\mu mol_{Trolox}\ g_{DB}^{-1}$) and (D) catechin content (*CA*, mg 100 g_{DB}^{-1}) as simultaneous functions of temperature (T, coded values) and ethanol concentration in the hydroalcoholic solution (Sc, coded values) in the *A. platensis* extracts obtained by high pressure/temperature extraction. GAE: gallic acid equivalents, CE: catechin equivalents.

Table 3
Correlation matrix with Pearson's correlation coefficients (*r*) for the responses investigated in *A. platensis* extracts obtained by high pressure/temperature extraction.

	TP[a]	*TF*[b]	ARP[c]	*GA*[d]	*CA*[e]	*VA*[f]	*SA*[g]	*EP*[h]	*FA*[i]
TP	1	0.958*	− 0.538	0.903*	0.895*	0.816*	0.924*	− 0.378	− 0.271
TF		1	− 0.297	0.870*	0.923*	0.942*	0.902*	− 0.367	− 0.167
ARP			1	− 0.410	− 0.245	− 0.045	− 0.376	0.240	0.301
GA				1	0.958*	0.751*	0.890*	− 0.359	− 0.006
CA					1	0.848*	0.852*	− 0.389	0.045
VA						1	0.779*	− 0.357	− 0.000
SA							1	− 0.242	− 0.392
EP								1	0.083
FA									1

[a] Total polyphenol yield.
[b] Total flavonoid yield.
[c] Antiradical power determined by the $ABTS^{\cdot+}$ radical scavenging method.
[d] Gallic acid.
[e] Catechin.
[f] Vanillic acid.
[g] Syringic acid.
[h] Epicatechin.
[i] Ferulic acid.
* $p < 0.05$.

platensis biomass. This result agrees with the observations of Esquivel-Hernández et al. [21], who reported an enhancement of thiamine, riboflavin, C-phycocyanin and A-phycocyanin in yields by microwave-assisted extraction from *A. platensis* when water/ethanol ratio was increased from 0.25 to 0.81 (v/v) at 40 °C for 55 min.

Besides, the low temperature range between 90 and 135 °C proved not to be efficient to recover polyphenols irrespectively of Sc, while a further T increase (180 °C) for Sc up to 60% resulted in a remarkable rise in *TP*. For instance, at Sc of 20%, *TP* increased by no less than 93 and 118% when T was increased from 90 to 135 °C and from 135 to 180 °C, respectively. As early mentioned (see Section 3.2), high temperature may have improved the extraction efficiency, because of enhanced diffusion rate and solubility of solutes [46,57,58], as well as the disintegration of cell wall and cell membrane [21].

As expected by the similarity of Eqs. (2) and (3), *TF* dependence on T and Sc (Fig. 4B) was very like that already discussed for *TP* (Fig. 4A). Temperature had a strong influence also on this response, in that the positive linear (+ 2.83) and quadratic (+ 0.98) terms of T were responsible for an exponential increase in *TF*, but the negative linear (− 1.89) and quadratic (− 0.38) terms of Sc together with the negative interaction term of both independent variables (− 1.21) led to a maximization of this response (10.25 ± 0.34 $mg_{CE}\ g_{DB}^{-1}$) at the lowest Sc (20%) and the highest T (180 °C) values. In particular, at this temperature, the *TF* increase was as higher as 115 and 149% when Sc was lowered from 100 to 60% and from 100 to 20%, respectively.

ARP of *A. platensis* extracts obtained by HPTE was influenced by T and Sc in a quite different way when compared with *TP* and *TF* (Fig. 4C). Being the negative linear regression coefficient of T the most significant term of the model (− 12.43), followed by the negative quadratic term of the same variable (− 10.44), the negative linear term of Sc (− 8.69), and the interaction one between them (− 6.19), ARP showed a parabolic trend and reached a maximum (67.77 ± 0.20 $\mu mol_{Trolox}\ g_{DB}^{-1}$ to 69.02 ± 1.11 $\mu mol_{Trolox}\ g_{DB}^{-1}$; $p > 0.05$) in runs 1, 2 and 7 carried out in the T and Sc ranges of 90–135 °C and 20–60%, respectively (Table 1).

However, at the highest T (180 °C), the negative influence of Sc became more marked, i.e., an ARP decrease by 44.1 and 58.2% took place when Sc was increased from 20 to 60% and from 20 to 100%, respectively, which suggests that the antioxidant compounds mainly responsible for this activity in *A. platensis* extract may be more soluble in water than in ethanol. This Sc effect was opposite to that observed by Maadane et al. [65] for ARP determined by the DPPH assay for SLE extracts of other microalgae (*Nannochlorpsis gaditana*, *Dunaliella* sp., *Dunaliella salina*, *Phaedactylium tricornutum*, *Isochrysis* sp., *Navicula* sp., *Chaeotoceros* sp., *Chlorella* sp. and *Tetraselmis* sp.).

As shown in Fig. 4D, catechin content showed a trend similar to those of *TP* and *TF* as function of T and Sc. In particular, the positive linear term of temperature (+ 1.08) was responsible for a 112.3% increase in *CA* when temperature was raised from 135 to 180 °C at Sc of 60%. But, the negative linear (− 0.68) and quadratic (− 0.92) terms of Sc, combined with the negative interaction between the independent variables (− 0.65), led to a maximization also of this response (3.45 ± 0.07 mg 100 g_{DB}^{-1} to 3.61 ± 0.01 mg 100 g_{DB}^{-1}; $p > 0.05$) for 20 ≤ Sc ≤ 60%.

3.5. Statistical correlations among responses

Because of some qualitative similarities among the above results, the values of the Pearson's correlation coefficient (*r*) [54] of linear equations relating the contents of different groups of phenolic compounds (*TP* and *TF*), individual phenolic compounds detected by HPLC (catechin, epicatechin, gallic, vanillic, syringic and ferulic acids) and ARP have been used to identify possible relationships among them (Table 3).

One can see from this table a strong positive ($r = 0.958$) and statistically significant ($p < 0.05$) correlation between *TP* and *TF* and satisfactory positive correlations between *TP* and most of the individual phenolic compounds detected by HPLC, namely *GA* ($r = 0.903$), *CA* ($r = 0.895$), *VA* ($r = 0.816$) and *SA* ($r = 0.924$). The same applies to the positive correlations of *TF* and these compounds ($r = 0.870$, 0.923, 0.942 and 0.902, respectively), whereas correlations between *TP* or *TF* with *EP*, *SA* and ARP were not statistically significant ($p > 0.05$).

To shed more light on these issues, either *TP* or *TF* determined by their respective colorimetric methods were plotted in Fig. 5 versus the sum of contents of individual phenolic compounds quantified by HPLC (SPC), with high Pearson's coefficients highlighting good linear correlation among the two assays. Statistically significant ($p < 0.05$) positive linear correlations of both *TP* ($r = 0.928$) and *TF* ($r = 0.960$) and these compounds (Figs. 5A and B, respectively) indicate that the phenolic compounds identified by HPLC were effective contributors to both groups of phenolics.

On the other hand, the Pearson's coefficients of linear equations relating *TP* with ARP and *TF* with ARP were not statistically significant ($p > 0.05$) for the extracts obtained under the operating conditions investigated in this study (Table 3). Since only polyphenols of a certain structure, particularly those carrying hydroxyl groups are responsible for antioxidant properties, which depend on the ability to donate hydrogen or electrons to free radicals [66], one can infer that the drastic

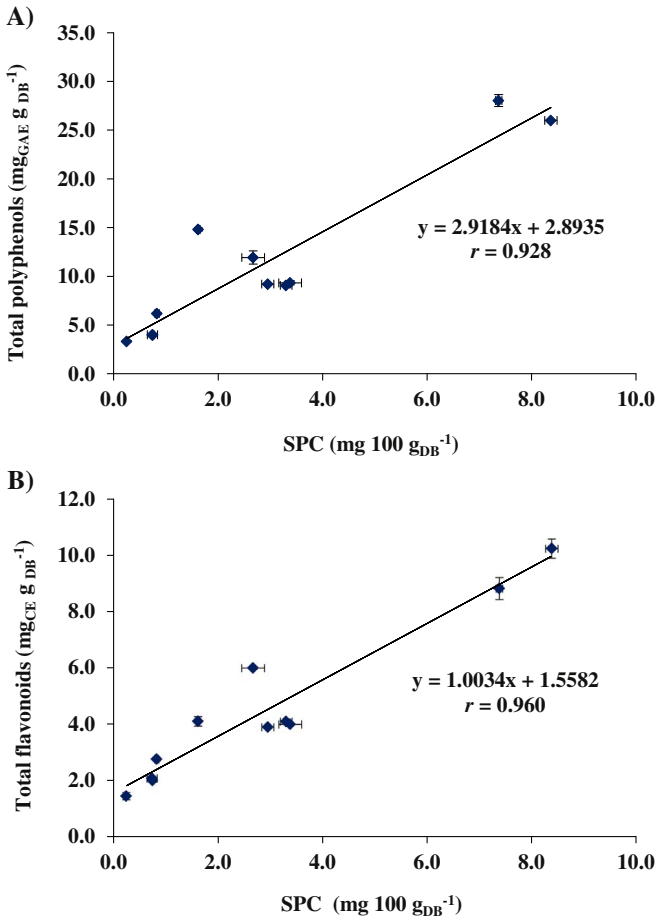

Fig. 5. Linear Pearson's correlations between the overall contents of total polyphenols (A) or total flavonoids (B), and the sum of contents of individual phenolic compounds quantified by HPLC (SPC) in *Arthrospira platensis* extracts obtained by HPTE. GAE: gallic acid equivalents, CE: catechin equivalents, HPTE: high pressure/temperature extraction. Data are expressed as means of three replicates (n = 3). Error bars indicate means ± standard deviations.

HPTE conditions might have somehow altered these structures. But a more likely reason is that photosynthetic microorganisms are able to produce a wide range of alternative antioxidants to protect them, including carotenoids, long-chain polyunsaturated fatty acids and polysaccharides [65,67]. For instance, Rodríguez-Meizoso et al. [68], who investigated the properties of *Haematococcus pluvialis* extracts obtained by subcritical water extraction, observed that the Trolox equivalent antioxidant capacity (TEAC) was correlated to vitamin E, together with simple phenols, Maillard reaction products obtained during high temperature-extraction (200 °C). Low correlations between antioxidant activity and phenolic contents (R^2 = 0.007–0.585) were also found in a relatively large number of microalgae extracts obtained by three-step sequential extraction with hexane, ethyl acetate and water [69], and the same occurred with ethanolic extracts of nine microalgae strains (R^2 = 0.154) [65].

On the contrary, it was reported that the phenolic content significantly contributed to the antioxidant capacity of *Isochrysis* sp., *Phaeodactylum* sp., *Chlorella* sp., *T. suecica*, *Dunaliella salina*, *Fischerella ambigua*, *Nostoc muscorum*, *Oocystis pusilla* and *Scenedesmus rubescens* [59,70]. These conflicting results may be explained with the fact that the production of phenolics as well as other antioxidant compounds by microalgae and cyanobacteria depends on growth conditions and nutritional and/or oxidative stress; therefore, this aspect should be considered when the results are being compared with those of other studies [71].

4. Conclusions

The high pressure/temperature extraction (HPTE) showed to be the most efficient extraction method to recover total polyphenols and flavonoids from *Arthrospira platensis* biomass as well as to obtain extracts with high antiradical power (ARP) using binary mixtures of green solvents (ethanol/water). Results obtained according to a 3^2-full factorial design and modeled by Response Surface Methodology clearly revealed that the extraction temperature and solvent (ethanol) concentration in the hydroalcoholic solution (Sc) had a strong influence on total polyphenol yield (*TP*), total flavonoid yield (*TF*) and ARP. The most suitable conditions to efficiently recover *TP* and *TF* were shown to be the highest temperature (180 °C) and Sc in the range 20–60%, while a different trend was observed for ARP, which kept always high in the whole tested temperature range ($90 \leq T \leq 135$ °C) at 20–60%. HPLC allowed identifying catechin and vanillic, gallic and syringic acids as the main phenolics in the extract. Linear correlations between the overall content of these compounds taken together and *TP* or *TF* suggested that they contributed significantly to either class of phenolics, whereas nothing similar was observed for ARP, suggesting that other cell constituents may play a major role in the antioxidant capacity of HPTE *A. platensis* extracts. This study demonstrates the potential of HPTE as a promising alternative technique to obtain high-ARP phenolic-rich hydroalcoholic extracts, and of *A. platensis* biomass as a potential natural source of compounds for functional food formulation or dietary supplement preparation. Furthermore, this work opens new avenues in the use of this emerging technology, for the recovery of other high-added value compounds from cyanobacterial biomass.

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ANEXO C – Artigo publicado apresentado no capítulo III desta tese

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A new bioenergetic and thermodynamic approach to batch photoautotrophic growth of *Arthrospira (Spirulina) platensis* in different photobioreactors and under different light conditions

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HIGHLIGHTS

- Batch *A. platensis* culture is studied in different photobioreactor configurations.
- Kinetics, bioenergetics and thermodynamics of *A. platensis* growth are evaluated.
- An increase in surface/volume ratio up to 1.94 cm^{-1} enhanced growth parameters.
- The influence of light intensity is investigated in the horizontal photobioreactor.
- The horizontal photobioreactor has the best configuration to perform batch culture.

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ABSTRACT

Photobioreactor configuration, mode of operation and light intensity are known to strongly impact on cyanobacteria growth. To shed light on these issues, kinetic, bioenergetic and thermodynamic parameters of batch *Arthrospira platensis* cultures were estimated along the time at photosynthetic photon flux density (PPFD) of 70 μmol m^{-2} s^{-1} in different photobioreactors with different surface/volume ratio (*S*/*V*), namely open pond (0.25 cm^{-1}), shaken flask (0.48 cm^{-1}), horizontal photobioreactor (HoP) (1.94 cm^{-1}) and helicoidal photobioreactor (HeP) (3.88 cm^{-1}). Maximum biomass concentration and productivity remarkably increased with *S*/*V* up to 1.94 cm^{-1}. HoP was shown to be the best-performing system throughout the whole runs, while HeP behaved better only at the start. Runs carried out in HoP increasing PPFD from 40 to 100 μmol m^{-2} s^{-1} revealed a progressive enhancement of bioenergetics and thermodynamics likely because of favorable light distribution. HoP appeared to be a promising configuration to perform high-yield indoor cyanobacterial cultures.

1. Introduction

The energy management in a living cell is called bioenergetics. When combined with the fundamental principles of thermodynamics, it has proven to be a highly useful tool for the analysis of biosystems (Küçük et al., 2015). Photosynthetic microorganisms have recently gained huge attention worldwide (Cheah et al., 2015), since they are one of the most promising renewable and neutral energy sources, i.e., by consuming carbon dioxide, their cultivation has the additional benefit of combining valuable biomass production with CO_2 emissions reduction (Belay, 2002; Rodrigues et al., 2011). Among the photosynthetic microorganisms with commercial importance, the filamentous cyanobacterium (blue-green alga) *Arthrospira (Spirulina) platensis* has widely been studied, because its biomass has a large number of industrial applications besides being considered a high-value food (Belay, 2002; Benelhadj et al., 2016). *A. platensis* production is in fact increasing worldwide owing to its high contents of highly-valuable proteins, amino acids, essential fatty acids (i.e., γ-linolenic acid, GLA), polysaccharides, vitamins and pigments (β-carotene, chlorophyll a and phycocyanin) (Pulz and Gross, 2004); in addition, it contains other phytochemicals that find application in several industrial

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segments like those of health foods and therapeutics (Belay, 2002; Pulz and Gross, 2004; Raposo and Morais, 2015).

A lot of parameters can be tuned to obtain high biomass yield, among which are the mode of operation, light intensity and reactor configuration, that can strongly impact on the performance of *A. platensis* cultivation. Different types of processes have been developed to get an optimal compromise between high productivity and low production costs. Among these are: (a) the fed-batch process that consists in periodically replacing a part of the exhaust medium with fresh medium to keep the culture volume constant; (b) the batch culture systems, which are the most widely applied because of their simplicity and flexibility, even though not necessarily the most efficient ones; and (c) large-scale continuous cultures, which have not been applied extensively up to now, due to several difficulties in their control, among which are higher risk of contamination, use of feeding pumps, lower yields, etc.

Light irradiance should be provided with care to indoor systems performed using artificial light, since excess light leads to a phenomenon called "photooxidation" or "photoinhibition". That is, cell concentration increases with light intensity until reaching a maximum threshold value at the so-called "saturation level", beyond which a further increase in light intensity provokes damage of cell photosynthetic apparatus (Bezerra et al., 2012).

The reactor configuration is an additional factor greatly influencing cell growth. Photobioreactors can reduce the cultivation area by a vertical distribution of the photosynthetic organism and enlarge the surface exposed to light, thereby ensuring high surface/volume ratios and increasing cell concentration. Light is better captured by cells in tubular photobioreactors when compared to the conventional open ponds, where, owing to a relatively high depth of culture medium, it has to go through thick layers to reach the inner cells (Converti et al., 2006; Rodrigues et al., 2010). Although the open-channel raceway ponds is the most widely used configuration for *A. platensis* commercial production, tubular photobioreactors have been deeply studied, not only because of their high cell productivity, but also of many other advantages, such as low levels of contamination and better CO_2 solubilization in the medium, better light distribution and then higher photosynthetic efficiency (Converti et al., 2006).

Bioenergetic studies based on the Gibbs energy dissipation may be applied to describe or predict the microbial growth yield, the energy flow to ATP production, the increase in enthalpic content and the heat released by living organisms (Bezerra et al., 2012; Sassano et al., 2004; Torre et al., 2003). All of them are quite important to optimize any bioprocess and even to design the most suitable bioreactor to perform it; but, unfortunately, only a few studies dealt with the bioenergetic aspects of the growth of photosynthetic microorganisms based on Gibbs energy balances. In particular, biomass yield constitutes one of the key parameters in any bioprocess or experiment involving microbial cultures, since it determines the final biomass concentration, which must be maximized to obtain high productivities (Von Stockar et al., 2006). In addition, the Gibbs energy dissipation per C-mol of biomass can be regarded as a simple thermodynamic measure of the amount of biochemical "work" required to convert the carbon source into biomass (Bezerra et al., 2012; Heijnen and Van Dijken, 1991, 1993; Liu et al., 2007).

Based on this background, if from one hand previous works demonstrated that the use of the fed-batch mode of operation is able to promote the growth of photosynthetic microorganisms minimizing a number of well-known adverse phenomena (shading, inhibitions related to excess salt level or osmotic pressure, and so on), from the other it masks their effects. Therefore, to shed light on these issues and because of a certain lack of detailed and recent theoretical studies on the mechanisms ruling *A. platensis* cultures, the main bioenergetic and thermodynamic parameters of the photoautotrophic batch growth of such a cyanobacterium were investigated in this study based on the model proposed by Torre et al. (2003). For this purpose, we investigated four different photobioreactor configurations with different surface/volume ratios, namely shaken flask, open pond, helicoidal photobioreactor and horizontal photobioreactor, and varying the light irradiance in the last, best-performing configuration.

2. Methods

2.1. Microorganism and culture conditions

A. (Spirulina) platensis UTEX 1926 was obtained from the Culture Collection of Algae of the University of Texas (Austin, TX, USA). To allow a large growth of biomass, the microorganism was maintained and cultivated in the culture medium suggested by Schlösser (1982) modified so as to have a nitrogen concentration equal to about 4-fold that of the original medium. The resulting medium had the following composition (per liter): 13.61 g $NaHCO_3$, 4.03 g Na_2CO_3, 0.50 g K_2HPO_4, 10.0 g $NaNO_3$, 1.00 g K_2SO_4, 1.00 g NaCl, 0.20 g $MgSO_4{\cdot}7H_2O$, 0.04 g $CaCl_2{\cdot}2H_2O$. All the nutrients were dissolved in distilled water containing (per liter): 6.0 mL of metal solution (97 mg L^{-1} $FeCl_3{\cdot}6H_2O$, 41 mg L^{-1} $MnCl_2{\cdot}4H_2O$, 5 mg L^{-1} $ZnCl_2$, 2 mg L^{-1} $CoCl_2{\cdot}6H_2O$, 4 mg L^{-1} $Na_2MoO_4{\cdot}2H_2O$, 750 mg L^{-1} $Na_2EDTA{\cdot}2H_2O$), 1.0 mL of micronutrient solution (50.0 mg L^{-1} Na_2EDTA, 618 mg L^{-1} H_3BO_3, 19.6 mg L^{-1} $CuSO_4{\cdot}5H_2O$, 44.0 mg L^{-1} $ZnSO_4{\cdot}7H_2O$, 20.0 mg L^{-1} $CoCl_2{\cdot}6H_2O$, 12.6 mg L^{-1} $MnCl_2{\cdot}4H_2O$, 12.6 mg L^{-1} $Na_2MoO_4{\cdot}2H_2O$) and 1.0 mL of B12 vitamin solution (0.15 mg L^{-1}).

Cells were grown batch-wise either at photosynthetic photon flux density (PPFD) of 70 μmol m^{-2} s^{-1} in different photobioreactor configurations with different surface/volume ratios (S/V), namely open pond (Sassano et al., 2004) (S/V = 0.25 cm^{-1}), shaken flask (Frumento et al., 2016) (S/V = 0.48 cm^{-1}), horizontal photobioreactor (Ferreira et al., 2010) (S/V = 1.94 cm^{-1}) and helicoidal photobioreactor (Bezerra et al., 2011; Frumento et al., 2016) (S/V = 3.88 cm^{-1}), or progressively increasing PPFD from 40 to 100 μmol m^{-2} s^{-1} in the horizontal photobioreactor that proved the best-performing configuration. For this purpose, fluorescent artificial light was ensured by a variable number of 36 W-lamps. Schematics of horizontal and helicoidal photobioreactors were illustrated in a previous study (Frumento et al., 2013).

Cultivations were carried out at temperature of 30 ± 2 °C, by incubating the equipment in a thermostated chamber, using an initial biomass concentration of 0.40 g L^{-1}. The pH was controlled daily at 9.5 ± 0.2 through the addition of pure CO_2 from a cylinder. After growth, once the stationary phase had been reached after about 9 days of cultivation, biomass was separated from the culture medium by centrifugation at 7500 rpm for 10 min using a centrifuge, model 42426 (ALC, Milan, Italy). Recovered cells were washed twice with distilled water, dried at 105 °C for 24 h, pulverized in a mortar and stored at −20 °C for subsequent analysis of its elemental composition.

2.2. Analytical procedures

Cell concentration of *A. platensis* was determined daily by measuring the optical density (OD) at 560 nm by a UV-Visible spectrophotometer, model Lambda 25 (PerkinElmer, Milan, Italy), and expressed in grams of dried biomass per liter of medium (g L^{-1}) through a calibration curve relating OD to dry biomass weight. All measurements were done in triplicate.

The elemental composition of dried biomass stored at the end of culture was determined by an elemental analyzer Flash EA1112 series (CE Instruments, Wigan, UK). Since biomass composition varied very little among the different photobioreactor configurations

and light intensities, its average value obtained from all the runs ($CH_iO_jN_k$ where $i = 1.59 \pm 0.14$, $j = 0.50 \pm 0.06$, $k = 0.10 \pm 0.01$) was used to estimate the bioenergetic and thermodynamic parameters of growth.

PPFD was measured in several points of the culture surface using a type sensor quantum/photometer/radiometer, model HD-9021 Delta OHM (Li-Cor Inc., Lincoln NE, USA).

3. Theory

3.1. Kinetic parameters of A. platensis growth

A. platensis cultures in different photobioreactor configurations under different light intensities has first been evaluated in terms of variations of the main growth parameters, namely the maximum cell concentration (X_{max}), the maximum specific growth rate (μ_{max}) and the maximum biomass productivity ($P_{X\ max}$).

To this purpose, μ_{max} was calculated by the equation:

$$\mu_{max} = \frac{1}{t_i - t_{i-1}} ln\left(\frac{X_i}{X_{i-1}}\right) \quad (1)$$

where X_i and X_{i-1} are biomass concentrations (g L^{-1}) at times t_i and t_{i-1} (days), respectively.

Analogously, $P_{X\ max}$ was defined as:

$$P_{Xmax} = \frac{X_i - X_{i-1}}{t_i - t_{i-1}} \quad (2)$$

It should be noticed that t_i and t_{i-1} are the cultivation times separately maximizing the functions μ_{max} and $P_{X\ max}$; therefore, they have not the same values in Eqs. (1) and (2).

3.2. Bioenergetic and thermodynamic parameters of A. platensis growth

To estimate the bioenergetics parameters of *A. platensis* growth under batch conditions, we applied the model proposed by Torre et al. (2003), according to whom, under ideal conditions, the energy carried by 8 photons at 580 nm is captured by photosynthetic pigments held in the "reaction centers" of the photosystem II (PSII) and used to transfer 4 electrons from 2 water molecules to the photosystem I (PSI). In fact, according to Richmond (1983), the photosynthetic organisms can only use light with wavelength in the range 400–700 nm, and cyanobacteria have an energetic input (Li et al., 2001) equivalent to that of 580 nm monochromatic light (206.2 kJ mol^{-1} of photons). However, the yield of this process decreases as the conditions differ from the ideal ones. An equal number of photons with less energy is then released as heat, while the 4 electrons, through the PSI electron transport system, are used to reduce $NADP^+$ to NADPH:

$$2H_2O + E + 2NADP^+ \rightarrow 2NADPH + O_2 + 2H^+ \quad (3)$$

and finally transferred from NADPH to CO_2 through the Calvin cycle to produce biomass constituents.

The dissipation of Gibbs energy for cell growth and maintenance ($1/Y_{GX}$) was estimated, according to Heijnen (2001), as:

$$\frac{1}{Y_{GX}} = \frac{1}{Y_{GX}^{max}} + \frac{m_G}{\mu} \quad (4)$$

where $1/Y_{GX}^{max}$ is the growth-related Gibbs energy requirement, equivalent to 986 kJ per C-mol of dry biomass for photoautotrophic cultures, μ the specific growth rate and m_G the specific rate of Gibbs energy dissipation for maintenance, equivalent to 7.12 kJ C-mol^{-1} h^{-1} at 30 °C.

The photoautotrophic growth can be described by an overall mass balance equation, where the nitrogen (NO_3^-) and carbon (HCO_3^-) sources, H_2O, O_2, and H^+ are involved in the formation of one C-mol of dry biomass:

$$aHCO_3^- + bNO_3^- + cH_2O + dO_2 + eH^+ + CH_{1.59}O_{0.50}N_{0.10} = 0 \quad (5)$$

where a, b, c, d and e are the stoichiometric coefficients of the chemical species involved in biomass formation expressed in mol C-mol^{-1} and $CH_{1.59}O_{0.50}N_{0.10}$ is the average elemental composition of biomass experimentally determined in this work.

The stoichiometric coefficients estimated through material balances of carbon, nitrogen, oxygen, charge, and reduction degree are listed in Table 1.

The following Gibbs energy balance should have been employed to estimate the moles of photons (Einsteins), n_{Ph}, to sustain the autotrophic growth of one C-mol of *A. platensis* biomass under biological standard conditions (298 K, pH = 7, 1 bar, concentrations of 1 mol L^{-1}):

$$n_{Ph} = -\frac{a\Delta g^{\circ\prime}_{f_{HCO_3^-}} + b\Delta g^{\circ\prime}_{f_{NO_3^-}} + c\Delta g^{\circ\prime}_{f_{H_2O}} + d\Delta g^{\circ\prime}_{f_{O_2}} + e\Delta g^{\circ\prime}_{f_{H^+}} + \Delta g^{\circ\prime}_{f_X} + 1/Y_{GX}}{\Delta g^{\circ\prime}_{Ph}} \quad (6)$$

where $\Delta g^{\circ\prime}_{f_{HCO_3^-}} = -587.2$ kJ mol^{-1}, $\Delta g^{\circ\prime}_{f_{NO_3^-}} = -111.4$ kJ mol^{-1}, $\Delta g^{\circ\prime}_{f_{H_2O}} = -237.3$ kJ mol^{-1}, $\Delta g^{\circ\prime}_{f_{O_2}} = 0$ and $\Delta g^{\circ\prime}_{f_{H^+}} = -39.87$ kJ mol^{-1} are the molar Gibbs energies of formation of the chemical species involved in the photosynthetic event, while $\Delta g^{\circ\prime}_{f_X} = -67.0$ kJ C-mol^{-1} and $\Delta g^{\circ\prime}_{Ph} = h\underline{c}N_A/\lambda = 206.2$ kJ mol^{-1} are those associated with the formation of biomass and the absorption of 1 mol of photons, respectively, being $h = 6.626 \cdot 10^{-34}$ J s the Planck constant, $\underline{c} = 2.99626 \cdot 10^8$ m s^{-1} the light velocity, $N_A = 6.022626 \cdot 10^{23}$ mol^{-1} the Avogadro number and $\lambda = 580$ nm the early-mentioned reference wavelength.

However, culture conditions were quite different from the biological standard ones, and the actual Δg_f values should have been used for this calculation. Nonetheless, as stressed by Torre et al. (2003), the actual Gibbs energies of formation do not differ at all

Table 1
Molar Gibbs energy of formation (Δg_f) and stoichiometric coefficients (a–e)[a] of the chemical species involved in *A. platensis* biomass formation on modified Schlösser medium.

Species	HCO_3^-	NO_3^-	H_2O	O_2	H^+	Biomass
Δg_f (kJ mol^{-1})	−587.2	−111.4	−237.3	0	−54.4[b]	−67.0[c]
Stoichiometric coefficients						
Symbol	a	b	c	d	e	γ_X[d]
Value (mol C-mol^{-1})	−1.000	−0.100	0.255	1.272	−1.100	5.09

[a] Stoichiometric coefficients estimated through material balances of carbon, nitrogen, oxygen, charge, and reduction degree, according to the bioenergetic model of Eq. (5).
[b] Value calculated at pH 9.5 through the Gibbs equation (Eq. (7)) using $\Delta g^{\circ\prime}_{f_{H^+}} = -39.87$ kJ mol^{-1} at pH 7.0.
[c] Value expressed in kJ C-mol^{-1} instead of kJ mol^{-1}.
[d] Reduction degree of biomass (dimensionless) referred to the average elemental composition of biomass ($CH_{1.59}O_{0.50}N_{0.10}$) experimentally determined in this study.

or differ by no more than 3% from those under standard biological conditions, with exception of $\Delta g_{f_{H^+}}$, which is by definition a function of pH; therefore, this parameter was recalculated ($\Delta g_{f_{H^+}} = -54.4$ kJ mol^{-1}) for the actual conditions (pH 9.5; 30 °C) by the well-known equation of Gibbs:

$$\Delta g_{f_{H^+}} = \Delta g^{\circ\prime}_{f_{H^+}} + RTln\frac{10^{-9.5}}{10^{-7.0}} \quad (7)$$

where T is the absolute temperature (K) and R the ideal gas constant (8.3145 J mol^{-1} K^{-1}).

Once n_{Ph} had been estimated, the molar Gibbs energy associated with the photosynthetic growth of one C-mol of biomass (ΔG_a) was calculated as:

$$\Delta G_a = n_{Ph}\Delta g_{Ph} = \Delta H + \Delta G_{ATP} + Q \quad (8)$$

and assumed to be equivalent to the sum of the energy fixed by the system to increase its own enthalpic content (ΔH), the Gibbs energy transformed into ATP and used for growth and maintenance (ΔG_{ATP}) and that released as heat (Q).

ΔG_{ATP} and ΔH were calculated from n_{Ph}, the Gibbs energy associated to one mol of ATP (286.2 kJ mol^{-1}) and the sum (E^0 = 1.236 V) of the average potential variations between PSI and PSII (E^0 = 0.580 V) and between PSI and NADPH (E^0 = 0.656 V) reported in the literature for most of synthetic organisms (Torre et al., 2003).

The rates of O_2 production (q_{O2}) and H^+ consumption (q_{H+}) were calculated by multiplying their respective stoichiometric coefficients by the cell concentration expressed in C-mol L^{-1}, by using the biomass elemental composition experimentally determined in this study ($CH_{1.59}O_{0.50}N_{0.10}$), and by dividing by the cultivation time.

4. Results and discussion

Previous works demonstrated that the mode of operation, the photobioreactor configuration and the light intensity strongly influence the performance of *A. platensis* cultivation. In particular, Ferreira et al. (2010) found that fed-batch addition of nitrate as nitrogen source in a horizontal photobioreactor working at PPFD of 60, 120 and 240 μmol m^{-2} s^{-1} led to photo-limitation, photosaturation and photoinhibition, respectively, hence confirming the leading role of light intensity. A subsequent study performed under comparable conditions but comparing different photobioreactor configurations under fed-batch addition of two different nitrogen sources (nitrate and urea), highlighted that also these factors, besides the light intensity, exert a strong influence on the growth of such a cyanobacterium acting on its bioenergetic parameters (Bezerra et al., 2012). However, if from one hand the use of the fed-batch mode of operation is able to promote the growth minimizing a number of adverse phenomena (shading, inhibitions related to excess salt level or osmotic pressure, and so on), from the other it join them masking their separate effects.

To shed better light on these effects, the main growth, bioenergetic and thermodynamic parameters have been calculated in this study from batch cultures of *A. platensis* carried out either at PPFD of 70 μmol m^{-2} s^{-1} in four photobioreactor configurations, namely open pond (OP), shaken flask (SF), horizontal photobioreactor (HoP), helicoidal photobioreactor (HeP), or progressively increasing PPFD from 40 to 100 μmol m^{-2} s^{-1} in HoP that proved to be the best-performing configuration.

4.1. Kinetic parameters of A. platensis growth

It can be seen in Fig. 1 that biomass concentration was remarkably influenced by the photobioreactor configuration, in that its maximum value (X_{max}) strongly raised from 2.14 ± 0.11 g L^{-1} to 3.56 ± 0.25 g L^{-1} and 7.11 ± 0.53 g L^{-1} when the surface to volume ratio (S/V) was increased from 0.25 cm^{-1} (OP) to 0.48 cm^{-1} (SF) and 1.94 cm^{-1} (HoP), respectively (Table 2). Such a behavior may be ascribed to a better light distribution in HoP along with the occurrence of the well-known shading effect of the outer cell layers on the inner ones in systems with insufficient exposition to light (SF and OP), which resulted in reduced light availably to cells (Danesi et al., 2004). A separate reasoning should be applied to HeP, which, despite a S/V ratio that was twice that of HoP, offered unexpectedly a sigmoidal growth curve, with better performance during the log phase but a X_{max} value about 15% lower (6.02 ± 0.27 g L^{-1}) (Table 2). Such an observation could be explained with a combination of different positive and negative factors in HeP compared with HoP, among which the most important may have been the higher S/V ratio, resulting in better growth up to 4 days, and a more intense agitation, leading to excess mechanical stress to cells responsible for a lower X_{max} value.

The main growth parameters of these cultures summarized in Table 2 clearly show that HoP was by far the best photobioreactor configuration in terms not only of maximum cell concentration but also of maximum specific growth rate (μ_{max} = 0.988 ± 0.012 day^{-1}), while HeP ensured the highest maximum cell productivity

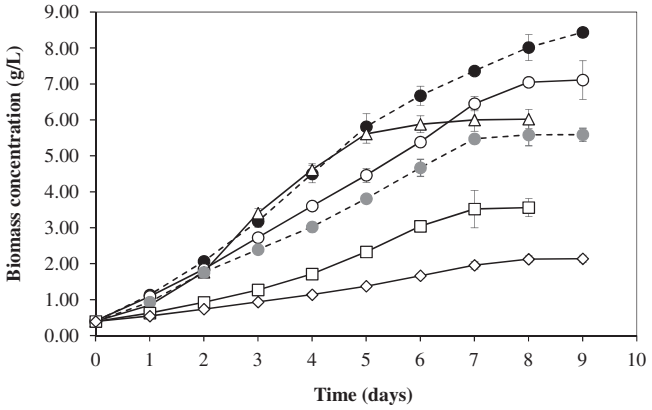

Fig. 1. Biomass concentration during *A. platensis* cultivations performed at PPFD of 70 μmol m^{-2} s^{-1} in different photobioreactor configurations with different S/V ratios: (◇) Open Pond, S/V = 0.25 cm^{-1}; (□) Shaken Flask, S/V = 0.48 cm^{-1}; (○) Horizontal Photobioreactor, S/V = 1.94 cm^{-1}; (△) Helicoidal Photobioreactor, S/V = 3.88 cm^{-1}. Cultivations carried out in the Horizontal Photobioreactor at different PPFD (μmol m^{-2} s^{-1}): (···●···) 40; (···●···) 100.

Table 2
Kinetic parameters of batch *A. platensis* cultures on modified Schlösser medium performed (a) in different photobioreactor configurations at photosynthetic photon flux density (PPFD) of 70 μmol m^{-2} s^{-1} and (b) in horizontal photobioreactor under different PPFD values.

	X_{max} (g L^{-1})[a]	$P_{X\ max}$ (g L^{-1} d^{-1})[b]	μ_{max} (d^{-1})[c]
(a) Photobioreactor			
HoP	7.11 ± 0.53	0.86 ± 0.03	0.988 ± 0.012
HeP	6.02 ± 0.27	1.12 ± 0.04	0.750 ± 0.011
SF	3.56 ± 0.25	0.45 ± 0.07	0.466 ± 0.006
OP	2.14 ± 0.11	0.22 ± 0.01	0.322 ± 0.005
(b) PPFD (μmol m^{-2} s^{-1})			
40	5.59 ± 0.18	0.73 ± 0.02	0.869 ± 0.052
70	7.11 ± 0.53	0.86 ± 0.03	0.988 ± 0.012
100	8.44 ± 0.13	1.08 ± 0.07	1.047 ± 0.006

HoP = Horizontal Photobioreactor; HeP = Helicoidal Photobioreactor; SF = Shaken Flask; OP = Open Pond.

[a] X_{max} = maximum cell concentration.

[b] $P_{X\ max}$ = maximum cell productivity. Values obtained after 7 days, except for runs carried out in HeP at PPFD of 70 μmol m^{-2} s^{-1} (5 days) and HoP at PPFD of 100 μmol m^{-2} s^{-1} (5 days).

[c] μ_{max} = maximum specific growth rate. All values obtained after 1 day.

($P_{X\ max}$ = 1.12 ± 0.04 g L^{-1} d^{-1}). These results confirm that, even though HeP was the best configuration at the beginning of runs, its performance worsened along the time; thus, such conditions may not be the best option for a slow-growth system like this, especially when used in long-term (continuous or fed-batch) operations.

As far as the effect of light intensity is concerned, the results of Table 2 also shows that *A. platensis* growth in the best photobioreactor configuration (HoP) was favored by a progressive increase in PPFD up to 100 μmol m^{-2} s^{-1}, reaching X_{max} = 8.44 ± 0.13 g L^{-1}, $P_{X\ max}$ = 1.08 ± 0.07 g L^{-1} d^{-1} and μ_{max} = 1.047 ± 0.006 day^{-1}. Lower X_{max} values were reported for a two-plane tubular photobioreactor for outdoor culture (4.2 g L^{-1}), elevated panels (5.0 g L^{-1}) and mainly open ponds (0.8 g L^{-1}), while a similar combined airlift-tubular photobioreactor system allowed reaching comparable X_{max} values (6.5-10.6 g L^{-1}) but lower productivity (0.29–0.62 g L^{-1} d^{-1}) under similar irradiation conditions (55 ⩽ PPFD ⩽ 120 μmol m^{-2} s^{-1}) (Converti et al., 2006). These results suggest that light intensity was the growth-limiting factor under the selected conditions, in agreement with previous findings that demonstrated the occurrence of photoinhibition in the same photobioreactor at PPFD as high as 240 μmol m^{-2} s^{-1} (Bezerra et al., 2012). The following bioenergetic study allowed shed light on these observations.

4.2. Bioenergetic parameters of A. platensis growth

Consistently with Westerhoff and Van Dam (1987), according to whom a high rate of Gibbs energy dissipation is related to high metabolic rates but low biomass yield, Bezerra et al. (2012) observed that the Gibbs energy dissipation for cell growth and maintenance ($1/Y_{GX}$) progressively increased in fed-batch *A. platensis* cultures as the likely result of the increased biomass level. The results of Fig. 2, which illustrates the decrease along the time of the reciprocal of this parameter (Y_{GX}), i.e. the biomass yield on Gibbs energy, confirm the suspicion of those authors that the increase in the energy requirements observed when the system approached the stationary growth phase may have been related to a dramatic decrease in the specific growth rate, owing to the difficulty in synthesizing biomass from given carbon and energy sources (Heijnen, 1994), rather than to biomass decay.

A comparison among the different photobioreactor configurations (Fig. 2A) reveals that the best-performing one (HoP) allowed delaying the quick fall of Y_{GX} almost to zero by several days with respect to SF and HeP. It is also noteworthy that this occurrence always took place when μ was less than 0.01 day^{-1} (results not shown). As suggested by Bezerra et al. (2012), under these stress conditions the growth is so strongly affected that most of the Gibbs energy is addressed to cell maintenance. In addition, it should be noticed that, in OP and SF, Y_{GX} was at the beginning of the runs about 23 and 14% lower than in HoP (0.863 C-mmol kJ^{-1}), which means that biomass production required more energy, thus resulting in poorer performances. On the other hand, the helicoidal configuration, which at the beginning showed comparable performance to HoP because of its higher S/V ratio (3.88 cm^{-1}), after about 4 days suffered a quick worsening likely due to excess mechanical stress. As earlier mentioned, a progressive increase in

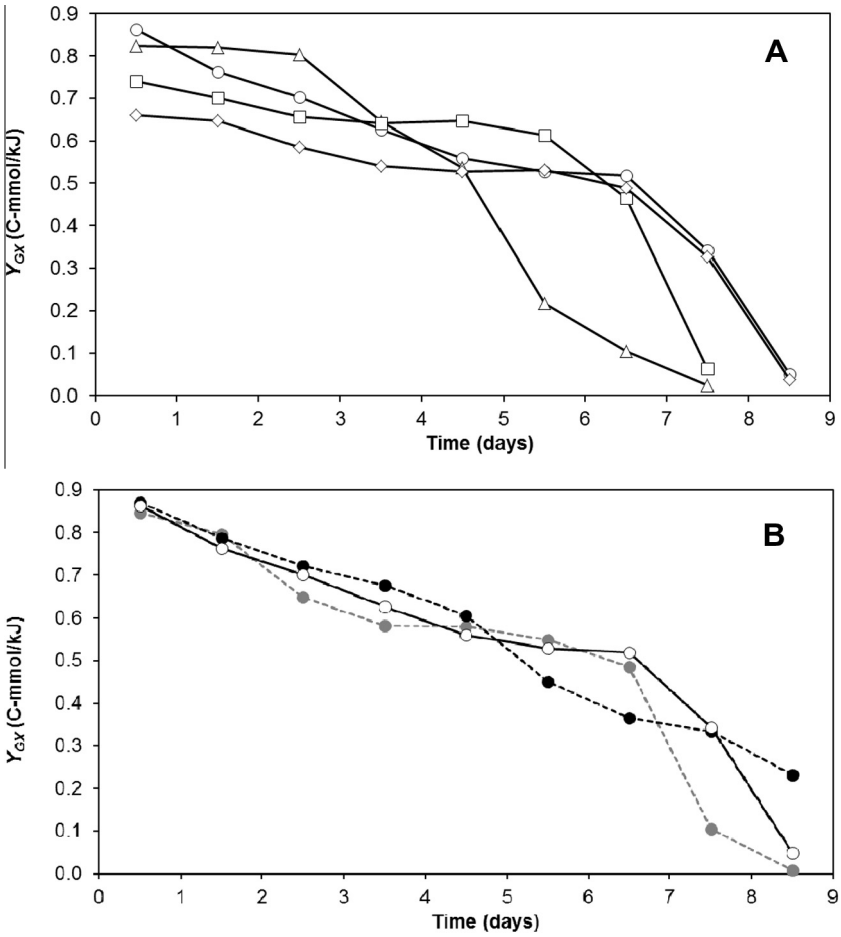

Fig. 2. Time behavior of biomass yield on Gibbs energy (Y_{GX}) during *A. platensis* cultivations performed: (A) at PPFD of 70 μmol m^{-2} s^{-1} in: (◇) Open Pond, S/V = 0.25 cm^{-1}; (□) Shaken Flask, S/V = 0.48 cm^{-1}; (○) Horizontal Photobioreactor, S/V = 1.94 cm^{-1}; (△) Helicoidal Photobioreactor, S/V = 3.88 cm^{-1}. (B) in the Horizontal Photobioreactor varying PPFD (μmol m^{-2} s^{-1}): (···●···) 40; (—○—) 70; (···●···) 100.

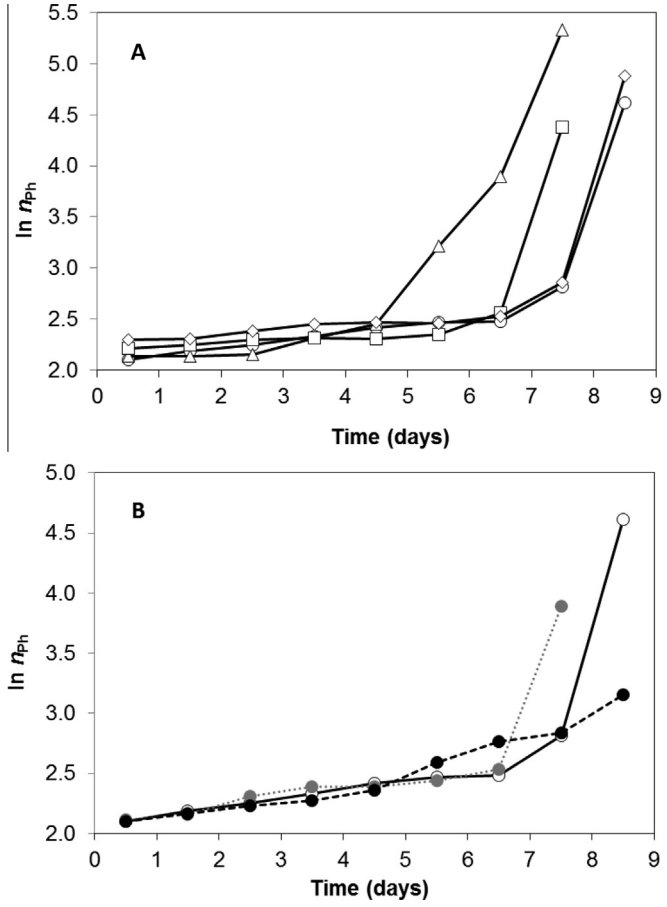

Fig. 3. Semi-log plot of the moles of photons required for the synthesis of 1 C-mol of *A. platensis* biomass (n_{Ph}, expressed in mol C-mol^{-1}) versus time during cultivations performed: (A) at PPFD of 70 μmol m^{-2} s^{-1} in: (◇) Open Pond, S/V = 0.25 cm^{-1}; (□) Shaken Flask, S/V = 0.48 cm^{-1}; (○) Horizontal Photobioreactor, S/V = 1.94 cm^{-1}; (△) Helicoidal Photobioreactor, S/V = 3.88 cm^{-1}. (B) in the Horizontal Photobioreactor varying PPFD (μmol m^{-2} s^{-1}): (···●···) 40; (—○—) 70; (···●···) 100.

light intensity in HoP favored cell growth because of photolimitation in the tested PPFD range; as a result, Y_{GX} fell sharply only after 6.5 days in runs carried out at PPFD of 40 and 70 μmol m^{-2} s^{-1}, while it decreased slightly even after 8.5 days at 100 μmol m^{-2} s^{-1} (Fig. 2B).

As shown in Fig. 3A, the moles of photons absorbed to produce one C-mol of biomass (n_{Ph}) progressively increased (in absolute value) throughout all the cultivations, just reflecting the decrease in Y_{GX} illustrated in Fig. 2. As expected, at the beginning of runs where the microorganism grew at the highest μ, this bioenergetic parameter approached values very close to that reported to sustain growth under ideal conditions, i.e. 8 mol of photons C-mol^{-1} ($\ln n_{Ph}$ = 2.08) of biomass (Richmond, 1983); the release of 1 mol of O_2 by the photosynthesis does in fact imply the absorption of at least 8 mol of photons and the fixation of 1 mol of CO_2 to produce 1 C-mol of carbohydrate (Osborne and Geider, 1987). Also in terms of such a bioenergetic parameter, HoP was shown to be by far the best photobioreactor configuration, requiring only 8.2–11.9 mol of photons ($\ln n_{Ph}$ = 2.10–2.48) for the growth of 1 C-mol of biomass up to 6.5 days. As already observed in previous works carried out under fed-batch conditions (Bezerra et al., 2012; Sassano et al., 2004), the additional energy requirement with respect to the above ideal situation was negligible at the beginning, but considerably increased only during the stationary phase (about 100 mol of photons C-mol^{-1} after 7.5 days; $\ln n_{Ph}$ = 4.60) indicating that most of the energy was lost. On the contrary, OP and SF were the less performing configurations within the first 6.5 days, requiring 9.1–13.0 mol of photons C-mol^{-1} ($\ln n_{Ph}$ = 2.21–2.56), which suggests that, under stress conditions like these, biomass used energy of absorbed photons preferably for maintenance rather than for growth. Disappointingly, HeP, after 4 days during which exhibited the best performance, started to have the highest photon requirement.

Confirming the observations made for Y_{GX}, a progressive increase in light intensity did not exert any appreciable influence on n_{Ph} up to 6.5 days, but had a remarkable effect on the time at which this parameter suddenly increased (7, 8 and 9 days for PPFD of 40, 70 and 100 μmol m^{-2} s^{-1}, respectively) (Fig. 3B). However, it is noteworthy that, irrespective of light conditions and configurations, the additional photons with respect to the theoretical requirement (8 mol of photons C-mol^{-1}) were partly utilized to reduce nitrate to ammonia (Hatori and Myers, 1966), which is the preferential form of nitrogen assimilated by photosynthetic microorganisms (Boussiba et al., 1984; Torre et al., 2003), and that such a reduction was accompanied by the oxidation of a portion of NADPH produced by the photosynthesis (Bruinenberg et al., 1983).

Fig. 4 shows the time behaviors of the rates of O_2 development and H^+ consumption during cultivations performed at PPFD of 70 μmol m^{-2} s^{-1} in the four photobioreactor configurations tested in this study (panel A), or progressively increasing PPFD from 40 to 100 μmol m^{-2} s^{-1} in the sole HoP (panel B). These trends clearly show that, as biomass is the main product of photosynthetic

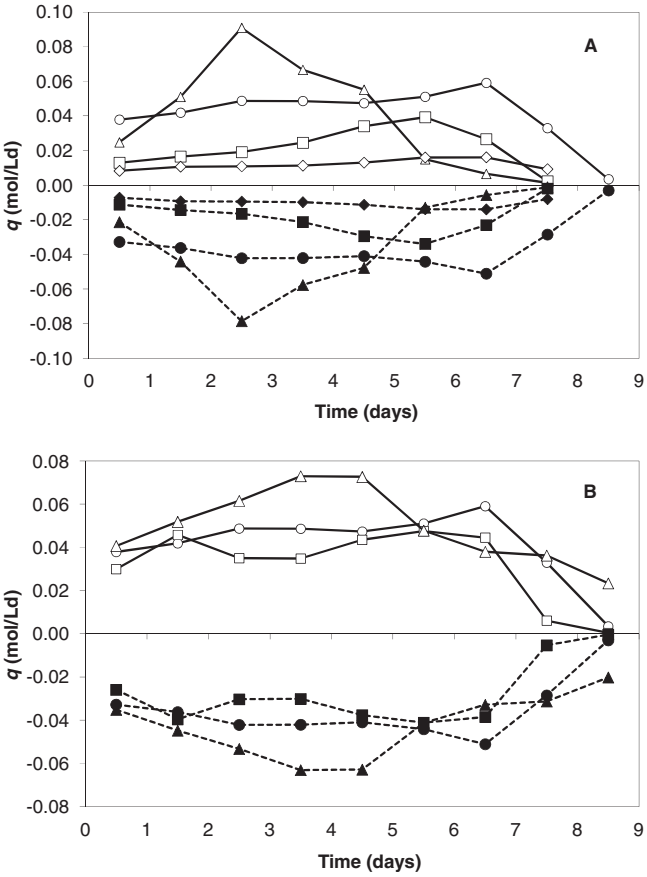

Fig. 4. Time behaviors of the rates of O_2 development (empty symbols and solid lines) and H^+ consumption (filled symbols and dotted lines) during *A. platensis* cultivations performed: (A) at PPFD of 70 μmol m^{-2} s^{-1} in: (◇) Open Pond, S/V = 0.25 cm^{-1}; (□) Shaken Flask, S/V = 0.48 cm^{-1}; (○) Horizontal Photobioreactor, S/V = 1.94 cm^{-1}; (△) Helicoidal Photobioreactor, S/V = 3.88 cm^{-1}. (B) in the Horizontal Photobioreactor varying PPFD (μmol m^{-2} s^{-1}): (□) 40; (○) 70; (△) 100.

metabolism, both rates faithfully followed cell growth, reaching maximum values in the late log phase, with exception of HeP where the maxima took place in the early log phase owing to the above-mentioned performance worsening along the time. Thus, consistently with the growth curves already illustrated in Fig. 1, the poorest O_2 release and H^+ consumption occurred in OP, while the highest rates were observed in HeP at the start or in HoP at the end of runs (Fig. 4A). As expected, both rates progressively increased with PPFD in HoP (Fig. 4B), hence confirming that the growth was light-limited and the system never suffered photoinhibition under the conditions tested in this work. However, an effect masked up to now was the speed up of both activities, which reached their maximum rates at PPFD of 100 μmol m^{-2} s^{-1} about 2.5 days before than at 40–70 μmol m^{-2} s^{-1}.

4.3. Thermodynamic parameters of A. platensis growth

As known, a portion of the Gibbs energy carried by the photons absorbed in PSII is used to transfer electrons to PSI and to create a proton-motive force across the plasma membrane, thus providing energy for ATP synthesis. In all the photobioreactor configurations, the highest values of the thermodynamic parameters taken into account in this estimation, i.e. the enthalpic content (ΔH), the energy recovered as ATP (ΔG_{ATP}) and that released as heat (Q), whose sum equals the total Gibbs energy effectively fixed by the photosynthesis (ΔG_a), were obtained in the stationary phase of growth (Table 3).

This means that, under these conditions, cell growth was so impaired that the synthesis of one C-mol of biomass required much more Gibbs energy than in the other growth phases, and this occurrence was less marked in HoP, likely because of its favorable configuration and less turbulent conditions. Reynolds numbers (N_{Re}) in the ranges 600–2500 and 10–600 were in fact estimated in a similar air-lift tubular photobioreactor at biomass concentrations around 0.05 and 10 g L^{-1}, respectively (Converti et al., 2006), while Carlozzi and Torzillo (1996) warned that a turbulence excess with respect to optimal turbulence regime ($2680 < N_{Re} < 4000$) is deleterious for the culture.

To make comparisons possible, times 0.5, 3.5 and 7.5 days were selected as representative of the mid lag phase, mid log phase and early stationary phase of growth, respectively. As in the modified medium of Schlösser (1982) used in this study all the nutrients were present in excess, the stress conditions suffered by the cyanobacterium in the stationary growth phase were the likely result of a shading effect depending on light availability to the system rather than of shortage of some nutrient, as it often happens in batch heterotrophic cultures.

As previously suggested (Bezerra et al., 2012), exposition of the system to excess light intensity means that a progressively more significant portion of ΔG_a is dissipated via a mechanism called Non-Photochemical Quenching (NPQ) (Mozzo et al., 2008) to protect the photosynthetic apparatus against photodestruction (Heber et al., 2006; Karapetyan, 2008). However, such a situation was likely not to take place in HoP, where an increase in PPFD from 40 to 70 μmol m^{-2} s^{-1} reduced the energy lost as heat, while a further PPDF increase did not lead to any significant variation in the thermodynamic parameters (Table 3). The system exhibited practically the same behavior in HoP up to the achievement of the stationary growth phase, which was fully established after 7 and 8 days at PPFD of 40 and 70 μmol m^{-2} s^{-1}, respectively, but no less than 9 days at 100 μmol m^{-2} s^{-1} (Fig. 1). These results as a whole confirm that in the PPFD range tested in this study, despite of a variation in the overall Gibb energy availability that influenced the duration of cultures, the system kept under photolimited conditions. Such a situation is consistent with that observed in fed-batch *A. platensis* cultures, where the highest values of the same thermodynamic parameters were observed at PPFD of 120 μmol m^{-2} s^{-1} and kept almost unvaried up to 240 μmol m^{-2} s^{-1}, because of light saturation occurrence (Bezerra et al., 2012).

Fig. 5A shows the time behavior of the percentages of absorbed light stored by the system as ATP (η_{ATP}) and fixed as enthalpy (η_H) in the different photobioreactor configurations at PPFD of 70 μmol m^{-2} s^{-1}, while Fig. 5B that of the same percentages in HoP at increasing PPFD. One can see that, in all the runs, both η_{ATP} and η_H decreased along the time, confirming the increasing difficulty of biomass to grow, and fell down after the achievement of the stationary growth phase. Whereas such decreases were almost regular for HoP, SF and OP, with higher values of both parameters in HoP ($6.4 \leqslant \eta_{ATP} \leqslant 13.2\%$; $13.8 \leqslant \eta_H \leqslant 28.2\%$), once more HeP exhibited the best performance at the beginning of the run up to 3.5 days ($10.7 \leqslant \eta_{ATP} \leqslant 12.8\%$; $22.9 \leqslant \eta_H \leqslant 27.3\%$), but behaved unsatisfactorily ($\eta_{ATP} = 0.52\%$ and $\eta_H = 1.12\%$) at the early stationary phase.

On the other hand, a progressive increase in light intensity did not imply in HoP any significant variation in these percentages up to the achievement of the early stationary phase (Fig. 5B), but, as earlier mentioned, it led to a simple delay, after which they fell down. As a matter of fact, whereas at PPFD of 40 μmol m^{-2} s^{-1} η_{ATP} fell to 2.2% after only 7.5 days, at higher PPFD this fall was less marked and was likely to occur after longer time (results not shown). These results taken together are consistent with photolimitation before the early stationary growth phase and a light-modulated shading effect after its appearance. Based on the proposed model, the percentage of absorbed Gibbs energy released as heat (η_Q) (results not shown) is the complement of η_{ATP} and η_H to 100%, which means that, consistently with the well-known low photosynthetic efficiency of every photosynthetic organisms, during all the cultivations most of the absorbed energy was

Table 3
Main thermodynamic parameters of *A. platensis* growth in different photobioreactor configurations and under different PPFD values.

	ΔG_{ATP} (kJ C-mol^{-1})			ΔH (kJ C-mol^{-1})			Q (kJ C-mol^{-1})		
Time (d)	0.5	3.5	7.5	0.5	3.5	7.5	0.5	3.5	7.5
(a) Cultures in different photobioreactors at PPFD of 70 μmol m^{-2} s^{-1}									
HoP	228	287	466	244	308	499	1218	1534	2488
HeP	235	281	5749	252	301	6161	1257	1500	30703
SF	254	282	2219	272	302	2378	1357	1505	11852
OP	276	321	485	296	344	519	1475	1716	2588
(b) Cultures in HoP at different light intensities (PPFD, μmol m^{-2} s^{-1})									
40	231	303	1358	248	325	1455	1235	1621	7251
70	228	287	466	244	308	499	1218	1534	2488
100	227	271	475	243	291	509	1210	1448	2538

HoP = Horizontal Photobioreactor; HeP = Helicoidal Photobioreactor; SF = Shaken Flask; OP = Open Pond; ΔG_{ATP} = Gibbs energy recovered as ATP; ΔH = enthalpy variation; Q = energy released as heat. Times 0.5, 3.5 and 7.5 days were selected as representative of the mid lag phase, mid log phase and early stationary phase of growth, respectively.

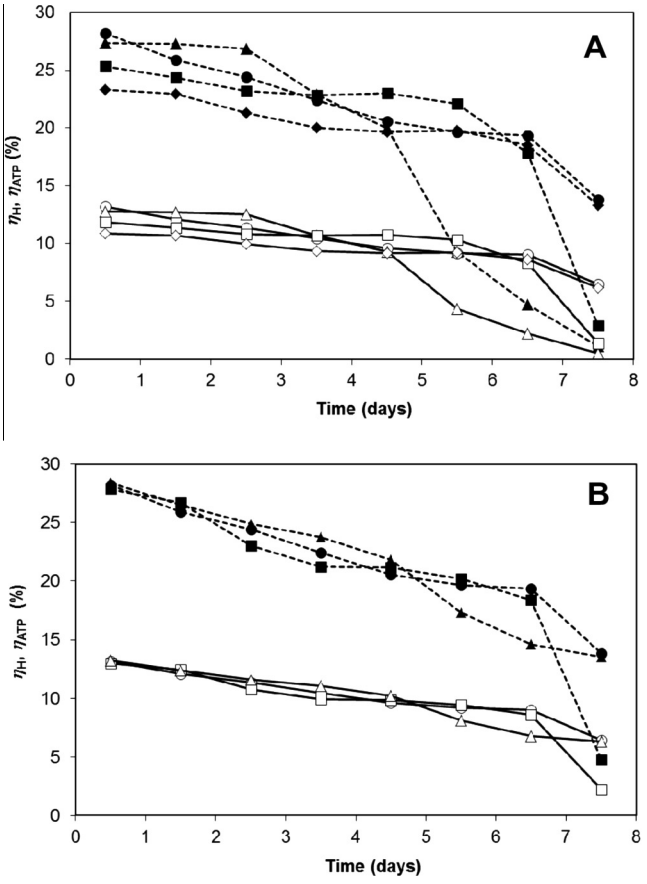

Fig. 5. Fractions of the absorbed light energy stored as ATP (η_{ATP}) (empty symbols and solid lines) and used to increase the enthalpic content of the system (η_H) (filled symbols and dotted lines) during *A. platensis* cultivations performed: (A) at PPFD of 70 μmol m^{-2} s^{-1} in: (◇) Open Pond, S/V = 0.25 cm^{-1}; (□) Shaken Flask, S/V = 0.48 cm^{-1}; (○) Horizontal Photobioreactor, S/V = 1.94 cm^{-1}; (△) Helicoidal Photobioreactor, S/V = 3.88 cm^{-1}. (B) in the Horizontal Photobioreactor varying PPFD (μmol m^{-2} s^{-1}): (□) 40; (○) 70; (△) 100.

released as heat, being this fraction much higher at the early stationary phase ($79.7 \leqslant \eta_Q \leqslant 93.0\%$) than at the mid lag phase ($58.4 \leqslant \eta_Q \leqslant 59.2\%$).

5. Conclusions

Kinetic, bioenergetic and thermodynamic parameters of batch *A. platensis* cultures were influenced by photobioreactor configuration, surface/volume ratio (S/V) and light intensity. The photobioreactor configuration had a strong impact on biomass concentration, in that its maximum value remarkably increased when S/V was raised. However, the helicoidal photobioreactor showed the best performance only at the beginning of runs because of its highest S/V ratio, but subsequently it behaved poorly owing to a time-depending mechanical stress. The horizontal photobioreactor, where the growth was favored by a progressive irradiance increase, proved to possess the best configuration and showed the best bioenergetic and thermodynamic parameters.

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ANEXO D – Publicações desenvolvidas durante o doutorado

- Resumo publicado em congresso
- Trabalhos completos aceitos para publicação em congresso
- Artigo publicado na revista *Biotechnology Progress*

XLIII Annual Meeting of SBBq

Sociedade Brasileira de Bioquímica e Biologia Molecular — SBBq

Foz do Iguaçu, PR, Brazil, May 17th to 20th, 2014

Influence of light intensity on the growth of microalgae *Chlorella vulgaris*

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Microalgae are ecologically important and have been used in food and medicines for years. Several strategies have been applied to improve microalgae growth, pigment and biochemical composition. These include light intensity, one of the most important parameters affecting algal growth. The aim of this study was to evaluate the influence of light intensity on the growth of microalgae *Chlorella vulgaris*. The *C. vulgaris*UTEX 1803 was originally obtained from the Culture Collection of Algae at the University of Texas (Austin, TX, USA). The microalgae was grownaseptically in Erlenmeyer flasks of 500 ml containing 200 ml of culture medium standard Bold's Basal, constantly aerated with an air pump, initial cell concentration of 50 mg.L^{-1}at two different light intensities (30 and 70 µmol m^{-2} s^{-1}) at 27 ± 1 °Ccultivation temperature. At the end of the exponential phase, cells were centrifuged. All assays were performed in duplicate. Maximum biomass concentration (Xmax, g.L^{-1}) and maximum productivity (Pmax, mg.L^{-}1.dia^{-1}) were evaluated. The results demonstrate that there is an influence of light intensity in relation to the parameters analysed, biomass concentration and cell productivity. It was observed that at the light intensity assays of 30 and 70 µmol fótons m^{-2} s^{-1}, the maximum biomass concentration was 0.57 g.L^{-1}after 15 days of cultivation and 0.70 g.L^{-1}after 9 days, respectively. The maximum cell productivity (72.01 mg.L^{-}1.day^{-1}) occurred in the light intensity of 70 µmol fótons m^{-2} s^{-1}, which also showed a higher biomass concentration. Increasing light intensity favoured the growth of microalgae in less cultivation time, reaching higher cell concentrations and productivities. Thus, the results obtained in this work show that for the cultivation of *Chlorella vulgaris* the light intensity studied (70 µmol fótons m^{-2} s^{-1}) is a viable alternative to reduce the cost of the production of microalgal biomass.

Keywords: microalge, light intensity, biomass.
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INFLUENCE OF PHOTOBIOREACTOR CONFIGURATION ON THE BATCH CULTIVATION OF Arthrospira (Spirulina) Platensis: BIOENERGETIC AND THERMODYNAMICS ASPECTS

autoria de

Milena Fernandes da Silva, Patrizia Perego, Raquel Pedrosa Bezerra, Attilio Converti, Ana Lúcia Figueiredo Porto

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INFLUENCE OF PHOTOBIOREACTOR CONFIGURATION ON THE BATCH CULTIVATION OF *Arthrospira (Spirulina) Platensis*: BIOENERGETIC AND THERMODYNAMICS ASPECTS

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RESUMO – *Bioenergetics when combined with the fundamental principles of thermodynamics proved to be a highly useful tool for the analysis of the biological systems. The objective of this study was to investigate the main bioenergetic and thermodynamic parameters of the batch culture of Arthrospira (Spirulina) platensis at light intensity of 70 µmol m^{-2} s^{-1} in four photobioreactor configurations, namely horizontal photobioreactor (HoP), helicoidal photobioreactor (HeP), shaken flasks (SF) and open pond (OP). The photobioreactor configuration had a strong impact on biomass concentration, in that its maximum value (X_{max}) remarkably increased from 2.14 ± 0.11 g L^{-1} to 3.56 ± 0.25 g L^{-1} and 7.11 ± 0.53 g L^{-1} when the surface (S) to volume (V) ratio was increased from 0.25 cm^{-1} (OP) to 0.48 cm^{-1} (SF) and 1.94 cm^{-1} (HoP), respectively. However, the HeP showed the best performance only at the beginning of runs because of its highest S/V ratio (3.88 cm^{-1}) resulting in better growth up to 4 days, but subsequently it behaved poorly, with a X_{max} value about 15% lower (6.02 ± 0.27 g L^{-1}), owing to a excess mechanical stress to cells. The best energy yields values on Gibbs energy necessary for cell growth and maintenance were achieved in up to 6.5 days of cultivation in HoP, indicating that it would be the optimum condition to maintain cell growth. This work demonstrate the potential HoP as a promising process for A. platensis cultures to obtain high growth yields.*

1. INTRODUCTION

Bioenergetics when combined with the fundamental principles of thermodynamics, it has proven to be a highly useful tool for the analysis of biosystems (Küçük *et al.*, 2015). Photosynthetic microorganisms have recently gained huge attention worldwide (Cheah *et al.*, 2015), since they are one of the most promising renewable and neutral energy sources, i.e., by consuming carbon dioxide, their cultivation has the additional benefit of combining valuable biomass production with CO_2 emissions reduction (Belay, 2002).

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Among the photosynthetic microorganisms with commercial importance, is the filamentous cyanobacterium (blue-green alga) *Arthrospira (Spirulina) platensis* whose production is increasing worldwide due to its high contents of highly-valuable proteins, amino acids, essential fatty acids (i.e., γ-linolenic acid, GLA), polysaccharides, vitamins and pigments (β-carotene, chlorophyll *a* and phycocyanin) which find application in several industrial segments like those of health foods and therapeutics (Belay, 2002; Pulz and Gross, 2004; Raposo and Morais, 2015; Benelhadj *et al.*, 2016).

A lot of parameters can be tuned to obtain high biomass yield, among which is the reactor configuration, that can strongly impact on the performance of *A. platensis* cultivation. The reactor configuration can greatly influencing on cell growth. Photobioreactors can reduce the cultivation area by a vertical distribution of the photosynthetic organism and enlarge the surface exposed to light, ensuring high surface/volume ratios, thereby increasing cell concentration. Light is better captured by cells in tubular photobioreactors when compared to the conventional open ponds, where, owing to a relatively high depth, it has to go through thick layers to reach the inner cells (Converti *et al.*, 2006; Rodrigues *et al.*, 2010). Although the open pond is the most widely used configuration for *A. platensis* commercial production, tubular photobioreactors have been deeply studied, not only because of their high cell productivity, but also of many other advantages, such as low levels of contamination and better CO_2 solubilization in the medium, better light distribution and then higher photosynthetic efficiency (Converti *et al.*, 2006).

Bioenergetic studies based on the Gibbs energy dissipation may be applied to describe or predict the microbial growth yield, the energy flow to ATP production, the increase in enthalpic content and the heat released by living organisms (Bezerra *et al.*, 2012; Sassano *et al.*, 2004; Torre *et al.*, 2003). All of them are quite important to optimize any bioprocess and even to design the most suitable bioreactor to perform it; but, unfortunately, only a few studies dealt with the bioenergetic aspects of the growth of photosynthetic microorganisms based on Gibbs energy balances. In particular, biomass yield constitutes one of the key parameters in any bioprocess or experiment involving microbial cultures, since it determines the final biomass concentration, which must be maximized to obtain high productivities (Von Stockar *et al.*, 2006). In addition, the Gibbs energy dissipation per C-mol of biomass can be regarded as a simple thermodynamic measure of the amount of biochemical "work" required to convert the carbon source into biomass. Only a few studies emphasized the bioenergetic aspects of the growth of photosynthetic microorganisms based on Gibbs energy balances (Bezerra *et al.*, 2012; Heijnen and Van Dijken, 1991; Liu *et al.*, 2007).

Based on this background, and because of a certain lack of detailed and recent theoretical studies on the mechanisms ruling *A. platensis* cultures, the objective of this study was to investigate the influence of photobioreactor configuration in the bioenergetics and thermodynamics of batch culture of *Arthrospira (Spirulina) platensis*. For this purpose, the main bioenergetic and thermodynamic parameters were evaluated at light intensity of 70 μmol m^{-2} s^{-1} in four photobioreactor configurations, namely horizontal photobioreactor (HoP), helicoidal photobioreactor (HeP), shaken flasks (SF) and open pond (OP).

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2. MATERIAL AND METHODS

2.1. *Microorganism and culture conditions*

Arthrospira (Spirulina) platensis UTEX 1926 was obtained from the Culture Collection of Algae of the University of Texas (Austin, TX, USA). To allow a large growth of biomass, the microorganism was maintained and cultivated in the culture medium suggested by Schlösser (1982) modified so as to have a nitrogen concentration equal to about four fold that of the original medium. The resulting medium had the following composition (per liter): 13.61 g NaHCO3, 4.03 g Na_2CO_3, 0.50 g K2HPO4, 10.0 g $NaNO_3$, 1.00 g K_2SO_4, 1.00 g NaCl, 0.20 g $MgSO_4 \cdot 7\ H_2O$, 0.04 g $CaCl_2 \cdot 2\ H_2O$. All the nutrients were dissolved in distilled water containing (per liter): 6.0 mL of metal solution (97 mg L-1 $FeCl_3 \cdot 6\ H_2O$, 41 mg L-1 $MnCl_2 \cdot 4\ H_2O$, 5 mg L-1 $ZnCl_2$, 2 mg L-1 $CoCl_2 \cdot 6\ H_2O$, 4 mg L-1 $Na_2MoO_4 \cdot 2\ H_2O$, 750 mg L-1 $Na_2EDTA \cdot 2\ H_2O$), 1.0 mL of micronutrient solution (50.0 mg L-1 Na_2EDTA, 618 mg L-1 H_3BO_3, 19.6 mg L-1 $CuSO_4 \cdot 5\ H_2O$, 44.0 mg L-1 $ZnSO_4 \cdot 7\ H_2O$, 20.0 mg L- 1 $CoCl_2 \cdot 6\ H_2O$, 12.6 mg L-1 $MnCl_2 \cdot 4\ H_2O$, 12.6 mg L-1 $Na_2MoO_4 \cdot 2\ H_2O$) and 0.15 mg L-1 of B_{12} vitamin.

Cells were grown batch-wise at light intensity of 70 μmol m^{-2} s^{-1} in different photobioreactor configurations, namely open pond (OP) (Sassano et al., 2004) S/V = 0.25 cm^{-1}, shaken flask (SF) (S/V = 0.48 cm^{-1}) (Frumento *et al.*, 2016), horizontal photobioreactor (HoP) (Ferreira *et al.*, 2010) (S/V = 1.94 cm^{-1}) and helicoidal photobioreactor (HeP) (Bezerra *et al.*, 2011; Frumento *et al.*, 2016) (S/V = 3.88 cm^{-1}). For this purpose, fluorescent artificial light was ensured by a variable number of 36 W-lamps. The cultivations were carried out at temperature of 30 ± 2 °C, using an initial biomass concentration of 0.40 g L^{-1}. The pH was controlled at 9.5 ± 0.2 through the daily addition of pure CO_2. After growth, once the stationary phase had been reached, about 9 days of cultivation, biomass was separated from culture medium by centrifugation (centrifuge ALC 42426, Milan, Italy) at 7500 rpm for 10 min. Recovered cells were washed twice with distilled water, dried at 105 °C for 24 h, pulverized in a mortar and stored at −20 °C for subsequent analysis of its elemental composition.

2.2. *Analytical procedures*

Cell concentration of *A. platensis* was determined daily by measuring the optical density (OD) at 560 nm by an UV-visible spectrophotometer, model Lambda 25 (PerkinElmer, Milan, Italy). All measurements were done in triplicate, and biomass concentration, expressed in grams of dried biomass per litre of medium (g L^{-1}) through a calibration curve relating OD to dry biomass weight.

The elemental composition of dried biomass stored at the end of culture was determined by an elemental analyzer Flash EA1112 series (CE Instruments, Wigan, UK). Since biomass composition varied very little among the different photobioreactor configurations and light intensities, its average value obtained from all the runs ($CH_{1.59}O_{0.50}N_{0.10}$) was used to estimate the bioenergetic and thermodynamic parameters of growth. Light intensity at the culture surface was measured using a type sensor quantum/photometer/radiometer, model HD-9021 Delta OHM (Li-Cor Inc., Lincoln NE, USA).

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2.3. Bioenergetic and thermodynamic parameters of *A. platensis* growth

To estimate the bioenergetic and thermodynamic parameters of *A. platensis* growth under batch conditions, we applied the model proposed by Torre *et al.* (2003) and Bezerra *et al.* (2012).

3. RESULTS AND DISCUSSION

The photobioreactor configuration was strongly influenced by the biomass concentration, in that its maximum value (X_{max}) remarkably increased from 2.14 ± 0.11 g L^{-1} to 3.56 ± 0.25 g L^{-1} and 7.11 ± 0.53 g L^{-1} when the surface (S) to volume (V) ratio was increased from 0.25 cm^{-1} (OP) to 0.48 cm^{-1} (SF) and 1.94 cm^{-1} (HoP), respectively (Figure 1).

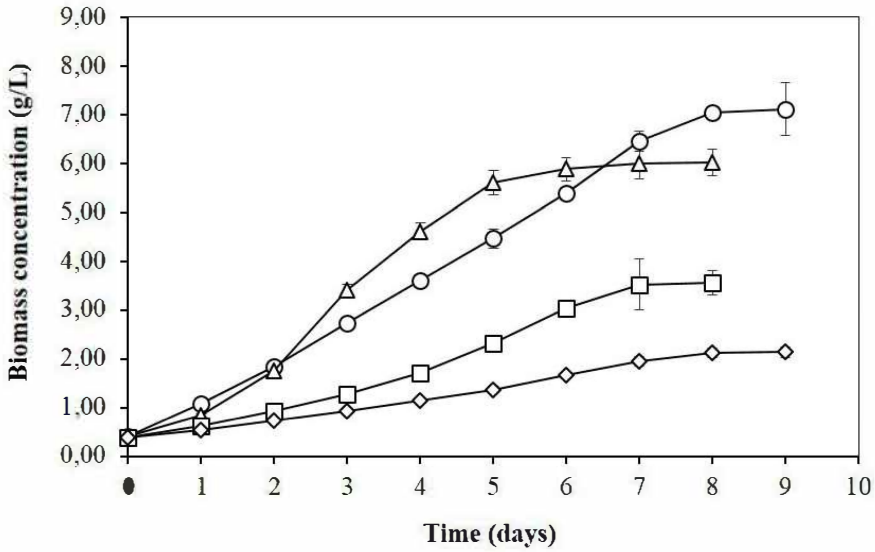

Figure 1 – Biomass concentration during *A. platensis* cultivations performed at light intensity of 70 µmol m^{-2} s^{-1} in different photobioreactor configurations with different S/V ratios: (□) Open Pond, S/V = 0.25 cm^{-1}; (□) Shaken Flask, S/V = 0.48 cm^{-1}; (△) Helicoidal photobioreactor, S/V = 3.88 cm^{-1}; (□) Horizontal photobioreactor, S/V = 1.94 cm^{-1}.

Such a behavior may be ascribed to a better light distribution in HoP along with the occurrence of the well-known shading effect of outer cell layers on the inner ones in systems with insufficient exposition to light (SF and OP), which resulted in reduced light availably to cells (Danesi *et al.*, 2004). A separate reasoning should be applied to HeP, which, despite a S/V ratio that was twice that of HoP, offered unexpectedly a sigmoidal growth curve, with better performance during the log phase but a Xmax value about 15% lower (6.02 ± 0.27 g L^{-1}). Such an observation could be explained with a combination of different positive and negative factors in HeP compared with HoP, among which the most important may have been the higher S/V ratio, resulting in better growth up to 4 days,

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and a more intense agitation, leading to excess mechanical stress to cells responsible for a lower X_{max} value. These results confirm that the HoP was the best photobioreactor configuration, even though HeP have been the best configuration at the beginning of runs, but its performance worsened along the time; thus, such conditions may not be the best option for a slow-growth system like this, especially when used in long-term (continuous or fed-batch) operations. The following bioenergetic study allowed shed light on these observations.

The Figure 2 illustrates the time behavior of biomass yield on Gibbs energy (Y_{GX}) during *A. platensis* cultivations performed. A comparison among the different photobioreactor configurations reveals that the best-performing one (HoP) allowed delaying the quick fall of Y_{GX} almost to zero by several days with respect to SF and HeP, respectively.

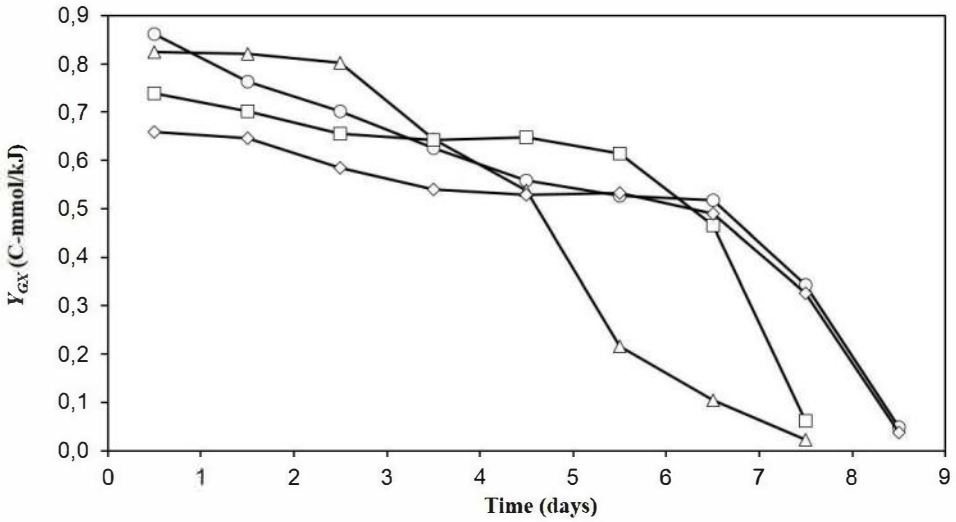

Figure 2 – Time behavior of biomass yield on Gibbs energy (Y_{GX}) during *A. platensis* cultivations performed: A) at light intensity of 70 μmol m^{-2} s^{-1} in: (□) Open Pond, S/V = 0.25 cm^{-1}; (□) Shaken Flask, S/V = 0.48 cm^{-1}; (△) Helicoidal Photobioreactor, S/V = 3.88 cm^{-1}; (□) Horizontal Photobioreactor, S/V = 1.94 cm^{-1}.

As suggested by Bezerra *et al.* (2012), under these stress conditions the growth is so strongly affected that most of the Gibbs energy is addressed to cell maintenance. In addition, it should be noticed that, in OP and SF, Y_{GX} was at the beginning of the runs about 23 and 14% lower than in HoP (0.863 C-mmol kJ^{-1}), which means that biomass production required more energy, thus resulting in poorer performances. On the other hand, the helicoidal configuration, which at the beginning showed better performance than HoP because of its higher S/V ratio (3.88 cm^{-1}), after about 4 days suffered a quick worsening likely due to excess mechanical stress.

As shown in Figure 3, the moles of photons absorbed to produce one C-mol of biomass (n_{Ph}) progressively increased (in absolute value) throughout all the cultivations, just reflecting the decrease in Y_{GX} illustrated in Figure 2. The beginning of runs where the microorganism grew at the highest μ, this bioenergetic parameter approached values very close to that reported to sustain growth under

ideal conditions, i.e. 8 moles of photons C-mol^{-1} ($\ln n_{Ph}$ = 2.08) of biomass (Richmond, 1983); the release of 1 mol of O_2 by the photosynthesis does in fact imply the absorption of at least 8 moles of photons and the fixation of 1 mol of CO_2 to produce 1 C-mol of carbohydrate (Osborne and Geider, 1987).

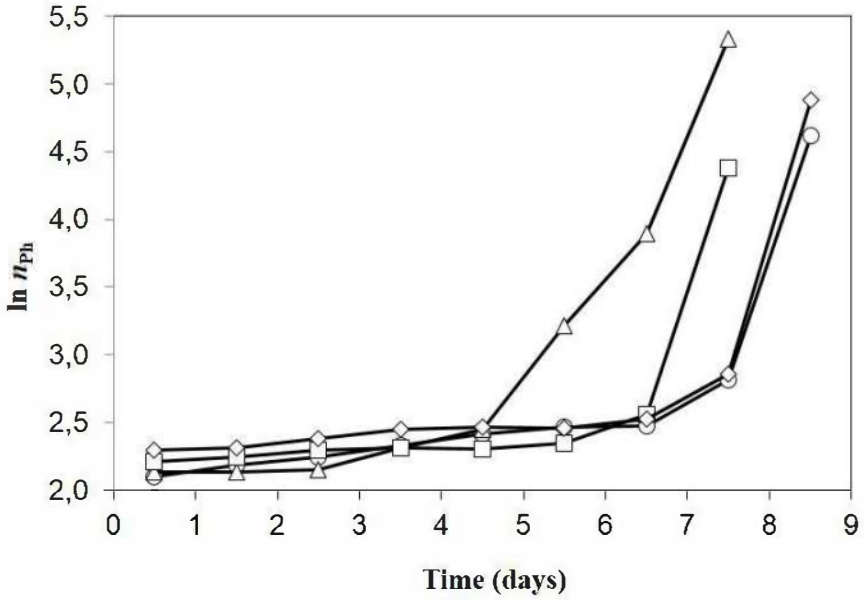

Figure 3 – Semi-log plot of the moles of photons required for the synthesis of 1 C-mol of *A. platensis* biomass (n_{Ph}, expressed in mol C-mol^{-1}) versus time during cultivations performed at light intensity of 70 µmol m^{-2} s^{-1} in: (◇) Open Pond, S/V = 0.25 cm^{-1}; (□) Shaken Flask, S/V = 0.48 cm^{-1}; (△) Helicoidal Photobioreactor, S/V = 3.88 cm^{-1}; (○) Horizontal Photobioreactor, S/V = 1.94 cm^{-1}.

Also in terms of such a bioenergetic parameter, HoP was shown to be by far the best photobioreactor configuration, requiring only 8.2-11.9 moles of photons ($\ln n_{Ph}$ = 2.10-2.48) for the growth of 1 C-mol of biomass up to 6.5 days. As already observed in previous works carried out under fed-batch conditions (Bezerra *et al.*, 2012; Sassano *et al.*, 2004), the additional energy requirement with respect to the above ideal situation was negligible at the beginning, but considerably increased only during the stationary phase (about 100 moles of photons C-mol^{-1} after 7.5 days; $\ln n_{Ph}$ = 4.60) indicating that most of the energy was lost. On the contrary, OP and SF were the less performing configurations within the first 6.5 days, requiring 9.1-13.0 moles of photons C-mol^{-1} ($\ln n_{Ph}$ = 2.21-2.56), which suggests that, under stress conditions like these, biomass used the energy of absorbed photons preferentially for maintenance rather than for growth. Disappointingly, HeP, after 4 days during which exhibited the best performance, started to have the highest photon requirement.

As known, a portion of the Gibbs energy carried by the photons absorbed in PSII is used to transfer electrons to PSI and to create a proton-motive force across the plasma membrane, thus providing energy for ATP synthesis. To make comparisons possible, times 0.5, 3.5 and 7.5 days were selected as representative of the mid lag phase, mid log phase and early stationary phase of growth, respectively. In all the photobioreactor configurations, the highest values of the thermodynamic

parameters taken into account in this estimation, i.e. the enthalpic content (ΔH), the energy recovered as ATP (ΔG_{ATP}) and that released as heat (Q), whose sum equals the total Gibbs energy effectively fixed by the photosynthesis (ΔG_a), were obtained in the stationary phase of growth. This means that, under these conditions, cell growth was so impaired that the synthesis of one C-mol of biomass required much more Gibbs energy than in the other growth phases, and this occurrence was less marked in HoP, likely because of its favorable configuration and less turbulent conditions.

4. CONCLUSIONS

The results of the current study indicate that the photobioreactor configuration had a strong impact on biomass concentration, in that its maximum value remarkably increased when S/V was raised. However, the helicoidal photobioreactor showed the best performance only at the beginning of runs because of its highest S/V ratio, but subsequently it behaved poorly owing to a time-depending mechanical stress. The horizontal photobioreactor proved to possess the best configuration and showed the best bioenergetic and thermodynamic parameters. This work demonstrate the potential HoP as a promising process for *A. platensis* cultures to obtain high growth yields.

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EFFECT OF LIGHT INTENSITY IN THE BIOENERGETICS AND THERMODYNAMICS OF BATCH Arthrospira (Spirulina) Platensis CULTURE

autoria de

Milena Fernandes da Silva, Attilio Converti, Raquel Pedrosa Bezerra, Patrizia Perego, Ana Lúcia Figueiredo Porto

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EFFECT OF LIGHT INTENSITY IN THE BIOENERGETICS AND THERMODYNAMICS OF BATCH *Arthrospira* (*Spirulina*) *platensis* CULTURE

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RESUMO – *The bioenergetics and thermodynamics of the batch culture of Arthrospira (Spirulina) platensis was investigated at different light intensity conditions (40, 70 and 100 µmol m^{-2} s^{-1}) in horizontal photobioreactor (HoP). The results indicate that the increase in biomass yield on Gibbs energy (Y_{GX}) was favored by a progressive increase in light intensity up to 100 µmol m^{-2} s^{-1}, reaching a maximum cell concentration of about 8.6 g/L. An increase in light intensity in the HoP favored cell growth because of photolimitation in the tested range, and the Y_{GX} fall was observed after 6.5 and 7.5 days in the cultivations carried out at 40 and 70 µmol m^{-2} s^{-1}, respectively, whereas no fall was detected at 100 µmol m^{-2} s^{-1} even after 8.5 days. A progressive increase in the light intensity did not exert any appreciable influence on number of moles of photons for the growth of 1 C-mol of biomass up to 6.5 days. These results demonstrate that light intensity was the growth-limiting factor under the selected conditions.*

1. INTRODUCTION

The production of the cyanobacterium *Arthrospira platensis* is increasing due to its high content of highly-valuable proteins, fundamental amino acids, vitamins, β-carotene and other pigments, mineral substances, essential fatty acids and polysaccharides, which find application in several industrial productions like those of health foods and therapeutics (Blinkova *et al*., 2001).

The energy management in a living cell is called bioenergetics. When combined with the fundamental principles of thermodynamics, it has proven to be a highly useful tool for the analysis of biosystems (Küçük *et al*., 2015). Photosynthetic microorganisms have recently gained huge attention worldwide, since they are one of the most promising renewable and neutral sources of energy, i.e., by consuming carbon dioxide, their cultivation has the additional benefit of combining valuable biomass production and CO2 emissions reduction (Belay, 2002; Cheah *et al*., 2015; Rodrigues *et al*., 2011).

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Environmental factors, such as light intensity, are well known to influence the growth of photosynthetic microorganism. In the absence of any other limiting factor, cell concentration increases with light intensity until reaching maximum biomass concentration that is denominated "saturation level". Light intensities higher than the saturation level provoke damage of cell photosynthetic apparatus due to a phenomenon called "photooxidation" or "photoinhibition" (Bezerra *et al*., 2012)

The environmental conditions imposed on a given microbial community in a reactor determine the total amount of Gibbs energy (Rodríguez *et al*., 2008). Bioenergetics studies can predict the energy flow to ATP production, to increase its own enthalpic content and dissipated as heat through living organism (Sassano *et al*., 2004) as well as determinate the condition of the microbial growth to obtain high cell energetic yields. The biomass yield constitutes one of the key parameters in any biotechnological process or experiment involving microbial cultures, since it determines the final biomass or cell concentration, which must be optimized in order to obtain reasonable productivities (Von Stockar *et al*., 2006). Typically, growth is quantified using well known parameters such as maximum biomass yield on substrate, maintenance requirements for substrate or electron donor and maximum specific growth rate. A practical problem is that the values for these parameters vary by one to two orders of magnitude, depending on the growth systems being considered (Heijnen and Van Dijken, 1991). To satisfy the requirements, Heijen and Van Dijken (1991; 1993) correlated the biomass yield in terms of Gibbs energy dissipation per amount of biomass grown. A simple correlation is found which provides the Gibbs energy dissipation per mol of carbon in the dry biomass as a function of the nature of the carbon source. Based on Heijenen model, some authors are exploring alternative, simpler correlations for the Gibbs energy dissipation, which can be used to predict biomass yields (Liu *et al.,* 2007).

Only a few studies emphasized the bioenergetic aspects of the growth of photosynthetic microorganisms based on Gibbs energy balances. The Gibbs energy dissipation per C-mol of biomass can be regarded as a simple thermodynamic measure of the amount of biochemical "work" required to convert the carbon source into biomass by the proper irreversible carbon-carbon coupling and oxidation/reduction reactions (Heijnen and Van Dijken, 1991). In this study we investigated the bioenergetics parameters of this cyanobacterium based on Heijnen model, when cultivated in tubular photobioreactor, under different photosynthetic photon flux densities ($60 \leq PPFD \leq 240$ mmol m^{-2} s^{-1}).

2. MATERIAIS E MÉTODOS

2.1. Microorganism and culture conditions

Arthrospira (*Spirulina*) *platensis* UTEX 1926 was obtained from the Culture Collection of Algae of the University of Texas (Austin, TX, USA). To allow a large growth of biomass, the microorganism was maintained and cultivated in the culture medium suggested by Schlösser (1982) modified so as to have a nitrogen concentration equal to about four fold the one of the original

medium. The resulting medium had the following composition (per liter): 13.61 g $NaHCO_3$, 4.03 g Na_2CO_3, 0.50 g K_2HPO_4, 10.0 g $NaNO_3$, 1.00 g K_2SO_4, 1.00 g NaCl, 0.20 g $MgSO_4 \cdot 7\ H_2O$, 0.04 g $CaCl_2 \cdot 2\ H_2O$. All the nutrients were dissolved in distilled water containing (per liter): 6.0 mL of metal solution (97 mg L^{-1} $FeCl_3 \cdot 6\ H_2O$, 41 mg L^{-1} $MnCl_2 \cdot 4\ H_2O$, 5 mg L^{-1} $ZnCl_2$, 2 mg L^{-1} $CoCl_2 \cdot 6\ H_2O$, 4 mg L^{-1} $Na_2MoO_4 \cdot 2\ H_2O$, 750 mg L^{-1} $Na_2EDTA \cdot 2\ H_2O$), 1.0 mL of micronutrient solution (50.0 mg L^{-1} Na_2EDTA, 618 mg L^{-1} H_3BO_3, 19.6 mg L-1 $CuSO_4 \cdot 5\ H_2O$, 44.0 mg L^{-1} $ZnSO_4 \cdot 7\ H_2O$, 20.0 mg L^{-1} $CoCl_2 \cdot 6$ H2O, 12.6 mg L^{-1} $MnCl_2 \cdot 4\ H_2O$, 12.6 mg L^{-1} $Na_2MoO_4 \cdot 2\ H_2O$) and 0.15 mg L^{-1} of B_{12} vitamin.

Cells were grown batch-wise in the horizontal photobioreactor (Ferreira *et al*., 2010) at light intensity from 40 to 100 µmol m^{-2} s^{-1}. For this purpose, fluorescent artificial light was ensured by a variable number of 36 W-lamps. Schematics of horizontal photobioreactors were illustrated in a previous study (Frumento *et al*., 2013).

Cultivations were carried out at temperature of 30 ± 2 °C and initial biomass concentration of 0.40 g L^{-1} (Soletto *et al*., 2008). The pH was controlled at 9.5 ± 0.2 through the daily addition of pure CO2. After growth, once the stationary phase had been reached after about 9 days of cultivation, biomass was separated from the culture medium by centrifugation (centrifuge ALC 42426, Milan, Italy) at 7500 rpm for 10 min. Recovered cells were washed twice with distilled water, dried at 105 °C for 24 h, pulverized in a mortar and stored at −20 °C conditions.

2.2. Analytical procedures

Cell concentration of A. platensis was determined daily by measuring the optical density (OD) at 560 nm by a UV-visible spectrophotometer, model Lambda 25 (PerkinElmer, Milan, Italy). All measurements were done in triplicate, and biomass concentration, expressed in grams of dried biomass per liter of medium (g L^{-1}), was estimated from OD data using a calibration curve relating the absorbance to dry biomass weight.

The elemental composition of dried biomass obtained at the end of culture was determined by an elemental analyzer Flash EA1112 series (CE Instruments, Wigan, UK). Since biomass composition varied very little among the different photobioreactor configurations and light intensities, its average value obtained from all the runs ($CH_{1.59}O_{0.50}N_{0.10}$) was used to estimate the bioenergetic and thermodynamic parameters of growth.

Light intensity was measured in several points of the culture surface using a type sensor quantum/photometer/radiometer, model HD-9021 Delta OHM (Li-Cor Inc., Lincoln NE, USA).

2.3. Bioenergetic and thermodynamic parameters of *A. platensis* growth

To estimate the bioenergetics parameters of *A. platensis* growth under batch conditions, we applied the model proposed by Torre *et al*. (2003) and Bezerra *et al*. (2012).

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3. RESULTS AND DISCUSSION

A. platensis growth was favored by a progressive increase in light intensity up to 100 µmol m^{-2} s^{-1}, reaching X_{max} = 8.44 ± 0.13 g L^{-1} (Figure 1). These results demonstrate that light intensity was the growth-limiting factor under the selected conditions, in agreement with previous findings that suggested the occurrence of photoinhibition in the same photobioreactor at light intensity as high as 240 µmol m^{-2} s^{-1} (Bezerra *et al.*, 2012). The following bioenergetic study allowed shed light on these observations.

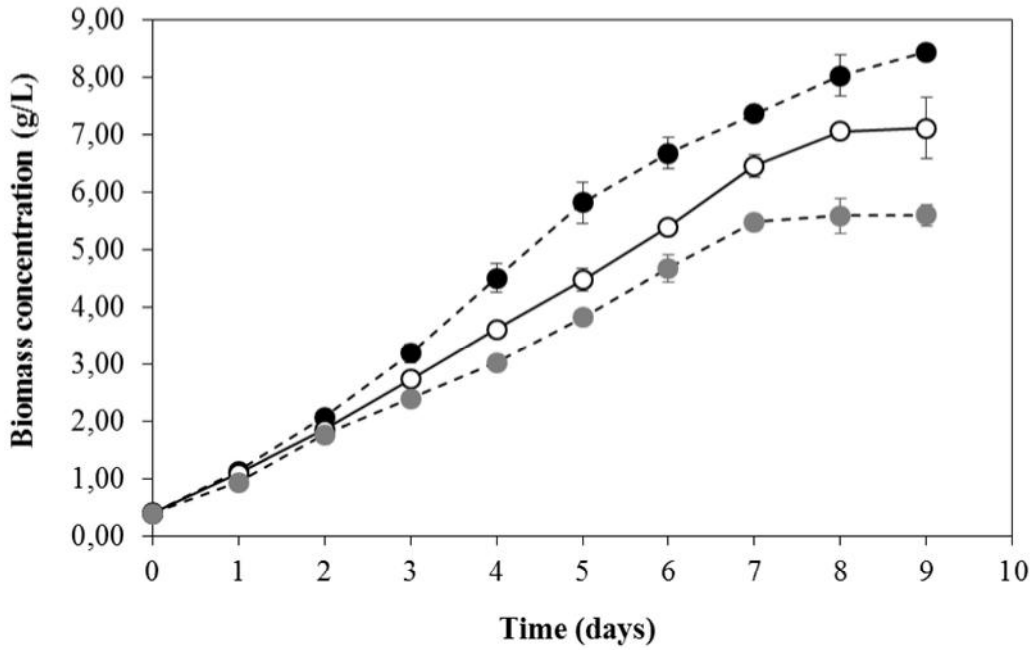

Figure 1. Biomass concentration during *A. platensis* cultivations performed in Horizontal photobioreactor varying light intensity (µmol m^{-2} s^{-1}): (···●···) 40; (—○—) 70; (···●···) 108.

Figure 2 illustrates the behavior of Gibbs energy dissipation for cell growth and maintenance, Y_{GX}, versus the time. As a general rule, this bioenergetic parameter decreased with the time under all the tested conditions, as a result of the increasing biomass level that was responsible for a decrease in the specific growth rate. As well known, a high rate of Gibbs energy dissipation leads to high metabolic rates but low biomass yield, while a low rate of Gibbs energy dissipation leads in general to the contrary (Westerho and Van Dam, 1987). An increase in PPFD up to 100 µmol m^{-2} s^{-1}, favored cell growth because of photolimitation and the Y_{GX} remarkably decreased after 8.5 days of cultivation, while Y_{GX} fell sharply only after 6.5 days in runs carried out at 40 and 70 µmol m^{-2} s^{-1}. In this conditions, the specific growth rate decreases and the Y_{GX} was mainly for cell maintenance than to cell growth. The low Y_{GX} values after 6.5 days are due to

the difficulty with respect to synthesizing biomass from given carbon and energy sources (Heijnen *et al*., 1994)

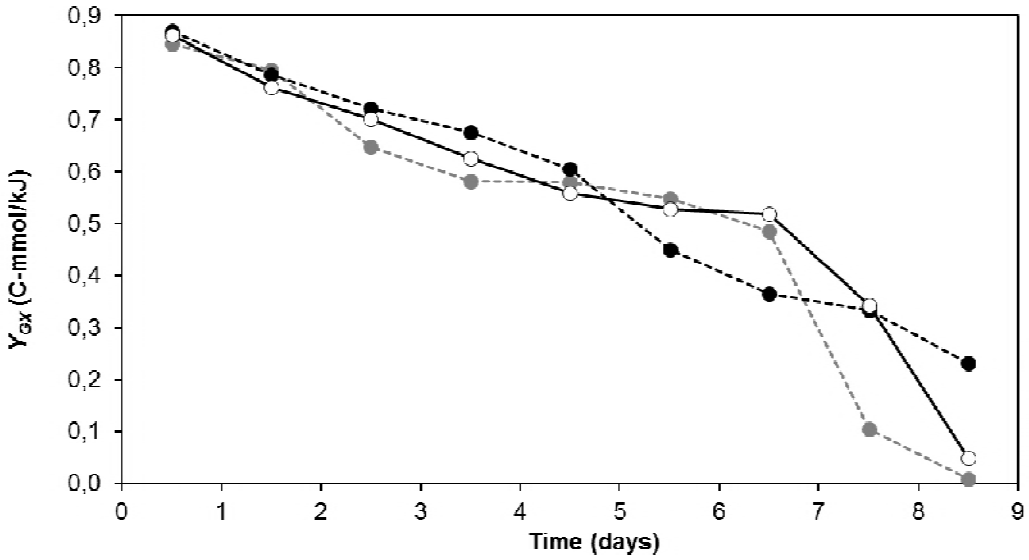

Figure 2 - Time behavior of biomass yield on Gibbs energy (Y_{GX}) during *A. platensis* cultivations performed in the Horizontal Photobioreactor varying light intensity (μmol m^{-2} s^{-1}): (···●···) 40; (—○—) 70; (···●···) 108.

As shown in Figure 3, the number of Einsteins (E) absorbed to produce one C-mol of biomass (n_{Ph}) progressively increased (in absolute value) throughout all the cultivations, just reflecting the decrease in Y_{GX} illustrated in Figure 2. As expected, at the beginning of runs where the microorganism grew at highest μ, this bioenergetic parameter approached values very close to that reported to sustain growth under ideal conditions, i.e. 8 E $C\text{-}mol^{-1}$ of biomass (Richmond, 1983); the release of 1 mol of O_2 by the photosynthesis does in fact imply the absorption of at least 8 E and the fixation of 1 mol of CO_2 to produce 1 C-mol of carbohydrate (Osborne and Geider, 1987).

Confirming the observations made for Y_{GX} (Figure 2), a progressive increase in the light intensity did not exert any appreciable influence on n_{Ph} up to 6.5 days, but remarkably influenced the time at which the subsequent strong increase in this parameter took place (7, 8 and 9 days for I = 40, 70 and 100 μmol m^{-2} s^{-1}, respectively) (Figure 3). However, it should be stressed that, irrespective of light conditions and configurations, the additional photons with respect to the theoretical requirement (8 E $C\text{-}mol^{-1}$) were partly utilized to reduce nitrate to ammonia (Hatori and Myers, 1966), which is the preferential form of nitrogen assimilated by photosynthetic microorganisms (Boussiba *et al*., 1984; Torre *et al*., 2003), and that such a reduction is accompanied by the oxidation of a portion of NADPH produced by the photosynthesis (Bruinenberg *et al*., 1983).

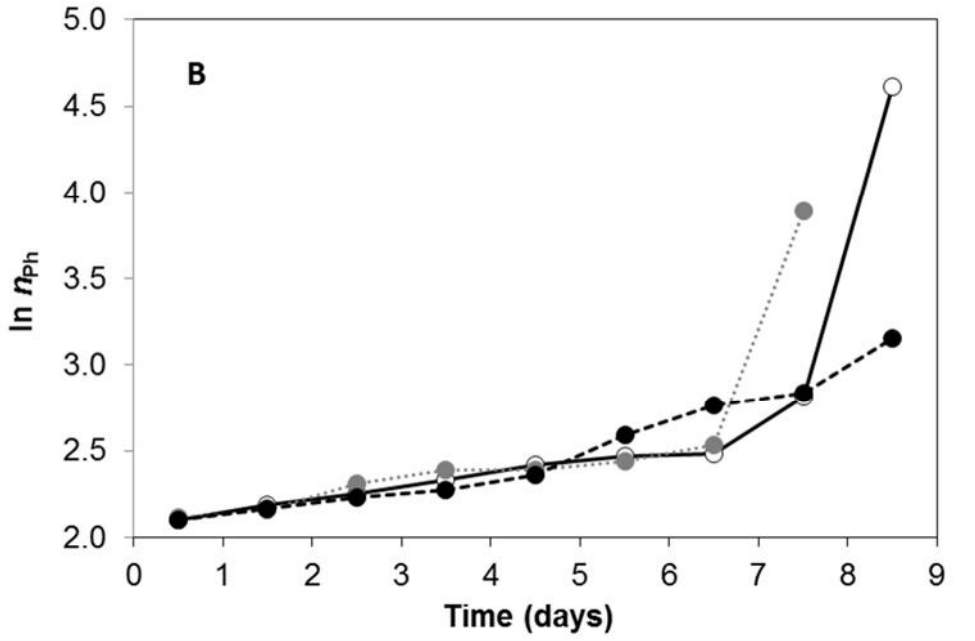

Figure 3 - Semi-log plot of the moles of photons (E) required for the synthesis of 1 C-mol of *A. platensis* biomass (n_{Ph}, expressed in mol C-mol^{-1}) versus time during cultivations performed in the Horizontal Photobioreactor varying light intensity (µmol m^{-2} s^{-1}): (···●···) 40; (—○—) 70; (···●···) 108.

The results of the current study indicate that the bioenergetics parameters of the blue-green cyanobacterium *Arthrospira* (*Spirulina*) *platensis* were influenced photosynthetic photon flux density (PPFD). In particular, the highest values of the Gibbs energy dissipation for cell growth and maintenance and ln of number of photons moles absorbed by the cell to produce one mol of carbon in the dry biomass were obtained 100 µmol m^{-2} s^{-1}.

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AIChE

Chlorella vulgaris as a Lipid Source: Cultivation on Air and Seawater-Simulating Medium in a Helicoidal Photobioreactor

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The freshwater microalga Chlorella vulgaris was cultured batchwise on the seawater-simulating Schlösser medium either in a 1.1-L-working volume helicoidal photobioreactor (HeP) or Erlenmeyer flask (EF) as control and continuously supplying air as CO_2 source. In these systems, maximum biomass concentration reached 1.65 ± 0.17 g L^{-1} and 1.25 ± 0.06 g L^{-1}, and maximum cell productivity 197.6 ± 20.4 mg L^{-1} day^{-1} and 160.8 ± 12.2 mg L^{-1} day^{-1}, respectively. Compared to the Bold's Basal medium, commonly employed to cultivate this microorganism on a bench-scale, the Schlösser medium ensured significant increases in all the growth parameters, namely maximum cell concentration (268% in EF and 126% in HeP), maximum biomass productivity (554% in EF and 72% in HeP), average specific growth rate (67% in EF and 42% in HeP), and maximum specific growth rate (233% in EF and 22% in HeP). The lipid fraction of biomass collected at the end of runs was analyzed in terms of both lipid content and fatty acid profile. It was found that the seawater-simulating medium, despite of a 56–63% reduction of the overall biomass lipid content compared to the Bold's Basal one, led in HeP to significant increases in both the glycerides-to-total lipid ratio and polyunsaturated fatty acid content compared to the other conditions taken as an average. These results as a whole suggest that the HeP configuration could be a successful alternative to the present means to cultivate C. vulgaris as a lipid source. © 2016 American Institute of Chemical Engineers *Biotechnol. Prog.*, 32:279–284, 2016

Keywords: Chlorella vulgaris, lipids, fatty acids, microalgae, Schlösser medium

Introduction

Microalgae are a potential biodiesel source[1] that may be used in various applications, i.e. wastewater treatment under mixotrophic or heterotrophic conditions,[2–4] production of pharmaceuticals and functional foods or as food for aquaculture.[5] A great variety of compounds can be extracted from microalgae such as fatty acids,[6,7] pigments,[8–10] and vitamins.[11,12] In particular, the microalga *Chlorella vulgaris*, an eukaryotic unicellular photosynthetic microorganism characterized by fast growth,[13] has been widely studied for the production of fatty acids. Among them, the saturated and monounsaturated fatty acids (e.g., palmitic, myristic, stearic, and oleic acids) are used in the biodiesel industry,[14] while the polyunsaturated ones can be incorporated into several products among which are feeds, foods, cosmetics, and pharmaceutics.[15] It has been assessed that stressful conditions are able to increase fatty acids synthesis by microalgal cells; therefore, the setup of such conditions is an important matter of study.[16]

C. vulgaris is usually cultured on the Bold's Basal medium (BBM), a nutrient solution that simulates freshwater composition, with the addition of B1 and B12 vitamins. Air or flue gases are injected into this culture medium to furnish carbon dioxide as the only carbon source.[17]

In previous studies on *C. vulgaris*-based biodiesel production, various methods have been adopted to increase lipid yield (growth conditions, extraction setup, etc.),[16] but only a few studies on culture medium composition have been published.[18] Šoštariè et al. studied the growth of *C. vulgaris* and its chlorophyll and lipid contents in three media, namely Jaworski's medium, a natural mineral water, and an enriched solution from modified Dual Solvay process, the best growth being observed in the last of them.[19] Yeh and Chang, who used either a nitrogen-rich (BBM) or a nitrogen-limited medium (Modified Bristol's medium, MBL) to culture *C. vulgaris* ESP-31, found that cell growth and lipid accumulation were greatly influenced by medium composition and cultivation conditions. Higher biomass production (2–5

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g L^{-1}) was obtained using BBM under various growth conditions, whereas higher lipid content (20–53%) was achieved in MBL.[20]

Because of this, for lab-scale lipid production by *C. vulgaris*, the present study was focused on the alternative use of the Schlösser medium (SM), a seawater-simulating nutrient solution commonly employed to culture cyanobacteria mainly belonging to the *Arthrospira* genus,[21] which is often supplemented with B12 vitamin and contains sodium bicarbonate and carbonate as inorganic carbon sources.

Based on this background, the aim of this work was to investigate the fatty acid profile of *C. vulgaris* biomass cultured under different conditions, so as to exploit it, accordingly, either for biodiesel production or for pharmaceutical or food applications. To this purpose, batch cultures were carried out in a 1.1-L helicoidal photobioreactor (HeP) using either BBM or SM continuously supplying air as CO_2 source, and the results compared with those obtained in Erlenmeyer flasks.

Materials and Methods

Microorganism and chemicals

The *C. vulgaris* CCAP 211 (Culture Collection of Algae and Protozoa, Argyll, UK) strain used in the present work was maintained using atmospheric CO_2 (about 300 ppm) as the only carbon source in the BBM,[17] having the following composition (per liter of distilled water): 250 mg $NaNO_3$, 75 mg $MgSO_4{\cdot}7H_2O$, 25 mg NaCl, 75 mg K_2HPO_4, 175 mg KH_2PO_4, 25 mg $CaCl_2{\cdot}2H_2O$, 11.42 mg H_3BO_3, 8.82 mg $ZnSO_4{\cdot}7H_2O$, 1.44 mg $MnCl_2{\cdot}4H_2O$, 0.71 mg MoO_3, 1.57 mg $CuSO_4{\cdot}5H_2O$, 0.49 mg $Co(NO_3)_2{\cdot}6H_2O$, 50 mg $Na_2EDTA{\cdot}2H_2O$, 31 mg KOH, 4.98 mg $FeSO_4{\cdot}7H_2O$, 1.84 mg H_2SO_4, plus 3.0 mg B1 and 2.5 mg B12 vitamins. Based on this composition, an osmolarity of 14.9 mOsm/L and a C/N ratio of 0.418 mol/mol have been calculated for this medium. The pH was finally regulated at 7.5 by addition of dilute HCl solution. Chloroform, methanol, hexane, and GC standards (methyl myristate, palmitate, stearate, oleate, linoleate, α-linolenate, and γ-linolenate and eicosapentaenoate) were purchased from Sigma-Aldrich (St. Louis, MO).

Media preparation and culture conditions

Batch runs were performed in duplicate at room temperature (20 ± 1°C) on two different culture media, namely the above BBM[17] and the Schlösser one (SM),[21] having the following composition (per liter): 13.61 g $NaHCO_3$, 4.03 g Na_2CO_3, 0.50 g K_2HPO_4, 2.50 g $NaNO_3$, 1.00 g K_2SO_4, 1.00 g NaCl, 0.20 g $MgSO_4{\cdot}7\ H_2O$, 0.04 g $CaCl_2{\cdot}2\ H_2O$. All the SM nutrients were dissolved in distilled water containing (per liter): 6.0 mL of metal solution (97 mg/L $FeCl_3{\cdot}6\ H_2O$, 41 mg/L $MnCl_2{\cdot}4\ H_2O$, 5 mg/L $ZnCl_2$, 2 mg/L $CoCl_2{\cdot}6\ H_2O$, 4 mg/L $Na_2MoO_4\ {\cdot}2\ H_2O$, 750 mg/L $Na_2EDTA{\cdot}2\ H_2O$), 1.0 mL of micronutrient solution (50.0 mg/L Na_2EDTA, 618 mg/L H_3BO_3, 19.6 mg/L $CuSO_4{\cdot}5\ H_2O$, 44.0 mg/L $ZnSO_4{\cdot}7\ H_2O$, 20.0 mg/L $CoCl_2{\cdot}6\ H_2O$, 12.6 mg/L $MnCl_2{\cdot}4\ H_2O$, 12.6 mg/L $Na_2MoO_4{\cdot}2\ H_2O$) and 0.15 mg/L of B12 vitamin. Based on this composition, an osmolarity of 559 mOsm/L and a C/N ratio of 10.9 mol/mol have been calculated for this medium. The pH was regulated and daily set at 7.5 by addition of dilute HCl solution.

Runs were carried out either in a HeP, whose experimental setup was previously illustrated,[18] or in Erlenmeyer flasks (EF) selected as control. Even though these systems had the same working volume (1.1 L), the ratio of light-exposed surface to volume (S/V) increased from 0.48 cm^{-1} in EF to 3.88 cm^{-1} in HeP. The photobioreactor, flasks and media were sterilized in autoclave for 20 min at 121°C in order to prevent any contamination during the early growth stages. Cultures were carried out under artificial light with about 5 klux intensity,[22,23] provided by eight 36 W-fluorescent lamps. Natural air was continuously supplied to both EF and HeP through M2K3 pumps (Schego, Offenbach, Germany) in order to provide the required agitation and CO_2, which in the case of BBM was the only carbon source.

Kinetic and yield parameters

The average specific growth rate was calculated by the equation:

$$\mu = \frac{1}{t} \ln \frac{X_f}{X_0} \tag{1}$$

where X_f and X_0 are the biomass concentrations at the end and the beginning of runs, respectively, and t is the cultivation time.

The average biomass productivity was defined as the ratio of produced biomass per unit volume between the end and the beginning of runs to the cultivation time:

$$Q_X = \frac{X_f - X_0}{t} \tag{2}$$

Both kinetic parameters were also calculated as maximum values (μ_{max} and $Q_{X,max}$, respectively) at the time corresponding to the maximum biomass concentration (X_{max}).

The lipid content of biomass was defined as:

$$Y_L = \frac{M_L}{M_D} \times 100 \tag{3}$$

where M_L and M_D are the masses of the extracted lipids and of the dry microalgal biomass collected at end of runs, respectively.

Finally, the average lipid productivity (Q_L) was calculated as the product of Q_X and M_L/M_D.

The glyceride content of the lipid fraction was roughly determined as the ratio of the total mass of fatty acid methyl esters to the total mass of the lipid fraction.[18]

Analytical procedures

Cell concentration was determined by optical density (OD) measurements at 625 nm using a UV–vis spectrophotometer, model Lambda 25 (Perkin Elmer, Milan, Italy). All measures were carried out in triplicate, and biomass concentration (X) was related to OD by the equation $X = 0.238$ OD ($R^2 = 0.990$).

After growth, biomass was centrifuged at 6,000g for 10 min in order to get rid of the culture medium, using a centrifuge, model PK131 (ALC, Milan, Italy). Biomass was dried at 105°C for 48 h, reduced to powder in a mortar and stored at −20°C to subsequently analyze the lipid fraction. The lipid fraction of dry biomass was extracted by a 2:1 chloroform/methanol solution according to Krienitz and Wirth,[24] i.e., by the method of Folch et al.[25] combined to the use of a

ultrasonic processor, model UP100H (Hielscher Ultrasonics, Teltow, Germany), and increasing the extraction time from 1.5 to 5.0 h. One portion of the lipid fraction, immediately after the lipid extraction, was transesterified by the method described by Zunin et al.[26] and rapidly analyzed by a gas chromatograph, model Ultra Trace (Thermo Finnigan, Milan, Italy), equipped with a ZB Vax column and a FID detector (Thermoscientific, Milan, Italy). To this purpose, methyl esters standard stock solutions were prepared using hexane as a solvent and stored at −20°C until use.

Optical microscopy observations of the algal morphology were made at ×200 magnitude throughout the runs, which excluded the absence of any bacterial contamination or change in cell size and morphology.

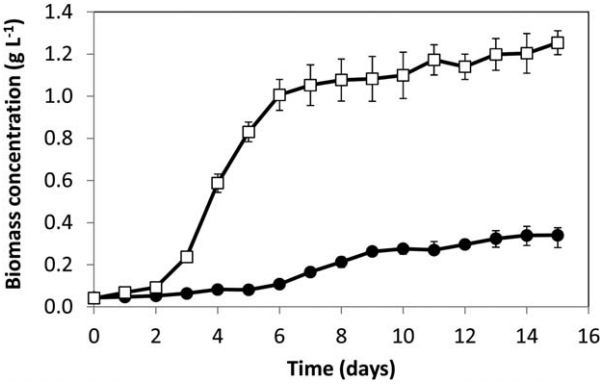

Figure 1. Time behavior of biomass concentration along *Chlorella vulgaris* cultivations carried out in Erlenmeyer flasks on different media: (●) Bold's Basal medium; (□) Schlösser medium.

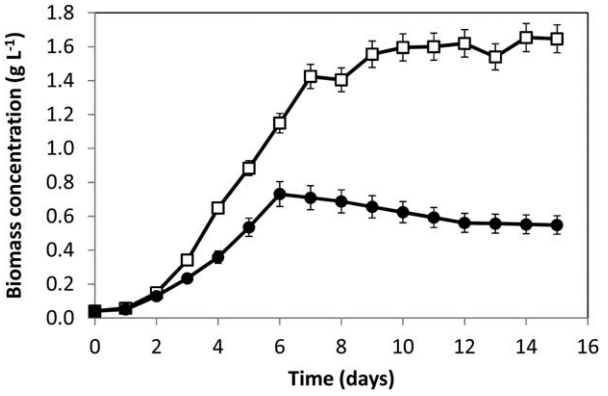

Figure 2. Time behavior of biomass concentration along *Chlorella vulgaris* cultivations carried out in the helicoidal photobioreactor on different media: (●) Bold's Basal medium; (□) Schlösser medium.

Statistical analysis

The effects of type of medium and photobioreactor configuration on biomass concentration and productivity, lipid content and productivity, average specific growth rate, and fatty acid profile were evaluated by the analysis of variance (ANOVA) and Tukey's *post hoc* test. Multiple comparisons of the mean values were made by the least significant difference test at $P < 0.05$. Statistically significant differences were labeled with different letters in tables. The "Statistica" software 6.0 (StatSoft, Tulsa, OK) was employed for this purpose.

Results and Discussion

Cultivations

Cultivations showed substantial differences in terms of both curve shape and biomass concentration depending either on the type of medium or the photobioreactor configuration.

First of all, in EF containing the Schlösser medium (SM), the exponential phase of *C. vulgaris* growth lasted about 7 days (Figure 1), i.e., about 6 days less than in the standard BBM. As can be seen in the Materials and Methods section, SM is characterized by much higher osmolarity (559 mOsm/L) and C/N ratio (10.9) than BBM (14.9 mOsm/L and C/N = 0.418), because of its higher content of salts, mainly carbonates and bicarbonates. Therefore, the better growth of *C. vulgaris* CCAP 211 in SM compared with BBM may be ascribed to a special resistance of this strain to high salinity as well as the high C/N ratio of SM owing to the presence of inorganic carbon sources (HCO_3^-/CO_3^{2-}).[18] The final biomass concentration in SM (1.25 ± 0.06 g L^{-1}) was at the stationary phase of growth about 30 times higher than the initial inoculum level and almost four times that obtained in BBM (0.34 ± 0.06 g L^{-1}) (Figure 1). This occurrence as well can be ascribed to a significant consumption of the inorganic carbon sources present in SM. On the other hand, the very low biomass concentration obtained in BBM could have also been due to limited light exposure during growth and/or possible limitation of CO_2 mass transfer.

Also the performance of the 1.1-L HeP, having a higher ratio of light-exposed surface to volume (S/V = 3.88 cm^{-1} instead of 0.48 cm^{-1}), was significantly different in these media (Figure 2). Under these conditions, maximum biomass concentration obtained in the run carried out in SM (1.65 ± 0.17 g L^{-1}) was more than twice that obtained in BBM, but a longer time was needed (14 days) to reach it (Figure 2). This result may be explained not only by the availability of inorganic carbon source as for the

Table 1. Main Kinetic Results of *Chlorella vulgaris* Cultivations Carried Out Under Different Conditions

Photobioreactor	Medium	μ (day^{-1})	μ_{max} (day^{-1})	$Q_{X,max}$ (mg L^{-1} day^{-1})	Y_L (%)	Q_L (mg L^{-1} day^{-1})
Flask	Bold's Basal	0.136 ± 0.010^a	0.199 ± 0.005^b	24.6 ± 1.1^a	13.11 ± 0.10^c	3.22 ± 0.17^a
Flask	Schlösser	0.227 ± 0.003^b	0.662 ± 0.018^a	160.8 ± 12.2^c	4.90 ± 0.09^a	7.88 ± 0.61^b
Helicoidal	Bold's Basal	0.172 ± 0.067^a	0.577 ± 0.033^c	114.9 ± 12.2^b	17.20 ± 0.51^d	19.82 ± 2.68^d
Helicoidal	Schlösser	0.245 ± 0.067^b	0.703 ± 0.034^a	197.6 ± 20.4^d	7.50 ± 0.31^b	14.88 ± 2.14^c

μ, average specific growth rate; μ_{max}, maximum specific growth rate; $Q_{X,max}$, maximum biomass productivity; Y_L, lipid content of biomass collected at the end of runs (15 days); Q_L, average lipid productivity based on dry biomass collected at the end of runs (15 days). Different letters (a–d) within each column mean statistically significant differences at $P < 0.05$.

Table 2. Fatty Acid Content (%), Quantified as Methyl Esters, of the Lipid Fraction of *Chlorella vulgaris* Biomass Obtained Under Different Culture Conditions

Photobioreactor	Medium	Myristic (C14:0) (RT* 8.62)	Palmitic (C16:0) (RT* 13.38)	Stearic (C18:0) (RT* 19.14)	Oleic (C18:1 cis-9) (RT* 19.64)	Linoleic (C18:2) (RT* 20.89)	Linolenic (C18:3)† (RT* 22.68)	Eicosapentaenoic (C20:5) (RT* 23.13)	Glycerides
Flask	Bold's Basal	2.56 ± 0.57^{a}	39.24 ± 1.3^{b}	2.01 ± 0.57^{c}	9.94 ± 0.22^{a}	$14.62 \pm 0.86^{a,b}$	20.39 ± 0.93^{a}	11.24 ± 0.02^{b}	14.24 ± 0.22^{a}
Flask	Schlösser	2.46 ± 0.34^{a}	31.06 ± 2.53^{a}	0.00 ± 0.00^{a}	16.99 ± 0.27^{c}	$14.31 \pm 0.31^{a,b}$	19.97 ± 0.18^{a}	$15.21 \pm 3.65^{a,b}$	15.48 ± 2.51^{a}
Helicoidal	Bold's Basal	$3.08 \pm 0.15^{a,b}$	30.94 ± 1.47^{a}	0.00 ± 0.00^{a}	13.11 ± 1.87^{b}	12.72 ± 2.59^{a}	21.81 ± 2.17^{a}	18.34 ± 0.65^{a}	16.90 ± 0.83^{a}
Helicoidal	Schlösser	4.77 ± 1.42^{b}	29.20 ± 0.16^{a}	0.76 ± 0.08^{b}	10.50 ± 0.01^{a}	17.41 ± 0.64^{b}	20.80 ± 0.66^{a}	16.56 ± 1.06^{a}	35.86 ± 4.01^{b}

*Retention time (min). †Sum of α and γ-linolenic acids.
Different letters (a–c) within each column mean statistically significant differences at $P < 0.05$.

corresponding flask run, but also by a larger light exposure ensured by the better reactor configuration, which was likely able to minimize the shading effect among photosynthetic cells.[27] An interesting observation is that, whereas cell concentration slightly decreased after only 6 days after the achievement of its maximum value in BBM, it continued growing abundantly in the SM one, probably because the buffering power of the HCO_3^-/CO_3^{2-} mixture was able to face the progressive alkalization of the medium, which is a peculiar feature of all the microalgal and cyanobacterial cultures.[28] Oppositely, the absence of any inorganic carbon source in BBM required large amount of HCl to control the pH at its optimum value (7.5), thus affecting the culture.

Finally, the statistically different results in terms of biomass production obtained in EF compared with HeP suggest the possible occurrence of photolimitation at a S/V ratio as low as 0.48 cm^{-1}.

Growth kinetics and lipid fraction

As a combined result of richer medium and higher S/V ratio, *C. vulgaris* cultivated in HeP showed average (μ) and maximum (μ_{max}) specific growth rates in SM ($\mu = 0.245 \pm 0.067$ day^{-1}; $\mu_{max} = 0.703 \pm 0.034$ day^{-1}) 8 and 6% higher than in EF and 42 and 22% higher than in BBM, respectively. A qualitatively similar behavior was observed for maximum biomass productivity ($Q_{X,max}$) that in the SM-containing HeP reached a value (197.6 ± 20.4 mg L^{-1} day^{-1}) 23% higher than in EF and 72% higher than in BBM.

Consistently with these results, the average lipid productivity (Q_L) of biomass cultured in EF was in SM (7.88 ± 0.61 mg L^{-1} day^{-1}) more than twice that in BBM, but, oppositely, that of biomass cultured in BBM-containing HeP (19.82 ± 2.68 mg L^{-1} day^{-1}) was one third higher than in SM. This finding can be justified by the fact that the microorganism cultured in SM spent most of the energy to grow, while in BBM the lipid content of biomass (Y_L) was about 2.5-fold that in SM (Table 1). These results are consistent with the lipid overproduction previously observed in the same *C. vulgaris* strain under different nutritional and environmental stress conditions.[16] One can suppose that the microorganism accumulates lipids under nutritional stress conditions as an energy reserve.

Lipid composition

As can be seen in Table 2, whereas the fatty acid profile was not statistically significantly influenced either by the photobioreactor configuration or the type of medium, glycerides were overproduced by *C. vulgaris* ($35.86 \pm 4.01\%$) in SM-containing HeP, remaining instead at around 15% under the other conditions. An inference that one can draw from these results is that the growth limitation associated to the lack of inorganic carbon source is likely to be modulated by light availability: conditions of carbon source starvation (BBM in both EF and HeP) may have led to a remarkable increase in the overall lipid fraction (Table 1), whereas under non-limited growth conditions (SM in HeP) *C. vulgaris* biomass had lower lipid content, but higher glyceride fraction (Table 2).

In order to shed some more light on these results, one can give a look in Table 2 at the percentages of fatty acid methyl esters (FAMEs). Even though all of them were similar in

biomass grown on BBM and SM with no statistically significant influence of the bioreactor configuration, the sum of saturated and monounsaturated fatty acids of biomass grown in EF was as an average (52.1%) higher than that of biomass grown in HeP (46.2%). These results suggest that, under conditions of high light irradiance ($S/V = 3.88\ cm^{-1}$) in HeP, *C. vulgaris* was likely to synthesize larger amounts of polyunsaturated fatty acids as antioxidants and scavengers of free radicals and reactive oxygen species.[29]

Taking all these results as a whole, one can see that, although *C. vulgaris* biomass cultured in SM using the HeP had a lipid content more than 50% lower than in BBM, its glycerides content was more than double (Table 1), although the percentages of FAMEs were comparable (Table 2).

Conclusions

Batch cultures of *C. vulgaris* were carried out on either the traditional BBM or the seawater-simulating SM, using two photobioreactor configurations with different ratios of light-exposed surface to volume (S/V), namely common EF with $S/V = 0.48\ cm^{-1}$ and a HeP with $S/V = 3.88\ cm^{-1}$, in which air was continuously supplied as CO_2 source. The collected results demonstrated that SM can successfully be utilized for the lab-scale cultivation of *C. vulgaris* because of its accelerating effect on the growth associated to the presence of HCO_3^-/CO_3^{2-} mixture as inorganic carbon source. In both EF and HeP, the lipid yield of biomass grown in SM was always lower than in BBM as a result of lipid overproduction under conditions of carbon source starvation. The glyceride content of biomass was around 15% and was not statistically influenced either by the photobioreactor configuration or the type of medium, except for *C. vulgaris* cultured in HeP that overproduced glycerides in SM and polyunsaturated fatty acids in both media likely to neutralize free radicals and reactive oxygen species.

The results of this work taken together demonstrated that the *C. vulgaris* strain used herein was able to effectively grow in saline media, thus behaving as a salt-resistant strain. Therefore, one can envisage its large-scale production in salty water as a source of biodiesel or of polyunsaturated fatty acids, depending on the conditions of light availability and medium composition, which need to be strictly optimized for each of these goals.

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