

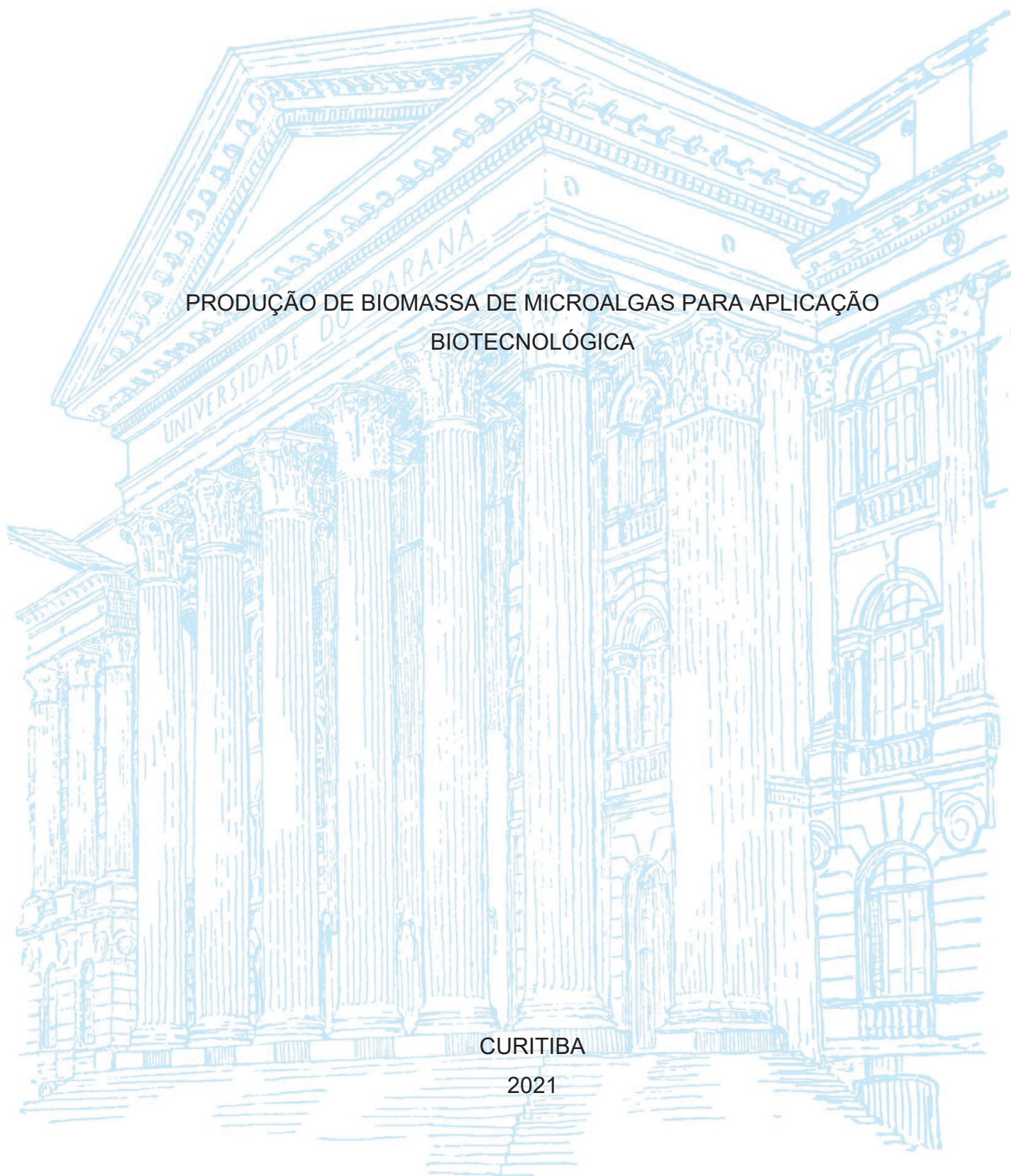
UNIVERSIDADE FEDERAL DO PARANÁ

GUILHERME AUGUSTO COLUSSE

PRODUÇÃO DE BIOMASSA DE MICROALGAS PARA APLICAÇÃO
BIOTECNOLÓGICA

CURITIBA

2021



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PRODUÇÃO DE BIOMASSA DE MICROALGAS PARA APLICAÇÃO
BIOTECNOLÓGICA

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Orientador: Prof. Dr. Miguel Daniel Nosedá

Coorientadores: Prof. Dr. Júlio Cesar de Carvalho. Profa. Dra. Maria Eugênia Rabello Duarte

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(As aventuras de Pi)

RESUMO

O desenvolvimento tecnológico associado ao crescimento populacional aprimoraram o estudo de novas metodologias na área da biotecnologia. Nesse contexto, o cultivo de microalgas para aplicações industriais apresenta diversas potencialidades de grande interesse ao setor. Com base nesse potencial, o presente estudo teve como objetivo avaliar de diversas formas o cultivo de microalgas para produção de biomassa e metabólitos de interesse industrial. A metodologia experimental consistiu primeiramente da influência de diversos meios de cultivo no crescimento de *Haematococcus pluvialis* e *Dunaliella salina*. Além da diferença significativa na produção de proteínas, carboidratos totais e lipídeos, os meios de cultivo apresentaram variação de custo de US\$2,61-18,36 cada 10³ L para o cultivo de *H. pluvialis*. Ainda, os resultados mostraram que os custos para produzir 1 kg de biomassa seca de *H. pluvialis* foram de US\$6,20 (meio WC), 21,73 (meio CHU) e 40,36 (meio BG-11). Além disso, vários fatores podem influenciar ainda mais o custo de produção e são encontrados em processos de *upstream* e *downstream* (carotenogênese e métodos de extração de biomoléculas, dentre vários outros). Os resultados referentes ao uso de meios de cultivo para cultivar *D. salina* apresentaram diferenças significativas no acúmulo de pigmentos e no custo de produção. A biomassa obtida através do emprego do meio Johnson apresentou 0,855 mg g⁻¹ de violaxantina e 14,255 mg g⁻¹ de clorofila *a*, enquanto 43% de teor de lipídeo foi encontrado na biomassa proveniente do meio Conway. Em geral, os custos da biomassa seca apresentaram variação de US\$4,64-301,61 por kg. Levando em consideração as características biológicas das microalgas, foi desenvolvido um bioprocesso para tratamento da vinhaça de arroz utilizando a cianobactéria *Synechococcus pevalekii* encapsulada em esferas de alginato. Logo após o tratamento biológico, a vinhaça tratada ainda foi utilizada para cultivo da microalga *D. salina*. Após o bioprocesso, foi observado a redução de nitrato (91%), sólidos totais (29%) e ions no sobrenadante, além da produção de clorofila_a (0,68 x 10⁻³ mg por esfera) e carotenóides totais (0,64 x 10⁻³ mg por esfera). O uso da vinhaça tratada no cultivo de *D. salina* aumentou a replicação celular em 175% apresentando viabilidade para estudo de aumento de escala. Além dos dados obtidos para produção de biomassa microalgal e tratamento biológico, foi realizado um estudo para avaliar pigmentos microalgais pouco convencionais explorados na literatura. A revisão apresentou pigmentos como echinenona, myxoxantofila, adonixantina, botrioxantina, entre outros associados com as principais espécies produtoras e propriedades biológicas. Além disso, o estudo apresenta alguns parâmetros de produção e métodos analíticos. Por último, foi avaliado e revisado moléculas alternativas aos pigmentos visando o processo de biorefinaria. A revisão de literatura avaliou diferentes tipos de polissacarídeos encontrados na parede celular de microalgas bem como a estrutura química e propriedades biológicas dos mesmos. Vale ressaltar que as tecnologias aplicadas ao cultivo de microalgas são fundamentais para a viabilidade econômica do processo e podem alavancar a produtividade. Estudos baseados em análises econômicas, projeções e busca por novos metabólitos podem eliminar os gargalos encontrados no cultivo de microalgas e possibilitar a inserção de novas empresas no ramo.

Palavras-chave: Microalgas. Bioprocesso. Meio de cultivo. Imobilização. Biorremediação. Biopigmentos. Polissacarídeos.

ABSTRACT

Technological development associated with population growth has improved the study of new methodologies in the area of biotechnology. In this context, the microalgae cultivation for industrial applications presents several potentials of great interest to the sector. Based on this potential, this study aimed to evaluate in different ways the cultivation of microalgae for the production of biomass and high value-added metabolites. The methodology consisted primarily of the influence of different culture media on the growth of *Haematococcus pluvialis* and *Dunaliella salina*. In addition to the significant difference in proteins, total carbohydrates and lipids content, the media showed a cost variation of US\$2.61-18.36 each 10^3 L for the cultivation of *H. pluvialis*. Furthermore, the results showed that the costs to produce 1 kg of dry biomass of *H. pluvialis* were US\$6.20 (WC medium), 21.73 (CHU medium) and 40.36 (BG-11 medium). Moreover, several factors can further influence the production cost and are found mainly in upstream and downstream processes (carotenogenesis and biomolecule extraction methods, among several others). The results regarding the use of culture media to cultivate *D. salina* showed significant differences in pigment accumulation and production cost. The biomass obtained through the use of Johnson medium presented 0.855 mg g^{-1} of violaxanthin and 14.255 mg g^{-1} of chlorophyll a , while 43% of lipid content was found in the biomass obtained from Conway medium. In general, dry biomass costs ranged from US\$4.64-301.61 per kg. Taking the biological characteristics of microalgae into account, a bioprocess was developed for the treatment of rice vinasse using the cyanobacterium *Synechococcus pevalekii* encapsulated in alginate beads. Soon after the biological treatment, the treated vinasse was used to cultivate the microalga *D. salina*. After the bioprocess, it was observed a reduction of nitrate (91%), total solids (29%) and ions in the supernatant, in addition to the production of chlorophyll a ($0.68 \times 10^{-3} \text{ mg}$ per bead) and total carotenoids ($0.64 \times 10^{-3} \text{ mg}$ per bead). The use of treated vinasse in the cultivation of *D. salina* increased cell replication by 175%, presenting feasibility for scale-up studies. In addition to the data obtained for the production of microalgal biomass and biological treatment, a study was carried out to evaluate lesser-known microalgal pigments explored in the literature. The review presented pigments such as echinenone, myxoxanthophyll, adonixanthin, botrioxanthin, among others associated with the main producing species and biological properties. In addition, the study presents some production parameters and analytical methods. Finally, alternative molecules to pigments were evaluated and reviewed for biorefinery process. The literature review evaluated different types of polysaccharides found in the cell wall of microalgae as well as their chemical structure and biological properties. It is noteworthy that the technologies applied to the cultivation of microalgae are fundamental for the economic viability of the process and can boost productivity. Studies based on economic analysis, projections and the search for new metabolites can eliminate the bottlenecks found in microalgae cultivation and enable the insertion of new companies in the field.

Keywords: Microalgae. Bioprocess. Culture medium. Cell immobilization. Bioremediation. Biopigments. Polysaccharides.

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LISTA DE ABREVIATURAS OU SIGLAS

WC	- <i>Water Culture</i>
BG-11	- <i>Blue-Green Medium</i>
NPK	- Nitrogênio (N), Fósforo (P) e Potássio (K)
FITR	- Espectroscopia no Infravermelho com Transformada de Fourier
ATR	- Reflexão Total Atenuada
TGA	- Termogravimetria
DTG	- Termogravimetria Derivada
BBM	- <i>Bold Basal Medium</i>
SUPRAS	- <i>Supramolecular solvent extraction</i>
VCA	- Value chain analysis
MW	- Molecular weight
Glc	- Glucose
Sor	- Sorbose
Ara	- Arabinose
Gal	- Galactose
Fuc	- Fucose
Xyl	- Xylose
Rha	- Rhamnose
GlcA	- Glucuronic acid
IdoA	- Iduronic acid
3,6-AnGal	- 3,6-anhydrogalactose

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INTRODUÇÃO

Atualmente a produção de microalgas para síntese de biomoléculas de interesse industrial, como biopigmentos, polissacarídeos e proteínas, é considerada objeto de pesquisa que visa principalmente a viabilidade econômica no processo produtivo e o aumento da biomassa produzida por área. A utilização desses microrganismos tornou-se um fator decisivo e muito pesquisado para a competitividade na produção de metabólitos para aplicação biotecnológica.

A estrutura da célula de microalga permite uma eficiente conversão de energia solar em energia química possibilitando o uso do processo bioquímico para produção de biomassa microalgal e, posteriormente, aplicação industrial de compostos biológicos gerados no processo. A produção de biomoléculas de interesse industrial proveniente dessa biomassa compreende diversas formas de produtos disponíveis no mercado como corantes, antioxidantes, ácidos graxos, enzimas, polímeros e produtos especiais como aminoácidos e esteróis.

Vale ressaltar que dentre os custos existentes no cultivo de microalgas, independente do tipo de cultivo, a fonte de nutrientes é considerado o segundo maior componente do custo de produção, visto que o uso específico dos nutrientes se faz necessário para a eficiência no cultivo. A facilidade de cultivo aliado ao rápido crescimento celular torna a produção de microalgas uma atividade com alto valor agregado, sendo que o uso alternativo de fontes de nutrientes vêm se tornando um aspecto promissor na viabilidade da cadeia produtiva.

Nesse contexto, efluentes agroindustriais possuem características desejáveis e que atendem às demandas exigidas pelas células em cultivos microalgais. Um dos exemplos de resíduo agroindustrial gerado a partir da destilação do etanol nas usinas sucroenergéticas é a vinhaça. No Brasil, a vinhaça é utilizada principalmente como matéria prima na fertirrigação da própria cultura da cana-de-açúcar, apresentando percentuais de macro e micronutrientes indispensáveis à manutenção do solo, porém prejudicial em alta concentração. Assim, o cultivo de microalgas associado ao emprego de vinhaça como meio parcial de cultivo é objeto de pesquisa fundamental para i) diminuição de elementos nocivos ao meio ambiente provenientes da vinhaça; ii) baratear

o custo de produção de microalgas devido a vinhaça ser um resíduo e sem custo; iii) produção de moléculas de alto valor agregado e de importância em diversos setores como farmacêutico, cosmético e alimentício provenientes do beneficiamento da biomassa microalgal produzida e iv) inserir a metodologia da economia circular em processos industriais onde um resíduo passa a ser matéria prima para geração de outros produtos com valor agregado.

JUSTIFICATIVA

Devido a grande diversidade de espécies de microalgas que podem ser empregadas no setor industrial e a produção de pigmentos naturais, há a necessidade da implementação de análises de custo do cultivo em escala de laboratório bem como a avaliação de novas metodologias de tratamento de efluente através do emprego de encapsulamento de células em matriz polissacarídica, com o objetivo de tornar o processo mais sustentável e economicamente viável.

OBJETIVOS

OBJETIVO GERAL

Diante do potencial biotecnológico das microalgas para produção de biomoléculas, o objetivo geral do trabalho consistiu no cultivo de microalgas para avaliação de parâmetros laboratoriais associados à otimização do processo e análise em escala laboratorial do emprego de microalgas para tratamento de efluente agroindustrial.

OBJETIVOS ESPECÍFICOS

Para atingir o objetivo geral, foram desenvolvidos os seguintes objetivos específicos:

- Realizar análise econômica entre meios de cultivo utilizados para produção de biomassa microalgal em escala de laboratório, bem como curva de crescimento e teores de pigmentos fotossintetizantes da microalga *Haematococcus pluvialis*;
- Realizar análise econômica entre meios de cultivo utilizados para produção de biomassa microalgal em escala de laboratório, bem como curva de crescimento e análise de perfil de pigmentos da microalga *Dunaliella salina*;
- Avaliar a metodologia empregada no tratamento biológico da vinhaça de arroz através do cultivo da cianobactéria *Synechococcus pevalekii* encapsulada em esferas de alginato cultivadas em biorreator de leito fluidizado;
- Caracterizar a vinhaça de arroz;
- Otimizar o cultivo de microalgas frente à adição de vinhaça tratada ao meio de cultivo;
- Avaliar e revisar pigmentos microalgais não convencionais com potencial propriedades biológicas;
- Revisar e compilar polissacarídeos da parede celular de microalgas com potencial atividade biotecnológica.

ESTRUTURA DO DOCUMENTO

Esta tese foi escrita em formato de capítulos com o intuito de apresentar os resultados compilados em formato de artigo científico. Vale ressaltar que quatro dos capítulos aqui apresentados foram publicados em periódicos internacionais, um na forma de capítulo de livro e outro ainda será submetido para publicação.

O **primeiro capítulo** apresenta a influência dos principais meios de cultivo na produção de biomassa microalgal de *Haematococcus pluvialis* associada ao custo de produção em escala de bancada. Os resultados foram publicados na revista *Bioresource Technology Reports* em 2019 (doi.org/10.1016/j.biteb.2019.100236).

O **segundo capítulo** avalia a produção de astaxantina obtida da microalga *H. pluvialis* e correlaciona etapas de *upstream* e *downstream* empregados no processo. O levantamento foi publicado em um capítulo de livro (Chapter 7: Production of astaxanthin by *Haematococcus pluvialis*: Lab processes to scale up including the cost considerations) no livro *Global Perspectives on Astaxanthin: From Industrial Production to Food, Health, and Pharmaceutical Applications*, editado por Gokare A. Ravishankar e Ambati Ranga Rao (2021, ISBN 978-0-12-823304-7).

O **terceiro capítulo** demonstra a influência de meios de cultura no cultivo da microalga marinha *Dunaliella salina* e associa o custo de produção em escala laboratorial aos principais pigmentos encontrados na biomassa microalgal na ausência da indução de carotenogênese. O artigo foi publicado na revista *Biotechnology Reports* em 2020 (doi.org/10.1016/j.btre.2020.e00508).

O **quarto capítulo** desta tese retrata a implementação de um bioprocesso inédito utilizando cianobactérias encapsuladas (*Synechococcus pevalekii*) em uma matriz polissacarídica para tratamento biológico da vinhaça obtida da destilação do etanol de arroz. Ainda, o projeto avalia o reuso da vinhaça tratada no cultivo de *D. salina* e analisa a redução de custo. Os resultados foram publicados na revista *Bioprocess and Biosystems Engineering* (doi.org/10.1007/s00449-021-02531-9), além de serem noticiados em diversos sites, incluindo a página da UFPR, e emissoras de rádio e tv.

O **quinto capítulo** revisa pigmentos pouco convencionais produzidos por microalgas. Há poucos dados na literatura reportando pigmentos como echinenone,

botrioxantnina, adonixantina, mixoxantofila, entre outros. Além disso, o trabalho apresenta algumas propriedades biológicas destes pigmentos e avalia métodos utilizados para caratectização química e parâmetros de cultivo. O trabalho está em processo de submissão na revista *Bioresource Technology*.

O **sexto capítulo** apresenta uma revisão de literatura baseada no potencial e na versatilidade de polissacarídeos obtidos principalmente da parede celular das microalgas. Este capítulo mostra a influência de parâmetros de cultivo na produção de polissacarídeos, as principais atividades biológicas relacionadas a essa classe de moléculas, apresenta e discute a diversidade estrutural dos polissacarídeos bem como as perspectivas futuras e principais barreiras encontradas na produção em escala industrial. A revisão bibliográfica foi publicada na revista *Critical Reviews in Biotechnology* (doi.org/10.1080/07388551.2021.1941750).

Vale ressaltar que todos os artigos aqui citados serão apresentados na língua inglesa e em suas versões pré-impressão formatados nas normas estabelecidas pela Universidade Federal do Paraná.

CHAPTER 1

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Media effects on laboratory scale production costs of *Haematococcus pluvialis* biomass



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ABSTRACT

The aim of this study was to conduct an economic analysis and evaluate the physiological features and growth performance of the biomass produced from *Haematococcus pluvialis* at vegetative stage using CHU, BG-11 and WC media under batch culture. Higher cell density was achieved by using CHU and BG-11 media by the 16th day of cultivation, whereas CHU medium promoted the highest pigment content during the microalgae cultivation period. Biochemical analysis showed an increase of 26.3% on protein production when cells were cultivated on BG-11 medium compared to WC medium cultivation. Economic analysis showed that the NPK components contributed to the major participation cost in the media composition. Media cost had shown a variation of US \$2.61–18.36 each 10³ L of produced medium with a biomass production cost of US\$6.20 (WC medium), US\$21.73 (CHU medium), and US\$40.36 (BG-11 medium)/kg in a dry basis.

1. Introduction

Microalgae culture has been demonstrated to produce valuable compounds, such as carotenoids, carbohydrates, proteins, and fatty acids, mainly described in biorefinery facilities (Borowitzka, 2013; Vanthoor-Koopmans et al., 2013). Several studies performed culture media optimization to maximize biomass and biomolecule production from microalgae species (Pábreas et al., 2000; Cheng et al., 2013). Likewise, an important factor to promote specific biomolecule synthesis is the culture medium used for microalgae cultivation, since different genera of microalgae demand different types of media, affecting directly its growth performance (Lananan et al., 2013). Nahidian et al. (2018) evaluated the effects of different media components on the growth of *Haematococcus pluvialis* TMU1. Nitrate and phosphate, iron and boron levels were reported to increase biomass productivity as well as normal cell function, respectively.

Besides biomass production, culture media also changes the metabolic pathways directly affecting the biochemical composition of cells (Nahidian et al., 2018; Kim et al., 2015; Carvalho and Malcata, 2000). Lourenço et al. (2000) reported biological effects obtained from different nitrogen sources on 10 microalgae species. Differences in protein, carbohydrate and chlorophyll contents and on polyunsaturated fatty

acid profiles were reported. The biochemical composition is crucial to determine which biomolecule of interest is considered a valuable compound and it might influence the production cost by media composition, upstream and downstream process (Li et al., 2011; Carvalho and Malcata, 2000). Since the specific use of nutrients is required for the efficiency in microalgae cultivation, the components from the culture media are considered the second largest component of the production costs (Vonshak, 1997).

H. pluvialis costs evaluation research is fundamental to produce chlorophylls and xanthophylls, mainly astaxanthin (3, 3'-dihydroxy- β , β' -carotene-4, 4'-dione), at a lower cost than the synthetic molecule that it is currently produced. The astaxanthin content in *H. pluvialis* cells might reach up to 3.8% in a dry weight basis (Ambati et al., 2014) and a theoretical assessment of an astaxanthin industrial plant from *H. pluvialis* was estimated around US\$ 718/kg of astaxanthin (Li et al., 2011).

The achievement of high-value products depends on the knowledge of the standard medium and its implication on the cell metabolic pathways of the vegetative phase. This study investigated the physiological effects of different freshwater media on *H. pluvialis* vegetative stage and correlated them with the production cost at laboratory scale. The experiments were done using one-stage cultivation with no influence of stress on the culture.

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MEDIA EFFECTS ON LABORATORY SCALE PRODUCTION COSTS OF *Haematococcus pluvialis* BIOMASS

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1.1 ABSTRACT

The aim of this study was to conduct an economic analysis and evaluate the physiological features and growth performance of the biomass produced from *Haematococcus pluvialis* at vegetative stage using CHU, BG-11 and WC media under batch culture. Higher cell density was achieved by using CHU and BG-11 media by the 16th day of cultivation, whereas CHU medium promoted the highest pigment content during the microalgae cultivation period. Biochemical analysis showed an increase of 26.3% on protein production when cells were cultivated on BG-11 medium compared to WC medium cultivation. Economic analysis showed that the NPK components contributed to the major participation cost in the media composition. Media cost had shown a variation of US\$2.61-18.36 each 10³ L of produced medium with a biomass production cost of US\$6.20 (WC medium), US\$21.73 (CHU medium), and US\$40.36 (BG-11 medium) per kilogram in a dry basis.

Keywords: Microalgae. Economic analysis. Pigment content. Biomass. High-valuable compounds.

1.2 INTRODUCTION

Microalgae culture has been demonstrated to produce valuable compounds, such as carotenoids, carbohydrates, proteins, and fatty acids, mainly described in biorefinery facilities (Borowitzka, 2013; Vanthoor-Koopmans, Wijffels, Barbosa, & Eppink, 2013). Several studies performed culture media optimization to maximize biomass and biomolecule production from microalgae species (Cheng, Ren, & Ogden, 2013; Fábregas, Domínguez, Regueiro, Maseda, & Otero, 2000). Likewise, an important factor to promote specific biomolecule synthesis is the culture medium used for microalgae cultivation, since different genera of microalgae demand different types of media, affecting directly its growth performance (Lananan, Jusoh, Ali, Lam, & Endut, 2013). Nahidian et al. (2018) evaluated the effects of different media components on the growth of *Haematococcus pluvialis* TMU1. Nitrate and phosphate, iron and boron levels were reported to increase biomass productivity as well as normal cell function, respectively.

Besides biomass production, culture media also changes the metabolic pathways directly affecting the biochemical composition of cells (Carvalho & Malcata, 2000; Kim et al., 2015; Nahidian et al., 2018). Lourenço et al. (2002) reported biological effects obtained from different nitrogen sources on 10 microalgae species. Differences in protein, carbohydrate and chlorophyll contents and on polyunsaturated fatty acid profiles were reported. The biochemical composition is crucial to determine which biomolecule of interest is considered a valuable compound and it might influence the production cost by media composition, upstream and downstream process (Carvalho & Malcata, 2000; J. Li, Zhu, Niu, Shen, & Wang, 2011). Since the specific use of nutrients is required for the efficiency in microalgae cultivation, the components from the culture media are considered the second largest component of the production costs (Venkataraman, 1997).

H. pluvialis costs evaluation research is fundamental to produce chlorophylls and xanthophylls, mainly astaxanthin (3, 3'-dihydroxy- β , β' -carotene-4, 4'-dione), at a lower cost than the synthetic molecule that it is currently produced. The astaxanthin content in *H. pluvialis* cells might reach up to 3.8% in a dry weight basis (Ambati, Phang, Ravi, & Aswathanarayana, 2014) and a theoretical assessment of an astaxanthin industrial plant

from *H. pluvialis* was estimated around US\$ 718 per kilogram of astaxanthin (J. Li et al., 2011).

The achievement of high-value products depends on the knowledge of the standard medium and its implication on the cell metabolic pathways of the vegetative phase. This study investigated the physiological effects of different freshwater media on *H. pluvialis* vegetative stage and correlated them with the production cost at laboratory scale. The experiments were done using one-stage cultivation with no influence of stress on the culture.

1.3 MATERIAL AND METHODS

Microalgae strain and stock culture maintenance

H. pluvialis (strain 015) was obtained from the culture collection of Federal University of São Carlos, Brazil, and maintained in test tubes using 10 mL CHU medium (ABNT, 1992). Media composition is shown in Table 1. To proceed with the experimental steps, a stock culture was prepared, photoautotrophically grown in a two-liter sterile Erlenmeyer flask (10% v/v inoculation), with a working volume of 1600 mL in the same media and maintained at 23 ± 1 °C under 12:12 light/dark cycle ($100 \mu\text{mol m}^{-2} \text{s}^{-1}$) for 19 days.

Media and experimental growth conditions

H. pluvialis was cultivated experimentally in three well known freshwater media previously described (Table 1), namely WC (R. R. L. Guillard & Lorenzen, 1972), CHU, and BG-11 (Stanier, Kunisawa, Mandel, & Cohen-Bazire, 1971).

Table 1 - Media composition (mM L^{-1}) and chemical characteristics

Components	WC	CHU	BG-11
NaNO ₃	1.000	2.941	17.649
MgSO ₄ .7H ₂ O	0.149	0.304	0.304
CaCl ₂	0.331	0.225	0.324
K ₂ HPO ₄	-	1.004	0.293

NaHCO ₃	0.150	-	-
KH ₂ PO ₄	0.064	0.551	-
Na ₂ EDTA	0.012	0.148	0.002
FeCl ₃ ·6H ₂ O	0.011	-	-
NaCl	-	0.427	-
KOH	-	0.552	-
FeSO ₄	-	0.032	-
ZnSO ₄ ·7H ₂ O	7.650 x 10 ⁻⁵	3.067 x 10 ⁻⁵	7.719 x 10 ⁻⁴
(NH ₄) ₅ Fe(C ₆ H ₄ O ₇) ₂	-	-	0.010
H ₃ BO ₃	0.016	0.184	0.046
C ₆ H ₈ O ₇	-	-	0.031
CoCl ₂ ·6H ₂ O	7.702 x 10 ⁻⁵	-	-
CuSO ₄ ·5H ₂ O	6.265 x 10 ⁻⁵	9.837 x 10 ⁻⁶	4.949 x 10 ⁻⁴
Co(NO ₃) ₂ ·6H ₂ O	-	1.683 x 10 ⁻⁶	1.697 x 10 ⁻⁴
MnCl ₂ ·4H ₂ O	9.095 x 10 ⁻⁵	7.276 x 10 ⁻⁶	0.009
Na ₂ CO ₃	-	-	0.188
Na ₂ MoO ₄ ·2H ₂ O	2.479 x 10 ⁻⁵	2.934 x 10 ⁻⁶	0.001
Pyridoxine	2.955 x 10 ⁻⁶	-	-
Thiamine	3.768 x 10 ⁻⁴	-	-
Cyanocobalamin	3.689 x 10 ⁻⁷	-	-
pH	7.5	7.5	7.5
Conductivity (mS cm ⁻¹)	0.83	2.84	6.92

The media were prepared individually in two-liter Erlenmeyer flasks with a working volume of 1600 mL. All the reagents used were of analytical grade. pH was adjusted at 7.5 and the flasks were autoclaved. When the stock culture from the previous step reached the late exponential phase, about 13×10^4 cells mL⁻¹, the cells were collected via centrifugation at 1330 x g for 5 min. The supernatant was discarded to avoid nutrient interferences, and inoculation was done in each medium based on 10% v/v final concentration. Each experiment was carried out for 22 days under 12:12 light/dark cycle with cool white fluorescent lamps by the side of the flasks providing 100 μ mol m⁻² s⁻¹. During all the incubation time, continuous compressed air was supplied at the bottom of each flask with a flow rate of 3 L min⁻¹ (without supplementary CO₂).

Measurement of cell growth and pigment content

All analyses were done in 48 h intervals. Cell counting measurements were carried out by optical microscopy with a Neubauer hemocytometer for cell growth and cell morphology analysis. pH and conductivity were measured to monitor the variation in the culture. Pigment content analyses were performed during the entire cultivation period. The biomass was prepared by filtration of 5 mL of culture using a Macherey-Nagel GF-1 glass-fiber filter and added into light-protected centrifuge tubes containing with 4 mL of 90% acetone solution. The tubes were maintained at 4 °C about 18 h for total extraction. The pigments were centrifuged (1915 x g for 10 min) to remove colorless filter fragments and the supernatant was collected. The optical absorbance of the supernatant was determined at 480, 510, 664 and 750 nm using a spectrophotometer BEL SP1105. Pheophytin _a determination demanded a 12µL addition of 0.25M HCl into the cuvette and it needed to be read again at 664 and 750 nm. Chlorophyll _a (Chl), pheophytin _a (Phe) and total carotenoid content (Car) were calculated according to Eqs. 1, 2 (Carl Lorenzen, 1967), and 3 (Strickland & Parsons, 1968), respectively:

$$[\text{Chl } (\mu\text{g L}^{-1})] = \frac{26.73 \times [(A_{664 \text{ nm}} - A_{750 \text{ nm}}) - (A_{664a \text{ nm}} - A_{750a \text{ nm}})] \times V_a}{(V_c \times c)} \quad (1)$$

$$[\text{Phe } (\mu\text{g L}^{-1})] = \frac{26.73 \times [1.8 \times (A_{664a \text{ nm}} - A_{750a \text{ nm}}) - (A_{664 \text{ nm}} - A_{750 \text{ nm}})] \times V_a}{(V_c \times c)} \quad (2)$$

$$[\text{Car } (\mu\text{g L}^{-1})] = \frac{[7.6 \times A_{480 \text{ nm}} - (3 \times A_{750 \text{ nm}}) - 1.49 \times A_{510 \text{ nm}} - (2 \times A_{750 \text{ nm}})] \times V_a}{(V_c \times c)} \quad (3)$$

where *A* corresponds to absorbance at the specific wavelength, *V_a* is the volume (L) of 90% acetone solution used for the extraction process, *V_c* is the culture volume (L) used for the filtration, *c* is the optical path (cm) from the cuvette and *a* is the reading after acid addition.

Biomass production and growth performance

Dry biomass production (g L^{-1}) was quantified by means of dry weight using filtration process. Culture samples (30 mL) were filtered using a pre-weighted Macherey-Nagel GF-1 glass-fiber filter and dried at $80\text{ }^{\circ}\text{C}$ overnight to determine biomass dry weight. Biomass productivity ($\text{g L}^{-1} \text{ d}^{-1}$) was calculated from Eq. 4:

$$\text{Productivity} = \frac{(X_f - X_i)}{(t_f - t_i)} \quad (4)$$

where X_f and X_i correspond to final and initial biomass (g L^{-1}), respectively related to final (t_f) and initial time (t_i). Microalgae growth performance was measured by the following equations 5-8 (R. Guillard, 1973; Wood, Everroad, & Wingard, 2005):

Growth rate

$$(r) = \frac{\ln(N_f/N_i)}{t_f - t_i} \quad (5)$$

Doubling time

$$(T_2) = \frac{0.6931}{r} \quad (6)$$

Divisions per day

$$(k) = \frac{r}{0.6931} \quad (7)$$

Maximum cellular yield

$$(R) = N_f - N_i \quad (8)$$

where N_f and N_i correspond to final and initial cell density, respectively related to their specific final (t_f) and initial time (t_i) in days.

Biochemical analyses

At the end of the experiments, *H. pluvialis* biomass was centrifuged and lyophilized for biochemical analyses. Protein content was determined by the Lowry method (Lowry, Rosebrough, Faar, & Randall, 1951) using bovine serum albumin as a standard, while total lipids were extracted by the Bligh and Dyer method (Bligh & Dyer, 1959) and quantified gravimetrically. Total carbohydrates were determined by the phenol-sulfuric method (DuBois, Gilles, Hamilton, Rebers, & Smith, 1956) and quantified by a standard glucose curve at 490 nm. Protein, lipid and total carbohydrate were expressed as percentage.

Fourier-transform infrared spectroscopy (FTIR) analysis

FTIR spectra were collected on a FTIR – ALPHA II Bruker® spectrometer equipped with an attenuated total reflectance (ATR) accessory. Approximately 2-4 mg of freeze-dried biomass were applied to the surface of the crystal and then analyzed between 4000 and 400 cm^{-1} at 4 cm^{-1} resolution and 24 scans. FTIR spectroscopy can be used as a molecular fingerprint to evaluate microalgae biomass and to identify its functional groups.

Thermogravimetric (TG) and derivative thermogravimetric (DTG) analysis

Thermogravimetric analyses were determined on a NETZSCH STA 449F3 Jupiter® simultaneous thermal analyzer. Baseline was corrected by subtraction of determined baseline with no sample analyzed at the same conditions. The biomass was heated from room temperature up to 800 °C following a heating rate of 10 °C min^{-1} .

Economic analysis

Media costs were obtained by evaluating the component price individually using quotations from regional laboratory companies. The minimum cost of each medium component was chosen by comparing the cost among the items from the quotations. The information was used to compare total media cost, individual component cost and biomass production cost at laboratory scale.

Statistical analysis

All experiments were carried out in triplicate and the results were expressed as mean $\pm$ SD (Standard Deviation). Significant differences were determined using one-way analysis of variance (ANOVA) at 95% confidence interval and Tukey's test ($p < 0.05$).

1.4 RESULTS AND DISCUSSION

H. pluvialis cell concentration in different media

All tested media have shown favorable conditions to grow *H. pluvialis* cells since they increased biomass production. In order to compare the growth conditions among the media used in each experiment, it was necessary to establish the same initial cell concentration in all flasks (2×10^4 cell mL^{-1}).

A similar pattern was found in cell density evolution between CHU and BG-11 media during the entire experimental time (22 days). The highest cell density was $33.25 \times 10^4 \pm 2.13$ and $31.33 \times 10^4 \pm 1.52$ cells mL^{-1} , at day 16 for each medium respectively (Fig.1). No significant difference was observed between CHU and BG-11 media in terms of cell density (see Table 2).

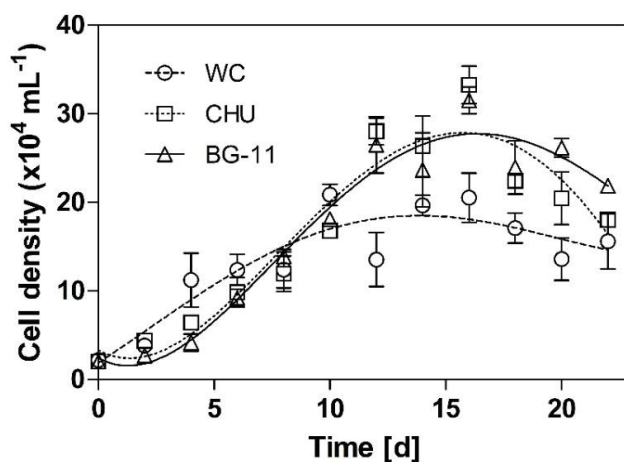


Fig. 1 - Growth curve of *Haematococcus pluvialis* during 22 days in three different media

At day 10, the cells cultured in WC medium started to decline and remained at this level until the end of the experiment. WC medium also showed altered cells at the end of

the cultivation presenting initial aplanospore formation such as the transition between green coccoid cells to red intermediate cells. This can be explained by the low nitrate concentration of WC medium when compared to CHU and BG-11 media (see Table 1). Low nitrate content can induce the formation of reddish palmella cells in *H. pluvialis* cultivation (Borowitzka, Huisman, & Osborn, 1991). Normal morphology was observed in cells grown in BG-11 and CHU media during the same period. Sipaúba-Tavares et al. (2015) showed cell density of 21×10^4 cells mL⁻¹ on 26 days of *H. pluvialis* cultivation using WC medium with no altered cells reported. Similar studies have shown that there is a preference of each microalgae species for a specific culture medium, affecting growth rate and pigment content (Lananan et al., 2013; Nahidian et al., 2018).

It should be noted that low cell density implies in low biomass productivity and consequently increases the facility production cost. Therefore, the correct medium directly influences the final cost of the product in addition to its downstream process total cost (J. Li et al., 2011).

Although there is available information related with the vegetative stage of *H. pluvialis*, it is difficult to compare those results due to the heterogeneity of culture media used, optimization processes that are applied to the process and the focus only on astaxanthin production (Lababpour et al., 2005; Ledda, Tamiazzo, Borin, & Adani, 2016). The present study showed that the CHU and BG-11 media provided the most suitable nutrient conditions for the development of *H. pluvialis* cells at the vegetative stage, as well as the highest cell density among the tested media.

Comparison of growth performance

All growth performance data were calculated for the same time intervals. According to Table 2, CHU and BG-11 media showed the highest *H. pluvialis* cellular density in the study (33.2 and 31.3×10^4 cells mL⁻¹, respectively), while WC media reached $20.8 \pm 1.23 \times 10^4$ cells mL⁻¹ at 10 days of cultivation (Fig.1). Fábregas et al. (2000) presented *H. pluvialis* cell density of 37.7×10^4 cells mL⁻¹ using an optimized culture medium. The results from the current study were lower due to the lack of optimization in the culture media since our aim was to verify differences among commonly used media in microalgae cultivation.

Table 2 - Growth parameters of *Haematococcus pluvialis* in different culture media and physicochemical parameters of culture media*

Parameters	WC	CHU	BG-11
Cellular density ($\times 10^4$ cells mL ⁻¹)	20.8 $\pm$ 1.23 ^a	33.2 $\pm$ 2.13 ^b	31.3 $\pm$ 1.52 ^b
Division per day (k)	0.26	0.31	0.30
Doubling time (T ₂)	3.81	3.22	3.28
Growth rate (r)	0.181	0.215	0.211
Maximum cellular yield (R) ($\times 10^4$ cells mL ⁻¹)	18.6	31.2	29.3
Dry biomass (g L ⁻¹)	0.40 $\pm$ 0.04 ^a	0.49 $\pm$ 0.05 ^a	0.45 $\pm$ 0.04 ^a
Productivity (g L ⁻¹ d ⁻¹)	0.020	0.024	0.022
Final pH	8.08 $\pm$ 0.09 ^a	8.76 $\pm$ 0.05 ^b	8.85 $\pm$ 0.14 ^b
Final conductivity (mS cm ⁻¹)	0.61 $\pm$ 0.05 ^a	1.98 $\pm$ 0.06 ^b	4.60 $\pm$ 0.11 ^c

*Average followed by the same letter do not significantly differ (p<0.05).

H. pluvialis cultured in different media can produce different cell concentrations. Dalay et al. (2007), reported a biomass production of 0.64 g L⁻¹ using BG-11 medium in comparison with fertilizer-based media to provide economical profit.

CHU medium showed the highest growth parameter in *H. pluvialis* cultivation. The cells presented more divisions per day, lower doubling time, higher growth rate and maximum cellular yield. WC media presented lower growth when compared with CHU and BG-11 results. The difference on NaNO₃ concentration among media was reported to decrease *H. pluvialis* growth rate during the exponential phase (Orosa, Franqueira, Cid, & Abalde, 2005).

The conductivity at the end of the study showed a significantly decrease of 26.0, 30.3 and 33.5% for WC, CHU and BG-11 media from the initial conductivity, respectively. The conductivity is commonly used for monitoring nutrient consumption in other research areas (Gent, 2003), and it is enticing to employ this parameter in microalgae cultivation.

pH values increased in all tested media. A significant difference was observed between pH from WC medium and pH from CHU and BG-11 media. Similar pH enhancements were reported previously (Borowitzka et al., 1991; Lababpour et al., 2005) and are probably due to the consumption of nitrate and phosphate from the culture medium.

Pigment content

Spectrophotometric measurements were carried out to check the effects of different media on the production of photosynthetic pigments during *H. pluvialis* cultivation (Fig. 2). The *H. pluvialis* cells cultivated in the CHU medium showed the highest chlorophyll *a* and pheophytin *a* contents (5.16 ± 0.31 and $1.57 \pm 0.15 \mu\text{g L}^{-1}$, respectively) at 16 days of cultivation. The chlorophyll *a* content was 29% higher than the result obtained using BG-11 medium. The pigment content can vary based on nutrients added or removed from the medium. Total chlorophyll content from *H. pluvialis* was reported to increase 28% by using an iron rich culture medium in the cultivation process (Nahidian et al., 2018).

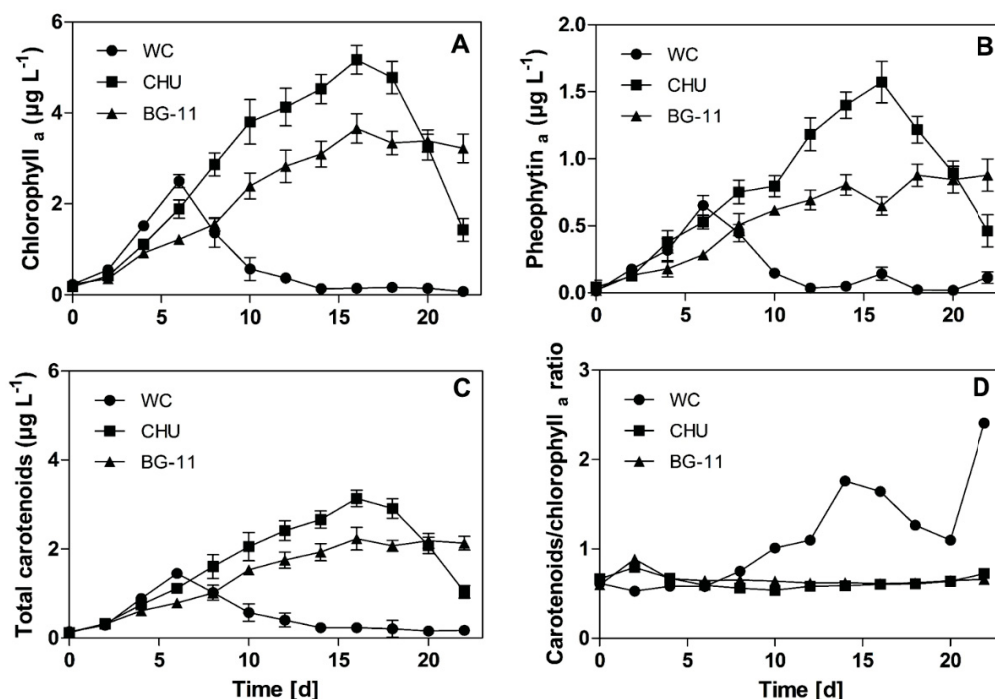


Fig. 2 - *Haematococcus pluvialis* pigment profiles in different media. Chlorophyll *a* (A), Pheophytin *a* (B), Total carotenoids (C), Carotenoids/Chlorophyll *a* ratio (D)

Biomass produced with BG-11 medium presented lower chlorophyll and pheophytin production compared with CHU medium. BG-11 medium showed a chlorophyll *a* production of $3.65 \pm 0.32 \mu\text{g L}^{-1}$ also at 16 days of cultivation. The pheophytin

a content reaches the maximum production around 14 days and maintains the same content until the end of the cultivation period.

WC medium showed a different pigment pattern when compared with the other tested media. Both chlorophyll *a* and pheophytin *a* contents reach the maximum production at 6 days of cultivation (2.49 ± 0.14 and $0.65 \pm 0.07 \mu\text{g L}^{-1}$, respectively). This may be due to the decline of microalgal cell density as shown in Fig. 1.

The decrease of chlorophyll content and increase of total carotenoids production (Fig. 2 C) depends on the cell encystment and nutrient availability from the medium. Sarada et al. (2002) reported a decrease of 90% of chlorophyll content in cultures that use potassium nitrate and ammonium nitrate as nitrogen sources and 50% in calcium nitrate cultures. Non-stressed microalgae cultivation maintains chlorophyll and carotenoid contents at constant level before N-deficiency, which can be seen by Figure 2D. After this point a massive chlorophyll decline is observed (Orosa et al., 2005). Therefore, media containing low nitrogen content can promptly inhibit the chlorophyll biosynthesis and lower the photosynthetic response from the cell, directly affecting the cell cycle and biomass production (Orosa et al., 2005; Scibila, Girolomoni, Berteotti, Alboresi, & Ballottari, 2015).

Microalgae biomass biochemical composition

Biomass biochemical composition was determined at the end of the cultivation period (Table 3).

Table 3 - Biochemical composition of *Haematococcus pluvialis* biomass*

Parameters (%)	WC	CHU	BG-11
Proteins	10.9 ± 1.09^a	34.2 ± 0.50^b	41.4 ± 2.02^c
Lipids	18.6 ± 1.15^a	11.3 ± 1.15^b	17.3 ± 2.30^c
Total carbohydrates	11.6 ± 0.44^a	17.2 ± 0.44^b	21.1 ± 1.68^b

*Average followed by the same letter do not significantly differ ($p < 0.05$).

Biomasses produced using CHU and BG-11 media showed the highest protein and carbohydrate contents, differing significantly from WC medium. Biomass from BG-11

medium presented an increase of protein content compared to WC and CHU media up to 26.3 and 17.3%, respectively. Biochemical composition of *H. pluvialis* sp. KORDI03 isolated in Korea showed 21.2% of protein content and 22.2% of total lipid using BG-11 as medium (Kim et al., 2015). Cell crude composition of *H. pluvialis* can vary from “green” to “red” stage cultivation mainly on proteins (29-45%) and carbohydrates content (15-17%) (Grewe & Griehl, 2012).

The carbohydrate content can increase massively in stressed *H. pluvialis* up to 63% in a dry weight basis (Boussiba & Vonshak, 1991). Biomass from BG-11 and CHU media increased the carbohydrate content up to 45 and 32.5%, respectively, when compared to biomass obtained with WC medium. The high carbohydrate content in the cell wall can be enticing since it can be explored to isolate polysaccharides and to study its potential bioactivity (Liu et al., 2018).

Biomass from WC medium presented higher lipid content (18.6%) among all media tested. Increase of lipid content in *H. pluvialis* cultivation can be explained by the physiological changes in microalgae cell by cellular stress and/or nutrient depletion (Damiani, Popovich, Constenla, & Leonardi, 2010; Xin, Hong-ying, Ke, & Ying-xue, 2010). In green stage, total lipids are predominantly deposited in the cells varying from 20 to 25% DW (Grewe & Griehl, 2012; Shah, Liang, Cheng, & Daroch, 2016), whereas, a lower lipid content at a non-stress *H. pluvialis* cultivation was reported (Damiani et al., 2010), which is consistent with the present study. The use of different microalgal media was found to have a significant effect on cell biochemical composition, mainly in lipid production as reported by George et al. (2014) on *Ankistrodesmus falcatus* cultivation.

FTIR spectroscopy

FTIR spectroscopy is widely used to rapidly analyze microalgal biomass and to compare biomass profiles. All biomass spectra showed similar bands with some differences on intensity. Data available in the literature were used to identify the main components as well as to compare the data (Haque, Dutta, Thimmanagari, & Chiang, 2017). The FTIR analysis of *H. pluvialis* biomass from this study reveals the presence of organic compounds with an aliphatic characteristic ($400\text{-}800\text{ cm}^{-1}$), containing phenol and

alcoholic groups ($1000\text{-}1400\text{ cm}^{-1}$), carbonyl groups ($1500\text{-}1700\text{ cm}^{-1}$), and hydroxyl groups ($3200\text{-}3400\text{ cm}^{-1}$) (Haque et al., 2017; Sudhakar & Premalatha, 2015).

The band located at 2920 cm^{-1} refers to asymmetric C-H stretching vibrations due to the methylene groups from lipids. There are variations at the bands within the $3000\text{-}2800\text{ cm}^{-1}$ region reflecting the differences on lipid production among the media tested. The intensity of the absorption at 1015 cm^{-1} (related to carbohydrates) is higher when compared to the 2918 cm^{-1} region (related to lipids). Similar results were reported from *H. pluvialis* biomass cultivated using bioethanol wastewater under $23 \pm 2\text{ }^{\circ}\text{C}$ and 12 h/12 h light/dark cycle (600 W Metal halide lamp) (Haque et al., 2017).

The protein content is represented at the $1800\text{-}1500\text{ cm}^{-1}$ region where there is a diversification on the intensity due to the higher protein production in biomasses cultivated in BG11 and CHU media in agreement with the results shown in Table 3. The spectra profiles are similar to those previously reported for microalgae biomass (Haque et al., 2017; Sudhakar & Premalatha, 2015).

TGA and DTG curves analysis

The thermogravimetric analyses (TGA) and the rate of weight loss (DTG) curves of *H. pluvialis* biomasses at a heating rate of $10\text{ }^{\circ}\text{C min}^{-1}$ are shown in Fig. 3. TG/DTG curves of all biomasses showed a similar pattern. Weight loss occurs in two steps for biomasses obtained from BG-11 and CHU media and in three steps when using WC medium (Fig. 3B). The small peak from DTG curves around $100\text{ }^{\circ}\text{C}$ represents residual water corresponding to the drying stage. The major peak around $300\text{ }^{\circ}\text{C}$ corresponds to weight loss due to thermal decomposition of organic molecules such as proteins and carbohydrates.

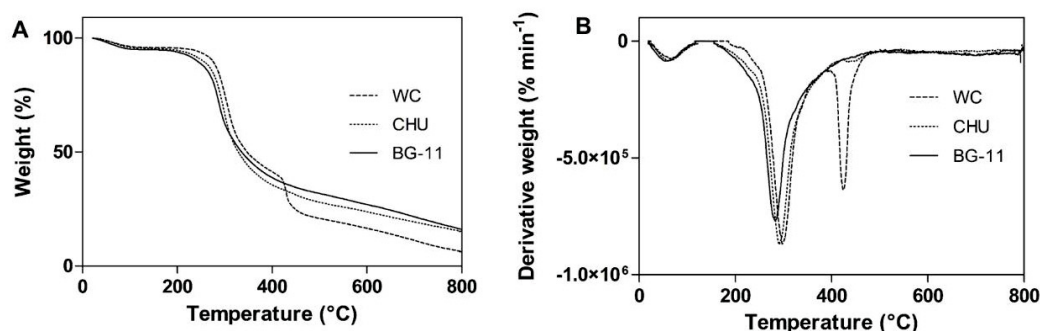


Fig. 3 - Thermochemical characteristics of *H. pluvialis* at heating rate of 10 °C min⁻¹. TGA (A) and DTG curves (B) at 10 °C min⁻¹

Thermal decomposition of chemical compounds follows the order of carbohydrate, protein and lipid. Lipid functional groups are already decomposed at 400 °C. The peak on DTG curve of the biomass from WC medium at 432 °C probably represents an initial astaxanthin production due to cellular stress (Fig. 2D). The color of biomass and cell characteristics could be an indicative of xanthophyll accumulation since there was no specific astaxanthin analysis in this study. Biomass from CHU and BG-11 media do not present altered color or changes in cell morphology.

The weight loss pattern of biomasses produced using BG-11 and CHU media was similar to results found from pyrolysis of *Chlorella* biomass (Hu, Jiang, & Ma, 2018). A similar pattern was also reported for the decomposition of *Tetraselmis suecica* (Chlorophyta) from non-stressed microalgae cultivation (F. Li, Srivatsa, Batchelor, & Bhattacharya, 2017).

Table 4 presents the initial, final and maximum decomposition of *H. pluvialis* biomasses as well as their volatile matter percent. Li et al. (2017) presented similar results for *T. suecica*, in terms of initial and maximum decomposition temperatures. However, the volatile matter was lower (55-70%) when compared with the results obtained in the present study (Table 4) indicating differences in the biomass chemical composition of these microalgae species.

Additionally, differences among thermal decomposition of the biomasses produced in the tested media were observed. TG/DTG curves of the biomass obtained in WC medium showed higher temperature values and volatile matter than those produced with

the other two media. These differences could be explained by changes in the microalga metabolism and the activation of xanthophyll biosynthesis and consequent accumulation during the cultivation period in WC medium.

Table 4 - TG decomposition parameters of *Haematococcus pluvialis* biomass grown in different media

Medium	T _i (°C) ^a	T _f (°C) ^b	T _{max} (°C) ^c	VM (%) ^d	AC (%) ^e
WC	186	471	297	93.7	6.3
CHU	157	464	292	84.8	15.2
BG-11	163	444	287	83.8	16.2

^a Initial temperature of the biomass main decomposition.

^b Final temperature of the biomass main decomposition.

^c Maximum decomposition temperature.

^d Volatile matter (from 20°C to 800°C).

^e Ash content

From Table 4, the ash content of *H. pluvialis* biomasses can be calculated (% DW). Ash is the final product of the biomass thermal process, including pyrolysis and gasification. Differences were observed in the ash content (6.3, 15.2 and 16.2%) of the biomasses produced in WC, CHU, and BG-11 media, respectively. A great variation of ash content found in *H. pluvialis* cultivation was reported in the literature (Pan & Chien, 2009; L. Sipaúba-Tavares, Millan, & Berchielli-Morais, 2013). Bi and He, (2013) showed a 13.8-18.8% ash variation from green microalgae on a dry weight basis. That is possibly related with the inorganic compounds present in the culture medium and the phase of the cell cycle occurring at a specific period of microalgae cultivation.

Media economical evaluation

The economical evaluation of the used culture media is shown in Table 5. All media costs were calculated based on batch-culture mode considering 10³ L medium and the estimated cost to produce 1 kilogram of dry biomass. Biomass produced using BG-11 medium presented a 10³ L laboratory cost around 86 and 37% higher than WC and CHU media, respectively. Sipaúba-Tavares et al. (2015) showed a 10³ L WC medium cost of US\$1.31 and compared with NPK addition to minimize the cost effect.

Even with differences in biomass production, the estimated biomass contribution of culture medium cost to biomass production cost followed the same pattern: BG-11 medium presented higher biomass cost when compared with WC and CHU media. WC medium showed a contribution to biomass cost of US\$ 6.20; even with a lower cell density and pigment content, the WC-produced biomass is three times cheaper than CHU-produced biomass. The total cost of *H. pluvialis* biomass, including operational cost, raw materials, utilities and labor, was reported through an economic assessment of a hypothetical plant to be around US\$ 18 per kilogram (J. Li et al., 2011). The estimated cost could be even lower depending where the facility is located.

Table 5 - Economical evaluation of media used in the experiments (in US dollars)^a

Cost	WC	CHU	BG-11
10 ³ L medium	2.61	11.55	18.36
Kg of dry biomass	6.20	21.73	40.36

^a Average exchange rate (February 2018) US\$1.00 = R\$3.22.

Macro and micronutrients from each medium play an important role in the economical evaluation. According to Fig. 4, the components with the major cost participation were macronutrients, mainly nitrogen (N), phosphorus (P) and potassium (K). Components that presented less than 0.1% of cost participation were not listed.

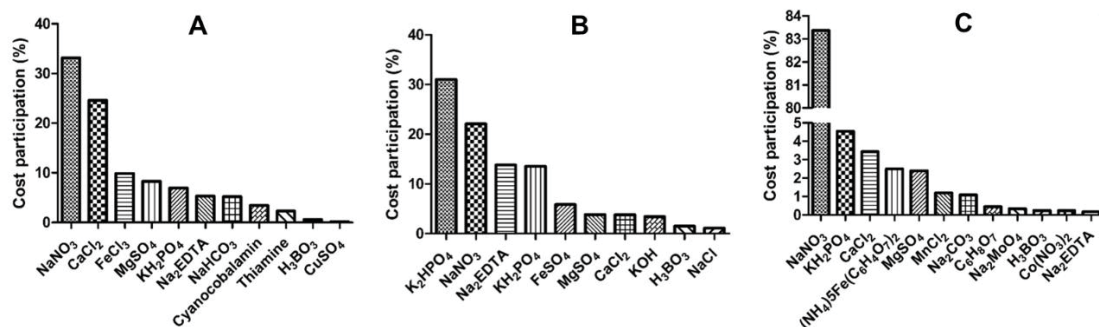


Fig. 4 - Component participation in the final media cost of WC (A), CHU (B) and BG-11 (C) media

The cost of each component is highly related to the regional market and it can differ depending on the place. Furthermore, the major cost variation among the media was caused by the NPK components used in their compositions. The components with the highest cost participation were NaNO_3 (33.1%), K_2HPO_4 (31%) and NaNO_3 (83.3%) at the WC, CHU and BG-11 media composition, respectively. Besides, WC and CHU media presented CaCl_2 (24.6%) and NaNO_3 (22.1%), respectively, which contribute to most of the medium cost at laboratory scale. NPK components are the most essential nutrients for cellular maintenance as well as for the synthesis of ATP, proteins, enzymes and nucleic acids (Stanbury, Whitaker, & Hall, 2003).

Medium components cost participation is crucial since it directly reflects in the final microalgae production cost. The addition of low-cost nutrients, mainly residues, in an interesting research area because it could decrease media cost and increase the production of valuable compounds from microalgae (Haque et al., 2017; Ledda et al., 2016; L. H. Sipaúba-Tavares et al., 2015).

1.5 CONCLUSIONS

Vegetative growth of *H. pluvialis* cultivation is influenced by the media used, mainly in cellular biochemical composition, pigment content and final production cost at a laboratory scale. Biomass obtained from CHU medium showed superior pigment content while BG-11 medium presented higher protein production. The higher cost among the media owed to NPK components that are used in the media composition. The use of specific media to produce microalgal biomass may allow further exploitation depending on the biomolecule of interest and it can be a suitable choice for the production of high-value products.

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Production of astaxanthin by Haematococcus pluvialis: Lab processes to scale up including the cost considerations

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Abbreviations

BBM	bold basal medium
BG-11	blue-green medium
NPK	nitrogen, phosphorus, and potassium
S_f/V	surface/volume ratio
SUPRAS	supramolecular solvent
WC	water culture
WT	weight

1 Introduction

The increase in population growth and market regulation about synthetic molecules has led to the exploitation of large-scale cultivation of microorganisms with an attempt to produce sustainable bioproducts. Among these natural molecules, astaxanthin (3,3'-dihydroxy- β,β' -carotene-4,4'-dione), a secondary carotenoid produced by several microorganisms, is widely used as a powerful antioxidant in food, pharmaceutical, and medical industries. The green biflagellate microalga *Haematococcus pluvialis* Flotow (Chlamydomonadales, Chlorophyta) is considered a cell factory of natural astaxanthin, which composes up to 3.8–5.0wt% of its total dry biomass [1], and it is cultivated to produce a great amount of astaxanthin in large scale worldwide.

Astaxanthin-producing *H. pluvialis* consists of two cultivation cycles, a green motile stage and a red nonmotile one. The former is related with the increase of cell concentration, while the

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2.1 ABSTRACT

The current global and economic scenario has driven the search for alternative source of natural colorants produced by microalgae. Astaxanthin (3,3'-dihydroxy- β,β' -carotene-4,4'-dione) is a secondary carotenoid widely used in food, pharmaceutical and aquaculture industries with antioxidant effects. The green microalga *Haematococcus pluvialis* is the richest source worldwide of natural astaxanthin, produced with lower cost than the synthetic molecules currently used. This chapter reviews some laboratory parameters related to *H. pluvialis* cell enhancement, astaxanthin production and cost. The review begins with an introduction about astaxanthin-producing *H. pluvialis* cells followed by upstream process conditions (e.g., microalgal media effects on biomass productivity and carotenogenesis). Subsequently, downstream process parameters are evaluated (e.g., astaxanthin extraction methods and efficiency) and laboratory production cost and future prospects of this topic are presented. Economic challenges and laboratory conditions of astaxanthin production by *H. pluvialis* have been reviewed and discussed in view of a cost-effective astaxanthin facility.

Keywords: Microalgae. Pigment. Astaxanthin cost. Biomass production. Challenges. Laboratory parameters.

2.2 GENERAL INTRODUCTION

The increase in population growth and market regulation about synthetic molecules has led to the exploitation of large-scale cultivation of microorganisms with an attempt to produce sustainable bioproducts. Among these natural molecules, astaxanthin (3,3'-dihydroxy- β,β' -carotene-4,4'-dione), a secondary carotenoid produced by several microorganisms, is widely used as a powerful antioxidant in food, pharmaceutical and medical industries. The green biflagellate microalga *Haematococcus pluvialis* Flotow (Chlamydomonadales, Chlorophyta) is considered a cell factory of natural astaxanthin which composes up to 3.8-5.0 wt% of its total dry biomass (Wayama et al., 2013) and it is cultivated to produce a great amount of astaxanthin in large scale worldwide.

Astaxanthin-producing *H. pluvialis* consists of two cultivation cycles, a green motile stage and a red non-motile one. The former is related with the increase of cell concentration while the latter is related to enhancement of astaxanthin inside the cell. In other words, astaxanthin accumulation is strictly dependent on cell density in culture medium. Moreover, astaxanthin can be increased by several stress cultivation conditions such as nutrient deprivation, increasing of temperature, light and salinity (Lorenz & Cysewski, 2000) and through genetic engineering (Galarza, Gimpel, Rojas, Arredondo-Vega, & Henríquez, 2018; M. E. Hong et al., 2018).

Furthermore, the cultivation system depends on optimal parameters from optimized laboratory conditions to overcome astaxanthin yield and biomass production associated to the reduction of production cost. Variables such as temperature (Giannelli, Yamada, Katsuda, & Yamaji, 2015), optimal pH and salinity (Sarada, Tripathi, & Ravishankar, 2002), light intensity and light wavelength (Ma et al., 2018) have been studied to create new approaches that can be applicable in a cost-effective *H. pluvialis* cultivation. Besides increase biomass production, these variables can interfere in cellular metabolic pathways promoting changes on molecules content, such as proteins, carbohydrates and lipids (Colusse, Duarte, de Carvalho, & Nosedá, 2019) and also

triggering the production of different molecules in the cell (Rashidi, Dechesne, Rydahl, Jørgensen, & Trindade, 2019).

Laboratory conditions and cost related to all production processes of astaxanthin are crucial to determine the success rate of a microalgal biorefinery. In this chapter, several laboratory parameters associated with the production of biomass and astaxanthin by *H. pluvialis* were evaluated and assessed. Besides, a cost evaluation of the main cultivation features was presented. In addition, new technologies on optimizing astaxanthin production and cost reduction were discussed and analyzed.

2.3 UPSTREAM LABORATORY PROCESS EVALUATION

Accurate laboratory parameters are essential to promote the scale up of process in an astaxanthin-producing *Haematococcus pluvialis* biorefinery. Existing parameters to increase cell density and induce cellular carotenogenesis include culture media, pH change, temperature, and increase of light intensity or salt addition. These parameters are scalable and represent great relevance on commercial production upstream processes.

The most common culture media used to cultivate *H. pluvialis* cells are Blue-Green Medium (BG-11) (Stanier, Kunisawa, Mandel, & Cohen-Bazire, 1971), Water Culture (WC) (Guillard & Lorenzen, 1972), CHU medium (ABNT, 1992) (i.e. a modified variant of BG-11), Bold Basal Medium (BBM) (Stein, 1973), and their modifications. The total cost production of BG-11, WC and CHU media was reported to vary from \$2.61-18.36 each 10³ L and it is mainly related to nitrogen, phosphorus and potassium components (Colusse et al., 2019). According to the authors, the components cost is strictly related to the regional market, place and purity of inorganic salt used in the formulation. Sarada et al. (Sarada, Tripathi, et al., 2002) found a great accumulation of astaxanthin by adding calcium nitrate into the medium culture. Therefore, the addition of alternative nutrients can ensure an optimal solution to reduce media cost. Sipaúba-Tavares et al. (L. H. Sipaúba-Tavares, Berchielli-Morais, & Scardoeli-Truzzi, 2015) described a medium based on addition of commercial fertilizer NPK (20-5-20) to produce *H. pluvialis* at high cell density and similar astaxanthin content compared to cultivation on common media.

The use of industrial effluents, and low-cost nutrients, can be a feasible approach to decrease the cost related to the microalgal culture medium and produce low cost astaxanthin (Ledda, Tamiazzo, Borin, & Adani, 2016).

Moreover, laboratory parameters such as pH also play an important role on *H. pluvialis* biomass production. pH can stimulate biomass production normally varying from 7 to 7.85 due to its effect on cellular biochemical processes including CO₂ availability for photosynthesis and nutrient consumption (Costa & de Moraes, 2014; Shah, Liang, Cheng, & Daroch, 2016). Santos and Lombardi (Santos & Lombardi, 2017) analyzed the interference from several pH values in microalgal cultivation. The authors reported that pH adjusted to 6.3 presented the highest cell density and best F_v/F_m values for *H. pluvialis* biomass production. Likewise, the optimal temperature range for *H. pluvialis* growth is considered to be between 20 and 28°C. Temperature around 30°C can induce astaxanthin accumulation and transition from green motile cells to red non-motile cellular stage (Shah et al., 2016), however high temperatures (up to 36°C) can inhibit astaxanthin production in photoautotrophic growth conditions (M.-E. Hong et al., 2015). Giannelli and colleagues (Giannelli et al., 2015) showed a 1.4 fold increase of astaxanthin productivity at 27°C associated with nitrogen starvation in the culture, besides elucidated a relationship between light harvesting capability and different temperature conditions.

Light intensity is another important laboratory parameter in *H. pluvialis* cultivation. The typical light intensity used to cultivate *H. pluvialis* is around 40-100 $\mu\text{mol photons m}^{-2} \text{s}^{-1}$ (Colusse et al., 2019; Shah et al., 2016). Vo and coworkers (Vo, Tran, Nguyen, & Mai, 2017) presented light intensities variation on *H. pluvialis* cultivation from 30 to 120 $\mu\text{mol photons m}^{-2} \text{s}^{-1}$. The authors presented an increase on total carotenoids content around 4.2 $\mu\text{g mL}^{-1}$ at 120 $\mu\text{mol photons m}^{-2} \text{s}^{-1}$ exposure after 24 days of cultivation. Light intensity is an essential parameter to promote astaxanthin accumulation acting directly at the astaxanthin biosynthetic pathway of *H. pluvialis* metabolism. Moreover, using the optimal light intensity could increase astaxanthin production in closed systems.

Also, astaxanthin accumulation can be induced by adding NaCl into the culture medium (Shah et al., 2016). However, values above 1% (w/v) could be devastating to the culture and should be maintained in a range between 0.25 and 0.63% (w/v) to induce accumulation of astaxanthin (13 mg L^{-1}) (Sarada, Bhattacharya, Bhattacharya, &

Ravishankar, 2002; Shah et al., 2016). According to Gao et al. (Gao et al., 2015), cellular salinity stress depends on the laboratory experimental conditions and physiological state of the microalgal culture to achieve better performance. Table 6 summarizes the optimal laboratory conditions used in *H. pluvialis* cultivation to increase biomass and astaxanthin production.

Table 6 - Optimal laboratory cultivation conditions for *H. pluvialis* biomass and astaxanthin production

Laboratory condition	Biomass production (green motile cells)	Astaxanthin production (red non-motile cells)
pH	6.0-8.0	Acidic pH, around 4.0
Temperature	20-28°C	around 30°C
Light intensity	30-100 $\mu\text{mol photons m}^{-2} \text{ s}^{-1}$	120-300 $\mu\text{mol photons m}^{-2} \text{ s}^{-1}$
NaCl addition	none	0.25-0.63% (w/v)

Adapted from Colusse et al., (2019); Hata, Ogbonna, Hasegawa, Taroda, & Tanaka, (2001); Orosa, Valero, Herrero, & Abalde, (2001); Shah et al., (2016) and Christian, Zhang, Sawdon, & Peng, (2018)

Laboratory parameters are scalable to be used in large scale astaxanthin production processes. Generally, the most widely commercial large-scale microalgal production is based on the use of closed or open systems such as photobioreactors and open ponds, respectively. Even though there is a possibility of scale-up from the optimal conditions to the large scale commercial production, additional parameters appear and become as important as laboratory parameters are. Table 7 presents a comparison between the two different growth systems and their main characteristics.

Table 7 - Comparison of closed and open cultivation systems

Characteristics	Closed systems	Open systems
Capital investments	High	Low
Water loss	Low	High
pH control	Easy	Difficult
Biomass productivity	High	Low

Astaxanthin yield	High	Low to average
Scalable	Easy	Difficult

Adapted from Costa & de Moraes, (2014) and Ranjbar, Inoue, Shiraishi, Katsuda, & Katoh, (2008)

Regarding laboratory parameters aforementioned, many other relevant features are crucial and can interfere positively or negatively in an astaxanthin-producing *Haematococcus pluvialis* growth system. Cost of land and energy, water availability and light source are also essential factors to create a cost-effectively microalgal industry (Costa & de Moraes, 2014). Another important feature related to an efficient production in a closed system is the surface/volume (S_f/V) ratio (Kroumov et al., 2013). This parameter is strictly related to the photobioreactor functioning and optimal design in terms of light availability. In a study conducted by Xi et al. (Xi, Kim, Roh, Choi, & Choi, 2016), astaxanthin was significantly increased by employing wavelength shift strategy (light-emitting diodes) in *H. pluvialis* cultures. Although there are several laboratory condition variations reported for astaxanthin accumulation in *H. pluvialis*, it is rather difficult to establish a standard protocol due to various methodologies used by researches worldwide.

2.4 *Haematococcus pluvialis* BIOMASS AND ASTAXANTHIN PRODUCTION COST

Albeit all upstream processes are extremely important for natural astaxanthin production, the cost involved in the biomass production cycle is crucial to determine the persistence of a microalgal positive economic status. Several studies have been developed to determine the total cost in different microalgal scale cultivation. Colusse and coworkers (Colusse et al., 2019) reported a cost variation of \$6-40/kg of dried *H. pluvialis* biomass cultivated under laboratory scale production, demonstrating that the laboratory conditions can impact on production process cost, positively or negatively. Furthermore, biomass production of *H. pluvialis* in laboratory scale seems to be more productive compared to pilot plant biomass production (L. Sipaúba-Tavares, Millan, & Berchielli-Moraes, 2013). The increase on microalgal cell number, higher doubling time and faster growth rate, decrease biomass cost even though expensive media are used in the cultivation. In spite of that, *H. pluvialis* biomass cost must be scalable and monitored

through scale production. A hypothetical astaxanthin plant was proposed by Li and colleagues (Li, Zhu, Niu, Shen, & Wang, 2011), where the authors presented a process economic analysis by which *H. pluvialis* biomass could be produced around \$18/kg in a dry weight basis.

The enhancement of *H. pluvialis* cells in cultivation process increases the astaxanthin yield through the red non-motile stage and decrease astaxanthin cost production. The production cost of natural astaxanthin from an industrial plant with a production capacity of 900 kg of astaxanthin per year was proposed and studied. It was found a total cost of \$718/kg of astaxanthin (Li et al., 2011). Several strategies to decrease costs of biomass and high-value microalgal products have been discussed, such as the addition of wastewater and flue gases (Acién et al., 2017) or the use of low-cost hydroponic fertilizers as part of the culture medium (Tocquin, Fratamico, & Franck, 2012).

Besides features related to biomass production cost, astaxanthin post-harvesting stage affects the entire production cost. Ahmed and coworkers (Ahmed, Li, Fanning, Netzel, & Schenk, 2015) reported a cost-benefit analysis of *H. pluvialis* biomass drying methodologies. The authors showed a 580% higher energy cost when producing freeze-dried powder compared to spray-dried powder in terms of 100 kg of dried biomass. However, astaxanthin degradation has to be taken into account since different methods can show different effectiveness for retention of this molecule (Raposo, Morais, & Morais, 2012; Takeungwongtrakul & Benjakul, 2016). Regarding biomass and astaxanthin production cost, astaxanthin extraction plays an important role on downstream processes mainly on techno-economic analysis. The use of optimal extraction conditions can improve a sustainable natural astaxanthin-producing company.

2.5 ASTAXANTHIN EXTRACTION LABORATORY CONDITIONS

Downstream process parameters and conditions applied in astaxanthin-producing *H. pluvialis* biorefinery have been studied in order to increase the extraction efficiency and decrease cost. Astaxanthin can be recovered through cell-wall disruption followed by extraction process, which implies in high-energy and cost-intensive processes (Kim et al.,

2016). Each extraction methodology presents a cost participation in the total cost and it is related to the efficiency of the extraction process. Table 8 correlates some conventional and recent astaxanthin extraction techniques with their extraction efficiency.

Table 8 - Relationship between astaxanthin extraction process and efficiency

Astaxanthin extraction process	Extraction efficiency (%)	General comments	Reference
Supercritical CO ₂ extraction	80-98	Most efficient, sustainable and widely used method	(Khoo, Lee, et al., 2019; Machmudah, Shotipruk, Goto, Sasaki, & Hirose, 2006)
Supramolecular solvent (SUPRAS) extraction	96	Cost-effective technique	(Salatti-Dorado et al., 2019)
Magnetic-assisted extraction	62	Emerging technology and effective alternative extraction technique	(Khoo, Lee, et al., 2019; Zhao et al., 2016)
Liquid biphasic flotation	99	Lower cost and shorter processing time compared to conventional methodologies	(Khoo, Chew, et al., 2019)

Different laboratory conditions can affect directly the astaxanthin extraction efficiency in a biorefinery. Aside from the extraction technique, variables such as extraction time, temperature, pH, solvent ratio and volume, pressure and gas flow rate are crucial to determine a cost-effective standard protocol applied to astaxanthin extraction. According to Machmudah et al. (Machmudah et al., 2006), supercritical CO₂ extraction coupled to ethanol as entrainer could increase astaxanthin yield. The laboratory conditions were performed at 55 MPa, 343 K and CO₂ flow rate of 3 mL min⁻¹. On the other hand, a supramolecular solvent (SUPRAS) extraction, an astaxanthin

transfer liquid phase vector, was carried out (Salatti-Dorado et al., 2019). The biomass-solvent mixture was vortex-shaken during 8 min at 2300 rpm followed by centrifugation (10,000 rpm, 10 min) and separation of SUPRAS-astaxanthin complex from the superior liquid phase. Furthermore, magnetic-assisted extraction condition was based on the use of liquid-solid ratio of 77:1 (v/w) with a magnetic intensity of 18 mT and 50 min of extraction time (Zhao et al., 2016) whereas liquid biphasic flotation system was done by using 15 mL of 100% 2-propanol, 350 g L⁻¹ of (NH₄)₂SO₄ and 15 min of flotation time (in terms of 10 mg of dried *H. pluvialis* biomass) (Khoo, Chew, et al., 2019).

These data highlights the influence of several extraction techniques coupled to its laboratory conditions on the recovery of astaxanthin from *H. pluvialis*. Besides the cost and laboratory conditions involved in the process, astaxanthin quality is also an important parameter to be evaluated in terms of cost-benefit (Ahmed et al., 2015). Furthermore, new technologies have been developed in this field with an emphasis on the cost of production and parameterization of new approaches in microalgal systems.

2.6 ASTAXANTHIN-PRODUCING *Haematococcus pluvialis*: BROADENING HORIZONS

Several technologies have been developed aiming to decrease cost in astaxanthin derived from *H. pluvialis*, enabling the emergence of new scientific reported studies. Hwang and coworkers (Hwang, Choi, & Sim, 2019) presented a new strategy to inhibit fungal contamination in *H. pluvialis* systems and also increased astaxanthin production using acidic condition in the culture (pH around 4). Besides fungal contamination prevention, astaxanthin was increased by 141-fold compared to contaminated cultures. Moreover, techniques to increase biomass and astaxanthin accumulation are efficient to overcome existing challenges found in astaxanthin commercial scale.

Innovations on astaxanthin extraction have been introduced in the *H. pluvialis* sector such as the cellular milking-mode technique. General microorganism milking-mode approaches have been reported previously (Frenz, Largeau, & Casadevall, 1989; Hejazi & Wijffels, 2004). Based on that, a remarkable strategy was developed by Samori and colleagues (Samori, Pezzolesi, Galletti, Semeraro, & Tagliavini, 2019). The authors

presented an astaxanthin extraction process with no cellular pre-treatment and 90% astaxanthin recovery. In their method, besides decrease extraction cost, the use of almond oil as solvent preserves the *H. pluvialis* cells alive, with no negative effect on photosynthetic activity. Processes coupled to application of green solvent to astaxanthin extraction are becoming appreciated and sustainable worldwide (Salatti-Dorado et al., 2019).

Current technologies to produce astaxanthin derived from *H. pluvialis* are divided between closed and open cultivation systems, and sometimes both of them in combination (Lorenz & Cysewski, 2000). In addition, continuous production of *H. pluvialis* cells and astaxanthin in a one stage cultivation process was proposed either by biofilm cultivation (Kiperstok, Sebestyén, Podola, & Melkonian, 2017) or the use of a novel double-region photobioreactor (Suh, Joo, & Lee, 2006), inciting astaxanthin industry development in terms of production and sustainability. Besides technologies used to increase cell number or improve growth systems, genetic engineering has also been showing alternative approaches to enhance *H. pluvialis* performance (Galarza et al., 2018; M. E. Hong et al., 2018; Sharon-Gojman, Maimon, Leu, Zarka, & Boussiba, 2015).

Based on these strategies, parameters set on a laboratory scale could be used in large scale production process to (i) attenuate/eliminate problems or bottlenecks from microalgal growth systems, (ii) increase astaxanthin content and accumulation based on cellular stress conditions and (iii) study new methodologies to increase a cost-effective scenario in microalgal biorefineries.

2.7 CONCLUSION

Astaxanthin-producing *H. pluvialis* is a complex system based on laboratory conditions divided on upstream and downstream processes that influence directly the total cost production. Parameters such as pH control/variation, nutrient availability, temperature, light intensity and salt addition are the mainly controlled conditions that are applicable in *H. pluvialis* production process. Furthermore, economic analysis standardization and techno-economic studies of each step are crucial to determine new decision-making and the success rate in a microalgal facility. New research development

in microalgae-based bioproducts is very important to meet tomorrow's challenges in terms of cost-effectiveness and to build a promising future for astaxanthin-producing *H. pluvialis*.

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CHAPTER 3

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Effects of different culture media on physiological features and laboratory scale production cost of *Dunaliella salina*

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ABSTRACT

Microalgal cultivation system depends on optimal parameters obtained from laboratory conditions to overcome high-value compounds production and decrease cost. In this study, the laboratory-scale cultivation of *Dunaliella salina* was performed to explore the cost and biochemical effects on biomass cultivated using F/2, Conway, and Johnson media. *D. salina* cultivation was monitored by cell counting, dry biomass measurements and biochemical analyses. Photosynthetic pigment profiles were identified and quantified through high-performance liquid chromatography (HPLC) whereas economic evaluation was based on media cost. Cellular growth parameters were significantly improved by Conway medium, whereas maximum accumulation of pigments, especially violaxanthin (0.855 mg g^{-1}) and chlorophyll *a* (14.255 mg g^{-1}) were observed when Johnson medium was used. Conway-biomass showed 43 % of total lipid content. This value represents 1.5 and 1.4 times higher than lipid content found in Johnson- and F/2-biomass, respectively. Furthermore, media cost presented a variation of US\$1.17–49.62 for each 10^3 L of media mainly due to NaNO_3 , KNO_3 and cyanocobalamin supplementation. Thus, biomass production showed a cost variation of US\$4.64–301.61 per kg on a dry weight basis. These outcomes emphasize the suitability of laboratory parameters and cost comparison among biomass produced by different media. © 2020 The Authors. Published by Elsevier B.V. This is an open access article under the CC BY license (<http://creativecommons.org/licenses/by/4.0/>).

1. Introduction

The unicellular green halophilic flagellate microalga, *Dunaliella salina*, is a well-organized cell factory and the most feasible source of natural β -carotene worldwide. Scientific knowledge about *D. salina* cultivation is crucial to determine how to increase biomass production at low cost, intensifying industrial production. Several studies have reported the importance of looking-forward technologies that increase microalgal biomass and pigment production in a cost-effectively economic scenario [1–3].

Even though *D. salina* cultivation can be a potential source of high-added value pigments, laboratory parameters and media costs are concerns to be overcome. Previous studies have shown that different microalgal medium and culture conditions can

affect the cellular metabolism directly and therefore the production cost [4–6]. Microalgal typical medium can cost from US\$ 2.6 to 18.3 each 10^3 L in laboratory-scale cultivation systems, and medium components such as NaNO_3 and K_2HPO_4 can represent 83.3 and 31 % respectively, in the medium cost [6]. Moreover, medium component replacement by economical sources can provide higher biomass production as well as an increase on protein, lipid and docosahexaenoic acid (DHA) content [7].

Furthermore, culture conditions can interfere directly with the microalgal metabolism, affecting the production of molecules such as pigments. Some studies have addressed significant effects of light and adaptive evolution [8], temperature [9] and gene expression responses to stress [10] on pigment accumulation from *D. salina* cells. In addition to cultivation features, strategies to enhance carotenoid biosynthesis using leaf extracts was described by Einali and colleagues (2017) [11]. However, few studies have demonstrated the correlation of microalgal biomass cultivated

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EFFECTS OF DIFFERENT CULTURE MEDIA ON PHYSIOLOGICAL FEATURES AND LABORATORY SCALE PRODUCTION COST OF *Dunaliella salina*

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3.1 ABSTRACT

Microalgal cultivation system depends on optimal parameters obtained from laboratory conditions to overcome high-value compounds production and decrease cost. In this study, the laboratory-scale cultivation of *Dunaliella salina* was performed to explore the cost and biochemical effects on biomass cultivated using F/2, Conway, and Johnson media. *D. salina* cultivation was monitored by cell counting, dry biomass measurements and biochemical analyses. Photosynthetic pigment profiles were identified and quantified through high-performance liquid chromatography (HPLC) whereas economic evaluation was based on media cost. Cellular growth parameters were significantly improved by Conway medium, whereas maximum accumulation of pigments, especially violaxanthin (0.855 mg g⁻¹) and chlorophyll *a* (14.255 mg g⁻¹) were observed when Johnson medium was used. Conway-biomass showed 43% of total lipid content. This value represents 1.5 and 1.4 times higher than lipid content found in Johnson- and F/2-biomass, respectively. Furthermore, media cost presented a variation of US\$1.17-49.62 for each 10³ L of media

mainly due to NaNO_3 , KNO_3 , and cyanocobalamin supplementation. Thus, biomass production showed a cost variation of US\$4.64-301.61 per kg on a dry weight basis. These outcomes emphasize the suitability of laboratory parameters and cost comparison among biomass produced by different media.

Keywords: Microalgae. Vegetative phase. Economic analysis. Photosynthetic pigments. Culture medium. Biomass production.

3.2 INTRODUCTION

The unicellular green halophilic flagellate microalga, *Dunaliella salina*, is a well-organized cell factory and the most feasible source of natural β -carotene worldwide. Scientific knowledge about *D. salina* cultivation is crucial to determine how to increase biomass production at low cost, intensifying industrial production. Several studies have reported the importance of looking-forward technologies that increase microalgal biomass and pigment production in a cost-effectively economic scenario (Banerjee & Ramaswamy, 2019; Benavente-Valdés, Aguilar, Contreras-Esquivel, Méndez-Zavala, & Montañez, 2016; Rizwan, Lee, & Gani, 2015).

Even though *D. salina* cultivation can be a potential source of high-added value pigments, laboratory parameters and media costs are concerns to be overcome. Previous studies have shown that different microalgal medium and culture conditions can affect the cellular metabolism directly and therefore the production cost (Ahmed et al., 2017; Colusse, Duarte, de Carvalho, & Nosedá, 2019; Srinivasan et al., 2018). Microalgal typical medium can cost from US\$ 2.6 to 18.3 each 10^3 L in laboratory-scale cultivation systems, and medium components such as NaNO_3 and K_2HPO_4 can represent 83.3 and 31% respectively, in the medium cost (Colusse et al., 2019). Moreover, medium component replacement by economical sources can provide higher biomass production as well as an increase on protein, lipid and docosahexaenoic acid (DHA) content (Camacho-Rodríguez et al., 2020).

Furthermore, culture conditions can interfere directly with the microalgal metabolism, affecting the production of molecules such as pigments. Some studies have addressed significant effects of light and adaptive evolution (Fu et al., 2013), temperature

(Wu, Duangmanee, Zhao, Juntawong, & Ma, 2016a) and gene expression responses to stress (Sánchez-Estudillo, Freile-Pelegrin, Rivera-Madrid, Robledo, & Narváez-Zapata, 2006) on pigment accumulation from *D. salina* cells. In addition to cultivation features, strategies to enhance carotenoid biosynthesis using leaf extracts was described by Einali and colleagues (2017). However, few studies have demonstrated the correlation of microalgal biomass cultivated under common media (Camacho-Rodríguez et al., 2020; Colusse et al., 2019; Nahidian, Ghanati, Shahbazi, & Soltani, 2018), pigment profile and laboratory cost in *D. salina* cultivation (no stress applied).

In this study, we evaluated the performance of different microalgal culture media on *D. salina* cultivation and analyzed their effects on biomass composition and laboratory cost production. The study evaluated crucial parameters related to microalgae cultivation: (i) cellular growth and physicochemical parameters, (ii) pigment profile in different media, and (iii) parameterizing cost under laboratory-scale production. The results of this study may provide useful information about the enhancement of microalgal biomass production related to the chosen medium and laboratory-scale production cost.

3.3 MATERIAL AND METHODS

In order to obtain a complete picture of the methodology used in this study, we presented a step-by-step approach as shown in Figure 5.

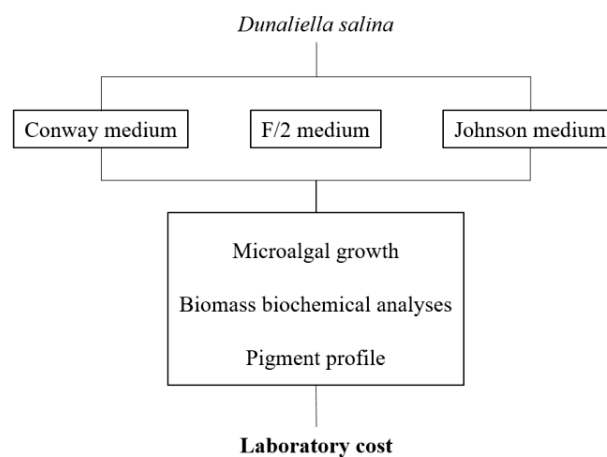


Fig. 5 - Schematic step-by-step approach of this study

***Dunaliella salina* strain and culture maintenance**

D. salina strain (BMAK 116) was obtained from the Aida & Kutner Collection Bank (BMA&K, Oceanographic Institute at the São Paulo University, Brazil). The strain was maintained photoautotrophically in Conway medium (Walne, 1966), using artificial seawater (35 g L⁻¹, Salinity[®] salt). To proceed with the experimental design, the inoculum was grown in a two-liter flask (10% v/v inoculation, initial concentration of 30 x10⁴ cells mL⁻¹) in the same medium at 23 ± 1 °C under 12:12 light/dark cycle (100 µmol m⁻² s⁻¹) for 5 days.

Experimental design

The microalga was cultivated in three common media described in the literature. Conway, F/2 (R. R. L. Guillard, 1975), and Johnson (Johnson, Johnson, MacElroy, Speer, & Bruff, 1968) media were prepared individually into two-liter flasks with a working volume of 1600 mL using artificial seawater (35 g L⁻¹, Salinity[®] salt). pH was adjusted to 7.5, and the flasks were autoclaved. Inoculation was done based on 10% v/v of stock culture from previous step after centrifugation (851 x g for 15 min) to avoid nutrient interferences on media (initial concentration of 16 x10⁴ cells mL⁻¹ after inoculation). The experiment was carried out during 15 days under 12:12 light/dark cycle with cool white fluorescent lamps providing 100 µmol m⁻² s⁻¹. During all cultivation period, continuous sterilized compressed air was provided with a flow rate of 2 L min⁻¹ (without supplementary CO₂). All components used were analytical grade.

Microalgal growth and offline measurement

Microalgal culture samples were taken every 24 h. Dry biomass quantification was determined by filtration using a pre-weighted Macherey-Nagel GF-1 glass-fiber filter and dried at 80 °C until constant weight. Biomass productivity (in terms of g L⁻¹ per day) was calculated using Eq. (1).

$$\text{Productivity} = \frac{(X_f - X_i)}{(t_f - t_i)} \quad (1)$$

where X_f and X_i correspond to final and initial biomass (g L^{-1}), respectively related to final (t_f) and initial time (t_i). Cell counting was performed by optical microscopy using a Neubauer hemocytometer for cell growth analysis. Microalgae growth performance was measured by the following equations 2-5 according to Guillard (1973) and Wood et al. (2005):

$$\text{Growth rate (r)} = \frac{\ln(N_f/N_i)}{t_f - t_i} \quad (2)$$

$$\text{Doubling time (T2)} = \frac{0.6931}{r} \quad (3)$$

$$\text{Division per day (k)} = \frac{r}{0.6931} \quad (4)$$

$$\text{Maximum cellular yield (R)} = N_f - N_i \quad (5)$$

where N_f and N_i correspond to final and initial cell density, respectively related to their specific final (t_f) and initial time (t_i) in days. pH and conductivity were measured to monitor variation in the culture during the whole experiment.

Photosynthetic pigment profile and quantification

All analyses were done on a daily basis. Briefly, a culture volume of 5 mL was filtered using a Macherey-Nagel GF-1 glass-fiber filter (47 mm, 0.7 μm). The biomass-filter was resuspended in an acetone-water mixture (4 mL, 90% v/v) and shaken vigorously. The tubes were maintained in the dark at 4 °C for 20 h to extract all the photosynthetic pigments. The supernatant was recovered through centrifugation (1915 x g for 10 min), and the optical absorbance was determined using a spectrophotometer BEL SP1105 at 480, 510, 664 and 750 nm. Chlorophyll_a (Chl), pheophytin_a (Phe) and total carotenoids (Car) were calculated according to spectrophotometric equations described in the literature (Carl Lorenzen, 1967; Strickland & Parsons, 1968). The analysis was performed in triplicate and the results were shown in mean $\pm$ SD (Standard Deviation).

Biomass was also centrifuged at the end of the experiment and lyophilized for pigment profile analyses. Moreover, biomass mix of all 3 replicates was analyzed using high-performance liquid chromatography (HPLC). Freeze-dried samples of 0.0042–0.0368 g were placed in a screw-cap tube with 6 mL of 95% cold-buffered methanol (2% ammonium acetate) containing 0.05 mg L⁻¹ trans- β -apo-8'-carotenal (Fluka, Buchs, Switzerland) as internal standard. The samples were sonicated for 5 min in an ice-water bath, placed at -20 °C for 1 h and centrifuged at 1100 g for 5 min at 3 °C. Supernatants were filtered through Fluoropore PTFE membrane filters (0.2- μ m pore size; Merck Millipore Ltd, Billerica, MA, USA) to remove cell debris. Immediately before injection, 1 mL of sample was mixed with 400 mL of Milli-Q water in 2-mL amber glass sample vials, and vials were placed in the HPLC cooling rack (4 °C). Method procedures for HPLC analyses (using a monomeric C8 column with a pyridine-containing mobile phase) are fully described in Zapata et al. (2000) [20]. The detection limit and quantification procedure of this method were done according to Mendes et al. (2007) [21]. Pigments were identified from both absorbance spectra and retention times from the signals in the photodiode array detector (SPDM20A) (Shimadzu Corporation, Kyoto, Japan). Peaks were integrated using LC-Solution software, but all peak integrations were checked manually and corrected where necessary. The HPLC system was previously calibrated with pigment standards from DHI (Institute for Water and Environment, Denmark). For correction losses and volume changes, the concentrations of the pigments were normalized to the internal standard.

Biomass biochemical analyses

Biomass were centrifuged and lyophilized for biochemical analyses. The spectrophotometric method of Lowry et al. (1951) was used to determine protein content in the dried biomass. Total carbohydrates were determined by the phenol-sulfuric methodology (Dubois, Gilles, Rebers, & Smith, 1956) and quantified spectrophotometrically using a standard glucose curve (490 nm), while total lipids were determined by the gravimetric method described by Blight and Dyer (1959). The analysis was performed in triplicate and the results were shown in mean $\pm$ SD (Standard Deviation).

Laboratory economic analysis

Medium component costs were obtained by several quotations from laboratory companies and chosen based on the minimum price. The contribution of microalgal media was evaluated based on the total media cost, individual media component cost, and indoor biomass production cost. The individual component cost was expressed as a percentage of the total medium cost. To evaluate laboratory pigment costs, the analysis was performed based on pigment content and dry biomass cost. The individual pigment cost was expressed in US\$ dollar and 1 kg basis of powdered pigment.

Statistical analysis

Each experiment was performed in triplicate, and the results were shown in mean $\pm$ SD (Standard Deviation). Results were analyzed by ANOVA at 95% confidence interval and Tukey's test with significance at $p < .05$.

3.4 RESULTS AND DISCUSSION

***Dunaliella salina* culture kinetics, pH and conductivity**

Differences in growth characteristics of *D. salina* during 15 days cultivation in microalgal common culture media in batch-culture mode are shown in Fig. 6. A total of $229 \pm 5.29 \times 10^4 \text{ mL}^{-1}$ cells was achieved when Conway medium was used in the cultivation. The highest cell density in the Conway medium was 1.7 and 1.9 times higher than cells cultivated in F/2 and Johnson media in the same period, respectively (Fig. 2A). Thus, F/2 and Johnson media presented 162 ± 10.69 and $121 \pm 3.05 \times 10^4 \text{ mL}^{-1}$ cells, respectively. No cellular morphology differences were observed during cultivation in the different media.

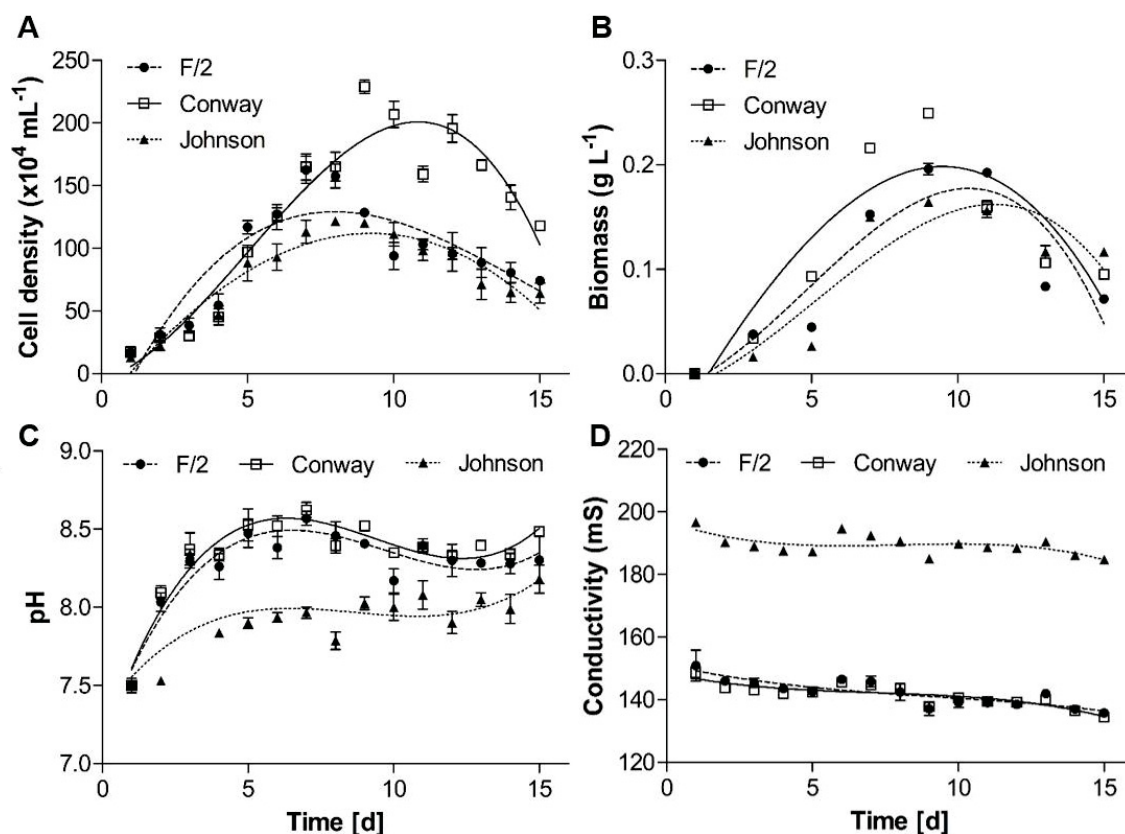


Fig. 6 - Characterization of growth behavior (cell density and biomass), pH and conductivity profiles of *D. salina* cultivated in F/2, Conway and Johnson media

Biomass production followed the same pattern as cellular growth (Fig 6B). Biomass was highest in Conway medium with 0.25 g L^{-1} followed by 0.19 g L^{-1} and 0.16 g L^{-1} for F/2 and Johnson media, respectively. Higher cellular concentrations are reported in *D. salina* and *D. tertiolecta* cultivation due to the presence of some additives in culture media such as pentoses (de Freitas et al., 2019) and growth hormone (El Arroussi, Benhima, Bennis, El Mernissi, & Wahby, 2015) presenting a biomass production of 1.25 and 0.8 g L^{-1} respectively. In comparison with other studies on microalgal medium evaluation, Nahidian and colleagues (2018) highlighted possible improvements on microalgae cultivation due to microalgal dependency to the medium nutrient content. Moreover, to increase *D. salina* production, Sathasivam and Juntawong (2013) presented a modified Johnson medium with altered MgSO_4 and NaHCO_3 concentrations, addition of NaVO_3

and elimination of MgCl_2 . The authors reported a production of 508×10^4 cells mL^{-1} . However, the present study exhibited characteristics related to the use of common media without any supplementation on laboratory scale.

According to Figure 6C, pH showed an increase in all media. Similar pH increase was reported and it might be favorable for the induction of carotenoids (Lababpour et al., 2005) and biomass production (Khalil, Asker, El-Sayed, & Kobbia, 2010). Conductivity (Fig 6D) showed a decrease of 10, 9.4 and 6% in F/2, Conway and Johnson media, respectively compared to the initial values. These results can be related to nutrient consumption by the cells over the cultivation period. Conductivity analysis is an useful approach in microalgae cultivation since it can be used to monitor nutrient availability (Gent, 2003) and microalgal dewatering performance (Larronde-Larretche & Jin, 2016).

Regarding *D. salina* kinetics, cellular growth parameters were measured to evaluate the media effects on cell metabolism (Table 9). Conway medium proved to have a significant positive impact on *D. salina* growth parameters when compared to biomass produced using F/2 and Johnson media.

Table 9 - Growth and physicochemical parameters for *Dunaliella salina* cultivation in different culture media at the end of experiment*

Growth parameters	F/2	Conway	Johnson
Maximum cellular density ($\times 10^4$ cells mL^{-1})	162 $\pm$ 10.69 ^b	229 $\pm$ 5.29 ^a	121 $\pm$ 3.05 ^c
Division per day (k)	0.53	0.54	0.51
Doubling time (T_2)	1.85	1.84	1.94
Growth rate (r)	0.373	0.375	0.356
Maximum cellular yield (R) ($\times 10^4$ cells mL^{-1})	145 $\pm$ 9.71 ^b	211 $\pm$ 3.05 ^a	108 $\pm$ 3.78 ^c
Dry biomass (g L^{-1})	0.19 $\pm$ 0.005 ^b	0.25 $\pm$ 0.002 ^a	0.16 $\pm$ 0.001 ^c
Productivity (g $\text{L}^{-1} \text{d}^{-1}$)	0.024	0.031	0.020
pH	8.30 $\pm$ 0.15	8.48 $\pm$ 0.02	8.18 $\pm$ 0.08
Conductivity (mS cm^{-1})	135.76 $\pm$ 0.35 ^b	134.46 $\pm$ 0.85 ^b	184.83 $\pm$ 0.65 ^a

*Averages followed by the same letter do not significantly differ ($p < .05$).

Productivity affects the cost of microalgal biomass and molecules of interest. As can be seen further on this study, parameters such as division per day, doubling time,

growth rate, dry biomass and biomass pigment composition from the evaluated media can affect directly the laboratory production cost. According to Chavoshi and Shariati, (2019), decrease on cell division and biomass production are related to cellular self-shading and photoinhibition observed in autotrophic cultures. Besides medium nutrients, variables such as quality and quantity of light also affect biomass productivity rate (Delavari Amrei, Ranjbar, Rastegar, Nasernejad, & Nejadebrahim, 2015).

KNO₃ and NaHCO₃ increase growth parameters in *D. salina* cultivation as previously reported (Khadim et al., 2018). These authors also evaluated microalgae cultivation by adopting an aeration rate of 0.5 L min⁻¹ and an irradiance of 70 μmol photons m⁻² s⁻¹. However, for this study we used an aeration rate of 2 L min⁻¹ and 100 μmol photons m⁻² s⁻¹ in all cultivation steps.

Several aspects concerning microalgal development parameters are essential to understand dynamic kinetic growth, and can be evaluated through model equations as described by Fachet and colleagues (2014) or to choose a specific culture medium related to the production of a molecule of interest. In this sense, the Conway medium provided the most suitable nutrient conditions for the growth of *D. salina* in vegetative stage cultivation. These outcomes indicate that further evaluation related to biomass enhancement and growth parameters with Conway medium would be interesting since it has presented better results than other culture media evaluated in laboratory scale from this study.

***D. salina* pigment quantification during cultivation**

In order to evaluate the pigment profile during cultivation, photosynthetic pigments were monitored once a day using spectrophotometric analysis (Fig 7). Pigments concentrations are shown in terms of wet basis (w/v). We found that the increase in cell concentration (previous step) leads to an increase in pigment content per cell, in particular chlorophyll *a* and pheophytin *a*.

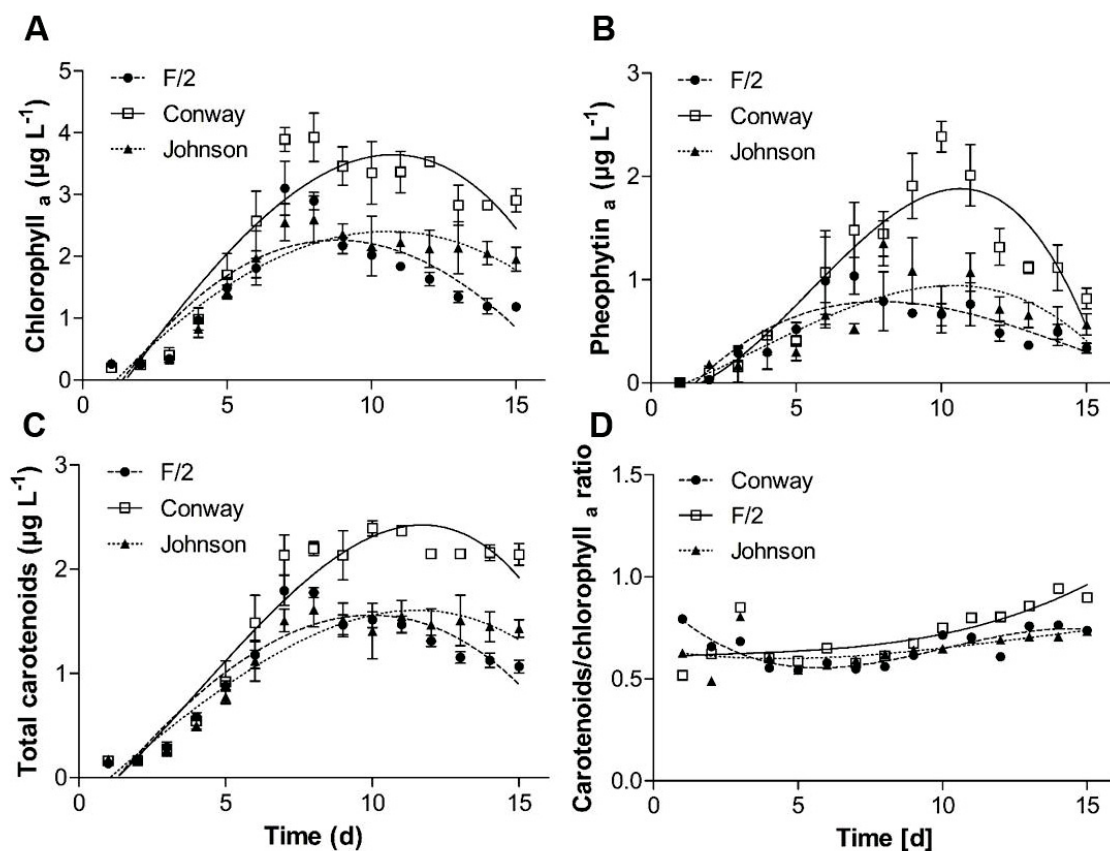


Fig. 7 - *D. salina* photosynthetic pigments profile, obtained by spectrophotometry, over cultivation period using F/2, Conway, and Johnson media

Conway medium showed the highest chlorophyll *a* content ($3.92 \pm 0.43 \mu\text{g L}^{-1}$) at day 7 of cultivation as presented in Figure 7A. It corresponds to an increase of 21 and 34% when compared with chlorophyll *a* content from F/2- and Johnson-biomass, respectively. According to Figure 7A and 3B, F/2-biomass presented chlorophyll *a* and pheophytin *a* contents of 3.10 ± 0.43 and $1.03 \pm 0.17 \mu\text{g L}^{-1}$, respectively, reaching the maximum pigment production at 7 days of cultivation. Furthermore, Johnson-biomass showed the maximum chlorophyll *a* and pheophytin *a* production at the 8th day of cultivation with 2.59 ± 0.37 and $1.35 \pm 0.21 \mu\text{g L}^{-1}$, respectively.

Moreover, several aspects on microalgal cultivation can increase (or decrease) pigment content. According to Wu and colleagues (2016), temperature and light intensity showed positive impact in *D. salina* pigment accumulation, mainly β -carotene. These

authors also discussed the interference of other factors such as nutrients availability (KNO_3 , $\text{CO}(\text{NH}_2)_2$ and NaHCO_3) and strain used. Furthermore, light emitting diodes (LED) can also influence pigment production in *D. salina* cultivation (Xu & Harvey, 2019).

Total carotenoids/chlorophyll *a* ratio is an indicator of stress in microalgal cultivation related to exposure to several biotic and abiotic factors. In this study, non-stressed microalgal cells present total carotenoid (Fig 7C) and chlorophyll *a* content at the same level during the cultivation period, as can be seen in Figure 7D. Cultivation variables such as light and nitrogen deprivation can induce carotenoid synthesis in microalgal cells (Scibilia, Girolomoni, Berteotti, Alboresi, & Ballottari, 2015) and can be monitored by the total carotenoids/chlorophyll *a* ratio (Xu & Harvey, 2019).

D. *salina* pigment profile at the end of the cultivation period

The effect of microalgal media on *D. salina* pigment profile was measured by HPLC at the end of microalga culture (Table 10). The usual approach to analyze pigment content from microalgal biomass is by colorimetric assays, however HPLC allows the identification and quantification of a wider range of pigments, and was used to gather information about pigment profile and consequent influences from culture media variables (Gao et al., 2016).

Table 10 - HPLC pigments (chlorophylls and carotenoids) quantification (mg g^{-1} of dry biomass) of *D. salina* at the end of the cultivation period*

Pigments (mg g^{-1})	F/2	Conway	Johnson
Neoxanthin	0.497	0.668	0.671
Violaxanthin	0.454	0.568	0.855
Zeaxanthin	0.159	0.115	0.007
Lutein	2.123	2.596	1.252
Chlorophyll <i>b</i>	3.284	4.247	5.974
Chlorophyll <i>a</i>	8.304	10.267	14.255
β -carotene	n/d**	n/d	n/d

* Biomass mix of all 3 replicates. **n/d: not detected.

We found that biomass obtained from Johnson medium presented the highest neoxanthin (0.671 mg g^{-1}), violaxanthin (0.855 mg g^{-1}), chlorophyll *a* (14.255 mg g^{-1}) and chlorophyll *b* (5.974 mg g^{-1}) content when compared with biomass from Conway and F/2 media. Furthermore, chlorophyll *a* was highly enriched in biomass produced in Johnson medium in a dry basis (w/w), differing from the previous section, in which Conway medium proved to increase this molecule during cultivation. It suggests that Johnson medium enhances chlorophyll *a* inside the cell, and no longer to cellular growth. Differences on pigment profile related to different nitrate concentration were reported by Gao and coworkers (2016). These authors showed an intrinsic relationship among photosynthetic pigments such as chlorophyll *a*, β -carotene, vaucheriaxanthin-ester and violaxanthin, from *Vischeria stellata*, and the nitrate concentration.

According to the results achieved in the present study, Conway medium induced lutein synthesis (2.596 mg g^{-1}). This pigment showed an increase of 18 and 51% compared to lutein concentration from F/2- and Johnson-biomass, respectively. Microalgal pigment variation is reported in literature. Srinivasan and colleagues (2018) [5] observed an increase in lutein and β -carotene content in *D. salina* cultivation under nutrient deficit conditions and sodium bicarbonate addition (100 mM). It reduced oxidative stress, lowered lipid peroxidation damage and improved antioxidant enzyme activities. Taking advantage of the fact that culture medium interferes in the microalgal pigment profile, we reasoned that it is an affordable and feasible approach to enhance specific pigment in a large scale microalgal facility.

Pigment profile plays an important role in microalgal species characterization as well as on the evaluation of cultivation features in microalgae production. Furthermore, medium components can be supplemented in order to increase certain pigment of interest since our experimental results have demonstrated that each medium has different impact on pigment production.

D. *salina* biomass biochemical composition

D. salina cells were grown for up to 15 d in different culture media. Biochemical parameters, such as lipids, proteins and carbohydrates were monitored in the biomass at the end of the cultivation period. Several studies reported that there is a change from

photosynthetic carbon flow to energy-rich chemical compounds such as lipids, proteins and carbohydrates during cultivation (He, Yang, Wu, & Hu, 2015; Pancha et al., 2014). These molecules act as storage components in the cellular mechanism and can be influenced by diverse cultivation features (Fazeli Danesh, Ebrahimi, Salehi, & Parsa, 2017; George et al., 2014; X. Wang et al., 2019). In the present study, *D. salina* biomass produced in different culture media showed significant differences on biochemical composition as can be seen in Table 11.

Table 11 - Effect of culture media on lipid, protein, and carbohydrate content from *D. salina* biomass*

Parameters	Culture media		
	F/2	Conway	Johnson
Lipids (%)	31.3 ±1.15 ^b	43.3 ±2.3 ^a	28.6 ±1.15 ^b
Proteins (%)	19.1 ±1.96 ^b	23.4 ±0.89 ^b	38.3 ±2.88 ^a
Carbohydrates (%)	5.6 ±0.28 ^b	6.9 ±0.13 ^b	11.3 ±0.45 ^a

*Averages followed by the same letter do not significantly differ ($p < .05$).

Biomass produced using Conway medium showed a 43.3% of lipid content. This value represents 1.5 and 1.4 times higher than lipid content obtained in Johnson- and F/2 biomass, respectively. According to Ahmed and colleagues (2017), lipid enhancement is strictly dependent on salt addition to the culture medium in *D. salina* cultivation. The authors recorded a 22.28% of total lipid content at optimum salinity level (2 M NaCl). In the present study we focused on microalgal effects by medium components; therefore, synthetic seawater (with artificial sea salt) was used equally in all media preparation and the salt effect was not analyzed.

Microalgal biomass obtained using Johnson medium presented the highest protein content, differing significantly from protein content obtained in F/2- and Conway biomass. It showed a 38.3% protein content, followed by 23.4 and 19.1% obtained from Conway and F/2 biomass, respectively. High-quality protein production potential from *D. salina* was reported by Sui et al. (2019). The authors reported that growth phase and light regime also affect protein content and essential amino acid levels on *D. salina* cultivation. These

results highlight the importance of studies that correlate biomass biochemical composition with microalgae cultivation features.

Carbohydrate content showed 11.3% in biomass obtained using Johnson medium. It presented a production of 1.6 and 2 times higher than carbohydrate content found in biomass produced by Conway and F/2 media. de Freitas and coworkers (2019) presented a carbohydrate content of 20.5% in *D. salina* cultivation. The authors studied the positive effects of D-xylose and L-arabinose addition into the culture media related to carbohydrate and protein content, and biomass production (1.38 g L^{-1}). Besides structural features in the microalgal cell, carbohydrates derived from microalgae have also received attention due to its biological application in medical and pharmaceutical areas such as antitumor properties (Sadovskaya et al., 2014), antitussive and bronchodilatory activity (Halaj et al., 2018), plant growth biostimulant action (Elarroussi et al., 2016), and antimicrobial property (Hafsa et al., 2017).

Different biochemical contents were also reported for microalgal biomass cultivated under different media (Colusse et al., 2019; Nahidian et al., 2018; Praveen, Abinandan, Natarajan, & Kavitha, 2018). The biochemical comparison among microalgal biomass suggests that medium components present an intrinsic relationship in cell metabolism promoting the increase of a specific molecule; therefore, it can be further used to promote the enhancement of biocompounds of interest.

Economic evaluation

To further compare culture media, we performed an economic analysis of each medium (Table 12). The basis considered for calculation was 10^3 L of culture medium, in batch-culture mode, and the cost to produce 1 kg of dry biomass.

Table 12 - Media economical evaluation (in US\$ dollars)*

Cost	F/2	Conway	Johnson
10^3 L medium	1.17	4.33	49.62
kg of dry biomass	4.64	16.24	301.62

*Average exchange rate (February 2020) US\$1.00 = R\$4.31.

Based on the economic analysis of the media in laboratory scale, F/2 medium showed a contribution to biomass cost of US\$4.64/kg whereas Conway medium presented a cost of US\$16.24/kg. For Johnson media, which had the highest cost not only for medium components but also for dry biomass, showed a production cost of US\$49.62 and US\$301.62, respectively. In our previous study, culture media showed a cost from US\$2.6 to 18.3 for 10³ L and dry biomass production cost from US\$6.2 to 40.3/kg (Colusse et al., 2019).

Moreover, Li and colleagues (2011) showed in an economic analysis of *Haematococcus pluvialis* cultivation, that the proposed biomass production cost was around US\$18/kg in a dry weight basis. Interestingly, media cost variation is dependent of several parameters and play an important role on microalgal production process. Besides energy consumption and harvesting methods (Pirwitz, Flassig, Rihko-Struckmann, & Sundmacher, 2015), nutritional components significantly contribute to final medium cost. Figure 8 presents the components with major cost participation in the tested media. Components that presented <0.1% of cost participation were not listed.

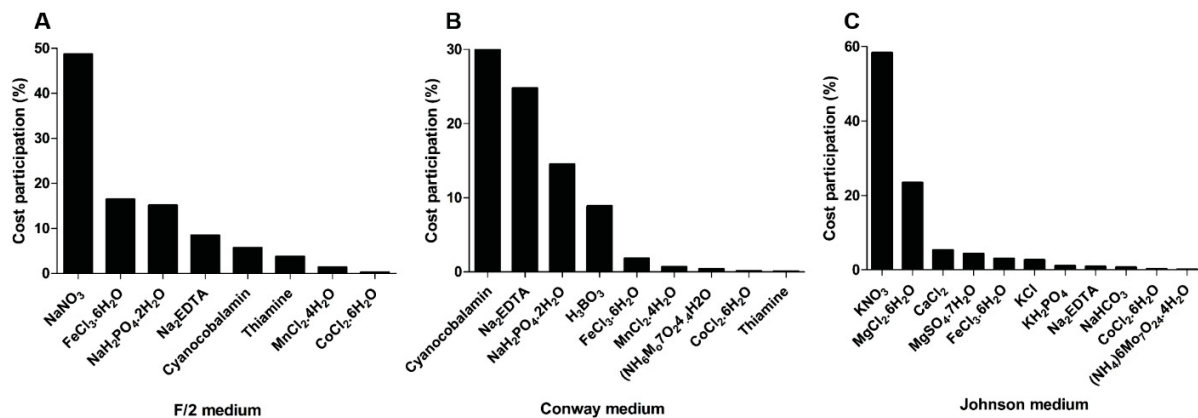


Fig. 8 - Component participation in the media cost of F/2, Conway and Johnson media. Medium component costs were obtained from quotations by laboratory companies.

The components with the highest cost participation were NaNO₃ (48.6%), cyanocobalamin (30%) and KNO₃ (58.3%) at the F/2 (Fig 4A), Conway (Fig 8B) and Johnson (Fig 8C) media composition, respectively. MgCl₂·6H₂O and KNO₃ content from

Johnson medium represents a total of 81% of total cost while cyanocobalamin, Na₂EDTA, NaH₂PO₄·2H₂O and H₃BO₃ content in Conway medium and NaNO₃, FeCl₃·6H₂O and NaH₂PO₄·2H₂O content in F/2 medium show a 78% and 80%, respectively, of participation on total cost in these media.

It is worth mentioning that some medium components could be easily replaced by low/null-cost effluents from the agribusiness industry. The use of low-cost nutrients or residues such as sugarcane vinasse (Santana et al., 2017) or anaerobic digestate (Uggetti, Sialve, Latrille, & Steyer, 2014) in microalgae cultivation could provide benefits to the process besides decreasing the medium cost. Wang and colleagues (2018) (S.-K. Wang et al., 2018) presented an increase on *Chlorella pyrenoidosa* productivity of 4.76 times than that cultured at BG-11 medium using tofu whey wastewater as basal medium.

In order to make the presented study applicable for industrial purposes, extended evaluations of low-cost nutrient addition and cultivation features such as CO₂ injection and light intensity studies would be required. However, detailed results from medium components participation presented here are essential to support information to be elucidated in upcoming studies.

Furthermore, several studies have examined different ways to decrease microalgal medium cost (Erkelens, Ward, Ball, & Lewis, 2014; Sipaúba-Tavares, Berchielli-Morais, & Scardoeli-Truzzi, 2015; S.-K. Wang et al., 2018), but few focused on pigment production and cost related with laboratory scale basis. In this sense, this study presented information of pigment cost based on the use of common media described in the literature, in their original concentration. To assess a relationship that may correlate biomass and pigment, estimations of pigment cost related to biomass production were taken to assess different economic effects resulting from microalgal media. Table 13 presents the estimation of pigment cost production derived from F/2, Conway and Johnson biomass in 1 kg basis of powdered pigment.

Table 13 - Estimated pigment economical evaluation based on laboratory production scale (1 kg basis of powdered pigment, US\$ dollars)

Pigments	F/2	Conway	Johnson
Neoxanthin	9,336.01	24,311.37	449,508.19
Violaxanthin	10,220.26	28,591.54	352,771.92
Lutein	2,185.58	6,255.77	240,910.54
Chlorophyll <i>b</i>	1,412.91	3,823.87	50,488.78
Chlorophyll <i>a</i>	558.76	1,581.76	21,158.89

In all biomass produced, pigment cost was excessively high. Johnson medium components increase pigment cost in all pigments analyzed compared with pigments extracted from F/2 and Conway biomass. The increase of pigment cost is related to biomass production and media component participation in the media. Chlorophyll *a* presented the lower cost among all pigment comparison since it was the most abundant molecule showed in all biomass (see Table 10).

Due to the toxic effects of synthetic pigments, there is an increase on the use of natural colorants obtained from microalgae in several promising applications such as diagnostics, biomedical research, dairy products, therapeutics, and colorings in cosmetics (Begum, Yusoff, Banerjee, Khatoon, & Shariff, 2016).

Studies showed that pigments play an important role mainly in biomedical areas. Brazionis and coworkers (2008) (Brazionis et al., 2008) suggested that pigments such as lutein can protect against diabetic retinopathy. Furthermore, neoxanthin was described to reduce cell viability on prostate cancer cell lines (Kotake-Nara et al., 2001) and violaxanthin presented antiproliferative activity on human mammary cancer cell line (Pasquet et al., 2011). In addition, chlorophyll is used as natural color in food, cosmetic and pharmaceutical products with high-added value (Begum et al., 2016).

In most microalgal mass cultures, particularly those of outdoor raceway ponds, pigments production as well as other molecules of interest have been studied with the aim of enhancing productivity and decreasing their costs (Faé Neto, Borges Mendes, & Abreu, 2018). Li and colleagues (2011) (Li et al., 2011) presented an economic evaluation of an industrial plant with a total capacity of 900 kg of natural astaxanthin per year. The

authors showed a total cost of US\$718/kg of powdered astaxanthin using the green biflagellate microalga *Haematococcus pluvialis* as a natural producer.

The addition of low-cost components such as ammonium sulfate, calcium superphosphate and urea can decrease medium cost and raise pigment content per unit of biomass (Faé Neto et al., 2018). It confirms that studies related to alternative cost-effective culture media are essential to enhance growth and pigment content in addition to reduce cost production. Moreover, information about laboratory-scale production is essential since it can be used as basic science to be part of the decision-making process and to overcome challenges faced in a large scale microalgal facility.

3.5 CONCLUSIONS

In our study, cultivating *D. salina* cells using F/2, Conway, and Johnson media showed several differences in growth, biochemical characteristics, pigment profile, and cost. Protein content (38.3%) and total carbohydrate (11.3%) were greatly improved in Johnson-biomass, whereas Conway-biomass showed higher lipid production (43.4%). Moreover, Johnson-biomass showed a 14.255 mg g⁻¹ of chlorophyll *a* content, while Conway medium presented a 2.596 mg g⁻¹ of lutein production besides allowed faster cell growth.

Media cost showed a variation of US\$1.17 – 49.62 each 10³ L and components such as NaNO₃, cyanocobalamin and KNO₃ represented 48.6, 30.0 and 58.3%, respectively in final medium cost participation. Experimentally, we verified that pigment laboratory costs, although unfeasible in a large production scale based on the results found in this study (US\$558.76 - 449,508.19, 1 kg basis of powdered pigment), presented enormous differences in terms of production only by changing the culture media.

Additionally, to further evaluate the effects of culture media on *D. salina* cultivation, future research might involve medium optimization based on the use of low-cost-added supplements related to the production of pigments as well as in-depth studies of scale-up outdoor experimental tests. Also, the increase of carbon dioxide to *D. salina* cultivation may yield greater biomass production and improvements for future prospects of this topic.

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CHAPTER 4

Bioprocess and Biosystems Engineering
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RESEARCH PAPER



Rice vinasse treatment by immobilized *Synechococcus pevalekii* and its effect on *Dunaliella salina* cultivation

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Abstract

The development of new strategies in microalgal studies represents an outstanding opportunity to mitigate environmental problems coupled with biomass production at a reduced cost. Here we present a combined bioprocess for the treatment of rice vinasse using immobilized cyanobacteria *Synechococcus pevalekii* in alginate beads followed by the use of the treated vinasse as a culture medium for *Dunaliella salina* biomass production. Cyanobacterial-alginate beads showed a chlorophyll *a* production of 0.68×10^{-3} mg bead⁻¹ and a total carotenoid production of 0.64×10^{-3} mg bead⁻¹. The first step showed a decrease in nitrate (91%), total solids (29%), and ions. Addition of treated vinasse into *D. salina* cultivation resulted in a significant increase in cell replication of about 175% (optimized cultivation). The use of natural seawater drastically reduced the medium cost to US\$4.75 per m³ and the addition of treated vinasse has the potential to reduce it even more (up to 69%). This study not only provides an insight on the use of cyanobacteria for rice vinasse treatment but also demonstrates a promising lower-cost medium for marine microalgal biomass production with biotechnological purposes.

Keywords Microalgae · Wastewater · Biotechnology · Ion removal · Alginate beads · Medium cost

Introduction

Increased biofuel production and the importance of preserving the environment have motivated new technology development worldwide. According to Lopes and colleagues (2016) [1], technologies to produce ethanol from various

feedstocks such as sugarcane and corn are available in Brazil and play an important role in the economic feasibility analysis. For these reasons, new feedstocks are necessary to support the ethanol production process. Rice cultivars with potential production of 14 t ha⁻¹ have been developed for ethanol generation and animal nutrition [2].

However, ethanol distilleries produce a highly polluting residue called vinasse. The inadequate disposal of vinasse can cause serious environmental damage such as alteration of physical, chemical, and biological soil properties [3], inhibition of seed germination, presence of harmful heavy metals [4], and genotoxic activity [5]. According to Parsaei et al. (2019) [6], 22.4 giga litres of vinasse are produced annually, and this residue could be used as an alternative nutrient source in biotechnological processes. Vinasse has a low carbon-to-nitrogen ratio and can be used as an additive in a complex medium as a nutrient source [6]. Montalvo et al. (2019) [7] reported the use of sugarcane vinasse for *Arthrospira maxima* biomass production with the aim of reducing Biochemical Oxygen Demand (BOD) and Chemical Oxygen Demand (COD) of the vinasse, coupled with peptide production. Furthermore, the bioremediation process could have increased efficiency by using microalgae

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RICE VINASSE TREATMENT BY IMMOBILIZED *Synechococcus pevalekii* AND ITS EFFECT ON *Dunaliella salina* CULTIVATION

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4.1 ABSTRACT

The development of new strategies in microalgal studies represents an outstanding opportunity to mitigate environmental problems coupled with biomass production at a reduced cost. Here we present a combined bioprocess for the treatment of rice vinasse using immobilized cyanobacteria *Synechococcus pevalekii* in alginate beads followed by the use of the treated vinasse as culture medium for *Dunaliella salina* biomass production. Cyanobacterial-alginate beads showed a chlorophyll *a* production of 0.68×10^{-3} mg bead⁻¹ and a total carotenoid production of 0.64×10^{-3} mg bead⁻¹. The first step showed a decrease in nitrate (91%), total solids (29%), and ions. Addition of treated vinasse into *D. salina* cultivation resulted in a significant increase in cell replication of about 175% (optimized cultivation). The use of natural seawater drastically reduced the medium cost to US\$4.75 per m³ and the addition of treated vinasse has the potential to reduce it even more (up to 69%). This study not only provides an insight on the use of cyanobacteria for

rice vinasse treatment, but also demonstrates a promising lower-cost medium for marine microalgal biomass production with biotechnological purposes.

Keywords: Microalgae. Wastewater. Biotechnology. Ion removal. Alginate beads. Medium cost.

4.2 INTRODUCTION

Increased biofuel production and the importance of preserving the environment have motivated new technology development worldwide. According to Lopes and colleagues (2016), technologies to produce ethanol from various feedstocks such as sugarcane and corn are available in Brazil and play an important role in economic feasibility analysis. For these reasons, new feedstocks are necessary to support the ethanol production process. Rice cultivars with potential production of 14 t ha⁻¹ have been developed for ethanol generation and animal nutrition (Embrapa, 2012).

However, ethanol distilleries produce a highly polluting residue called vinasse. The inadequate disposal of vinasse can cause serious environmental damage such as alteration of physical, chemical, and biological soil properties (Christofolletti, Escher, Correia, Marinho, & Fontanetti, 2013), inhibition of seed germination, presence of harmful heavy metals (Ale, Jha, & Belbase, 2008), and genotoxic activity (Yesilada, 1999). According to Parsaee et al. (2019), 22.4 gigalitres of vinasse are produced annually, and this residue could be used as an alternative nutrient source in biotechnological processes. Vinasse has a low carbon-to-nitrogen ratio and can be used as an additive in a complex medium as a nutrient source (Parsaee et al., 2019). Montalvo et al. (2019) reported the use of sugarcane vinasse for *Arthrospira maxima* biomass production with the aim of reducing Biochemical Oxygen Demand (BOD) and Chemical Oxygen Demand (COD) of the vinasse, coupled with peptide production. Furthermore, the bioremediation process could have increased efficiency by using microalgae encapsulation (Kozieł, Jaromin-Gleń, Polakowski, Walkiewicz, & Bulak, 2019) because of its advantages in terms of biomass recovery.

Immobilized microalgae are commonly reported in the literature as a potent and feasible technology that efficiently produces high-value metabolites (Desmet et al., 2014)

and are also studied for their use in enhancement of lipid production (Kitcha & Cheirsilp, 2014), wastewater treatment (Zeng et al., 2015), and nitrogen retention in soils (Kozieł et al., 2019). No standard methodology can be assigned to treat vinasse using microalgae beads because the efficacy of a chosen method relies on the species and immobilization technique. According to Moreno-Garrido (2008), active and passive cell immobilization can be applied in microalgal cell entrapment. After biological treatment, the vinasse can be used for microalgal free-living cell cultivation.

Marine microalgae play an important role in industrial metabolite production. The unicellular halotolerant green microalga *Dunaliella salina* is a rich source of biomolecules such as β -carotene (Borowitzka & Borowitzka, 1990), lutein, violaxanthin, antheraxanthin, zeaxanthin, and lycopene (Fu et al., 2013; Jin & Melis, 2003). Processes related to the potential of new isolates and biomass production of *D. salina* have been widely studied (Khadim et al., 2018), although the high operational cost remains a limiting factor when considering a profitable microalgal facility. The reduction of microalgal medium cost could be achieved by the use of wastewater as an alternative nutrient source (Acién Fernández, Fernández Sevilla, & Molina Grima, 2019).

In this study, the hypothesis was that immobilized cyanobacteria could be used to treat rice vinasse and that the resulting treated vinasse could be utilized as culture medium for marine microalga cultivation with the aim of decreasing the cost of the medium. The goals were: (a) to conduct biological treatment of rice vinasse using encapsulated *Synechococcus pevalekii* in a fluidized bed reactor, (b) to determine the cyanobacterial cell growth and photosynthetic pigment production of cells inside the alginate beads, and (c) to study the cell growth and impact on medium cost of *D. salina* cultivation using diluted treated rice vinasse. As far as we know, there are no reports related to the use of rice vinasse for this kind of approach.

4.3 MATERIALS AND METHODS

Microalgae strains and culture maintenance

S. pevalekii (BMAK163) and *D. salina* (BMAK 116) were obtained from the Aidar & Kutner Collection Bank (BMA&K, Oceanographic Institute at the São Paulo University,

Brazil). *S. pevalekii* inoculum was cultivated in sterilized BG-11 medium as previously described (Stanier, Kunisawa, Mandel, & Cohen-Bazire, 1971) with pH adjusted to 8.5. *D. salina* inoculum was cultured using sterilized Conway medium (Walne, 1966) in its original composition with the addition of 35 g L⁻¹ of artificial marine salt (Salinity®) and pH adjusted to 7.5. The cultures were grown photoautotrophically and maintained individually in 250 mL flasks at 23 ± 1°C under a 12:12 light/dark cycle (100 µmol m⁻² s⁻¹). Batch cultures were used for all experiments.

Rice vinasse physicochemical analysis

Rice vinasse was obtained from Bio4-Soluções Biotecnológicas located in Curitiba, Brazil. The pH (5400-H), total solids (2540-B), volatile total solids, fixed solids (2540-E), COD (5220-C), BOD (5210-B), ammoniacal nitrogen (NH₃-N), nitrate/nitrite and total nitrogen content (4500) of crude vinasse were analyzed according to APHA methods (APHA, 2005), and the total protein content was determined by the Kjeldahl method (Kirk, 1950). All tests were performed in triplicate. Ion analyses were carried out using a 761 Compact IC Ion Chromatograph (Metrohm) with eluent automatic injection equipped with a cation column (Metrosep C3 250/4.0, Metrohm) for the analysis of K⁺, Na⁺, Mg²⁺, and Ca²⁺ ions, and an anion column (Metrosep A Supp 5 250/4.0, Metrohm) for Cl⁻ and PO₄³⁻. The calibration curves were linear over a range of 1–10 mg L⁻¹. Protein content and ion quantification were analyzed after 0.22 µm filtration.

Cyanobacteria *S. pevalekii* immobilization and bioreactor setup

S. pevalekii immobilization via entrapment was achieved according to de-Bashan et al. (2002) with some modifications. Na-alginate (2.5 g, Sigma-Aldrich, CAS: 9005-38-3, low viscosity) was dissolved directly in late exponential cyanobacterial cell suspension (100 mL, initial biomass concentration of 0.38 g L⁻¹). The alginate-cyanobacteria mixture was dropped using a Peristaltic Pump P-1 (Pharmacia Biotech) with a flow rate of 270 mL h⁻¹ into a 5% CaCl₂ solution. The suspension was stirred to prevent cell bead aggregation. Next, the cyanobacterial Ca-alginate beads (~5 mm) were stabilized in CaCl₂ solution for 30 min at 23°C, then rinsed twice in 0.85% NaCl solution and once in distilled water. To

produce Ca-alginate blank beads without cells, the same standard methodology was applied but the cell suspension was replaced with the same volume of distilled water.

To proceed with the experiment, a fluidized bed reactor with 3 columns (38.5/2.2 cm) was built containing autoclaved BG-11 medium of 10% (v/v) crude rice vinasse (pH 8.5) added. The reactor inoculation was done using 2,000 beads L⁻¹ with a working volume of 70 mL in each column. The experiment was carried out for 10 days under a 12:12 light/dark cycle with illumination of 50 µmol m⁻² s⁻¹ and air supply at a flow rate of 2 L min⁻¹. Supernatant and bead samples were collected every 48 h for 10 days for analyses. The experiments were performed in triplicate.

Cyanobacterial *S. pevalekii* bead analyses

Cell growth in the Ca-alginate beads was investigated using scanning electron microscopy (SEM) (JSM-6360 LV, JEOL). For this analysis, the beads were dehydrated with increasing concentrations of ethanol solution (30–95% v/v) for 15 min for each solution. Samples were dried with CO₂ in a critical point dryer (CPD 030, Bal-Tec), metalized with gold in a sample coater (SCD 050, Bal-Tec), and then analyzed by SEM. Photographs were taken to compare cell development. The SEM analyses were performed before and after the fluidized bed experiment.

To proceed to pigment analysis of entrapped cells, cyanobacterial-alginate beads were solubilized in 2% NaHCO₃ (5 beads in 5 mL) and filtered using a Macherey-Nagel GF-1 glass-fiber filter. The retained cells were submitted to extraction in 90% acetone solution (4 mL) at 4°C for 18 h. The resulting extracts were then centrifuged (1915 x g, 10 min) and the pigments analyzed by spectrophotometry (Spectra max PLUS-384, Molecular Devices) at 664 and 750 nm before and after acidification with one drop of 0.25 M HCl in the cuvette for chlorophyll *a* (Chl *a*), and at 480, 510, and 750 nm for total carotenoids (Car). Chl *a* and Car were calculated according to Eqs. 1 (Carl Lorenzen, 1967) and 2 (Strickland & Parsons, 1968), respectively:

$$[\text{Chl } a \text{ (}\mu\text{g L}^{-1}\text{)}] = \frac{26.73 \times [(A_{664 \text{ nm}} - A_{750 \text{ nm}}) - (A_{664a \text{ nm}} - A_{750a \text{ nm}})] \times V_a}{(V_c \times c)} \quad (1)$$

$$[\text{Car } (\mu\text{g L}^{-1})] = \frac{[7.6 \times A_{480 \text{ nm}} - (3 \times A_{750 \text{ nm}}) - 1.49 \times A_{510 \text{ nm}} - (2 \times A_{750 \text{ nm}})] \times V_a}{(V_c \times c)} \quad (2)$$

where A corresponds to absorbance at the specific wavelength, V_a is the volume (L) of acetone solution used for the extraction process, V_c is the culture volume (L) used for the filtration, c is the optical path (cm) from the cuvette, and a is the reading after acid addition.

A total of 12 cyanobacterial beads (beads containing cells and blank beads without cells) were taken every 48 h for dry weight measurements (g bead^{-1}). The beads were dried at 80°C overnight to determine dry weight content. All pigment analyses were performed in triplicate.

Treated rice vinasse chemical measurements

Aliquots of the supernatant from the fluidized bed reactors were taken every 48 h for nitrate analysis. Nitrate content was determined according to Collos et al. (1999). Supernatant was filtered ($0.22 \mu\text{m}$) and analyzed by spectrophotometry (Spectra max PLUS-384, Molecular Devices) at 220 and 275 nm. The absorbances were compared to a standard nitrate curve using NaNO_3 . Total solids were evaluated in the supernatants according to the methodology described by APHA (2005). All tests were performed in triplicate. The supernatants from days 0 and 10 were also analyzed by ion chromatography, according to subsection 2.2, for analysis of the following ions: K^+ , Mg^{2+} , Ca^{2+} , Cl^- , and PO_4^{3-} .

Dunaliella salina cultivation, growth parameters, and pigment analysis

D. salina was cultivated in varying compositions of the medium to determine the effect of treated vinasse on the culture. For this, five different compositions were studied, as follows: 100% Conway, 75% Conway + 25% treated vinasse, 50% Conway + 50% vinasse, 25% Conway + 75% treated vinasse and 100% treated vinasse. The mixed media were prepared individually in 250 mL Erlenmeyer flasks with a working volume of 200 mL and pH adjusted to 7.5 followed by autoclaving. Inoculation was done in each flask based on 10% v/v final concentration from stock culture (see 2.1). The experiment

was carried out for 7 days under batch culture without aeration at a 12:12 light/dark cycle with cool white fluorescent lamps ($100 \mu\text{mol m}^{-2} \text{s}^{-1}$).

All growth and pigment analyses were done in the first and seventh day of the experiment. Cell growth measurements were carried out by cell counting in a Neubauer hemocytometer by optical microscopy. pH and conductivity were measured to monitor the effect of treated vinasse on the microalgal culture. Pigment analysis was performed according to the previous subsection. In addition to Chl *a* and Car, pheophytin *a* content (Phe *a*) was also calculated according to Eq. 3 (Carl Lorenzen, 1967). After measurements for chlorophyll, the samples were acidified by direct addition of 12 μL of 0.25 M HCl into the cuvette for Phe *a* determination based on readings at 664 and 750 nm.

$$[\text{Phe } a (\mu\text{g L}^{-1})] = \frac{26.73 \times [1.8 \times (A_{664a \text{ nm}} - A_{750a \text{ nm}}) - (A_{664 \text{ nm}} - A_{750 \text{ nm}})] \times V_a}{(V_c \times c)} \quad (3)$$

where *A* corresponds to absorbance at the specific wavelength, *V_a* is the volume (L) of acetone solution used for the extraction process, *V_c* is the culture volume (L) used for the filtration, *c* is the optical path (cm) from the cuvette, and *a* is the reading after acid addition. All tests were performed in triplicate.

Parameter optimization and experimental validation

A third order polynomial curve was applied for all four parameters studied in the previous step using Microsoft Excel® software. Optimized vinasse concentration was calculated from the derived equation when $y' = 0$ and the concentrations were averaged for experimental validation. The validation experiment was done in quadruplicate and under the aforementioned cultivation parameters.

Growth medium economic analysis

Conway medium costs were estimated by evaluating the nutrient price individually using quotes from regional laboratory companies. The total medium cost was compared

to the optimized modified medium with addition of treated vinasse used in the *D. salina* cultivation in terms of cost-effectiveness.

Statistical analysis

All experiments were carried out in triplicate and the results of all repetitions were expressed as the mean $\pm$ SEM (standard error of the mean) or $\pm$ SD (standard deviation). Significant differences were determined using one-way analysis of variance (ANOVA). Differences between mean values were tested using Tukey's test. Data with a $p < .05$ were considered statistically significant. All graphs were created using GraphPad - Prism 5.

4.4 RESULTS AND DISCUSSION

In this section, the crude rice vinasse characterization and bioprocess using immobilized cyanobacterial cells are presented, followed by the results of treated diluted rice vinasse addition in *D. salina* cultivation. The first step is related to the biological treatment of diluted rice vinasse using immobilized *S. pevalekii* in alginate beads. In the second step, different concentrations of treated diluted rice vinasse medium were used for *D. salina* cultivation and their impacts on cell growth and economic medium cost were evaluated.

Crude rice vinasse characterization

Table 14 summarizes the physicochemical characterization of crude rice vinasse and its comparison with sugarcane vinasse. As shown, rice vinasse has an acidic pH of 2.92 ± 0.01 . This result is directly related to the type of raw material used in the ethanol production process. pH variation between 2.9 and 5.4 for vinasses obtained from cane juice, cane molasses, grapes, agave, sweet sorghum, and beet molasses have been reported (Christofolletti et al., 2013; España-Gamboa et al., 2011). The rice vinasse showed total solids and protein content of 4.51 g L^{-1} and 23.0%, respectively. Differently, according to España-Gamboa et al. (2011), most of the nitrogen in corn and barley vinasses can be found as amino acids and peptides rather than as proteins.

COD and BOD content showed 64,028 and 15,458 mg L⁻¹, respectively. Several studies reported different values of COD and BOD content from sugarcane vinasse. Montalvo and colleagues (2019) reported a COD and BOD content of 24,605 and 7,642 mg L⁻¹, respectively from sugarcane vinasse used for microalgal cultivation. The authors also reported a nitrate content of 20 mg L⁻¹. Moreover, nitrite content showed a very low value in rice vinasse presumably due to reduction state from nitrogen cycle as described by Stein and Klotz (2016).

Table 14 - Physicochemical characterization of crude rice vinasse and its comparison with sugarcane vinasse

Parameter	Rice vinasse (this study)*	Sugarcane vinasse**
pH	2.92 ± 0.01	4.15 - 8.60
Total solids (g L ⁻¹)	4.51 ± 0.20	10.36 - 36.8
Volatile solids (g L ⁻¹)	4.08 ± 0.25	23.8
Fixed solids (g L ⁻¹)	0.42 ± 0.14	-
Protein (%)	23.01 ± 0.74	2.92
COD (mg L ⁻¹)	64,028 ± 322	24,605 - 42,990
BOD (mg L ⁻¹)	15,458 ± 824	7,642 - 36,847
NO ₂ ⁻ (mg L ⁻¹)	0.00345 ± 0.00015	-
NO ₃ ⁻ (mg L ⁻¹)	7.74 ± 0.31	20
NH ₃ -N (mg L ⁻¹)	7.20 ± 0.44	-
Total nitrogen (mg L ⁻¹)	410.97 ± 12.05	144
K ⁺ (mg L ⁻¹)	40	1,620 – 4,451
Na ⁺ (mg L ⁻¹)	20	8.6 - 91
Mg ²⁺ (mg L ⁻¹)	20	162 - 426
Ca ²⁺ (mg L ⁻¹)	160	1,166 – 3,160
Cl ⁻ (mg L ⁻¹)	500	59.4
PO ₄ ³⁻ (mg L ⁻¹)	300	10 - 560

*Data are presented as mean value ± SD (*n* = 3). **Adapted from Montalvo et al. (2019), Nitayavardhana et al. (2013), dos Santos et al. (2013), Dowd et al. (1994) and Paz-Pino et al. (2014).

High amounts of constituent elements such as Cl^- (500 mg L^{-1}) and PO_4^{3-} (300 mg L^{-1}) can be observed in Table 1. Different results were reported by Wilkie et al. (2000) on vinasse from ethanol distillery of conventional and cellulosic feedstock. Christofolletti et al. (2013) showed a total phosphorus content variation of 83–1,990 mg L^{-1} (254–6,101 mg L^{-1} in terms of phosphate) and magnesium content of 1.23–237 mg L^{-1} in sugarcane vinasse. This study also showed the presence of K^+ , Na^+ , Mg^{2+} , and Ca^{2+} at 40, 20, 20, and 160 mg L^{-1} , respectively. As shown in Table 14, differences in ion content from vinasse composition can be attributed to cultivation techniques, regional climate, variation in soil management, as well as different raw materials used in the ethanol production process (de Godoi et al., 2019) and storage conditions. Furthermore, all major components found in rice vinasse might be beneficial in microalgae and cyanobacteria cultivation systems.

Cyanobacteria-alginate bead analysis

Cyanobacteria-alginate beads collected at days 0 and 10 were submitted to SEM analysis to verify cell replication. Immediately after cell immobilization, individual trapped cells localized in the alginate matrix were observed (Fig. 9a).

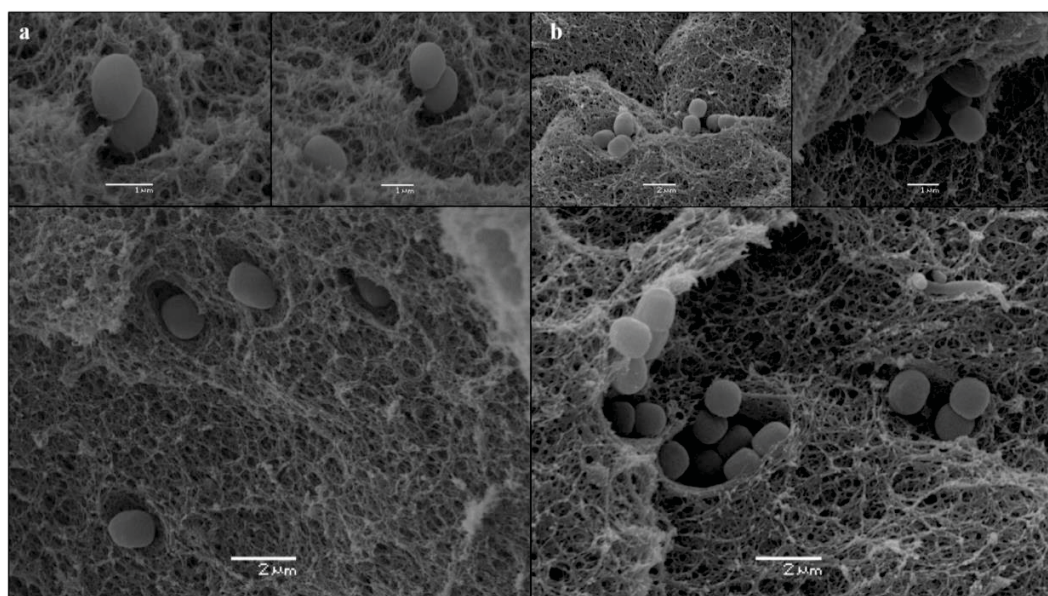


Fig. 9 - Scanning electron microscopy analysis of fractured beads at days 0 (A) and 10 (B) during *Synechococcus pevalekii* cultivation

After 10 days of cultivation (Fig. 9b), the cells presented an increased population inside of the beads, showing a colony aspect. The alginate matrix acted as a physical barrier affecting cell dispersion into the bead. No cells were observed in the supernatant. Several studies have reported an increase in cell density on immobilized cyanobacteria (Ruiz-Güereca & Sánchez-Saavedra, 2016) and enhancement of cell survival and enzyme activity (Pansook, Incharoensakdi, & Phunpruch, 2016). Covarrubias et al. (2012) reported significant cell enhancement of immobilized *Chlorella sorokiniana* and *Azospirillum brasilense* suspended in municipal wastewaters.

Moreover, immobilized cyanobacterial cultivation in Ca-alginate beads plays an important role in biomass recovery from downstream processes. In this case, beads were recovered easily through a conventional filtration process, implying a reduced cost in microalgal cultivation or wastewater treatment facilities and promoting normal metabolite production since immobilized cells are not affected by the alginate matrix. According to Lv et al. (2017), the immobilization process does not cause damage or toxicity to cells as expected considering its excellent nutrient permeability. Additionally, this technique is applicable in the treatment of secondary effluents to remove pollutants (mainly nitrogen and phosphorus), accumulating biomass in the process (Lv et al., 2017).

Cyanobacterial beads were also analyzed for wet and dry weight to monitor *S. pevalekii* growth rates during the experiment. Fig. 10 shows the differences between wet/dry weights of entrapped cells (a, b) compared to blank beads (c, d). Fig. 10a shows an increase in entrapped bead weight over cultivation time; however, Fig. 10c and 10d indicate a loss in blank bead weight in the late experimental cultivation phase. Fig. 10b presents a weight loss of 14% from the initial dry weight, while Fig. 10d shows a weight loss of 70%. This difference could be explained, respectively, by the increase in cell numbers inside the entrapped beads, and Ca-alginate bead partial disintegration, evidenced by the great increase of calcium content in the supernatant medium after 10 days of cultivation (see Table 15).

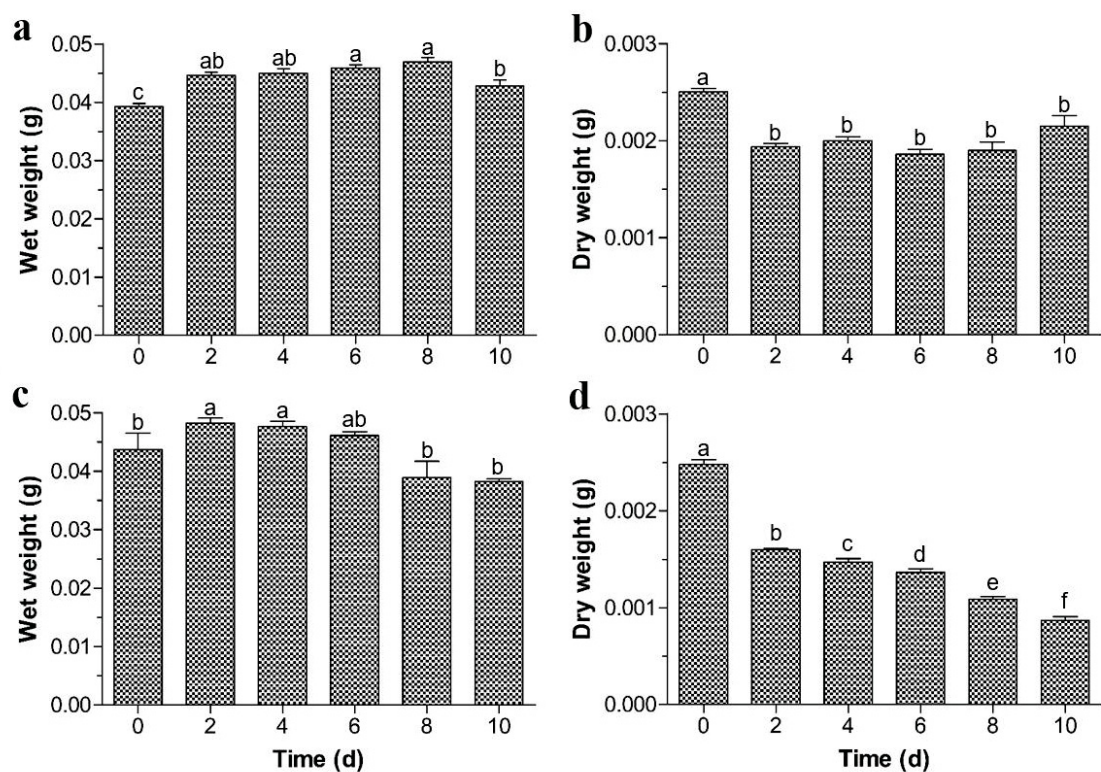


Fig. 10 - Wet/dry weight of loaded cells beads (A, B) and blank beads (C, D). Data are shown as mean $\pm$ SEM (n = 3). Bars followed by the same letters do not differ significantly (Tukey's test, $p < .05$)

Increase in cell growth and biomass in entrapped cells is reported in the literature to represent higher yields compared to free-living cell cultures. *Botryococcus braunii* showed an entrapped cell biomass production of 1.05 g L^{-1} compared to 0.73 g L^{-1} in free cell cultures in 24 days of cultivation using polyester wadding as a fixing matrix (Giraldo Calderón, Díaz Bayona, & Atehortúa Garcés, 2018). In this case, the cell density inside the bead was responsible for weight maintenance over the experimental cultivation period.

Kube et al. (2019) reported that alginate presents a positive effect on cell growth, in addition to being a cheap and effective option for wastewater treatment, which makes it a successful cell entrapment system for microalgae cultivation. For *Scenedesmus obliquus*, mixotrophic cultivation showed better performance than heterotrophic

cultivation systems (Liu et al., 2019), promoting higher biomass yields. Besides biomass accumulation, entrapped cyanobacterial cells can be used for wastewater treatment (Zeng et al., 2015) and to produce bio-hydrogen in higher rates compared to free cell cultivation (Touloupakis et al., 2016).

Fig. 11 shows the photosynthetic pigment production from entrapped cells during rice vinasse treatment. The highest production content occurred at day 8, with a Chl *a* production of $0.68 \times 10^{-3} \text{ mg bead}^{-1}$ and a total carotenoid (Car) production of $0.64 \times 10^{-3} \text{ mg bead}^{-1}$. The photosynthetic pigment production is related to cell growth (Tam & Wong, 2000). Ji et al. (2019) reported three distinct phases of culture development, i.e., an adjustment period, a log phase, and a stationary or decay phase. The cell culture profile corroborates with the pigment curves obtained from entrapped cells on Ca-alginate matrix (Fig. 11). After day 8, a decline of 36.7% and 7.8% of Chl *a* and Car was observed, respectively, probably as a result of the late cell phase of experimental cultivation. Photosynthetic pigments of microalgal cells are considered specific markers for autotrophic microorganisms and their concentration is directly affected by cellular death rates (Zhang, Wang, Wang, Zhang, & Zhao, 2008).

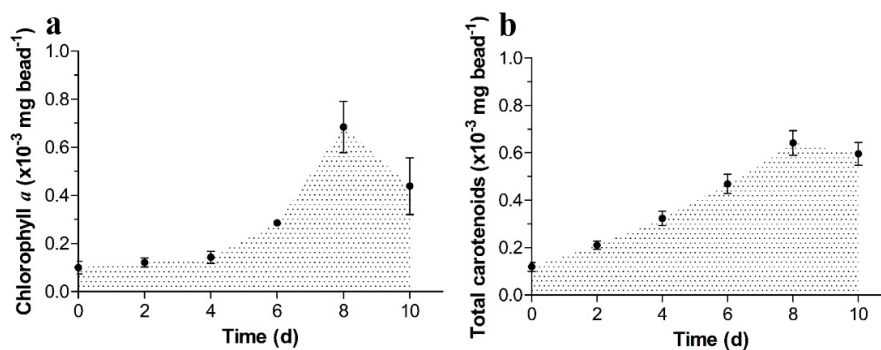


Fig. 11 - Photosynthetic pigment production by cyanobacterial entrapped cells. Data are shown as mean $\pm$ SEM ($n = 3$)

Calcium alginate beads can retain their integrity for 30 days with no leakage of cells to the medium and no interference with photosynthetic activity (Touloupakis et al., 2016). In addition, cells entrapped in Ca-alginate beads present a higher specific growth

rate than free cells (Soo, Chen, Bojo, & Hii, 2017) and show an increase in Chl *a* content compared to free cell cultivation because of the shading effect from the immobilization matrix (Ruiz-Güereca & Sánchez-Saavedra, 2016; Vichez & Vega, 1994). Thus, the Chl *a* content increase (Fig. 11a) in entrapped cells can be related to cell replication and self-shading (Vichez & Vega, 1994; Tam & Wong, 2000; this study). The total carotenoid content (Fig. 11b) was related to Chl *a* production rate and it expresses non-stressed cells during the experiment.

In this study, Ca-alginate matrix not only provided a successful culture of *S. pevalekii*, but it also led to carotenoid production which may be exploited to scale-up processes. Furthermore, high quality alginate could be a costly biopolymer, however, for the purpose of the present study, there is no need of a high purity alginate since the main objective is only to create an immobilization matrix. Further approaches are in progress in our research group to evaluate the impact that different polysaccharides have in bead formation, stability and economic costs. Moreover, the production of high-value compounds from entrapped cells was previously reported (Desmet et al, 2014; Kitcha & Cheirsilp, 2014), and it could be used synergistically with the wastewater treatment process.

Diluted rice vinasse medium characterization

The diluted rice vinasse medium was submitted to nutrient analysis to evaluate the removal efficiency of the cyanobacterial bead system. According to Fig. 12a-d, the bead systems presented a drastically decrease in nitrate content and total solids, and after 10 days of immobilization, cyanobacterial beads showed a nitrate and total solid removal of 91 and 29%, while blank beads showed 80 and 25%, respectively (Fig. 12f).

Comparing the removal percentage, the total solid removal for cyanobacterial beads was observed to be 1.2 times higher than alginate beads without cells (Fig. 12f). An increase in NO_3^- removal of 6.4, 3.1, and 2.3 times was observed on days 2, 4 and 6, respectively, than in blank beads during the same cultivation period (Fig. 12e). The increase in NO_3^- removal during the late phase of the blank bead cultivation period (Fig. 12e) may be related to the denitrification process, in which NO_3^- is reduced to N_2 by denitrifying bacteria since the air was not sterilized. Heterotrophic denitrifying bacteria

have been described to remove nitrogen sources in wastewater under aerobic conditions (He, Xie, Li, Ni, & Sun, 2017; Lei, Wang, Liu, Xi, & Song, 2016; Ye, Zhao, An, & Huang, 2016). Moreover, some microorganisms use organic substrate and energy to convert ammonium (NH_4^+) into nitrogenous gas (N_2) (Zhao, An, He, & Guo, 2012). This process was planned to be further studied as a scale-up option for wastewater treatment; thus, non-sterilized atmospheric air was provided to the system (no measurement of CO_2 or O_2 was taken).

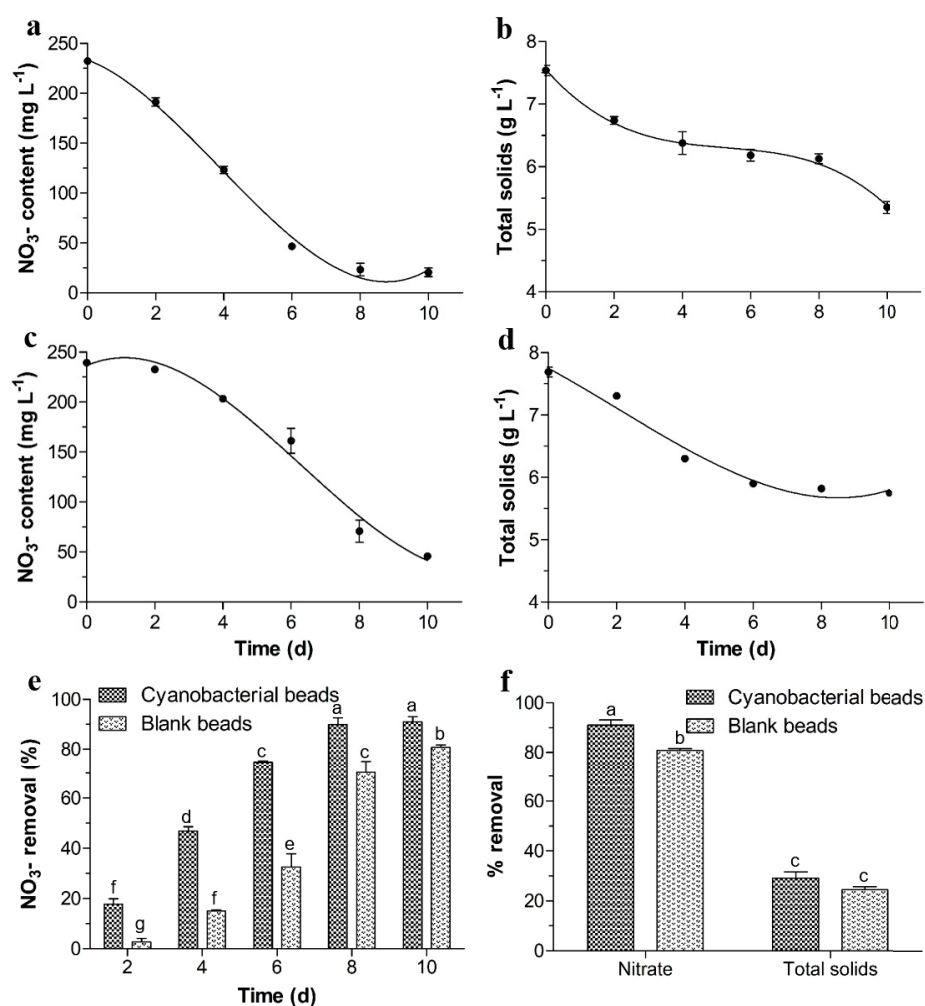


Fig. 12 - Nitrate concentration and total solids in diluted rice vinasse medium with cyanobacterial beads (A, B), blank beads (C, D), and total pollutant removal (E, F). Data are shown as mean $\pm$ SEM ($n = 3$). Bars followed by the same letters do not differ significantly (Tukey's test, $p < .05$)

Although there is a slight difference in removal rates (mainly in total solids), it is well-established that encapsulated cyanobacteria produce relevant metabolites with biotechnological applications in addition to wastewater treatment (Desmet et al., 2014; Tan, Du, & Lu, 2015; Weiss, Young, & Ducat, 2017), adding value to the process. The metabolite production potential from encapsulated cyanobacteria was not analyzed in our study.

Regarding nitrate and total solids removal, the ion absorption from the supernatant of entrapped cyanobacterial cells was also studied. The comparison of the ion composition in the diluted rice vinasse medium before and after treatment with cyanobacterial beads is summarized in Table 15. The results showed a reduction of 30% in both K^+ and Mg^{2+} contents. Cl^- and PO_4^{3-} showed initial concentrations of 74 and 17 $mg\ L^{-1}$, and after the biological treatment were below the limit of detection.

Table 15 - Performance of ion removal from diluted rice vinasse medium treatment at day 0 (D0) and day 10 (D10)

Sample	Ion ($mg\ L^{-1}$)				
	K^+	Mg^{2+}	Ca^{2+}	Cl^-	PO_4^{3-}
D0	73	10	19	74	17
D10	52	7	255	LoD	LoD

Ion standard curves were linear over the range of 1–10 $mg\ L^{-1}$. LoD: Under limit of detection.

Microorganisms were successfully cultivated to reduce nutrients, ions, and suspended solids, as previously reported, using immobilized cells for wastewater treatment. Ji et al. (2013) showed the potential of microalgae cultivation in tertiary municipal wastewater for biomass production and nutrient removal. Hodges et al. (2017) reported an increase in the removal of suspended solids in petrochemical wastewater using microalgal biofilm. The high removal rate of entrapped cells was related to the increase in cell density in alginate beads, promoting greater efficiency of nutrient removal (Zhang et al., 2008) and nitrogen assimilation by the cells, since this is closely dependent on photosynthetic electron flow (Vega, Menacho, & León, 1991).

According to Liu et al. (2019), immobilized *Scenedesmus obliquus* was used for ammonium removal from synthetic wastewater. The authors reported a 96% removal in 50 mg L⁻¹ of NH₄⁺-N in mixotrophic conditions. de-Bashan et al. (2002) reported the removal of 99% of ammonium and 75% of phosphorus from wastewater with the use of coimmobilization of *Chlorella vulgaris* and *Azospirillum brasilense*, while Ruiz-Güereca and Sánchez-Saavedra (2016) reported a phosphorus removal rate of 32% using immobilized *Synechococcus elongatus*.

In this study, unlike the other ions, the Ca²⁺ content was 13.4 times higher at day 10 in comparison with day 0 of cultivation. This indicates that Ca²⁺ was released from the inoculated Ca-alginate beads (see Table 2). Similar results were also reported by Kube et al. (2019) in wastewater treatment by *C. vulgaris* immobilized in Ca-alginate beads.

Efficiency rate of environmental pollutant reduction from microalgal processes is strictly dependent on several variables such as microalga species, residue characteristics, experimental working volume and microalgal growth system. Santana and colleagues (2017) selected a productive microalgae strain to be cultivated in sugarcane vinasse using a 15-L flat-plate photobioreactor. The authors presented a successful cultivation using undiluted vinasse with high biomass productivity. However, vinasse can present organic and inorganic composition variation regarding harvesting season and raw material used (Wilkie et al., 2000) (see Table 1) that can interfere in microalgal process efficiency (this study).

All of the other ions analyzed in the diluted rice vinasse medium showed lower concentrations when compared to day 0. Moreover, the application of cyanobacterial beads in wastewater treatment provides a desirable choice to reduce ions from aqueous solutions. With the aim to further evaluate the diluted rice vinasse medium previously treated by immobilized cyanobacterial cells, the medium was added to *D. salina* cultivation to analyze its impact on growth performance and microalgal marine medium cost.

Cell growth evaluation of *Dunaliella salina* culture

After rice vinasse treatment by entrapped cyanobacterial cells, Conway medium was supplemented with diluted treated rice vinasse medium to cultivate *D. salina*. This

study was focused on the vegetative growth phase of *D. salina* to evaluate the impact of treated rice vinasse addition. Stress conditions and specific carotenoids were not evaluated. Fig. 13a compares the microalgal cell density when different concentrations of diluted treated rice vinasse medium were added to the Conway medium. Cell density increased to 522 and 493 $\times 10^4$ cell mL^{-1} when 50 and 75% of treated vinasse were added to the Conway medium, respectively. This implies (a) a significant increase of cell development in the culture of 170% (at 50% vinasse) and 160% (at 75% vinasse) and (b) a considerable cost reduction of the medium. It must be emphasized that no CO_2 was added to the cultivation. Several studies have reported the use of different types of vinasses in microalgae cultivation (Barrocal, García-Cubero, González-Benito, & Coca, 2010; Ramirez, Farenzena, & Trierweiler, 2014).

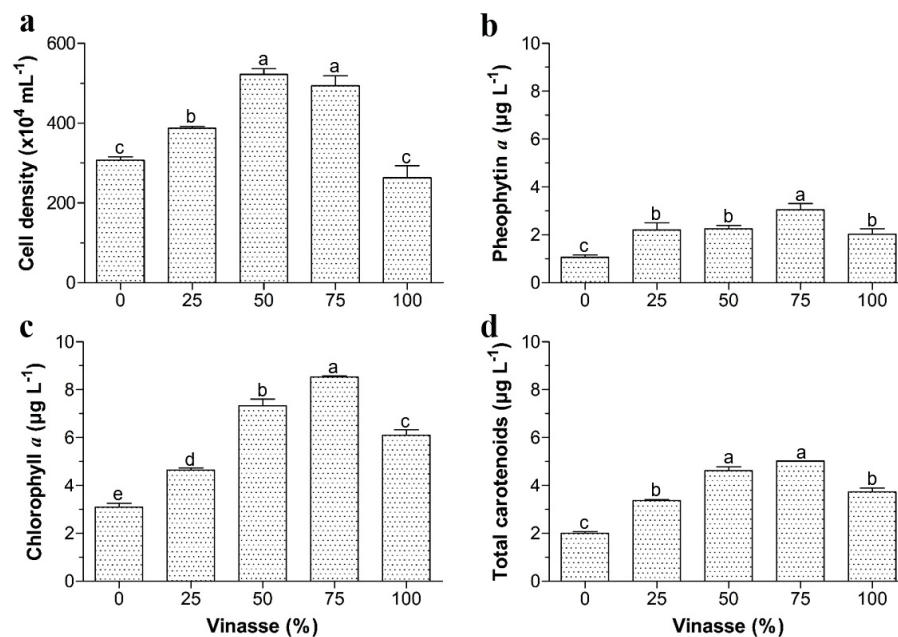


Fig. 13 - *Dunaliella salina* cell density (A) and photosynthetic pigment production (B, C, and D) by treated rice vinasse addition. Data are shown as mean $\pm$ SEM ($n = 3$). Bars followed by the same letter do not differ significantly (Tukey's test, $p < .05$)

Positive effects were observed on *Spirulina maxima* cultivation when crude vinasse from beet molasses was used as an alternative nutrient source (Barrocal et al.,

2010). Nonetheless, the addition of high vinasse concentration can inhibit microalgal cell development (Barrocal et al., 2010). Ramirez et al. (2014) reported that addition of more than 40% crude sugarcane vinasse on *Scenedesmus* sp. cultivation can decrease biomass production. These results differ from the present study since diluted treated rice vinasse was used for *D. salina* cultivation.

Aside from biomass production, the addition of vinasse to microalgae cultivation may contribute to the minimization of problems related to distillery byproducts by recycling hazardous ions from vinasse and producing high-value compounds. As can be seen in Fig. 13b, c, and d, photosynthetic pigment production is positively affected by treated rice vinasse addition in different concentrations. Chl *a*, Phe *a*, and Car content showed higher production at 8.53, 3.04, and 5 $\mu\text{g L}^{-1}$, respectively, when treated vinasse was added up to 75% (v/v). The use of 100% treated vinasse did not show any improvement in pigment synthesis when compared to the addition of other concentrations of treated vinasse in the culture. The treated rice vinasse addition at all concentrations showed higher pigment production content compared to 100% Conway medium.

Morais and Bastos (2019) presented an increase in *Aphanothece microscopica* cellular growth and phycocyanin production using sugarcane vinasse (9% v/v) in a mixotrophic cultivation system. However, it has been shown in the literature that the addition of digested vinasse decreased *Spirulina platensis* growth due to the presence of organic compounds (Budiyono, Syaichurro, Sumardiono, & Sasongko, 2014), which was not observed in the present study.

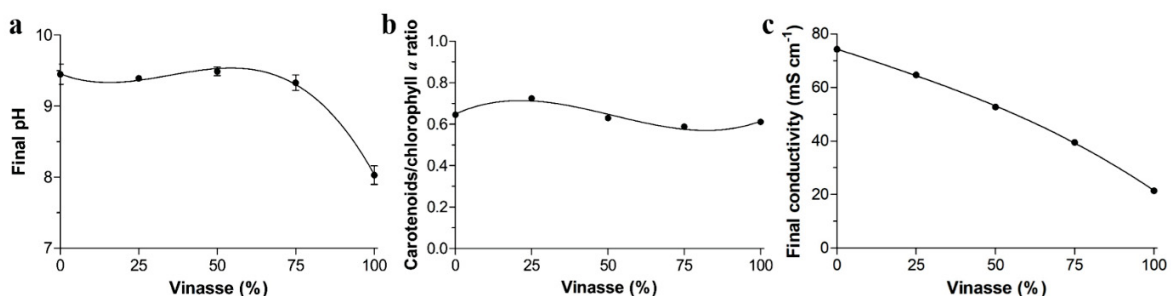


Fig. 14 - Effect of the addition of different percentages of treated rice vinasse on final pH (A), pigment production ratio (B), and final conductivity (C) from *D. salina* cultivation. Data are shown as mean $\pm$ SEM ($n = 3$)

Fig. 14 shows the offline measurements related to *D. salina* cultivation. The pH values showed stability up to 75% vinasse addition in the medium. The pH plays an important role in microalgae cultivation. According to Bartley et al. (2014), the ideal pH in the microalgal cultivation can maximize cellular growth, encourage high cell densities, maximize lipid accumulation, and reduce undesired microorganisms. It was also observed that the pH dropped when 100% vinasse was added in *D. salina* cultivation (Fig. 14a). Despite differences in cell density (Fig. 13a), experimental results showed that the cells were not stressed by lack of nutrients or light interference according to Fig. 14b. In general, a great amount of carotenoid production was not seen compared to Chl *a*.

At the end of cultivation, conductivity showed a decline as the treated rice vinasse concentration in the medium increased (Fig. 14c). This result can be explained by the decrease in artificial salt with the incremental increase of treated vinasse volume in Conway medium. The conductivity is commonly used to monitor nutrient availability on hydroponics (Gent, 2003) as well as for monitoring performance for microalgae dewatering (Larronde-Larretche & Jin, 2016). Furthermore, the use of cyanobacterial beads for wastewater treatment and the reuse of treated vinasse for microalgae cultivation may be useful as a biorefinery process because of its application and feasibility.

Microalgal culture optimization and validation process

After cell growth evaluation, an optimization process was performed with the intention of maximizing cell density and pigment content at a low cost as a result of treated rice vinasse addition. Table 16 shows the equations and optimized parameters achieved from third order polynomial curves. The optimized vinasse concentration (optimum value) was obtained from the x value when $y' = 0$. The third order polynomial curves can be seen in Online Resource 1.

Cell density, chlorophyll *a*, total carotenoids, and pheophytin *a* presented the best production when 61, 62, 83, and 70% of treated rice vinasse was added, respectively. This value was averaged (69%) to validate the optimization process. Similar results are not discussed or assessed in the literature because of the use of crude vinasse with no biological treatment directly in microalgal cultivation systems. According to Ramirez and

colleagues (Ramirez et al., 2014), the addition of 40% v/v crude sugarcane vinasse into microalgae cultivation can increase the biomass produced.

Table 16 - Optimization process from growth parameter data

Parameters	Equation	y'	Optimized vinasse concentration*
Cell density	$y = -0.0014x^3 + 0.1163x^2 + 1.658x + 304.85$	$y' = -0.0042x^2 + 0.2326x + 1.658$	61%
Chlorophyll <i>a</i>	$y = -3 \times 10^{-5}x^3 + 0.0027x^2 + 0.0105x + 3.1$	$y' = -9 \times 10^{-5}x^2 + 5.4 \times 10^{-3}x + 0.0105$	62%
Total carotenoids	$y = -1 \times 10^{-5}x^3 + 0.0012x^2 + 0.0076x + 2.6261$	$y' = -3 \times 10^{-5}x^2 + 0.0024x + 0.0076$	83%
Pheophytin <i>a</i>	$y = -3 \times 10^{-6}x^3 + 6 \times 10^{-5}x^2 + 0.0355x + 1.1268$	$y' = -9 \times 10^{-6}x^2 + 1.2 \times 10^{-4}x + 0.0355 + c$	70%

*Optimized treated vinasse concentration was calculated according to the derived equation ($y' = 0$) obtained from third order polynomial curves.

After optimized vinasse achievement, a validation experiment was proposed to confirm the obtained mathematical models. Table 17 presents the predicted and experimental values obtained in this study when 69% of treated rice vinasse was added to microalgal cultivation, according to the equations.

Table 17 - Comparison between predicted and experimental values

Parameters	Predicted value	Experimental value*
Cell density ($\times 10^4$ mL ⁻¹)	513	538 ± 17
Chlorophyll <i>a</i> (µg L ⁻¹)	6.82	7.89 ± 0.46
Total carotenoids (µg L ⁻¹)	5.57	5.03 ± 0.35
Pheophytin <i>a</i> (µg L ⁻¹)	2.87	2.88 ± 0.38

*Data are shown as mean ± SEM ($n = 4$).

The experimental values demonstrate similarity when compared to predicted data obtained from the previous equations. A 175% increase in cell number was observed when compared to cells cultivated in 100% Conway medium (see Fig. 13a). The addition of raw material derived from downstream processes (such as vinasse or even digestate obtained from anaerobic digestion) in microalgal processes is becoming more common because of the reduced/null cost and fewer environmental issues (Xia & Murphy, 2016). In spite of the overall high yields reported for *D. salina* cultivation using chemically well-defined medium (Tran, Doan, Louime, Giordano, & Portilla, 2014), the present study provides an environmentally sustainable technology development that combines microalgal biomass production with agroindustrial residue use. Besides cellular parameters, optimized *D. salina* culture showed a pH value of 10.1 ± 0.04 and conductivity of $26.2 \pm 0.22 \text{ mS cm}^{-1}$ after the optimization experiment. The advantages of using treated rice vinasse for *D. salina* cultivation include an increase in cell density and photosynthetic pigments and a decrease in the medium cost.

Medium cost in *D. salina* cultivation

As marine microalgae require a similar environment to their natural habitat, it is fundamental to promote synthetic or natural seawater for cell cultivation. While natural seawater requires proximity to the sea, synthetic seawater can be used in continental areas although it is expensive. The use of residues such as vinasse can prove to be an economic alternative.

Price evaluation was done based on the production of 1 m^3 of culture medium containing synthetic and natural seawater. According to regional quotes, the total medium cost using synthetic seawater is about US\$309 per m^3 . “Artificial” sea salt, used to maintain identical characteristics to seawater, represents 98% of the medium cost. The use of natural seawater can reduce the medium cost drastically down to US\$4.75 per m^3 ; therefore, this data was used to further evaluate the impacts on medium cost.

Table 18 shows the comparison between synthetic seawater and natural seawater medium costs by component distribution. After redistribution of medium components (without artificial sea salt), cyanocobalamin corresponds to 30.9% of the total medium cost, followed by Na_2EDTA (24.7%), NaNO_3 (17.5%), and $\text{NaH}_2\text{PO}_4 \cdot 2\text{H}_2\text{O}$ (14.5%).

Sipaúba-Tavares et al. (2015) showed a 77% medium cost reduction with addition of NPK (20-5-20) fertilizer in *Haematococcus pluvialis* cultivation.

In the previous study, it was found that the microalgal medium components with major cost participation were macronutrients, mainly nitrogen, phosphorus, and potassium (Colusse, Duarte, de Carvalho, & Nosedá, 2019). Regarding the cyanocobalamin cost, in the present study its value in the medium cost was significantly higher compared to other components. Microalgal medium optimization can improve high-value compound production and reduce nutrient requirements such as P, Mo, Mn, and thiamine (Camacho-Rodríguez, Cerón-García, Fernández-Sevilla, & Molina-Grima, 2015), reducing the total medium cost.

Table 18 - Conway medium component cost in marine microalgae cultivation*

Medium component	Synthetic seawater (%)	Seawater (%)
Artificial sea salt	98.4	-
NaNO ₃	0.3	17.5
NaH ₂ PO ₄ ·2H ₂ O	0.2	14.5
Na ₂ EDTA	0.4	24.7
FeCl ₃ ·6H ₂ O	0.0	1.8
H ₃ BO ₃	0.2	8.8
CoCl ₂ ·6H ₂ O	0.0	0.1
MnCl ₂ ·4H ₂ O	0.0	0.7
(NH ₄) ₆ Mo ₇ O ₂₄ ·4H ₂ O	0.0	0.4
Thiamine	0.0	0.1
Cyanocobalamin	0.5	30.9

*The component cost was calculated according to regional quotes and expressed as a percentage.

According to Acién Fernández and coworkers (2019), to reduce the microalgal production cost, it is necessary to decrease medium cost by using wastewater as an alternative nutrient source. The authors reported a total cost reduction of 1.25 times compared to the use of inorganic medium. Generally, the cost of microalgal medium

represents 15–20% of the final cost in large-scale microalgal cultivation processes (Vonshak, 1997). Furthermore, in this study, the addition of 69% treated rice vinasse into the microalgal cultivation could reduce the total medium cost by 69% since rice vinasse is considered a residue and has no market value.

4.5 CONCLUSIONS

The first reported rice vinasse biological treatment using cyanobacteria was conducted. Diluted crude rice vinasse was successfully treated by *S. pevalekii* entrapped cells promoting ion removal and positively affecting the growth of *D. salina* in mixotrophic cultivation. The results confirmed impressive K^+ , Mg^{2+} , Cl^- , and PO_4^{3-} removal from diluted crude vinasse medium. 30% of both K^+ and Mg^{2+} was removed and values for Cl^- and PO_4^{3-} were below the limit of detection, in addition to nitrate and total solid removal of 91 and 29%, respectively. After process optimization, a 175% increase in cell number was achieved in *D. salina* cultivation grown in the medium containing treated vinasse (69% v/v) when compared to 100% Conway medium, and there was significant enhancement of photosynthetic pigment content. This treated rice vinasse addition decreased the medium cost by as much as 69%.

To further evaluate the potential of rice vinasse microalgal biological treatment, future research might involve the optimization of continuous scale-up fluidized bed reactors and outdoor experimental tests as well as in-depth study of biorefinery strategy applications. Also, the addition of carbon dioxide to *D. salina* treated vinasse cultivation may yield greater biomass production and improvements for future prospects of this topic.

The combined process could become a practical option for vinasse pollutant removal and microalgal medium cost reduction.

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CHAPTER 5

LESSER-KNOWN MICROALGAL PIGMENTS: A DISCUSSION ABOUT PRODUCING SPECIES, BIOLOGICAL APPLICATION, AND POTENTIAL HEALTH BENEFITS

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5.1 ABSTRACT

Microalgae are an important source of biopigments. The health benefits of these molecules have made them a focus the biotechnology industry, thus increasing the demand for microalgal pigments that are more economically feasible. Pigments such as β -carotene, astaxanthin, and phycocyanin have been extensively explored and described in the literature. However, assessments of lesser-known microalgal pigments are rare and could provide new insights to optimize and develop innovative technologies. In this context, the present review provides an overview of lesser-known microalgal pigments associated with the main advances on microalgae cultivation, such as myxoxanthophyll, adonixanthin, echinenone, cryptoxanthin, and botryoxanthin, as well as their potential

health benefits and application in the superfood industry. Production parameters and analytical methods are summarized and reviewed together with the economics of producing lesser-known microalgal pigments and their impact on the industrial processes. Several studies regarding the prospects of microalgal pigments in terms of their industrial production potential and application to superfood are presented, highlighting the bottlenecks in the large-scale production process.

Keywords: Microalgae. Echinenone. Biological activities. Microalgal cultivation. Bioactive compounds.

5.2 INTRODUCTION

Concerns regarding microalgae potential and versatility have spurred interest in the search for novel bioactive metabolites to be used in the food industry. The interest in microalgae as a renewable and sustainable feedstock for the production of natural molecules has inspired new strategies based on cultivation parameters and unfamiliar molecules that can strengthen the industrial sector. Furthermore, microalgae are photosynthetic microorganisms able to produce molecules with high added value, such as proteins (Becker, 2007), polysaccharides (Colusse et al., 2021a), polyunsaturated fatty acids (Lang et al., 2011), and, primarily, pigments (Begum et al., 2016).

Microalgal pigments have been used for many applications. Some of them have anti-inflammatory, expressive antioxidant, and anti-obesity properties (Sathasivam and Ki, 2018), and others might prevent cataracts and macular degeneration associated with age (Raposo et al., 2015). Based on current knowledge, microalgal pigments have been used in several areas, such as the cosmetics, pharmaceutical, and food industries.

However, few studies have presented information about lesser-known microalgal pigments and their potential biological effects. Comprehensive analyses and market-trend studies have demonstrated that chlorophylls, phycocyanins, astaxanthin, and β -carotene are the most-commonly reported microalgal pigments (Silva et al., 2020). In this context, lesser-known microalgal pigments such as echinenone, botryoxanthin, braunixanthins, and cryptoxanthin are rarely discussed in the literature, and not much information is provided regarding their potential health benefits.

Moreover, the understanding of this category of molecule is crucial in terms of ecology and taxonomy. Some pigments can be used as biomarkers in pigment chemotaxonomic techniques because they can be associated with a determined specie by its presence or absence in the microalgae sample (Lima et al., 2019). It is possible to create a detailed map of microalgae communities' distributions across different continents. Furthermore, the advancement of new technologies based on microalgae species from several environments (freshwater, marine, polar, and tropical) can increase the amount of attention paid to molecules with lesser-known microalgal pigments.

Producing pigments from microalgae has several advantages: (i) a high growth rate compared to commercial crops, (ii) the use of non-arable land for cultivation, (iii) efficient CO₂ mitigation, and (iv) the potential use of agro-industrial residues as a culture medium (D'Alessandro and Antoniosi Filho, 2016). Nevertheless, despite the significant potential of microalgae, some challenges need to be addressed to establish an efficient microalgae-based pigment production chain for commercial deployment (Gifuni et al., 2019). High operational costs related to nutrient availability and replacement, biomass recovery, and difficult control of microbial contamination are a few challenges related to large-scale production that can prevent a cost-effective production process (Colusse et al., 2021a).

In this context, this review aims to elucidate lesser-known microalgal pigments associated with the main advances in microalgae cultivation, their potential health benefits, and their application in the superfood industry. Moreover, we also present aspects associated with microalgal cultivation parameters and extraction procedures to further improve the understanding of these molecules in an industrial context.

5.3 TYPICAL CHARACTERISTICS OF LESSER-KNOWN MICROALGAL PIGMENTS

Generally, three classes of photosynthetic pigments can occur in microalgae: chlorophylls (0.5%–4.0% of dry weight), phycobilins (up to 8% of dry weight), and carotenoids (0.1%–0.2% of dry weight) (Christaki et al., 2015; de Jesus Raposo et al., 2013; Guedes et al., 2011; Harun et al., 2010). Briefly, chlorophylls are greenish, fat-soluble pigments with a porphyrin ring; phycobilins are soluble in water and easy to isolate

and purify; and carotenoids (carotenes or xanthophylls) are fat-soluble molecules that can present as brown, red, orange, or yellow (D'Alessandro and Antoniosi Filho, 2016). According to a 10-year bibliometric survey conducted by Silva et al. (2020), microalgal pigments such as chlorophylls, phycocyanin, astaxanthin, and β -carotene are the most-commonly studied and relevant pigments associated with the microalgal pigment market. At this point, lesser-known microalgal pigments such as myxoxanthophyll, canthaxanthin, echinenone, and cryptoxanthin might present several industrial applications (Grama et al., 2014), but are rarely discussed in the literature.

In-depth information about the understanding of lesser-known pigments can be obtained by non-photochemical quenching (Deore et al., 2020). Based on an investigation of quenching efficiency, pigments such as diadinoxanthin and diatoxanthin obtained from *Phaeodactylum tricornutum* can be manipulated in several growth conditions (Schumann et al., 2007). Furthermore, the relationship between microalgae and certain pigments is unique and must be explored.

Microalgae species such as *Bathycoccus prasinos*, *Micromonas pusilla*, and *Ostreococcus tauri* present specific pigments like uriolide, micromonal, and prasinoxanthin (Latasa et al., 2004), that can be evaluated and incorporated into the market as potential alternative molecules. They could also provide new insights to optimize and develop innovative technologies based on strategic approaches. According to Mc Gee et al. (2018), microalgae species such as *Brachiomonas submarina*, *Rhodella* sp., and *Amphora* sp. have been subjects of great interest when it comes to carotenoid production and could be candidates for pigment biorefinery. In this context, Table 19 presents several lesser-known microalgal pigments and their features in microalgae cultivation.

Table 19 - Main features of some lesser-known microalgal pigments

Lesser-known microalgal pigments	Pigment content (mg g ⁻¹ dried biomass)	Microalga	Extraction procedure	Reference
Adonixanthin	<i>not described</i>	<i>Chlorococcum</i> cells (Chlorophyta)	Cell pellets were milled and soaked with dichloromethane (25%) and methanol (75%).	(Yuan et al., 2002)

Botryoxanthin A	0.16	<i>Botryococcus braunii</i> Berkeley (Chlorophyta)	The extract was saponified and analyzed by HPLC and RP-HPLC. Freeze-dried cells were sonicated in acetone. The acetone extract was subjected to silica gel column chromatography and eluted with n-hexane/benzene (2:1). The fraction was submitted to chromatography followed by HPLC analysis.	(Okada et al., 1996)
Botryoxanthin B	0.09	<i>Botryococcus braunii</i> Kawaguchi- 1 (Chlorophyta)	Lyophilized cells were sonicated in Me₂CO. Me₂CO extract was submitted to silica gel column chromatography, ODS chromatography, and fractionated with MeCN–CH₂C₁₂–MeOH (1:2:7–1:3:7–0:1:0). The fraction was submitted to HPLC analysis.	(Okada et al., 1998)
α-botryoxanthin A	0.11			
Braunixanthin	0.57	<i>Botryococcus braunii</i> Kawaguchi- 1 (Chlorophyta)	Freeze-dried cells were sonicated in acetone. The acetone extract was subjected to silica gel column chromatography and eluted with n-hexane. The remaining matters were eluted with MeOH, followed by ODS column chromatography. The retained sample was eluted with CH₂Cl₂ and purified by normal and reverse-phase HPLC.	(Okada et al., 1997)
Cryptoxanthin	1.34	<i>Selenastrum bibrainum</i> (Chlorophyta)	Freeze-dried cells were extracted using hexane/ethanol mixture (1:1). The mixture was left in the ultrasonic bath for 40 min at room	(Soares et al., 2019)

			temperature. Next, 7.5 ml Na ₂ SO ₄ 10% aqueous solution was added, and the upper phase was concentrated, dissolved in 2 ml methanol, and submitted to HPLC analysis.	
Echinenone	0.59	<i>Aphanothece microscopica</i> Nageli (Cyanobacteria)	Lyophilized cells were extracted by ethyl acetate and methanol followed by centrifugation. The supernatant was mix with petroleum ether/diethyl ether [1:1 (v/v)] and saponified overnight with 10% (w/v) methanolic KOH. The samples were evaluated with HPLC–PDA–MS/MS analysis.	(Patias et al., 2017)
Myxoxanthophyll	0.01	<i>Phormidium autumnale</i> (Cyanobacteria)	Freeze-dried biomass was submitted to ethyl acetate and methanol extraction followed by centrifugation. The sample was filtered, concentrated, suspended in a mixture of petroleum ether/diethyl ether [1:1 (v/v)], and saponified overnight (16 h) with 10% (w/v) methanolic KOH at room temperature. The alkali was removed, and the extract was concentrated and flushed with N ₂ .	(Rodrigues et al., 2015)
Uriolide Prasinoxanthin	0.0004 0.002	<i>Prasinococcus capsulatus</i> (Prasinodermato phyta)	Lyophilized cells were treated with phosphate buffer/acetone [1:2 (v/v)] and extracted with acetone/methanol [7:3 (v/v)].	(Egeland et al., 1997)

Furthermore, microalgae of the genus *Botryococcus* have been extensively evaluated due to their hydrocarbon-accumulating property. These species produce rare and unique secondary carotenoids called botryoxanthins and braunixanthins that have never been found in other microalgae species (Cheng et al., 2019). Chemical analyses of several strains have led to the appearance of three distinct *Botryococcus* races, classified into A, B, and L, based on the type of hydrocarbon produced (Eroglu et al., 2011). Briefly, race A yields C₂₅ to C₃₁, odd-numbered n-alkadienes and alkatrienes (Banerjee et al., 2002), race B produces differently methylated triterpenes known as botryococcenes (Rao et al., 2007), and race L yields a single C₄₀ isoprenoid hydrocarbon derivative of lycopadiene (Metzger and Casadevall, 1987).

According to Okada et al. (1996), botryoxanthin A (C₇₄H₁₁₂O₂) was the first example of a new class of carotenoids that present a C₄₀-carotenoid, a squalene-related compound, and contain a tetramethylsqualene moiety. The authors also confirmed that this carotenoid exists in the extracellular matrix, like any other secondary carotenoid, and contributes to the color of the cell. Carotenoids like botryoxanthin B (C₇₄H₁₁₀O₃) and α-botryoxanthin A (C₇₄H₁₁₂O₂), however, present 4-keto-β and ε-end groups instead of the β-end group encountered in botryoxanthin A (Okada et al., 1998). Besides botryoxanthin groups, *Botryococcus* produces another carotenoid called braunixanthin. Braunixanthins present a series of alkylphenol derivatives, and the molecular formula can present as C₁₁₁H₁₇₈O₈ and C₁₁₃H₁₈₂O₈ with the occasional presence of an echinone-type carotenoid moiety (Okada et al., 1997). Figure 15 represents a general chemical structure of carotenoids presented in this review. It is worth pointing out that no study has investigated whether an increase of these carotenoids is associated with any sort of cultivation parameter in the culture.

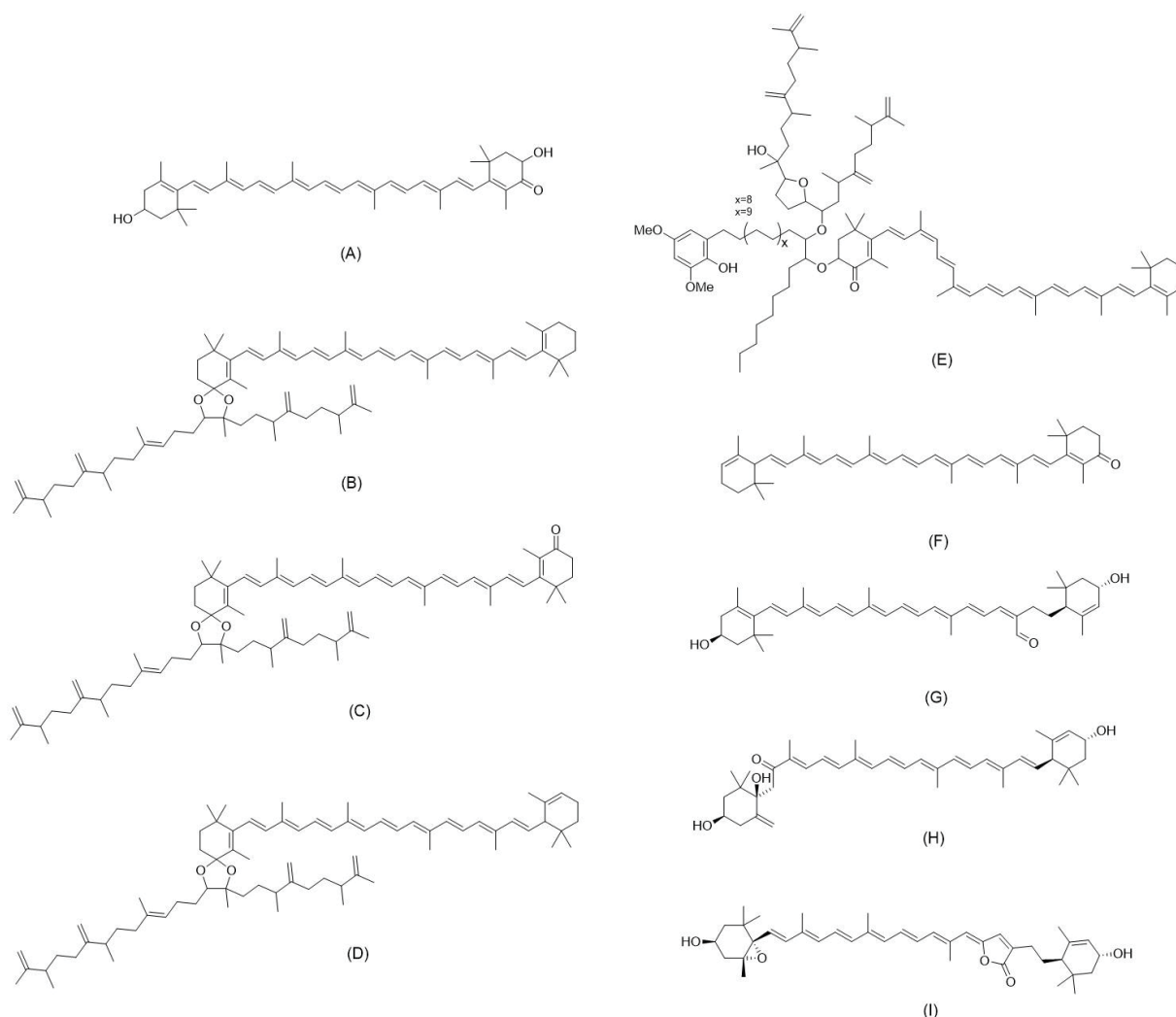


Fig. 15 - Chemical structure of some lesser-known microalgal pigments. (A) adonixanthin (4-ketozeaxanthin) obtained from *Chlorococcum* cells (Yuan et al., 2002); (B) botryoxanthin A extracted from the Chlorophyta *Botryococcus braunii* Berkeley (Okada et al., 1996); (C) botryoxanthin B and (D) α-botryoxanthin A isolated from the microalga *Botryococcus braunii* Kawaguchi-1 (Okada et al., 1998); (E) braunixanthin also obtained from *Botryococcus braunii* Kawaguchi-1 (Okada et al., 1997); (F) echinenone isolated from the Cyanobacteria *Aphanothece microscopica* Nageli; (G) micromonal extracted from the green microalga *Micromonas pusilla*; (H) uriolide and (I) prasinoxanthin isolated from the Prasinodermatophyta *Prasinococcus capsulatus* (Egeland et al., 1997)

Chlorococcum cells accumulate pigments such as adonixanthin and cantaxanthin. These pigments are fundamental for the biosynthesis of well-established pigment

production, such as that of astaxanthin; however, the presence of adonixanthin in *Chorococcum* cells indicates that astaxanthin could be synthesized from β -carotene from a different pathway (Yuan et al., 2002).

Another type of microalgae pigment hardly discussed in the literature is myxoxanthophyll. This molecule is a nonphotosynthetic pigment that acts as a natural sunscreen to protect the photosynthetic apparatus (Miskiewicz et al., 2000). It is also considered a major carotenoid that presents a unique glycoside linkage, and it is encountered only in cyanobacteria (Takaichi and Mochimaru, 2007). Myxoxanthophyll was first isolated from the cyanobacteria *Oscillatoria rubescens* in 1936 (Hertzberg and Liaaen-Jensen, 1969; Takaichi and Mochimaru, 2007), but it has been also reported in *Synechocystis* sp. PCC 6803 (Takaichi et al., 2001) and *Phormidium autumnale* (Rodrigues et al., 2015) biomass. It is worth mentioning that myxoxanthophyll presents a hydroxyl group at C-2 of the Ψ end group of the carotenoids and is bonded to glycoside in a (2'S)-configuration (Takaichi and Mochimaru, 2007). According to Hertzberg and Liaaen-Jensen (1969), the structure of myxoxanthophyll was ultimately determined to be 2'-rhamnoside.

The pigments diadinoxanthin and diatoxanthin are involved in the thermal energy dissipation cycle through nonphotochemical quenching induction (Schumann et al., 2007). According to the authors, these molecules exist as a free pigment in the thylakoid membrane and are bounded to the light-harvesting proteins (Schumann et al., 2007). It is worth noting that the diatoxanthin increases as microalgae cells become less healthy (Serive et al., 2017). Despite their importance in the photosynthetic pathway, these molecules are rarely discussed in the literature and might present biotechnological potential based on their biological properties.

5.4 POTENTIAL HEALTH BENEFITS IN THE INDUSTRIAL SECTOR: BIOLOGICAL ACTIVITY AND FURTHER APPLICATIONS

In addition to those of well-known pigments reported in the literature, lesser-known microalgal pigments also possess promising biological properties. Carotenoids, chlorophylls, and phycobilins extracted from microalgae biomass have health-promoting

properties and thus can be used as innovative functional ingredients. Several studies have shown that these molecules have numerous potential commercial applications, including in the food, pharmaceutical, and cosmetic industries, with biological properties, including a vitamin precursor capacity, antioxidant properties, immune enhancers, an anti-obesity capacity, and anti-aging factors (Christaki et al., 2015).

Potential pigments obtained from microalgae have been evaluated by several authors (Begum et al., 2016; Gouveia and Empis, 2003; Nwoba et al., 2020; Régnier et al., 2015; Sathasivam et al., 2019; Sathasivam and Ki, 2018; Wang et al., 2015). These molecules show a high added value and interesting biological properties that can be useful in several important fields. A study conducted by do Nascimento et al. (2021) shows the bioaccessibility and intestinal uptake of several carotenoids obtained from the microalga *Scenedesmus obliquus*. According to the authors, the most abundant carotenoids found in the dried biomass were *trans*- β -crocoxanthin (17.34%) and all-*trans*-echinenone (7.55%). Furthermore, a carotenoid extract containing pigments such as myxoxanthophyll and echinenone showed a potent peroxy radical scavenger capacity (Patias et al., 2017; Rodrigues et al., 2015) and can prevent light-induced damage in cells (Inoue et al., 2017), while others such as alloxanthin and crocoxanthin showed antiproliferative activity against A2058 cells. Moreover, these molecules promoted growth inhibition, decreased cell migration, and induced apoptosis; thus, the carotenoid alloxanthin potentiated the cytotoxic activity of vemurafenib (de Oliveira-Júnior et al., 2020).

In addition, chlorophyll molecules (a, b, c, d, and f types) have been investigated as potential candidates for the industrial scenario. Chlorophylls can be used in the cosmetics and food industries due to their strong green pigment and consumers' demand for natural products (da Silva Ferreira and Sant'Anna, 2017; Harun et al., 2010). Chlorophyll also possesses a chelating agent activity that can be used in ointments, as well as the ability to reduce the occurrence and severity of facial acne vulgaris lesions (Song et al., 2014), and has been shown to be an effective topical treatment for hemostatic eczema and ulcers (Yaakob et al., 2014; Zeligman, 1949). Consequently, based on the use of natural, chlorophyll-based coloring rather than artificial pigments, the use of microalgal biomass

to increase health benefits is encouraging the development of this niche market; however, some parameters could affect its bioaccessibility (Fernandes et al., 2021).

Several lesser-known pigments obtained from microalgae species have the potential to be used as food additives due to their antioxidant, anti-hyperglycemic, and provitamin A properties (Ghosh et al., 2016; Lederer and Moore, 1936). In this sense, the potential of these molecules to add value to natural molecule production worldwide could be explored.

A study conducted by Lauritano et al. (2016) is one of the few to simultaneously test a wide range of microalgae species using the same cultivation techniques, extraction procedures, and testing methodologies for bioactivity assays. They presented specific anti-inflammatory, anticancer, and anti-biofilm activities. Moreover, the authors did not perform characterization analysis; however, taking into account the chemical extraction and pre-fractioning steps, the biological effects were primarily caused by pigments. Furthermore, many studies need to be conducted to develop new methodologies to understand the full potential of lesser-known microalgal pigments. Thus, several unknown microalgal pigments could potentially be used to treat human pathologies and to increase foods' nutritional value.

5.5 BIOECONOMIC ASSESSMENT OF LESSER-KNOWN MICROALGAL PIGMENTS: THE INDUSTRIAL SCENARIO

The worldwide industrial production of microalgae is based mainly on functional food (supplements, dairy products, desserts, pasta, and oil-derivatives) and feed industries (for cattle, poultry, shellfish, and fish) (Camacho et al., 2019). It is worth mentioning that the market size for biopolymers and bioactive molecules (including microalgae-based commodities) corresponds to €14.2 billion in the European economy (Barsanti and Gualtieri, 2018) and the global algal/microalgal product market is projected to reach approximately US\$44.6 billion by 2023 (CredenceResearch, 2017). The commercial production and application of specific molecules such as echinenone, braunixanthins, and botryoxanthin are rarely explored since no information is available in the literature.

Li et al. (2011) presented a hypothetical plant to produce microalgal pigment (900 kg of astaxanthin per year) at a low cost. Based on their analysis, the production cost of astaxanthin and microalgal biomass could be as low as \$718/kg and \$18/kg, respectively. The pigment content plays an important role in production cost since it is the key to reaching financial viability. Moreover, a microalgae specie that produces lesser-known pigments at low concentrations should be optimized to increase its pigment content. On the other hand, cyanobacteria such as *Synechocystis* sp. CCNM 2501 is known to produce myxoxanthophyll, a potent antioxidant (see the previous section), as the major component from the extract ($2.21 \pm 0.43 \text{ mg g}^{-1}$) (Ghosh et al., 2016). Moreover, researches in the industrial sector might evaluate all the laboratorial parameters to enhance the production of a specific molecule.

Limitations encountered in microalgae cultivation such as productivity, biomass recovery, and shelf life extension, as well as the high cost of production and the extraction of the molecule of interest, have been analyzed and optimized with the aim of developing strategies and to create progressive technologies.

Otherwise, these parameters are based mainly on the upstream (i.e., cultivation) and downstream (i.e., extraction) processes. The two most common methods of microalgae cultivation are open-raceway pond and closed photobioreactor systems. To date, the open-raceway pond is the most economical cultivation system available due to its low energy input, no requirement of high-technology to control parameters, and simplicity. However, the system presents major challenges, such as water evaporation, no control of parameters, and a low yield. Furthermore, large microalgae cultivation can result in cross-contamination with microalgae species and infestation by ciliates (Moreno-Garrido and Cañavate, 2001) and amoebae (Post et al., 1983).

Closed photobioreactors can be operated at highly controlled parameters, but this cultivation system presents some disadvantages, such as bio-fouling, overheating, benthic algae growth, and high capital costs (Molina Grima et al., 1999). However, a third option presented by Narala et al. (2016) might be an interesting approach in terms of cost-effectiveness. The two-stage hybrid system combines exponential biomass production in air-lift-driven bioreactors using a separate synchronized high-lipid induction phase in nutrient-depleted open raceway ponds. The authors concluded that this system

is superior for the production of high-added-value molecules from microalgae; however, further techno-economic analyses should be performed to test its economic feasibility.

Besides the cultivation system, many other parameters related to the industrial scenario can affect the bioeconomic assessment of lesser-known microalgal pigments. To avoid or minimize these effects, an environmentally friendly method of biorefining microalgal biomass is an object of study in the literature (Chew et al., 2017; Nobre et al., 2013; Venkata Mohan et al., 2020). The aim is to take advantage of microalgae potential by reusing resources, thus implementing a circular economy (Fuentes-Grünwald et al., 2021). As such, the process can increase its size and move from niche markets to large-scale production. Figure 16 presents an industrial approach to produce lesser-known microalgal pigments associated with the production of other high-added-value molecules.

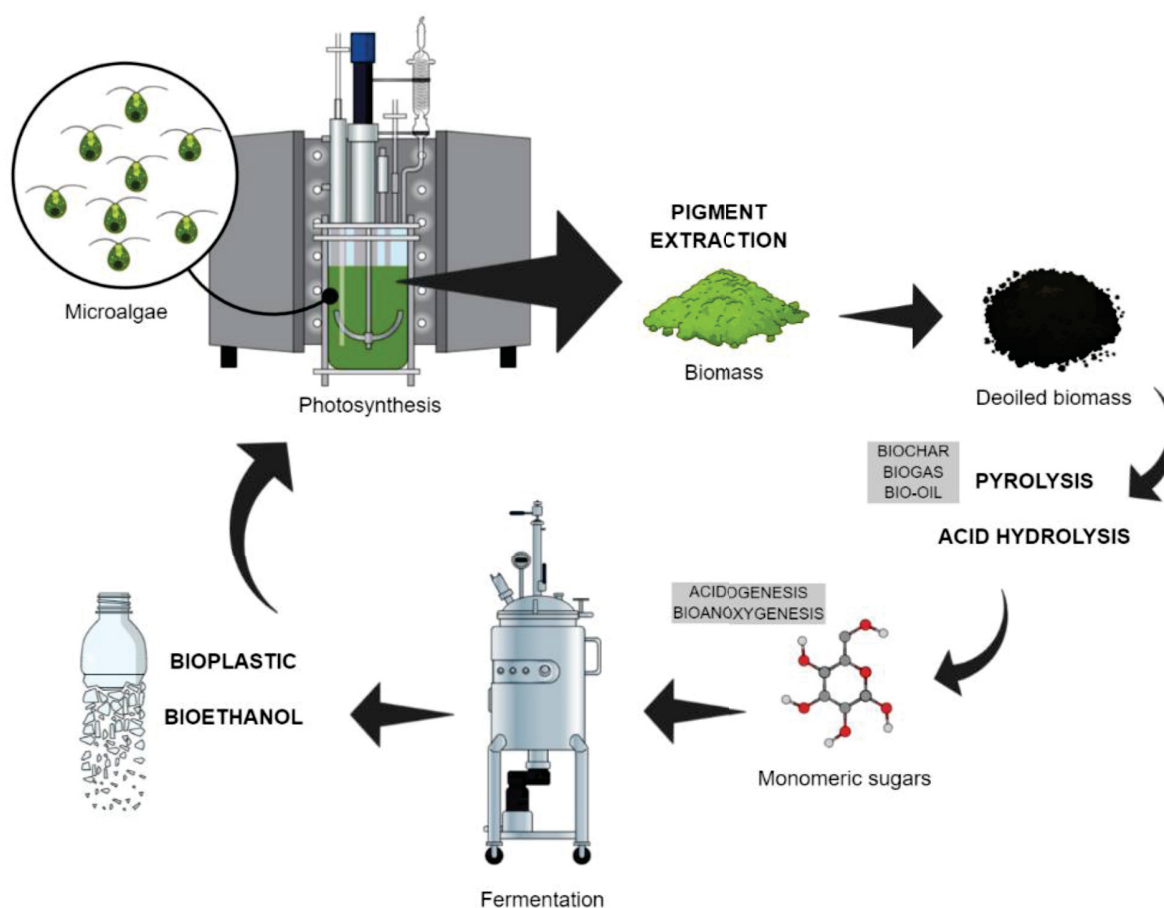


Fig. 16 - Biorefining microalgal biomass using economical and environmentally friendly approaches. Adapted from Venkata Mohan et al. (2020)

The biorefinery approach for lesser-known microalgae pigments is fundamental to integrate the production cycle in a sustainable way. According to its low yield, each gram of the pigment of interest will result in several kilograms of other microalgae products such as fatty acids, protein, and polysaccharides. In addition, the extraction steps would be the key feature in a large-scale pigment production facility. These steps would determine the pigment purity, cost related to extraction protocols, excessive use of solvent, and efficiency associated with pigment yield. Khoo et al. (2020) classified some extraction techniques applied to microalgae biomass based on conventional solvent methods and recent advances in extraction techniques. These extraction systems could be integrated into large-scale facilities that produce lesser-known microalgal pigments to improve the bioeconomic assessment in the industrial scenario. Table 20 presents the newest extraction procedures and their main characteristics, as described in the literature.

Table 20 - Recent advances in extraction techniques of microalgal carotenoids

Extraction method	Extraction efficiency	Overall comments	Disadvantages	References
Supercritical fluid extraction	80–98%	Environmentally friendly, non-toxic, use of chemical inert solvent, no solvent residue found in the extraction product, no need for a high temperature.	Low solubility of polar molecules, high cost, complex operating equipment.	(Khoo et al., 2020, 2019b)
Ionic liquids	90–100%	Thermal stability, non-volatility, synthetic flexibility, recyclability, capability of extracting molecules from both wet and dry microalgal biomass.	Non-environmentally friendly, thermal degradation resistance, toxicity, lack of studies related to its toxicity on scaling up.	(Choi et al., 2014; Jessop, 2011; Khoo et al., 2020)
Switchable solvents	53–61%	Easier recovery of solvents, low energy demand, no need to dry or mill the biomass since it can extract from wet biomass with no prior treatment.	Technical feasibility might be investigated for large industrial scale.	(Du et al., 2013; Khoo et al., 2020)

Several others methodologies were also performed on a laboratorial scale, such as the use of supramolecular solvents (Salatti-Dorado et al., 2019), liquid biphasic flotation (Khoo et al., 2019a), and a magnetic-assisted extraction procedure (Zhao et al., 2016). However, the choice of an upstream and downstream process remains in particular associated with the target of interest (in this case, the pigment), its characteristics, and the area it will be marketed.

5.6 LABORATORIAL PARAMETER EVALUATION AND PIGMENT CHARACTERIZATION FEATURES

Several strategies can enhance lesser-known pigment production in microalgae cultivation. These parameters can be grouped into two categories: nutrimental (change in media composition) and physical (change in operational conditions and external factors such as salinity, pH, temperature, light wavelength, light intensity, and salt addition) (Benavente-Valdés et al., 2016). For example, *B. braunii* Berkeley was cultivated with a modified Chu 13 medium under the illumination of 240 $\mu\text{E}/\text{m}^2\cdot\text{s}$ on a 12:12 (light/dark) cycle at 25°C and aerated with sterile air containing 2% CO_2 to produce botryoxanthin A (Okada et al., 1996). Taking these parameters into account, stress-based strategies have been used to increase production and efficiency in microalgae cultivation. Nutrient-related stresses such as phosphate, nitrogen, and sulfur limitation; phosphatidylglycerol depletion; and light, temperature, and salinity stress affect cellular performance and thus affect the biochemical path of pigment synthesis (Domonkos et al., 2009; Gacheva and Gigova, 2014; Shi et al., 2020). Furthermore, high light exposure in microalgae cultures can enhance certain pigments. For example, the use of a light regime of 100 $\mu\text{mol m}^{-2} \text{s}^{-1}$ in *Phaeodactylum tricornutum* cultivation increased diadinoxanthin and diatoxanthin by 72% compared to a low light regime of 7 $\mu\text{mol m}^{-2} \text{s}^{-1}$ (Schumann et al., 2007).

The differences in carotenoid composition are also due to the presence or absence of certain genes, different enzyme characteristics, and even the addition of organic compounds to the culture medium (Takaichi and Mochimaru, 2007; Yuan et al., 2002). The addition of organic compounds to the culture medium has been shown to improve pigment production. Yuan et al. (2002) reported a significant increase in carotenoid

content, mainly in adonixanthin and canthaxanthin, based on 0.3% glucose addition. In this context, the formation of carotenoids and chlorophylls is dependent on glucose concentration in the culture (Yuan et al., 2002).

Despite laboratorial approaches, the characterization technique used in the qualitative analysis presents an enormous input to the production process, as it contributes to the evaluation of pigment content, pigment composition of microalgae, structure characterization, and analysis of purity. It also helps identify unknown pigments through community analysis of pigment patterns (Serive et al., 2017) to establish the biotechnological capacity of alternative molecules such as lesser-known microalgal pigments. Furthermore, the chemical structure can be used to discover and explain the biological properties of these pigments, providing sufficient knowledge to create new compounds for the market. Table 21 presents some essential characterization techniques that are useful for pigment qualitative analysis.

Table 21 - Different processes that can be used in microalgal pigment separation and characterization

Approaches	Main advantages	Main disadvantages
<u>UV-Vis spectroscopy</u> Used to obtain the absorbance spectra of a pigment in solution.	-Simple -Fast -Inexpensive in terms of both equipment and analysis -Accurate compared to conventional chromatographic techniques.	-Poor resolution in sample containing many pigments -Lack of information about absorbance of some molecules -Pigment concentration is based on equation from literature
<u>NMR techniques</u> Applies a magnetic field to an atomic nucleus. This technique shows the interaction of radiofrequency electromagnetic radiations with the nuclei of pigments placed in a strong magnetic field.	-Nondestructive -High accuracy and reproducibility -Does not require separation and purification steps -Fast	-Not feasible in small laboratories or production facilities -High maintenance cost -Demanding qualified labor -Dependence on results from literature
<u>GC-MS</u>	-Sensitive response to most pigments	-Not feasible in small laboratories or production facilities

A combination of the features of gas-chromatography and mass spectrometry to identify and quantify different chemicals present in the sample.	-High resolution capacity -Provides information of the chemical structure -High separation capacity	-Demanding qualified labor -Slow -Significant sample preparation with chemical modification
<u>HPLC</u> Used to separate, identify, and quantify compounds that are dissolved in solution based on a standard solution.	-Relatively easy to use -Fast -High sensitivity and resolution -Technique of choice for identification and quantification of minor pigments	-Lack of an ideal universal detector -Less separation efficiency compared to others techniques -Still arduous for regulated testing -Poor comparison of retention times obtained from different columns
<u>TLC</u> Used to separate and identify molecules from a mixture through a thin layer of powered silica gel.	-Inexpensive in terms of analysis and equipment -Streamlined sample preparation -Easy preparative separation -Simultaneously analyses many samples	-Not very accurate -Possibility of errors during the analysis -Limited resolution capability -Absence of fully automated systems

NMR=Nuclear Magnetic Resonance; HPLC=High Performance Liquid Chromatography; GC-MS=Gas Chromatography–Mass Spectrometry; TLC=Thin Layer Chromatography. Adapted from Dong (2013), Hatzakis (2019), Minyuk and Solovchenko (2018), Serive et al. (2017), Sherma (2006), and Vicario et al. (2020).

Several approaches have been used to determine pigment characterization in both laboratorial and industrial scenarios. Each technique has certain advantages and disadvantages associated with cost, time, accuracy, and the need for qualified labor. Moreover, the performance of these approaches could indicate might be used as a rapid analytical method for pigment determination in several microalgae species (Chen et al., 2017; Henriques et al., 2007; Palmer et al., 2021; Wang et al., 2018). For example, an extraction protocol that allows for the environmentally friendly, non-damaging extraction of pigments was developed by Van Heukelem and Thomas (2001) and used by Serive et al. (2017).

The use of combined techniques, such as negative stain electron microscopy and cryo-electron microscopy, also assisted in the understanding of pigments in a cell through the study of three-dimensional structure of phycobilisomes in *Nostoc flagelliforme*, as shown by Yi et al. (2005). Rodrigues et al. (2015), however, identified the carotenoid

myxoxanthophyll in *Phormidium autumnale* through its UV-Vis characteristics. Chemical and physical methods, including NMR and mass spectra, have been also used (Hertzberg and Liaaen-Jensen, 1969). Furthermore, NMR techniques such as ^1H , ^{13}C , COSY45, HOHAHA, HSQC, HMBC, and PSNOESY seem to provide useful data to determine the molecular formula of botryoxanthins (botryoxanthin A, botryoxanthin B, and α -botryoxanthin A) (Okada et al., 1998, 1996).

The ultra-high-performance liquid chromatography coupled with quadrupole time-of-flight mass spectrometry (UPLC–PDA–MS) method can separate up to 41 different carotenoids in less than 15 minutes (Palmer et al., 2021). Briefly, dried extracts were resuspended in the elution buffer and then injected into an Acquity UPLC System equipped with a photodiode array detector (Acquity PDA) directly coupled with a quadrupole time-of-flight mass spectrometer (XEVO–QTOF–MS) in series (Waters). This innovative approach allowed pigment compounds to be rapidly identified. In addition, high-performance liquid chromatography coupled with diode array and mass spectrometry detectors (HPLC–PDA–MS/MS) was used for the first time to determine the composition of carotenoids and chlorophylls of microalgae (Fernandes et al., 2020).

Even though the characterization related to chlorophylls molecules (a well-known microalgal pigment) obtained from microalgae has been reported (Amin et al., 2018; Fernandes et al., 2020; Hosikian et al., 2010), there is a lack of information about the qualitative and quantitative profile of the a, b, c, d, and f chlorophyll types. This lack might be associated with the need for specific tools, such as NMR equipment and HPLC coupled with mass spectrometry, or a lack of qualified labor to obtain more reliable results.

Moreover, the integration system based on the use of laboratorial parameters and both conventional and new approaches to characterize pigments (in this case, lesser-known microalgal pigments) is obtained from the already established studies combined with new, untested ones that overcome the need of new molecules in this area.

5.7 LESSER-KNOWN MICROALGAL-PRODUCED PIGMENTS: BROADENING HORIZONS

The technology associated with microalgae cultivation, specifically how it relates to pigment production, is increasing worldwide. Production bottlenecks encountered in microalgal facilities have yet to be addressed. These include (i) the isolation, culturing, and characterization of new microalgae strains and their potentialities; (ii) upstream and downstream processing; (iii) cost; (iv) environmental aspects; (v) culture optimization in a large-scale production system; and (vi) increasing market demand by incorporating microalgal biomass into common products or to commercialize isolated pure compounds.

Several strategies have been reported with the aim of increasing pigment content and reducing the cost associated with microalgal production. A feasible solution for cost reduction in large-scale microalgal cultivation is to use industrial wastewater as a nutrient source, as proposed by Arashiro et al. (2020), Colusse et al. (2021), and Wang et al. (2016). The use of wastewater for microalgae cultivation could become a feasible approach to produce high-value pigments (Arashiro et al., 2020). Another option thoroughly discussed in the literature is the re-use of industrial CO₂ in the microalgae culture (Mohler et al., 2019), thereby increasing microalgal biomass and reducing the cost.

Changes in parameters from the nutrimental group are also subject to analysis in order to understand how medium nutrients interfere with biomass production (Colusse et al., 2020, 2019; Pandey and Pandey, 2008) and, consequently, pigment content. Moreover, laboratorial variables have been studied in this context (see previous section).

Furthermore, a study conducted by Deore et al. (2020) showed that the use of non-photochemical quenching monitoring could be used to avoid and minimize microalgal culture crash by microorganism contamination since it is a serious concern pertaining to open-raceway pond cultivation systems. However, it is worth mentioning that an important tool to select microalgae for pigment production is bioprospecting in an extreme environment associated with the use of molecular biology. The genes involved in lesser-known microalgal pigments should be further evaluated since most of them have not yet been identified. Saini et al. (2020) reviewed effective strategies based on metabolic and

genetic engineering to enhance microalgal pigment production that could be implemented in this area. Moreover, the search for new techniques, alternative methodologies, and optimized extraction procedures will enhance the research and production of lesser-known microalgal pigments in large-scale production systems.

5.8 CONCLUSIONS

This overview aims to provide the reader with an assessment of several pigments obtained from microalgae that present significant biotechnological potential but are rarely discussed in the literature. The study and production of lesser-known microalgal pigments are vital to identify opportunities for improvements and additions to the existing market. Nonetheless, the commercial production of these molecules is highly dependent on laboratorial parameters and data collection provided in scientific studies, which influences the feasibility producing these molecules in large-scale production systems. Some parameters, such as the optimization of pigment production, the cultivation of new microalgal strains, and the development of alternative biorefining facilities, are fundamental to benefit the market needs. Moreover, research on the large-scale production of lesser-known microalgal pigments and the incorporation of these molecules into already established microalgal facilities are crucial for the development of alternatives for the market. Moreover, the biological properties and increased content in cellular metabolism of these pigments warrant further study, which could allow them to move from niche markets to large-scale production.

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CHAPTER 6

CRITICAL REVIEWS IN BIOTECHNOLOGY
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REVIEW ARTICLE



Advances in microalgal cell wall polysaccharides: a review focused on structure, production, and biological application

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ABSTRACT

Microalgae have been shown to be useful in several biotechnological fields due to their feasible cultivation and high-value biomolecules production. Several substances of interest produced by microalgae, such as: proteins, lipids, and natural colorants, have already been explored. Based on the continuing demand for new natural molecules, microalgae could also be a valuable source of polysaccharides. Polysaccharides are extremely important in aquaculture, cosmetics, pharmaceutical, and food industries, and have great economic impact worldwide. Despite this, reviews on microalgal polysaccharide production, biological activity, and chemical structure are not abundant. Moreover, techniques of microalgal cultivation, coupled with carbohydrate production, need to be clarified in order to develop forward-looking technologies. The present review provides an overview of the main advances in microalgal cell wall polysaccharide production, as well as their associated potential biological applications and chemical structure. Several studies on future prospects, related to microalgae are presented, highlighting the key challenges in microalgal polysaccharide production.

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Introduction

Carbon dioxide emissions [1] and population growth [2] are two main global concerns that encourage researchers to investigate alternative biotechnological fields. In this context, microalgae cultivation has been highly explored in recent years [3–7]. Microalgae are photosynthetic microorganisms with significant genetic diversity and a high-efficiency photosynthetic complex that converts light energy and carbon dioxide into molecules of interest. The cultivation of these microorganisms depends on the irradiated light and the substrate used in the growth system to enhance growth rates and biomass yields [8]. From a practical point of view, microalgae can be cultured in areas unsuitable for agricultural purposes, and produce several high-value biological derivatives with commercial applications.

Many studies have explored the use of microalgae as natural colorants [7,9], for single-cell protein production [4,10–14], pollutant removal [15,16], biofuel production [17,18], and in the context of facility cost analysis of

upstream and downstream processes [6,19]. On the other hand, few studies have presented information about microalgal cell wall polysaccharides and their importance in the context of diversity, application, and other features.

Polysaccharides are natural polymers used as storage or structural materials in the cell [20]. Although most commercial polysaccharides are derived from land plants or macroalgae, they can potentially be sourced from microalgae. In the last decades, several microalgal polysaccharides have been studied, characterized, and biologically evaluated, focusing on their pharmacological activities as means of improving human and animal health. Although these macromolecules play an important role in diverse biotechnological applications, there is only scattered scientific review of the subject. Therefore, the present work aims to review available information regarding the structure, production, and biological application of microalgal cell wall polysaccharides, as well as their commercial aspects and future prospects.

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ADVANCES IN MICROALGAL CELL WALL POLYSACCHARIDES: A REVIEW FOCUSED ON STRUCTURE, PRODUCTION, AND BIOLOGICAL APPLICATION

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6.1 ABSTRACT

Microalgae have been shown to be useful in several biotechnological fields due to their feasible cultivation and high-value biomolecules production. Several substances of interest produced by microalgae, such as proteins, lipids, and natural colorants, have already been explored. Based on the continuing demand for new natural molecules, microalgae could also be a valuable source of polysaccharides. Polysaccharides are extremely important in aquaculture, cosmetic, pharmaceutical, and food industries, and have a great economic impact worldwide. Despite this, reviews on microalgal polysaccharide production, biological activity, and chemical structure are not abundant. Moreover, techniques of microalgal cultivation, coupled with carbohydrate production, need to be clarified in order to develop forward-looking technologies. The present review provides an overview of the main advances in microalgal cell wall polysaccharide production, as well as their associated potential biological applications and chemical structure. Several studies on future prospects related to microalgae are presented, highlighting the key challenges in microalgal polysaccharide production.

Keywords: Bioactive compounds. Biological activity. Biotechnological application. Cell-wall polysaccharides. Chemical structure. Microalgae.

6.2 GENERAL INTRODUCTION

Carbon dioxide emissions (Jiang, Ye, & Liu, 2019) and population growth (Anson, Bartl, & Kulczycki, 2019) are two main global concerns that encourage researchers to investigate alternative biotechnological fields. In this context, microalgae cultivation has been highly explored in recent years (Barka & Blecker, 2016; Enzing, Ploeg, Barbosa, & Sijtsma, 2014; Santana et al., 2017; Tan et al., 2018; Zhang, Wang, Wang, & Liu, 2014). Microalgae are photosynthetic microorganisms with significant genetic diversity and a high-efficiency photosynthetic complex that converts light energy and carbon dioxide into molecules of interest. The cultivation of these microorganisms depends on the irradiated light and the substrate used in the growth system to enhance growth rates and biomass yields (Patel, Joun, Hong, & Sim, 2019). From a practical point of view, microalgae can be cultured in areas unsuitable for agricultural purposes, and produce several high-value biological derivatives with commercial applications.

Many studies have explored the use of microalgae as natural colorants (Rahman, Sarian, van Wijk, Martinez-Garcia, & van der Maarel, 2017; Zhang et al., 2014), for single-cell protein production (Barka & Blecker, 2016; Buchmann, Brändle, Haberkorn, Hiestand, & Mathys, 2019; Chew, Chia, Lee, Zhu, & Show, 2019; Karan, de Boeck, Roles, Hankamer, & Ross, 2019; Koyande et al., 2020; Phong, Le, Show, Chang, & Ling, 2017), pollutant removal (Colusse et al., 2021; Matamoros, Uggetti, García, & Bayona, 2016), biofuel production (Milano et al., 2016; Morales et al., 2019), and in the context of facility cost analysis of upstream and downstream processes (Acién Fernández, Fernández Sevilla, & Molina Grima, 2019; Tan et al., 2018). On the other hand, few studies have presented information about microalgal cell wall polysaccharides and their importance in the context of diversity, application, and other features.

Polysaccharides are natural polymers used as storage or structural material in the cell (Bernaerts et al., 2018). Although most commercial polysaccharides are derived from land plants or macroalgae, they can potentially be sourced from microalgae. In the last

decades, several microalgal polysaccharides have been studied, characterized, and biologically evaluated, focusing on their pharmacological activities as means of improving human and animal health. Although these macromolecules play an important role in diverse biotechnological applications, there is only scattered scientific review of the subject. Therefore, the present work aims to review available information regarding the structure, production, and biological application of microalgal cell wall polysaccharides, as well as their commercial aspects and future prospects.

6.3 EFFECTS OF CULTIVATION ON MICROALGAL POLYSACCHARIDES

The effect of technological parameters of the microalgae cultivation process might increase, decrease, or even modify the polysaccharide content in the cell. In a detailed study on microalgal cell wall composition by Rashidi et al. (Rashidi, Dechesne, Rydahl, Jørgensen, & Trindade, 2019), it has been shown that the green microalga *Neochloris oleoabundans* presents differences in monosaccharide composition with variation in the culture medium used. The cultivation using freshwater medium gave cell walls composed mainly of rhamnose, galactose, and glucuronic acid, whereas cultivation of *N. oleoabundans* in seawater medium yielded cell walls with rhamnose, galactose, and glucose as the main monosaccharides in its composition.

Several studies have reported that in microalgal cultivation under stressed conditions, photosynthetic carbon flow is transformed into energy-rich chemical compounds such as lipids and carbohydrates (He, Yang, Wu, & Hu, 2015; Pancha et al., 2014). These molecules act as storage components in the cell, and their concentrations can be influenced by diverse variables (Fazeli Danesh, Ebrahimi, Salehi, & Parsa, 2017; George et al., 2014; Wang et al., 2019). An increase in light intensity can reduce the carbohydrate content in the cell (He et al., 2015), besides inducing physiological changes. In contrast, microalgal cells under nitrogen starvation conditions showed higher starch production, up to 50% on a dry weight basis (Fazeli Danesh et al., 2017). Table 22 shows the differences in the carbohydrate content of several microalgae species related to different cultivation parameters applied to the culture.

Table 22 - Microalgal carbohydrates content variation related to the laboratorial cultivation parameters

Microalga	Total carbohydrates content variation (%dw)*	Stress condition	Growth media	Light intensity ($\mu\text{mol m}^{-2} \text{s}^{-1}$)	Photoperiod (light:dark) (h:h)	Reactor volume (mL)	Reference
<i>Ankistrodesmus falcatus</i>	6-25 7-18 5-8	Growth medium Light intensity Photoperiod	BG-11, BBM, CHU-10, and ZM (at 60 $\mu\text{mol m}^{-2} \text{s}^{-1}$ and 12:12 photoperiod)	30, 60, and 150 (BG-11 medium and 12:12 photoperiod)	12:12, 18:6, 6:18, 24:0, and 0:24 (BG-11 medium at 60 $\mu\text{mol m}^{-2} \text{s}^{-1}$)	300	(George et al., 2014)
<i>Chlorella</i> sp.	27-34	Light intensity	BG-11 with half of NaNO_3 content	40, 80, and 100 (first two days) 40, 200, and 400 (following ten days of culture)	24:0	400	(He et al., 2015)
<i>Monoraphidium dybowskii</i>	17-31						
<i>Scenedesmus</i> sp.	18-45	N-starvation	BG-11 with different initial nitrate content (247, 123.5, 61.75, 30.87, 15.43, and 0 mg L^{-1})	Not informed	12:12	500	(Pancha et al., 2014)
Mixed microalgae	28-50 28-28 24-33	N-starvation S-starvation P-starvation	Two-stage cultivation: Complete nutrient medium followed by nitrogen free, sulfur free or phosphorus free medium	175	24:0	500	(Fazeli Danesh et al., 2017)
<i>Microactinium</i> sp.	17-28	Agro-industrial residue addition	BBM medium, sugarcane vinasse: diluted (50%), and clarified (100%)	400	12:12	13x10 ³	(Santana et al., 2017)
<i>Chlamydomonas biconvexa</i>	11-31						
<i>Chrysothrix carterae</i>	21-33	Salinity variation	Natural seawater + sterile nutrient solution with a salinity range of 35-233 ppt	150	12:12	150	(Ishika, Bahri, Laird, & Moheimani, 2018)
<i>Chaetoceros muelleri</i>	10-26						
<i>Nannochloropsis</i> sp.	12-21						

<i>Phaeodactylum</i>	17-30
<i>tricornutum</i>	
<i>Tisochrysis lutea</i>	14-24
<i>Tetraselmis</i>	16-34
<i>suecica</i>	
<i>Amphora</i> sp.	15-43
<i>Navicula</i> sp.	11-28
<i>Dunaliella salina</i>	10-19

*Variation based on different laboratorial cultivation parameters tested. BBM=Bold's Basal Medium; ZM=Zarrouk's Medium

Slight variations in stress conditions, such as a gradual increase in salinity or different light intensities, can substantially modify carbohydrate content in microalgal cells. Commercialized microalgal species such as *Chlorella vulgaris* and *Spirulina platensis* present an innate carbohydrate content of 12-17% and 8-14%, respectively, when cultivated under standard conditions (Harun & Danquah, 2011). However, cultivation parameters such as CO₂ concentration, light intensity, and nitrogen content can increase carbohydrate content in *Chlorella* sp. up to 77% (D. Cheng et al., 2017), while phosphorus starvation can enhance carbohydrate content up to 63% in *S. platensis* (Markou, Chatzipavlidis, & Georgakakis, 2012).

Alteration of microalgal culture media can positively or negatively affect the carbohydrate content of microalgal biomass. Total carbohydrate content variations of 11-21% and 5.6-11.3% were reported, respectively, in *Haematococcus pluvialis* and *Dunaliella salina* cultures under batch mode conditions using different standard media (Colusse, Borges Mendes, Rabello Duarte, de Carvalho, & Nosedá, 2020; Colusse, Duarte, de Carvalho, & Nosedá, 2019). Nutrient availability plays an important role in microalgal composition without negatively affecting the photosynthetic performance of photosystem II (PSII) (Hanifzadeh, Garcia, & Viamajala, 2018). Besides, differences in polysaccharide content and its derivatives were observed in *H. pluvialis* cellular stages (flagellated cell, green coccoid cell, intermediate cells and aplanospore) (Baudeflet, Ricochon, Linder, & Muniglia, 2017). Moreover, the possibility to control some microalgal cultivation parameters is becoming an affordable approach to maximize polysaccharide yields.

6.4 THE DIVERSITY OF MICROALGAL CELL WALL POLYSACCHARIDES

It is well established that there is a great variety of polysaccharides localized in the microalgal cell wall (Arad & Levy-Ontman, 2010; Baudeflet et al., 2017; Chanda, Merghoub, & EL Arroussi, 2019; Piro et al., 2000). Moreover, it has been observed that microalgal culture media components have an intrinsic relationship with the monosaccharide composition and glycosidic linkage configuration. Few chemical structures of microalgal cell wall polysaccharides have been described in the literature,

not only due to the complexity of these macromolecules, but also because they are difficult to extract (Arad & Levy-Ontman, 2010; Le Costaouëc, Unamunzaga, Mantecon, & Helbert, 2017). Figure 17 presents the general laboratorial process to determine the chemical structure of microalgal polysaccharides and their biological activities.

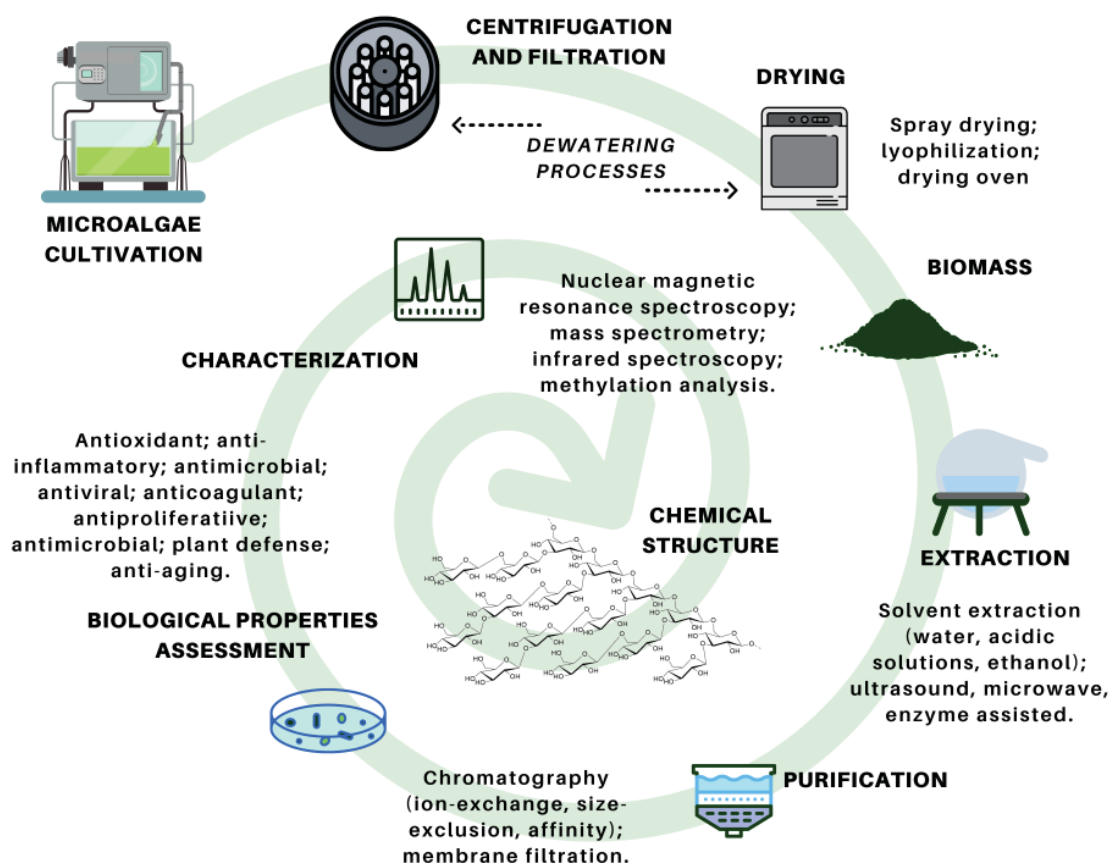


Fig. 17 - Laboratorial process for polysaccharide isolation and characterization

A full chemical characterization of complex polysaccharides includes, besides the determination of monosaccharide composition, their sequence and absolute configuration, the type of glycosidic linkage between units, and the identification, substitution pattern, and positioning of non-carbohydrate substituents. This challenging research demands a combination of different chemical, spectrometric, and spectroscopic methods. Methylation analysis and/or nuclear magnetic resonance (NMR) spectroscopy are used to determine the linkage and substitution patterns. In many cases, partial

depolymerization is necessary to determine the monosaccharide sequence, through partial hydrolysis (acid or enzymatic) or Smith degradation. The depolymerized products must be fractionated and purified by chromatographic techniques, and their structures determined by the above mentioned methods (Le Costaouéc et al., 2017; Reyes Suárez et al., 2008; Suárez et al., 2005). Furthermore, all these analyses are required to determine the chemical and physicochemical properties of polysaccharides found in microalgal cell wall. Figure 18 presents the enormous differences in cell wall structure found in several microalgal species.

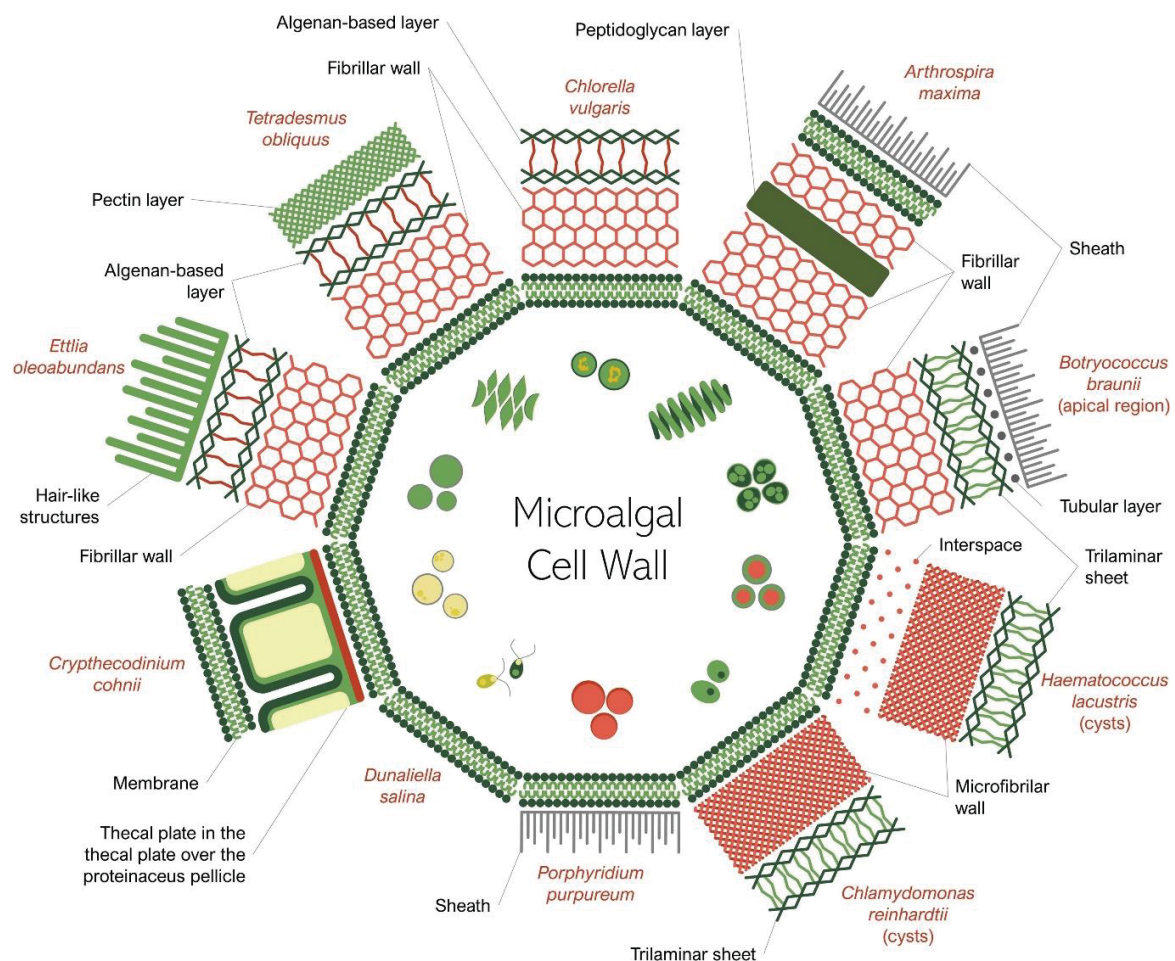


Fig. 18 - Cell wall structures of several important microalgae species. Reprinted from (de Carvalho et al., 2020), with permission from Elsevier.

Moreover, the high complexity of microalgal polysaccharide structures and the great diversity of microalgae species enhance several possibilities of chemical structure studies. Diatoms (Phylum Bacillariophyta) commonly present a sulfated glucuronomannan as the main structural polysaccharide (Le Costaouëc et al., 2017). An excellent example of the combined use of the available analytical techniques for the elucidation of microalgae polysaccharide structure is the study by Le Costaouëc et al. (Le Costaouëc et al., 2017). These authors described the complex chemical structure of the main cell wall polysaccharide found in diatoms, a sulfated β -D-glucurono- α -D-mannan (Table 23, Figure 19).

Also, complex polysaccharides containing branched α -L-rhamnan and xylogalactofuranan parts were found in *Parachlorella kessleri* HY1 (Chlorophyta) (Table 2, Figure 3) (Sushytskyi et al., 2020). The authors also reported a low molecular fraction containing O-2- β -xylo-(1 $\rightarrow$ 3)- β -galactofuranan found in the same species. Furthermore, Mirzaie et al. (Mirzaie, Tabarsa, & Safavi, 2020) showed a homogeneous polysaccharide with low molecular weight, low structural compactness composed of ($\rightarrow$ 1)-, (1 $\rightarrow$ 3), (1 $\rightarrow$ 3,6)-galactopyranose and α -glucopyranose residues obtained from *C. vulgaris* biomass. *Chlorella* spp. (Chlorophyta) have a rigid cell wall, mainly composed of glucosamine in a chitin-like polysaccharide (Baudet et al., 2017; Kapaun & Reisser, 1995) and also show the presence of uronic acid, dependent on the increase of CO₂ supply up to 2% (Y.-S. Cheng, Labavitch, & VanderGheynst, 2014). Polysaccharides such as β -(1 $\rightarrow$ 4)-glucans and/or β -(1 $\rightarrow$ 3)-glucans have been described in cell walls of *Botryococcus braunii* (Chlorophyta) (Weiss et al., 2012).

Other detailed studies were conducted by Suárez et al. (Reyes Suárez et al., 2008; Suárez et al., 2005, 2006), who reported the first description of cyclic β -(1 $\rightarrow$ 2)-D-glucans, an arabinogalactan constituted by [$\rightarrow$ 2)- α -L-Araf-(1 $\rightarrow$ 3)-[α -L-Araf-(1 $\rightarrow$ 4)]- β -D-Galp-(1 $\rightarrow$], and a new galactofuranan formed by 1 $\rightarrow$ 2-linked β -D-galactofuranose units (Table 2, Figure 3). These studies were performed via a hot water extraction from the green microalga *C. pyrenoidosa*, and demonstrated the considerable complexity that a single microalgal species can present.

From a different microalgae phylum, Pandeirada et al. (Pandeirada et al., 2019) described that the water-soluble fractions extracted from *Nannochloropsis oculata*

(Ochrophyta) were composed of (β 1 $\rightarrow$ 3, β 1 $\rightarrow$ 4)-glucans, (α 1 $\rightarrow$ 3)-, (α 1 $\rightarrow$ 4)-mannans, and anionic sulfated heterorhamnans.

Furthermore, Arad et al. (Arad & Levy-Ontman, 2010) presented an excellent review about red microalgal cell wall polysaccharides. The authors showed a full chemical and physicochemical characterization of an anionic polysaccharide (Table 2, Figure 3) using *Porphyridium* sp. (Rhodophyta) as study model. Also, red microalgae such as *P. aerugineum* and *Dixoniella grisea*, presented a similar sulfated polysaccharide as described by Liberman et al. (Netanel Liberman, Ochbaum, Mejubovsky-Mikhelis, Bitton, & (Malis) Arad, 2020). However, studies on chemical structures of polysaccharides obtained from red microalgae are limited. According to Arad et al. (Arad & Levy-Ontman, 2010), studies performed on red microalgal polysaccharides have been devoted mainly to four species: *Porphyridium* sp. (seawater), *P. cruentum* (seawater), *P. aerugineum* (freshwater), and *Dixoniella grisea* (brackish water). Figure 19 presents some chemical structures of polysaccharide obtained from microalgae cell wall.

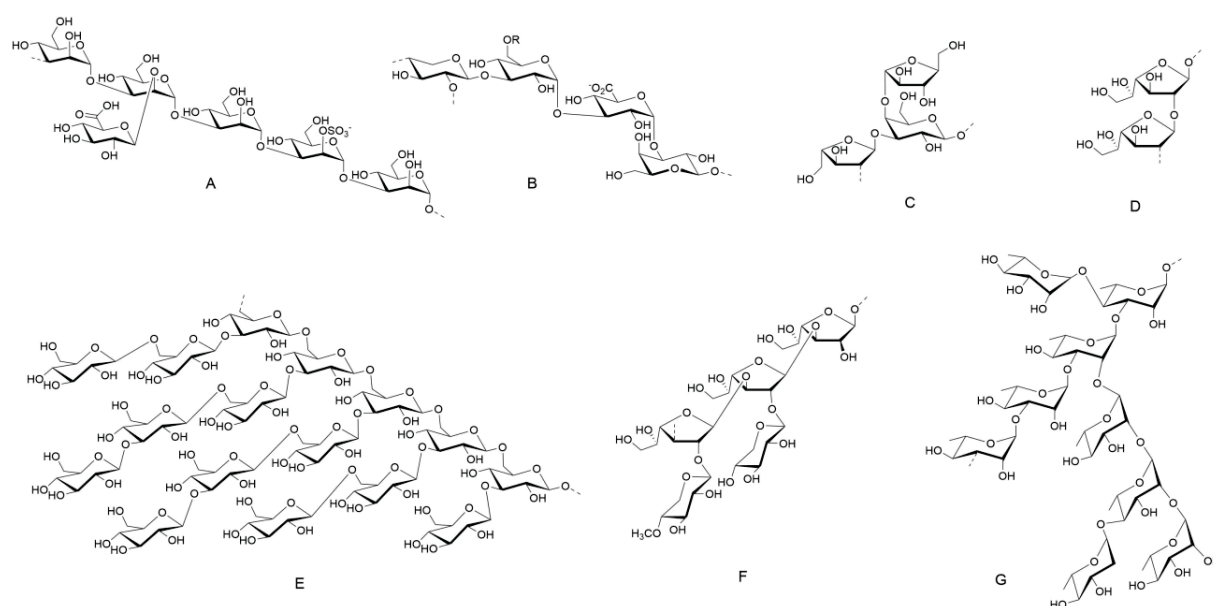


Fig. 19 - Microalgal polysaccharide chemical structures. (A) sulfated glucuronomannan isolated from the cell wall of the diatom *Phaeodactylum tricornutum* (Le Costaouëc et al., 2017); (B) an acidic heteropolysaccharide [(2 or 4)- β -D-Xylp-(1 $\rightarrow$ 3)]_m- α -D-Glcp-(1 $\rightarrow$ 3)- α -D-GlcpA-(1 $\rightarrow$ 3)-L-Galp-(1 $\rightarrow$) extracted from the red microalga *Porphyridium* sp. R= SO₃⁻ or H (Geresh et al., 2009); (C) arabinogalactan constituted by [$\rightarrow$ 2)- α -L-Araf-(1 $\rightarrow$ 3)-[α -L-Araf-(1 $\rightarrow$ 4)]- β -D-Galp-(1 $\rightarrow$)]

isolated from green microalga *Chlorella pyrenoidosa* (Suárez et al., 2005); (D) 1→2-linked β-D-galactofuranan obtained from *C. pyrenoidosa* (Suárez et al., 2006); (E) highly branched (1→3,1→6)-β-D-glucan from Haptophyta *Isochrysis galbana* (Sadovskaya et al., 2014); (F) branched β-D-xylo-β-D-galactofuranan and (G) 1→3-linked α-L-rhamnan branched at C2 and C4 obtained from green microalga *Parachlorella kessleri* (Sushytskyi et al., 2020).

Table 23 summarizes the main structural features, biomass extraction procedures and analytical techniques used for the study of some polysaccharides isolated from microalgae species.

Table 23 - Extraction process and characterization of polysaccharides isolated from microalgae

Phylum Microalgae (general environment)	Biomass extraction procedure	Methodology of characterization	Cell wall Polysaccharides	Reference
Bacillariophyta				
<i>Phaeodactylum tricornutum</i> (Seawater) ^a	Lyophilized cells were submitted to lipid and pigments removal, aq. extr. (80°C, 2 h, 3x), bleached with chlorite (1.7%, 80°C, 3 h), and alkaline extr. (4% NaOH, 100°C, 45 min)	HPSEC; GC; methylation/GC-MS; Smith degradation; Partial acid hydrolysis; GPC; HPAEC; NMR spectroscopy	Sulfated glucuronomannan: (1→3)-linked α-D-mannan backbone, partially decorated with sulfate and β-D-glucuronic acid at C-2. GlcA:Man, 1.0:4.5	(Le Costaouëc et al., 2017)
Chlorophyta				
<i>Neochloris oleoabundans</i> (Terrestrial) ^a	Sequential extr. using CDTA (50 mM, pH 7.5), and 4 M NaOH (with 0.1% NaBH ₄)	Microarray polymer profiling with Monoclonal antibodies	β-(1→4)-D-mannan	(Rashidi et al., 2019)
<i>Chlorella pyrenoidosa</i> (Freshwater) ^a	Aq. extr. (80°C, 1 h, 2x), centr. Followed by ethanol prec.	GLC-MS; SEC; NMR spectroscopy;	arabinogalactan constituted by [→2)-α-L-Araf-(1→3)-[α-L-Araf-(1→4)]-β-D-Galp-(1→]	(Suárez et al., 2005)
		Fract. using AEC and SEC; NMR spectroscopy	1→2-linked β-D-galactofuranan	(Suárez et al., 2006)
<i>C. vulgaris</i> (Freshwater) ^a	Cells were soaked with 2:1 benzene:ethanol, washed with hot water (80-85°C), followed by KOH addition, ethanol prec. and dialyzed	Acid hydrolysis; NMR spectroscopy.	3-O-α-D-glucopyranuronosyl-L-rhamnopyranose	(Ogawa, Yamaura, Ikeda, & Kondo, 1998)

<i>Parachlorella kessleri</i> (Freshwater/Terrestrial species) ^a	Biomass was treated with org. solvs. for removal of small molecules. Aq. extr. (100°C, 7 h, 2x). Removal of proteins	Fract. using AEC and SEC; HPSEC; Methylation analysis; GC-MS; FTIR; NMR spectroscopy	β -xylo- β -galactofuranan and α -L-rhamnan	(Sushytskyi et al., 2020)
Cyanophyta <i>Spirulina platensis</i> (Freshwater) ^a	Fresh microalgal biomass was mixed with H ₂ O (100°C, 2 h) followed by ethanol prec., centr., evap. & drying	HPLC; FTIR	Sulfated polysaccharide, mainly glucuronic acid and galactose	(Abd El-Hanaa, & El-Latife, 2013)
Haptophyta <i>Isochrysis galbana</i> (Marine species) ^a	Lyophilized cells were soaked with 85% ethanol (overnight) at rt, followed by heating in water bath (70°C, 10 min, 2x), centr. and supn. collection	Fract. using AEC and GPC; Smith degradation; Methylation analysis; GC-MS; ESI-MS and MALDI-TOF MS; NMR spectroscopy	Highly branched (1 $\rightarrow$ 3, 1 $\rightarrow$ 6)- β -D-glucan	(Sadovskaya et al., 2014)
<i>Pavlova gyrams</i> <i>P. lutheri</i> (Marine species) ^a	Aq. extr. (80°C, 2 h, 2x), ethanol prec. Fract. of the extract by freeze-thawing process	GC-MS; TGA; Methylation analysis; NMR spectroscopy	Branched (1 $\rightarrow$ 3, 1 $\rightarrow$ 6)- β -glucans and linear (1 $\rightarrow$ 3)- β -glucans	(Machado, Rodrigues, Moro, Duarte, & Nosedá, 2020)
Ochrophyta <i>Nannochloropsis oculata</i> (Brackish species) ^a	Defatted biomass, Aq. extr. (65°C, 3.5 h, 2x), centr. Ultrafiltration (cut-off 10 kDa). Ethanol fract. (50 and 85% v/v)	Fract. using AEC; GC; SEC; Methylation analysis; GC-MS; Carbohydrate microarray analysis	(β 1 $\rightarrow$ 3, β 1 $\rightarrow$ 4)-glucans, (α 1 $\rightarrow$ 3)-, (α 1 $\rightarrow$ 4)-mannans, and anionic sulphated heterorhamnans	(Pandeirada et al., 2019)
Rhodophyta				

<i>Porphyridium</i> sp. (Marine and freshwater species) ^a	Microalgal medium prec. with NaOH (2.5%), followed by H ₂ O and HCl (2 M) addition and ethanol prec. CTAB treatment, complex dissolved with 2.5 M NaCl.	Fract. by IEC; Methylation analysis; GC-MS; Partial acid hydrolysis; HPSEC; GC; Smith degradation; NMR spectroscopy	Acidic heteropolysaccharide: [(2 or 4)-β-D-Xylp-(1→3)] _m -α-D-Glcp-(1→3)-α-D-GlcpA-(1→3)-L-Galp(1→)	(Geresh et al., 2009)
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Aq.= aqueous; centr.= centrifugation; CDTA= 1,2-diaminocyclohexanetetraacetic acid; CTAB= Cetyltrimethylammonium bromide; extr.= extraction; Fract.= fractionation; org. solvs.= organic solvents; prec.= precipitation; rt= room temperature; supn.= supernatant. C: Chromatography; AEC: Anion-Exchange; IEC: Ion-Exchange; SEC: Size-exclusion; ESI: Electrospray Ionization; FTIR: Fourier transform-infrared; GC: GasC; GPC: Gel-Permeation; GC-MS: Gas chromatography-mass spectrometry; HPLC: High-Performance LiquidC; HPAEC: High-Performance Anion-Exchange; HPSEC: High-Performance Size-Exclusion; MALDI-TOF: Matrix Assisted Laser Desorption Ionization Time of Flight; MS: Mass Spectrometry; NMR: Nuclear Magnetic Resonance; TGA: Thermogravimetric Analysis. ^a According to Guiry, 2021 (Guiry & Guiry, 2021).

Understanding how polysaccharide structures are affected by controllable variables of microalgal growth systems such as nutrients, light intensity, salinity, and CO₂ supply is essential to establishing efficient industrial microalgal biorefinement on a large scale.

6.5 IMPORTANCE AND POTENTIAL OF MICROALGAL POLYSACCHARIDE PRODUCTION AND CULTIVATION SYSTEMS

Several risks and benefits of microalgae-based polysaccharide production processes were analyzed. Risks of large-scale microalgal cultivation are the potential for cross-contamination in open pond systems and the high energy input in downstream processes (mainly in microalgal biomass recovery) (Kadir, Lam, Uemura, Lim, & Lee, 2018). Benefits of microalgal cultivation include its potential to increase the amount of a particular metabolite of interest used for medical or pharmaceutical purposes. Many investigations have successfully used microalgal polysaccharides in biological applications or as part of biorefinery processes, such as the production of bioethanol (Chew et al., 2017; Sanchez Rizza, Sanz Smachetti, Do Nascimento, Salerno, & Curatti, 2017), biohydrogen (Kumar et al., 2018), biogas (Solé-Bundó, Salvadó, Passos, Garfí, & Ferrer, 2018), syngas, char, bio-oil (Azizi, Keshavarz Moraveji, & Abedini Najafabadi, 2018), and other derived products. These applications can improve the energy matrix by reducing the strong global dependence on fossil fuels.

According to Sanchez Rizza et al. (Sanchez Rizza et al., 2017), microalgae present advantages over conventional plant crops used for biotechnological applications, including their higher biomass productivity, non-competitiveness for arable land, use of agroindustrial residues as a source of alternative nutrients, and reduced greenhouse gas emissions.

Depending on the cultivation process, microalgal cellular metabolism could produce high-value polysaccharides as the main component of the cell, up to 70% on a dry weight basis (Sanchez Rizza et al., 2017). This efficiency is mainly related to the microalgal species cultivation system, as well as the induction of stress conditions (see Table 1). The most common methods of microalgae cultivation are open pond systems

and controlled closed systems (Narala et al., 2016). Open systems (unstirred pond, raceway ponds, and tanks) are the cheapest methods for producing microalgal biomass, and require minimal capital and operating costs, besides lower energy input to culture mixing. Raceway ponds have been extensively used for large-scale commercial production, mainly to cultivate *Spirulina*, *Dunaliella*, *Chlorella*, and *Haematococcus* (Milledge, 2011). This system uses 15-50 cm deep ponds, and the mixing is done by paddlewheels to promote cell exposure to sunlight (Chew et al., 2018). The use of closed systems in large-scale microalgal facilities can be 15 times more expensive in overall production cost than open systems (raceway pond) of the same volume, and 20 times higher in maintenance fees (J. Li, Zhu, Niu, Shen, & Wang, 2011).

Notwithstanding, closed systems (vertical, helical or horizontal tube photobioreactors, plastic bags, stirred tank photobioreactor, and flat panel photobioreactor) generally provide high illumination surface areas, high biomass productivity, efficient temperature control, and improved mass transfer and gas solubility, when compared to open systems (Chew et al., 2018; Silva Benavides, Torzillo, Kopecký, & Masojídek, 2013). Table 24 presents comparisons among biomass productivities and total costs of different cultivation systems, based on one acre of cultivation area.

Table 24 - Installed capital investment based on cultivation systems*

Cultivation system	Productivity	Total cost (US\$/acre)
Open pond	Low to average	33,042
Horizontal tube photobioreactor	High	43,748
Helical tube photobioreactor	High	377,892
Flat-panel photobioreactor	High	510,617
Plastic bag	Average to high	97,124

*Adapted from Chew et al. (2018) and Clippinger, Davis, Clippinger, & Davis (2019)

However, closed systems present the risk of biomass adhesion to photobioreactor walls and biomass flocculation due to low turbulence (Klinthong, Yang, Huang, & Tan, 2015). Although both systems have pros and cons, open systems are generally used to produce biomass with low-value, whereas closed systems are used to produce high-value

molecules (see Table 26). According to Bosma et al. (Bosma et al., 2014), it is not clear which cultivation system is the most appropriate for large-scale commercial application, although it is generally assumed that open systems such as raceway ponds are the cheapest.

As mentioned previously, microalgal productivity is a key parameter that influences the production process. According to Kumar et al. (Kumar et al., 2018), microalgal productivity can reach up to 2.57 g L⁻¹ using swine manure as an alternative nutrient added to the culture. Cultivation in this study was done using a transparent plastic bag with CO₂ injection and light intensity between 8000-9000 lux (12:12 light/dark) at 23°C. The system is considered to be environmentally-friendly, and has a favorable techno-economic analysis in the context of wastewater remediation, production of microalgal biomass, and biohydrogen.

6.6 POTENTIAL BIOLOGICAL ACTIVITY OF MICROALGAL POLYSACCHARIDES

Several biological activities have been observed for microalgal polysaccharides, such as antitussive and bronchodilatory activity (Halaj et al., 2018), adhesion to enterobacteria (Machado et al., 2020), plant growth biostimulant action (Rachidi, Benhima, Sbabou, & El Arroussi, 2020), plant defense (Farid et al., 2019), and antimicrobial properties (Hafsa et al., 2017), which reflect the wide range of possible applications of these molecules. Moreover, a single polysaccharide may express more than one biological activity (Abd El et al., 2013; Hafsa et al., 2017).

Antioxidant activity is one of the most explored biological activities of microalgal polysaccharides (Abd El et al., 2013; Burg & Oshrat, 2015; Y. X. Chen, Liu, Xiao, Huang, & Liu, 2016; Fimbres-Olivarria et al., 2018; Hafsa et al., 2017; Shen et al., 2018; L. Sun, Wang, Li, & Liu, 2014; Y. Sun, Wang, Guo, Pu, & Yan, 2014). Table 4 summarizes recent studies that explored this activity, which include radical scavenging, metal ion chelation, reducing potential, and induction of antioxidant enzyme gene expression. These results suggest the use of microalgal polysaccharides as antioxidants for pharmaceutical purposes (e.g., treatment of oxidative disorders such as arthritis) and in food, cosmetics, and biotechnological applications. The antioxidant activity of these polysaccharides

depends on several factors, such as polysaccharide chemical structure and molecular weight (L. Sun et al., 2014). It is also influenced by the addition of salts to the polysaccharide solution. Salt ions are able to change the three-dimensional structure of the polysaccharide to better expose antioxidant sites (Burg & Oshrat, 2015). Unfortunately, there is a lack of information on the specific antioxidant mechanisms of polysaccharides, which should encourage new studies of this subject.

Some *in vitro* studies have suggested that microalgal polysaccharides may also have antitumor properties, as observed for a (1→3,1→6)-β-D-glucan isolated from *Isochrysis galbana*, which inhibited the proliferation of a human leukemic monocyte lymphoma cell line (U937) (Sadovskaya et al., 2014). This property can be attributed to the direct action of polysaccharides on tumor cells or to enhancement of the organism immune response, as reported for polysaccharides obtained from *Pavlova viridis* (L. Sun, Chu, Sun, & Chen, 2016). Some studies concluded that chemical structure, in particular high molecular weight, is key to this activity (Qi & Kim, 2017, 2018). Thus, microalgal polysaccharides exhibit an interesting potential as new antitumor and immunomodulatory agents, and could be important as synergic or alternative treatment drugs. Examples of biological activities of microalgal polysaccharides are presented in Table 25.

Table 25 - Cell wall polysaccharides from microalgae, their main structural features, and biological application

Biological activity	Microalga	Polysaccharide main structural features	Reference
Antioxidant	<i>Pavlova viridis</i>	MW 3645 kDa; Sulfate (16.6%). Glc (60.8%), D-Fru (21.9%), Man (10.6%), and Rha (6.6%)	(L. Sun et al., 2014)
	<i>Sarcinochrysis marina</i>	MW 2595 kDa; Sulfate (16.1%). Glc (48.6%), L-Ara (42.6%), and D-Fru (8.8%)	
	<i>Isochrysis galbana</i>	MW 15.93 kDa; uronic acids (25.6%), sulfate (54.9 µg/mg). Contains primarily mannose, D-galactose, D-glucose and L-rhamnose	(Y. Sun et al., 2014)

Antitumor	<i>Navicula</i> sp.	MW 17 kDa; Contains rhamnose (35.3%), galactose (24.5%), glucose (15.5%), xylose (9.3%), and mannose (4.9%)	(Fimbres-Olivarria et al., 2018)
	<i>Porphyridium</i> sp. <i>P. aerugineum</i>	Soluble, sulfated	(Burg & Oshrat, 2015)
	<i>Nostoc flagelliforme</i>	MW 1.02×10^3 , 1.12×10^3 , 1.33×10^3 kDa Main monosaccharides: mannose, galactose, glucose, glucuronic acid, ribose	(Shen et al., 2018)
	<i>Chlorella pyrenoidosa</i>	Contains L-rhamnose, D-arabinose, D-xylose, D-glucose, D-galactose, and D-mannose	(Y. X. Chen et al., 2016)
	<i>Spirulina platensis</i>	Soluble, sulfated Main constituent: glucuronic acid; contains galactose, glucose, rhamnose and arabinose	(Abd El et al., 2013)
	<i>S. platensis</i>	<i>not described</i>	(Kurd & Samavati, 2015)
	<i>I. galbana</i> <i>Nannochloropsis oculata</i>	Glucose (56.9%); mannitol (38.8%) Glucose (68.3%), inositol (20.3%)	(Hafsa et al., 2017)
	<i>I. galbana</i>	(1→3,1→6)-β-D-glucan	(Sadovskaya et al., 2014)
	<i>Pavlova viridis</i>	MW 386.96 kDa, 15.0% sulfate, 5.9% uronic acids, contains D-fructose, glucose, and mannose MW 54.99 kDa, 17.8% sulfate, 8.8% uronic acids, contains D-fructose, glucose, mannose, and L-rhamnose	(L. Sun et al., 2016)
	<i>Dictyosphaerium pulchellum</i> <i>D. tetrachotomum</i> <i>P. viridis</i>	MW > 1000 kDa Main constituents: rhamnose, galactose, xylose and glucose MW 386.96 kDa, sulfate (15.0%), uronic acids (5.9%), contains D-Fru, Glc, and Man MW 54.99 kDa, sulfate (17.8%), uronic acids (8.8%), contains D-Fru, Glc, Man and L-Rha	(Halaj et al., 2019)
Immunomodulation	<i>C. ellipsoidea</i>	MW 126.9 kDa; MW 237.0 kDa Main constituent: glucose; also contains galactose, rhamnose, mannose, and uronic acids	(Qi & Kim, 2017)
	<i>C. pyrenoidosa</i>	MW 47 kDa. Main constituent: arabinose and galactose (2:1 ratio)	(Suárez et al., 2006)
	<i>Parachlorella kessleri</i>	MW 3.05×10^5 and 9.84×10^4 g mol ⁻¹ complex polysaccharides containing α-	(Sushytskyi et al., 2020)

Antitussive	<i>Dictyosphaerium chlorelloides</i>	L-rhamnan and xylogalactofuranan parts in different ratios MW 1069 kDa 1,3 and 1,4 linked galactose units branched in O-6, O-4, and O-3; also contains rhamnose, mannose	(Halaj et al., 2018)
Anticoagulant, Antiviral, and Antimicrobial	<i>S. platensis</i>	Soluble, sulfated Main constituent: glucuronic acid; also contains galactose, glucose, rhamnose and arabinose	(Abd El et al., 2013)
Antimicrobial and Anticholinesterase	<i>I. galbana</i> <i>Nannochloropsis oculata</i>	Glucose (56.9%); mannitol (38.8%) Glucose (68.3%), inositol (20.3%)	(Hafsa et al., 2017)
Anti-aging	<i>C. pyrenoidosa</i>	MW 9.950 kDa Contains L-rhamnose, D-arabinose, D-xylose, D-glucose, D-galactose, and D-mannose	(Y. Chen et al., 2018)
Plant biostimulant	<i>S. platensis</i> <i>Arthrospira platensis</i> <i>Dunaliella salina</i> <i>Porphyridium sp.</i>	Aqueous extraction (121 °C), ethanol precipitation Water-soluble polysaccharides. Aqueous extraction (90 °C, 2 h, 3x), ethanol precipitation	(Elarroussi et al., 2016) (Rachidi et al., 2020)
Plant defense	<i>C. vulgaris</i> , <i>C. sorokiniana</i> , <i>C. reinhardtii</i>	Aqueous extraction (95 °C), ethanol precipitation ^a	(Farid et al., 2019)
Adhesion capacity to enterobacteria	<i>Pavlova gyrans</i> <i>P. lutheri</i>	Linear (1→3)-β-glucans	(Machado et al., 2020)

Besides being applied in the pharmaceutical and medical sectors, microalgal polysaccharides have been used in the food and cosmetic industries. In the food industry, it have been utilized as viscosifiers, stabilizers, flocculants, and hydrating and gelling agents to improve quality and texture (de Jesus Raposo, de Moraes, & de Moraes, 2014; Sathasivam, Radhakrishnan, Hashem, & Abd_Allah, 2019). According to Bernaerts et al. (Bernaerts et al., 2018), some species, such as *A. platensis*, *C. vulgaris*, *Diacronema lutheri*, *Tisochysis lutea*, *Nannochloropsis sp.*, *Odontella aurita*, *Phaeodactylum tricornutum*, *Porphyridium cruentum*, and *Tetraselmis chuii*, present great potential as functional food ingredients based on the composition of the cell wall related polysaccharides that can be incorporated in pastas, snacks, candy bars, and even in drink mixes and beverages (Matos, Cardoso, Bandarra, & Afonso, 2017).

Microalgal polysaccharides can also be used in cosmetic and cosmeceutical formulations to support skin structure and function, as mentioned by Hamed et al.

(Hamed, 2016) and reviewed by Mourelle et al. (Mourelle, Gómez, & Legido, 2017). The registered product “alguronic acid” is an example of a cosmeceutical product obtained from polysaccharides extracted from microalga *Parachlorella kessleri* (Borowitzka, 2013; 2012/0202768, 2012). This field has recently received much attention because microalgal biomass is relatively easy to produce (Bernaerts, Gheysen, Foubert, Hendrickx, & Van Loey, 2019). Microalgae-based β -1,3-glucans can prevent external signs of aging and, due to its anti-inflammatory properties, be incorporated into formulations for sensitive and reactive skin types (Mourelle et al., 2017). Moreover, lesser-known applications such as biolubricants have been described, based on rheological properties of a sulfated polysaccharide produced by *Porphyridium* sp. (Arad et al., 2006).

Since the cost of large-scale microalgal polysaccharide facilities is still high (see Table 24), several studies have relied on outdoor pilot-scale microalgal production (Bosma et al., 2014; de Jesus et al., 2019; H. Li et al., 2011; Marazzi et al., 2019). This strategy has the potential to overcome bottlenecks in microalgae cultivation and reduce cost by optimization of process parameters. An interesting example of a microalgal pilot facility is the AlgaePARC (Algae Production and Research Center) at Wageningen UR (University & Research center) (Bosma et al., 2014). The facility consists of four cultivation systems (raceway pond, horizontal tube photobioreactor, vertical tube photobioreactor, and flat panels), which have been used to compare the performance and productivity of these growth systems under identical climatological conditions. These results could promote escalation in the decision-making process.

6.7 REGULATION AND MARKET

Regulations on food safety applicable to microalgal biomass and its components were summarized by Rani et al. (Rani, Sandal, & Sahoo, 2018). In the European Union, there are three main classes of regulations pertinent to the marketing of microalgal biomass and its derivatives: (a) regulation on food safety, (b) regulation on novel food and novel food ingredients, and (c) regulation on nutrition and health claims for food. The US market for microalgal products is subject to two laws, the Federal Food, Drug, and Cosmetic Act (FD&C) and the Dietary Supplement Health and Education Act (DSHEA)

(Rani et al., 2018). Government regulations play an important role in industrial development, by driving increases in specific market niches and determining warranty of merchantability.

Microalgal biomass presents potential in many categories of foods, such as single-cell proteins, natural colorants, carotenoids, and polysaccharides. However, the small-scale production of microalgal products, coupled with established commodities, may be constraining market penetration. The current estimated raw microalgal biomass selling price is about 1,000 € t⁻¹ (Barsanti & Gualtieri, 2018). However, the market size of microalgae-based bioproducts has expanded over the years, greatly adding value. The market size for biopolymers and bioactive molecules, of which microalgae-based commodities form a part, corresponds to 14.2 billion € in the European economy (Barsanti & Gualtieri, 2018), and the projection of the global algal product market is about US\$44.6 billion by 2023 (CredenceResearch, 2017). Table 26 summarizes the main selling prices of polysaccharides extracted from microalgal biomass.

Table 26 - Microalgal polysaccharide selling prices*

Polysaccharide application	Price (€ t⁻¹)
Immunostimulant	200,000
Biostimulant for plants	10,000
Moisturizing agents	2,500
Bioethanol/biohydrogen	400

*Adapted from (Barsanti & Gualtieri, 2018)

The incorporation of microalgal biomass into food and nutraceutical products has presented economic value in areas outside of human and animal health. According to Koyande et al. (Koyande et al., 2019), some microalgae species have been incorporated into common products in an effort to increase market demand and enhance food nutritional and techno-functional properties. Microalgal biomass addition to bread, cookies, extruded snacks, pasta, processed cheese, and vegetarian food gels are a few examples (Koyande et al., 2019). Several of these functional properties are related to the polysaccharide content of the microalgal cell (Burg & Oshrat, 2015; Y. X. Chen et al.,

2016; Fimbres-Olivarria et al., 2018; Shen et al., 2018). Not surprisingly, microalgal polysaccharides that present biological activity have a higher price due to their therapeutic value in the pharmaceutical/biomedical sectors. Conversely, polysaccharide-based biofuels present lower values relative to average selling prices, reflecting the low aggregated value of this purpose.

A large-scale hypothetical microalgal plant was developed by Li et al. (J. Li et al., 2011). The authors found that microalgal biomass might be produced for as low as US\$18 per kg. Although this study was focused on *H. pluvialis* biomass to obtain astaxanthin, the results are very promising, considering the fact that the biomass production process is very similar regardless of species. Moreover, microalgae cultivation production costs are strictly dependent on geospatial location, productivity, and optimal laboratory parameters (Banerjee & Ramaswamy, 2019). There is limited available information on polysaccharide selling price as related to large-scale cultivation bottlenecks. Microalgal biomass production and polysaccharide extraction optimization should be pursued to overcome challenges and difficulties associated with the operation of a high-value microalgal biocompound facility.

6.8 MICROALGAL POLYSACCHARIDES: CHALLENGES AND FUTURE PROSPECTS

Challenges related to polysaccharide production by microalgae cultivation are crucial to understanding the entire production process. Different approaches are applied in microalgae cultivation to obtain polysaccharides; however, there are common bottlenecks that prevent a cost-effective production process. These issues include:

- Limited data from industrial-scale microalgal polysaccharide production, in addition to insufficient guidance on extrapolation of parameters from laboratory-scale to commercial-scale production
- High operational costs of microalgal production related to nutrient availability and replacement, CO₂ addition, electric power consumption, biomass recovery, and trained labor

- Feasibility of small-scale microalgal production system competitiveness with traditional commodities (Vigani et al., 2015)
- Difficult control of microbial contamination in large-scale microalgal growth systems
- Heavy inorganic nutrient requirements and intensive energy input for microalgae cultivation and biomass drying (Lam, Lee, & Mohamed, 2012)
- Limited information on the relationship between microalgae species and polysaccharide of interest
- Low microalgal biomass productivity compared to common microbial fermentations
- Need for further polysaccharide research based on *in vivo* experiments of potential biological activities (X. Chen et al., 2019)

It is worth mentioning that it is difficult to integrate new products into the existing market. The process may face cultural barriers, a long and expensive evaluation cycle, restrictions, monopoly of the production process of a specific molecule, lack of incentive laws, country legislation (Godman et al., 2018), and—specifically in this case—the high cost attributed to the inherent disadvantages of microalgal cultivation. Moreover, likewise bacterial polysaccharides, which can be produced at low cost and great amount, polysaccharides derived from microalgae also face difficulties related with already established polysaccharides in the market. Nonetheless, increased investment in basic research (Khakoo, Yurgin, Eisenberg, & Fonarow, 2019) and optimization of polysaccharide extraction and purification is poised to overcome these limitations and improve efficiency. Several studies have already reported innovations in microalgal polysaccharide production processes (Alhattab, Kermanshahi-Pour, & Brooks, 2019; Dixon & Wilken, 2018; Ekelhof & Melkonian, 2017; Johnson et al., 2018). Recently, media nutrient optimization has been shown to be key in microalgal cultures to the extent that it can increase total carbohydrate content from cell walls and storage polysaccharides, as well as decrease media costs. Calcium and magnesium-limited media were shown to increase carbohydrate cell content by 50% when compared to control cultures (Hanifzadeh et al., 2018). In addition, infrastructure optimization may facilitate an increase in microalgal growth rate and polysaccharide yield. Several advancements in the design of photobioreactors for autotrophic microalgal cultivation (Yen, Hu, Chen, Nagarajan, &

Chang, 2019) and modelling of the impact of contamination in large-scale microalgal cultures (Deruyck, Thi Nguyen, Decaestecker, & Muylaert, 2019) promise to be useful for even further developments in this area.

Furthermore, studies on microalgal-based bioproducts using polysaccharides are developing new strategies and modern process routes to create sustainable products. Mathiot et al. (Mathiot et al., 2019) designed a starch-enriched microalgal process that could be used for the development of bioplastics. Farid et al. (Farid et al., 2019) reported a promising potential of microalgal polysaccharides in plant defense, relying mainly on β -1,3-glucanase, phenylalanine ammonia lyase, and lipoxygenase, which are key defense enzymes. In addition, studies on microalgal polysaccharides have presented interesting results related to crucial biomedical areas such as immunostimulation, anticancer activities (X. Chen et al., 2019), and antimicrobial hydrogels (Netanel Liberman, Ochbaum, Bitton, & (Malis) Arad, 2021). Moreover, genetically modified microalgae have been shown in laboratory-scale experiments to enhance biofuel and bio-based chemicals (Faraloni & Torzillo, 2018; Ng, Tan, Kao, Chang, & Chang, 2017; Vargas, Kava, Balmant, Mariano, & Ordonez, 2016). To the best of our knowledge, however, there are as of yet no available studies on the increase of polysaccharide production using genetic engineering of microalgae.

The study of microalgae-derived polysaccharides plays an important role in biorefinery strategies, since they represent a highly diverse group of biomacromolecules with a wide variety of biological applications and still-untapped potential for exploitation. Advances in this area are urgently required to create new methods and processes that overcome bottlenecks in microalgal polysaccharide production.

6.9 CONCLUSIONS

Due to their biological activities, microalgal polysaccharides possess great potential in the pharmaceutical, medical, and agroindustrial sectors. These properties, such as antioxidant, antitumoral, immunomodulatory, and/or plant biostimulatory capacities, make microalgal polysaccharides a welcome addition to the existing market. The production of these macromolecules is highly dependent on media effects, which

may influence both the carbohydrate and monosaccharide content. For microalgal polysaccharide exploitation to become feasible, limitations such as cross-contamination, lack of parameter control, and high cost will have to be overcome. Nonetheless, from a research perspective, polysaccharide-based microalgal processes are poised to greatly enhance the biotechnological sector over future decades in a cost-effective way. Even though there is a healthy rate of development in this area, technological advances in microalgae cultivation and polysaccharide extraction merit further attention. Moreover, research on selling prices and cultivation bottlenecks are crucial for the development of sustainable polysaccharide-based microalgae facilities that are capable of overcoming market and performance challenges from well-known companies in the food/pharmaceutical sectors.

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7 CONCLUSÕES FINAIS

Os resultados obtidos no presente trabalho permitem concluir que o uso de diferentes meios de cultivo influenciou a fase vegetativa, a composição bioquímica e o teor de pigmentos da biomassa microalgal de *Haematococcus pluvialis*. Além disso, os resultados indicaram que o principal custo do meio de cultivo está associado principalmente aos nutrientes NPK.

Em relação à produção de astaxantina a revisão realizada mostrou que os principais custos estão relacionados com os processos de *upstream* e *downstream*. Ainda, que condições ótimas de intensidade luminosa, pH, temperatura, adição de sal ao meio e sistemas de cultivo são fundamentais para a cultura de *H. pluvialis* e indução de carotenogênese. Além disso, foi possível verificar que os processos de extração de astaxantina estão associados à eficiência de extração, e por isso influenciam diretamente no custo final do processo.

A avaliação de diferentes meios de cultivo para o crescimento da microalga *Dunaliella salina* apresentou variações na composição bioquímica celular, além de influenciar o custo da biomassa e pigmentos. Os resultados demonstraram que os componentes NaNO_3 , cianocobalamina e KNO_3 representam os maiores custos de produção dos meios de cultivo avaliados. O custo de produção dos pigmentos em escala laboratorial, embora inviáveis economicamente, demonstraram diferenças significativas apenas trocando o meio de cultivo.

A partir da utilização do bioprocesso para tratamento da vinhaça de arroz foi possível concluir que a cianobactéria *Synechococcus pevalekii* conseguiu reduzir parâmetros como teor de nitrato e íons presentes na vinhaça. Além do tratamento biológico, foi visto que a adição da vinhaça tratada ao cultivo de *D. salina* proporcionou aumento significativo no crescimento celular. Desta forma, o bioprocesso apresentou viabilidade de escalonamento e uso para tratamento de outros resíduos agroindustriais, como a vinhaça proveniente da destilação do etanol de cana-de-açúcar.

Através do estudo da literatura científica sobre pigmentos pouco convencionais obtidos de microalgas, foi possível avaliar novas oportunidades de inclusão de moléculas baseado nas propriedades biológicas. Ainda, vale ressaltar que a produção comercial

dessas moléculas mostrou-se extremamente dependente de novos estudos científicos associados à otimização de processos, análises de atividade biológica e avaliação da produção em larga escala.

A partir da revisão bibliográfica dos polissacarídeos de parede celular das microalgas, foi possível verificar que há um grande potencial em aplicações dessas moléculas nas áreas farmacêutica, médica e agroindustrial devido às propriedades biológicas (antioxidante, antitumoral, imunomodulatória ou estimulante vegetal). Além disso, os resultados mostraram que a produção de polissacarídeos é diretamente associada aos parâmetros de cultivo adotados no processo.

8 PERSPECTIVAS PARA PRÓXIMOS TRABALHOS

Após o estudo e avaliação dos resultados obtidos no trabalho, é possível verificar algumas oportunidades em novas pesquisas visando a continuação do trabalho. Sendo assim, algumas das possibilidades a serem abordados por trabalhos futuros incluem:

- Suplementação do meio de cultivo com fontes alternativas de nutrientes visando a análise econômica para comparação com o custo de meios sintéticos;
- Estudo de polissacarídeos e exopolissacarídeos obtidos do cultivo de microalgas;
- Análise de atividade biológica dessas biomacromoléculas, tais como atividade antimicrobiana, leishmanicida e tripanocida, entre outras;
- Avaliação do perfil de pigmentos visando a obtenção e estudo de pigmentos pouco convencionais;
- Escalonamento do tratamento de vinhaça utilizando microalgas encapsuladas através da realização de experimento em fotobiorreatores tubulares, a fim de emular de forma mais realista o uso no setor agroindustrial.

Desta forma, seria possível conciliar os resultados com os dados obtidos para a melhora no entendimento em relação ao cultivo de microalgas para produção de metabólitos de interesse industrial bem como melhora em aspectos do cultivo e uso em processos industriais.

ANEXO I - AUTORIZAÇÃO PARA REPRODUÇÃO DO ARTIGO PUBLICADO



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ANEXO II - CAPÍTULO ACEITO PARA PUBLICAÇÃO NO LIVRO: SUSTAINABLE GLOBAL RESOURCES OF SEAWEEDS - VOLUME 2

CHALLENGES AND RECENT PROGRESS IN SEAWEED POLYSACCHARIDES FOR INDUSTRIAL PURPOSES

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ABSTRACT

Important advancements in seaweed production have driven the search for potential alternative molecules with great biotechnological interest. Several seaweed species have been cultivated worldwide and are exploited as a source of platform chemicals and specialty molecules such as polysaccharides. Seaweed polysaccharides have presented several applications in the industrial sector, mainly due to their capacity to form gels and viscous solutions. This chapter highlights the progress in seaweed polysaccharides production, as well as its potential, chemical structure, and biological activity. Furthermore, some economic aspects and commercial importance are summarized and discussed. We also address advanced technologies in massive seaweed production and their impact on the industrial scenario. The topics here studied

include some of the main challenges of sustainable production of seaweed-derived polysaccharides.

Keywords: Seaweed-derived polysaccharide. Macroalgae. Challenges. Market. Offshore cultivation. Industry.

INTRODUCTION

Natural molecule producers associated with biotechnological processes have increased scientific interest due to the global population growth. Macroalgae or seaweeds are natural, reliable, and sustainable organisms that have been used for direct human consumption or processed into functional foods, nutraceuticals, cosmetics, and medicines worldwide (Abu-Ghannam and Cox, 2013; Hasan, 2017). In 2019, the global seaweed market reached a production value of US\$ 8.8 billion, and the market is expected to increase by 7.5% during 2020-2025 (IMARC Group, 2020). Seaweeds are marine organisms that have been explored as promising sources of novel molecules such as significant amounts of proteins, minerals, and mainly polysaccharides (Lafarga et al., 2020; Salehi et al., 2019). Seaweeds harbor several polysaccharides that are extremely important in medical, pharmaceutical, cosmetic, and nutraceutical areas. These polysaccharides have a wide range of chemical structures and biological applications (Cosenza et al., 2017).

In biotechnological fields, seaweed production and seaweed-derived polysaccharides have presented immense potential. Moreover, the use of non-digestible carbohydrates as a source of dietary fiber (Garcia-Vaquero et al., 2017), biological activity such as anticoagulant effect from sulfated fucoidan isolated from *Sargassum vulgare* (Dore et al., 2013), and anti-tumor activity of galactofucan obtained from *Alaria angusta* (Menshova et al., 2015) are few examples of seaweed-derived polysaccharides importance. Seaweeds were also investigated as natural manipulation of rumen fermentation, mitigating ruminal methane production (Maia et al., 2016).

Current information and economic assessment are essential to reinforce the quality of the decision-making process in the industry. In this chapter, the industrial seaweed-derived polysaccharide potential was evaluated and assessed through its

chemical structure and biological activity in several key areas. Besides, seaweed cultivation features and their economic impacts were also evaluated to present a current vision of the global scenario. Also, advanced technologies for massive seaweed production were discussed and analyzed.

POTENTIAL OF SEAWEED POLYSACCHARIDE PRODUCTION

Since marine macroalgae present a wide range of natural chemicals with several potential applications, the development of large-scale seaweed aquaculture becomes essential for biomass production. Targeted research and monitoring are required to help the decision-making, improvement on cultivation, and market development during a large project in this area (Campbell et al., 2019). Moreover, in industrial seaweed-derived polysaccharide production, several biological and environmental variables can cause influences on macroalgae tissue biochemical content and bioactivity. Factors such as reproductive stage, blade age, salinity, sampling season, light climate, nutrient availability, sampling location, biomass density, water motion, temperature, and grazing pressure can directly influence the algal metabolism (Hafting et al., 2015).

According to Santelices (1999), seaweeds can be cultivated in one or multiple-step farming systems. Several seaweed industrial propagation techniques have been used, such as fragmentation followed by propagation directly for growth in the cultivation system for *Gracilaria* and *Kappaphycus*, or the use of a hatchery for the propagation of unitary seaweeds such as kelp (Buschmann et al., 2017). Seaweed propagation is done at different production scales: intensive on-land tanks, ponds, or open-sea culture systems. Also, the farming method and agronomic requirements are strictly associated with the target seaweed species. A few examples are nets at surface level (*Porphyra* and *Ulva*), bottom planting (*Gracilaria* and *Sarcodiotheca*), middle water (*Kappaphycus*), and rafts in deeper water (*Macrocystis*, *Laminaria*, and *Undaria*) (Santelices, 1999). It is worth highlighting that open-sea culture systems are considered the most commercially successful system due to lower operational and capital costs (Sahoo and Yarish, 2005).

Polysaccharides can represent up to 76% of most seaweeds, on a dry weight basis (Holdt and Kraan, 2011). According to the authors, the highest contents are found in

Ascophyllum, Porphyra, and Palmaria. Furthermore, brown algae (Laminaria, Fucus, Ascophyllum, and Sargassum) present a polysaccharide content variation from 35 to 70% on a dry weight basis. Also, green algae (Ulva) show a 15-65% variation, whereas red algae (Chondrus, Porphyra, Gracilaria, and Palmaria) represent a variation on polysaccharide content from 40 to 74% (Holdt and Kraan, 2011; Venugopal, 2019). Holdt and Kraan (2011) reviewed and described some seaweed-derived polysaccharides such as alginic acid, fucoidan, laminaran, porphyran, and floridean starch. According to the authors, these polysaccharides present variation in the cellular content based on climate change. Taking the large polysaccharide content into account, several methodologies are used to extract, purify, and characterize these molecules. Figure 20 describes an industrial process to obtain high-value molecules from macroalgae biomass. Here we focused on a general schematic diagram of polysaccharide production.

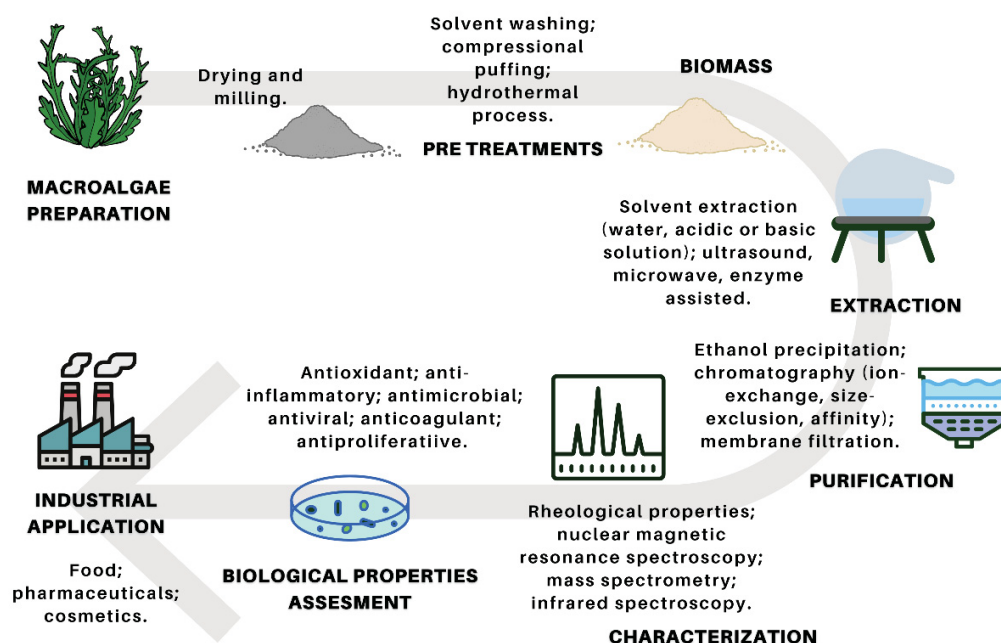


Fig. 20 - Steps in seaweed-derived polysaccharide extraction and purification

To obtain a good quality polysaccharide, extraction, purification, and quality control are necessary. The extraction of polysaccharides requires fine-tuning several operating

parameters such as extraction time, the ratio of solvent to sample, temperature, microwave power, and type of solvent followed by a rigid purification step. DEAE-Sephadex A-50 and A103S ion-exchange chromatography are a few examples of existing techniques of polysaccharide purification (He et al., 2016; Shi et al., 2018).

In downstream processes, methodologies are necessary to characterize the polysaccharide molecule. Usov (2011) reviewed and explained several important techniques used to characterize polysaccharides obtained from seaweeds, mainly red algae. Infrared spectroscopy, gas chromatography, and high-performance gel permeation chromatography (He et al., 2016) are useful to identify functional groups, evaluate the monosaccharide composition, and molecular weight (MW), respectively. Total sugars and uronic acids hint at the polysaccharide chemical structure (Rioux et al., 2007). Also, nuclear magnetic resonance (NMR) spectroscopy (1D and 2D analyses) is an essential analytical technique in resolving the polysaccharide structure, as mentioned by Ferreira et al. (2019) and explored by Gonçalves et al. (2002). To determine the pattern of glycosidic linkages present in the polysaccharide, it is common to use the methylation approach that includes, permethylation of the polysaccharide followed by total hydrolysis and GC-MS analysis of the partially methylated derivatives (Shi et al., 2018). In addition to polysaccharide chemical structure, several studies showed the potential of these macromolecules in pharmaceutical, food, and medical areas (de Jesus Raposo et al., 2015; Holdt and Kraan, 2011; Venugopal, 2016).

CHEMICAL STRUCTURE AND BIOLOGICAL ACTIVITY

Generally, a polysaccharide biological activity is associated with several chemical characteristics such as monosaccharide composition, functional groups, molecular weight (MW), anomeric configuration (α or β), and branching degree (Duarte et al., 2004; Guo et al., 2017; Lahaye, 2001; Shi et al., 2018; Wu et al., 2012). A polysaccharide (synonym: glycan) consists of several units of monosaccharides linked by glycosidic bonds. Heteropolysaccharides have two or more different types of monosaccharides in their composition, while homopolysaccharides have just one type of monosaccharide unit. Polysaccharides may be neutral (e.g. laminaran) or acidic, with the presence of sulfate

groups (e.g., carrageenans, fucoidans, and ulvan) or carboxylic acid groups (as in alginate and ulvan) (Ngo and Kim, 2013), or present other substituent groups, such as methyl or pyruvyl (Venugopal, 2019).

The polysaccharide chemical structure, as well as its above-mentioned features, are strictly associated with a particular biological activity. Ulvan represents the main polysaccharide found in green seaweeds from *Ulva* genus (Figure 26.2). This sulfated heteropolysaccharide is constituted by rhamnose, xylose, and glucuronic and iduronic acids (Jiao et al., 2011; Lahaye and Ray, 1996). According to Costa and colleagues (2010), biological effects as antiproliferative efficacy of polysaccharide correlate with its sulfate content. A variety of other sulfated seaweed-derived polysaccharides are synthesized by green macroalgae (Ghosh et al., 2004; Matsubara et al., 2001), such as a branched heteropolysaccharide from *Caulerpa racemosa*, that presents in vitro anti-herpetic activity. This polysaccharide contains 1,3- and 1,3,6-linked galactose, 1,3,4-linked arabinose, 1,4-linked glucose, and terminal- and 1,4-linked xylose residues (Ghosh et al., 2004). Figure 21 shows the disaccharide repeating units (diads) of the green macroalgae polysaccharide ulvan.

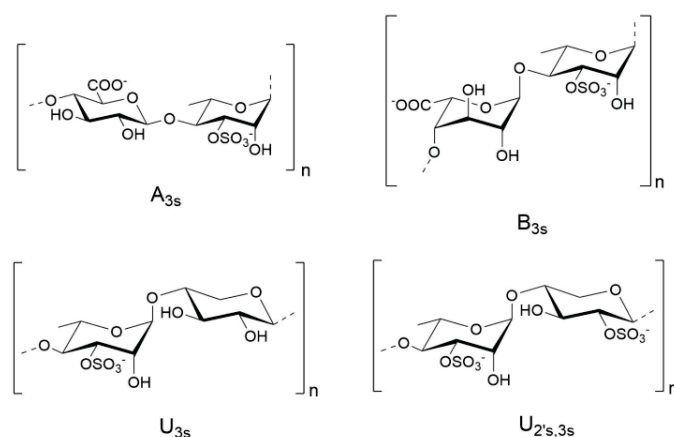


Fig. 21 - Diads present in ulvan, sulfated heteropolysaccharide isolated from green seaweeds of *Ulva* genus. A_{3s}: [→4)-β-D-GlcAp-(1→4)-α-L-Rhap 3-sulfate-(1→];

B_{3s}: [→4)-α-L-IdoAp-(1→4)-α-L-Rhap 3-sulfate-(1→];

U_{3s}: [→4)-α-L-Rhap 3-sulfate-(1→4)-β-D-Xylp-(1→];

U'_{2's,3s}: [→4)-α-L-Rhap 3-sulfate-(1→4)-β-D-Xylp 2-sulfate-(1→]. These structures were elucidated by Lahaye's group (Lahaye and Robic, 2007).

Additionally, chemical modification of the native polysaccharides such as sulfation, partial depolymerization, over-sulfation, and cyclization reaction can enhance the polysaccharide biological activity or rheological properties. The latter one (formation of 3,6-anhydro- α -galactose or 3,6-anhydro- α -galactose 2-sulfate units from α -galactose 6-sulfate or α -galactose 2,6-disulfate, respectively) is an important reaction used in carrageenans and agarans to increase their rheological properties and it was applied to several polysaccharides from red seaweeds (Ciancia et al., 1993, 1997; Nosedá et al., 2000; Nosedá and Cerezo, 1995; Viana et al., 2004; Zibetti et al., 2005, 2009). Carlucci and colleagues (1997) showed the relationship between the native carrageenans obtained from *Gigartina skottsbergii* and their cyclized derivatives with antiviral activity. The authors reported that the sulfate position in carrageenans molecules can influence the antiviral behavior. Furthermore, according to Patel (2012), chemical modification on carrageenan oligosaccharides increases its anti-tumor effect and anti-tumor immunity, whereas depolymerization and over-sulfation of fucoidan enhance its anti-angiogenic and anti-tumor activities (Koyanagi et al., 2003; Silchenko et al., 2018). Furthermore, polysaccharide features and biological activity present an intrinsic relationship that is essential to understand the biochemical pathway in biological assays. Table 27 presents a list of polysaccharide traits, structural features, and their biological activity.

Table 27 - Summary of existing seaweed-derived polysaccharides, main structure features, and biological application

Seaweed specie	Polysaccharide main structural features	Biological activity	Reference
<i>Sarcodia ceylonensis</i> (Plocamiales, Rhodophyta)	MW 466 kDa. Main monosaccharides: Man, Glc, Sor and Ara (molar ratio: 14.367:5.339:2.829:1.213)	Antioxidant activity	(He et al., 2016)
<i>Ulva lactuca</i> (Ulvales, Chlorophyta)	MW 404 kDa. Main monosaccharides: Man, Glc, Ara, Sor, Gal, and Fuc (molar ratio: 6.659:1.931:0.519:0.461:0.277:0.222:0.194)		

<i>Durvillaea antarctica</i> (Fucales)	MW 482 kDa. Main monosaccharides: Glc, Man, Sor, Fuc, and Xyl (molar ratio: 26.238:2.936:2.704:1.060:0.892)		
<i>Gracilaria lemaneiformis</i> (Gracilariales, Rhodophyta)	MW 591 kDa. Main monosaccharides: Gal, Fuc, Glc, and Xyl (molar ratio: 18.76:5.968:4.48:1.811) GLP1 (MW 5.5 kDa), GLP2 (MW 85 kDa, 10.8% sulfate) and GLP3 (MW 82 kDa, 23.2% sulfate). All fractions were mainly composed of Gal and had a (1→3)-Galp and (1→6)-Galp backbone.	Anti-tumor activity	(Shi et al., 2018)
	MW > 152 kDa and contains 3,6-anhydrogalactose (8.23%), sulfate (11.26%) and protein (0.98%).	Anti-allergy	(Liu et al., 2016)
<i>Laurencia dendroidea</i> (Ceramiales, Rhodophyta)	DHS-4 ($181.3 \times 10^3 \text{ g.mol}^{-1}$, 21.3% of NaSO_3) presented unit A 2-sulfated (18.9 mol%), nonsubstituted (15.3 mol%) and 6-O-methylated (10.1 mol%), and unit B composed mainly by galactose 6-sulfate precursor units (19.2 mol%) and 3,6-anhydrogalactose (13.8 mol%).	Snake antivenom	(Ferreira et al., 2019)
<i>Ulva fasciata</i> (Ulvales, Chlorophyta)	F2 (NaSO_3 14.1%; COO^- 1.23 mmol.g ⁻¹ ; MW 8.1 kg.mol ⁻¹) presented a compact sphere conformation with a helical motif as secondary structure. C3 showed a NaSO_3 21.0%; COO^- 1.81 mmol.g ⁻¹ ; MW 49 kg.mol ⁻¹ whereas C3b presented NaSO_3 14.1%; COO^- 1.23 mmol.g ⁻¹ ; MW 8.1 kg.mol ⁻¹ and C3c showed NaSO_3 21.0%; COO^- 1.81 mmol.g ⁻¹ ; MW 18 kg.mol ⁻¹	Anticoagulant	(de Carvalho et al., 2020)
<i>Ulva rigida</i> (Ulvales, Chlorophyta)	MW 56.7 kDa, 41% neutral sugars, 34% uronic acids and 4% proteins. Main monosaccharides: Glc/Gal (12.2 mol%), Xyl (8 mol%), Rha (42.6 mol%), GlcN (6.9 mol%) and GlcA/IdoA (30.3 mol%).		(Adrien et al., 2019)

<i>Sphaerococcus coronopifolius</i> (Gigartinales, Rhodophyta)	MW 308,700 Da. Main monosaccharides: Gal (33.1%), Xyl (1.8%) and Glc (1.7%), contains 3,6-AnGal (11%), GlcA (6.7%), GalA (1%), sulfate (24%) and pyruvic acid (0.34%).	Antiviral	(Bouhlal et al., 2011)
<i>Boergeseniella thuyoides</i> (Ceramiales, Rhodophyta)	MW 360,300 Da. Main monosaccharides: Gal (25.4%), Xyl (2.8%), Glc (3%) and Rha (0.3%), contains 3,6-AnGal (16%), GalA (3.2%), sulfate (7.6%) and proteins (6%).		

Note: GlcN=glucosamine; GalA= galacturonic acid; GlcA= glucuronic acid; IdoA= iduronic acid; 3,6-AnGal= 3,6-anhydrogalactose.

Anticoagulant activity is the most studied biological activity from seaweed-derived sulfated polysaccharides (Costa et al. 2010; Ciancia, Quintana, and Cerezo 2010). Moreover, sulfated polysaccharides isolated from *Padina tetrastrum* and *Gracilaria cervicornis* exhibited anti-angiogenic properties (Jose and Kurup, 2017) and antidiarrheal activity (Bezerra et al., 2018), respectively, whereas polysaccharide obtained from *Laminaria japonica* presented renoprotective effect in rats (Li et al., 2017). Furthermore, therapeutic agents for atherosclerosis are also reviewed and reported associated with the use of fucoidan, sulfated laminaran, and ulvan (Patil et al., 2018).

Most polysaccharides are considered non-toxic, these molecules have been studied in several biological assays in pharmaceutical and medical areas (Bilal and Iqbal, 2019). Undoubtedly, seaweed-derived polysaccharides enhance the possibilities of new drug development and therefore contribute to the environment by carbon storage and the reduction of greenhouse emissions during seaweed cultivation (Buschmann et al., 2017; Chung et al., 2011). Additionally, industrial steps – mainly downstream processes – are critical to support a high-quality polysaccharide extraction in a large-scale seaweed facility.

DOWNSTREAM PROCESS: A CRITICAL STEP ON INDUSTRIAL SEAWEED-DERIVED POLYSACCHARIDE PRODUCTION

Downstream processes are essential for any biotechnological bioprocess due to their influence on total production cost, energy input, and product quality. For seaweed

polysaccharides, these activities comprise biomass pre-treatment, extraction procedures, purification, enzymatic modification processes, marketing, trading, and transportation (Nor et al., 2019; Zayed and Ulber, 2020). According to Zayed and Ulber (2020), in a seaweed-derived fucoidan facility, the downstream process composes pre-treatment (washing, drying, and milling), followed by extraction procedures (hot- or cold-water incubation and solvent precipitation), separation techniques, and purification to obtain a high-quality final product. Figure 22 shows manufacturing process flow charts for carrageenan and alginate production.

Different extraction and purification methodologies can directly influence the quantitative yield and quality of seaweed-derived polysaccharides. The choice of these parameters is based on the physicochemical properties of the polysaccharide and its interactions with the cell wall, as well as the seaweed species, the period of collection, and the biomass conservation (Robic et al., 2009). Furthermore, variables such as extraction temperature, solvents, solvent to biomass ratio, biomass particle size, and extraction time are also extremely important to limit the co-extraction of macromolecular impurities and reduce the necessity of refined purification techniques (Kidgell et al., 2019).

A relevant example of an industrial downstream process concern is the correct distribution of seaweed intended for the processing versus that destined to be used as seedlings. Malaysia's seaweed industry is based on 90% dried *Kappaphycus* spp. to be used in industry on carrageenan production and 10% fresh seaweed as seedlings to maintain the cultivation cycle (Nor et al., 2019). In summary, the decision-making process in these industrial production steps requires a great understanding of seaweed processing, innovative technologies, and lab-scale experimental tests.

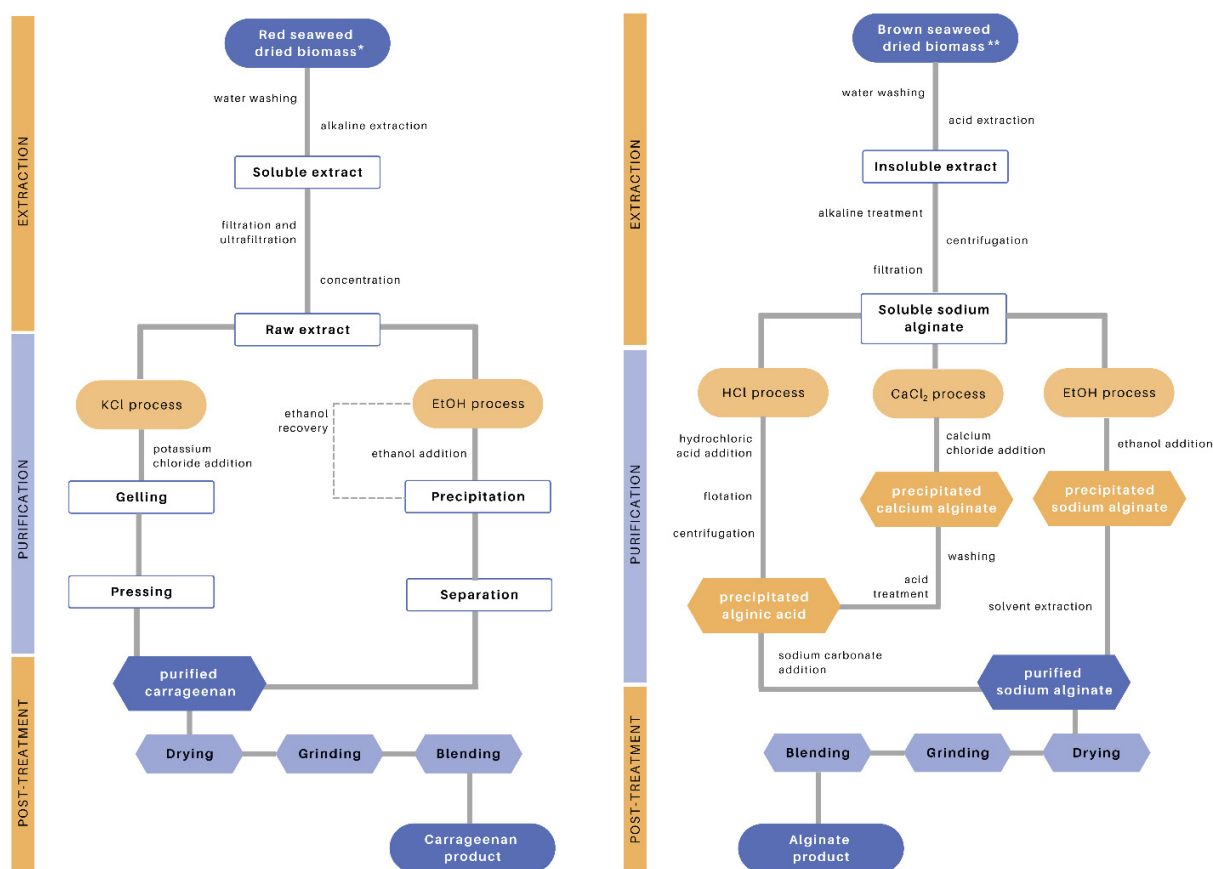


Fig. 22 - General extraction and purification technology applied on carrageenan and alginate manufacturing (adapted from Blakemore and Harpell, 2010; Peteiro, 2018).

ECONOMIC IMPACT AND COMMERCIAL IMPORTANCE

The global market of industrial marine biotechnology applications is expected to grow to US\$6.4 billion by 2025 (Hurst et al., 2016). This increase is mainly due to the potential use of polysaccharides in food, nutraceuticals, and pharmaceutical industries in Asian, Western, and European countries (Tanna and Mishra, 2019). Seaweed-derived polysaccharide production by the large-scale cultivation at open sea or using a biorefinery approach is dependent on the sustainable development of the process economy. According to the European Commission, concepts such as food security, guarantee sustainable use of resources, climate impact reduction, job creation, and competitiveness are key factors to strengthening the economy (Balina et al., 2017; Mathijs et al., 2015).

Like all natural product industries, large-scale seaweed market requires a level of standardization, efficacy, and traceability that can directly influence the scenario (Hafting et al., 2015). It is therefore not surprising the increase of scientific studies based on the seaweed-derived polysaccharide biological properties, improvement of quality, and cost of commodity-type products since traditional “in sea” cultivation presents the advantage of low-cost, and studies are expected to grow (Hafting et al., 2015; Hurst et al., 2016). Here we will briefly discuss important concepts such as species, characteristics, and biological properties of some established industrial seaweed-derived polysaccharides.

Carrageenans

Most commercial carrageenans are obtained by *Kappaphycus alvarezii*, *Eucheuma spinosum*, *Chondrus crispus*, *Gigartina stellata*, and other *Gigartina* spp. and *Chondrus* spp. (Chang et al., 2017; McHugh, 2003). This family of polysaccharides is constituted by sulfated galactans containing 15 to 40% of ester-sulfate and presenting molecular weights from 10^5 to 10^6 Da (Necas and Bartosikova, 2013; Venugopal, 2019). Carrageenans are constituted by alternative units of β -D-galactose and α -D-galactose (or 3,6-anhydro- α -D-galactose) joined by 1,3 and 1,4-glycosidic linkages (Usov, 2011). Moreover, it presents several biological activities as anti-tumor (Zhou et al., 2006), anti-viral (Carlucci et al., 1997, 2004), and immunomodulatory properties (Zhou, 2004), whereas applications in the non-food industry such as textile, pharmaceutical, and cosmetic sectors have also been reported (Balina et al., 2017). The use of carrageenan is approved by the Food and Drug Administration (FDA) in the United States (Borges Watson, 2008) and it is widely used in many sectors with different applications such as the addition in sausages (Ayadi et al., 2009) and application in drug delivery (Li et al., 2014). Figure 26.4 represents the disaccharide repeating units of the three main commercial carrageenans, kappa-, iota-, and lambda-carrageenans.

Agarans

Agarans are extracted and purified from seaweed species belonging to the Rhodophyceae (red algae), mainly *Gelidium sesquipedale*, *Hydropuntia cornea*, and *Gracilaria* species (Carmona et al., 1998; Martínez-Sanz et al., 2019; Pereira-Pacheco et

al., 2007; Rodríguez et al., 2009). Agarans are constituted by a disaccharide-based repeating unit $[\rightarrow 3)\text{-}\beta\text{-D-galactopyranose-(1}\rightarrow 4)\text{-}\alpha\text{-L-galactopyranose-(1}\rightarrow]$. The $\alpha\text{-L-Galp}$ units can be partially or totally substituted by 3,6-anhydro- $\alpha\text{-L-galactopyranose}$. This backbone can be partially substituted in different positions by variable degrees of one or more of the following substituents, sulfate, methyl, pyruvyl, and glycosyl groups. Agar and agarose are the two main types of industrially relevant agarans and are those presenting predominantly neutral structures (Figure 23).

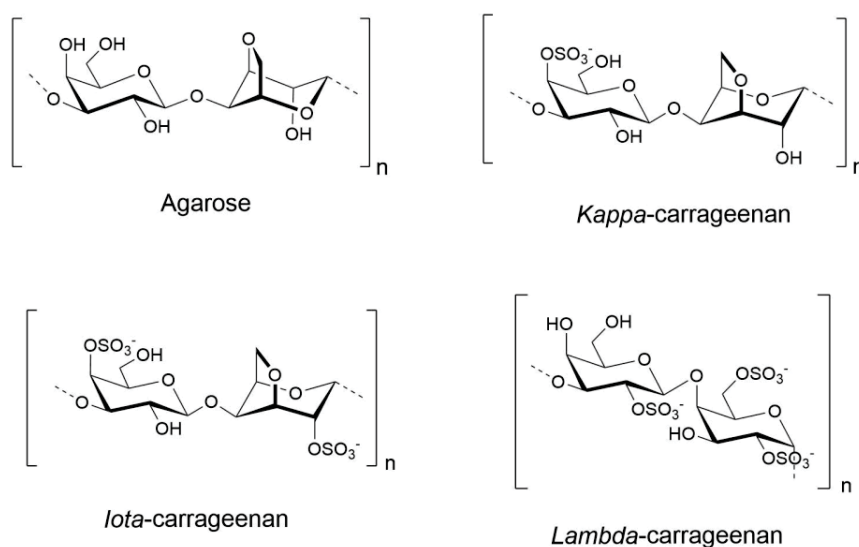


Fig. 23 - Red seaweed polysaccharides. Main industrial types of agarans and carrageenans (Usov, 2011).

Agar is considered an extremely important polysaccharide to the industrial sector due to its excellent thickening and gelling properties (Pereira-Pacheco et al., 2007). Alternative purposes, such as the development of biodegradable films and encapsulation structures, have been also reported (Alehosseini et al., 2018; Kanmani and Rhim, 2014).

Alginate

Alginate is a linear polyuronide constituted by 1,4-linked $\beta\text{-D-mannuronic acid}$ (M) and 1,4 $\alpha\text{-L-guluronic acid}$ (G) residues (Tanna and Mishra, 2019). The residues are distributed in blocks, G-block, M-block, and MG-block according to Figure 24.

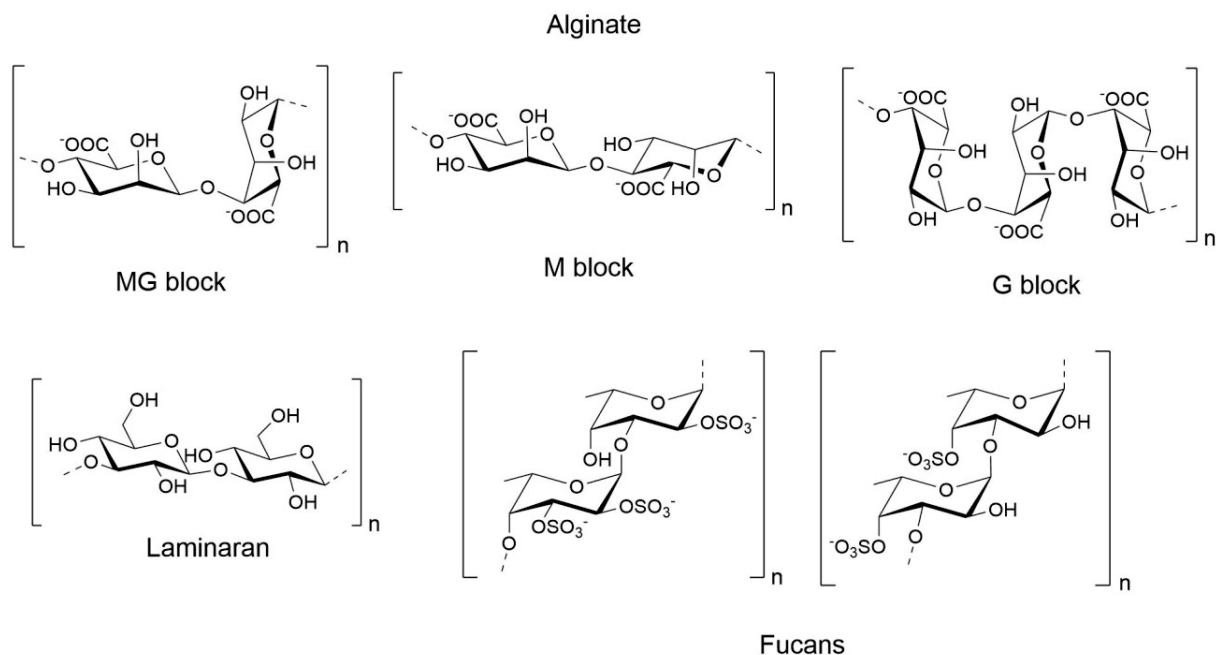


Fig. 24 - Main polysaccharides produced by brown seaweeds. Alginate, blocks distribution is species specific (Duarte et al., 1991); Laminaran, storage polysaccharide (Rinaudo, 2007); Fucans from: *Ascophyllum nodosum*/*Fucus vesiculosus* (left) and *Ecklonia kurome* (right) (Berteau and Mulloy, 2003).

The copolymer composition and block distribution are species-specific. The commercial application of alginate is mainly in the food and pharmaceutical industries. It can be used as an emulsifier, stabilizer, flavoring adjuvant, surfactant, viscosity increasing agent, tablet disintegrant, and diluent in capsule formulation (Colusse et al., 2021; Nause et al., 2009). Alginate is obtained from edible brown seaweed, particularly from *Cystoseira barbata* (Trica et al., 2019), *Macrocystis pyrifera*, *Laminaria hyperborea*, and *Ascophyllum nodosum* (Szekalska et al., 2016).

Laminaran

Laminaran is a β -glucan, with low molecular weight (approx. 20-30 glucosyl residues), constituted by β -1,3-linked-D-glucopyranose units (see Figure 26.5). This backbone can also present some interspersed β -1,6-linked units and β -1,6-linked glucose side chains (Rinaudo, 2007). Laminaran is the carbohydrate reserve of brown seaweeds

and can be obtained from species such as *Laminaria hyperborea*, *L. digitata*, *Ascophyllum nodosum*, and *Eisenia bicyclis* in average levels of 35% on a dry weight basis (Jönsson et al., 2020; Kadam et al., 2015; Maeda and Nisizawa, 1968; Read et al., 1996). Commercial applications of laminaran are limited, however, products with this polysaccharide claiming plant defense stimulant capacity, such as Vacciplant® are available in the agricultural market (Stadnik and Freitas, 2014; UPL, 2021). Several authors have studied and reported the disease resistance elicitor property of laminaran (Aziz et al., 2003; Tziros et al., 2021; Xin et al., 2019). Moreover, laminaran also presents the potential for functional foods, nutraceuticals, and cosmeceuticals (Jönsson et al., 2020), thus showing biological properties such as antioxidant, anti-inflammatory, and anticoagulant activities (Kadam et al., 2015).

Potential seaweeds-based biorefinery has been described based on the use of agar, cellulose, laminaran, and starch to be converted into bioethanol through the saccharification process (Balina et al., 2017). An important tool to be used to assess the commercial viability of seaweed aquaculture in a biorefinery approach is the value chain analysis (VCA) (Nor et al., 2019). This tool can identify the most valuable activities and improvements, help with financial accounts related to consumption, recycling, and disposal. Furthermore, the economic impact of those polysaccharides is strictly related to biological application and large-scale production. However, it has been suggested the use of smaller seaweed-derived polysaccharide facilities that may contribute to define molecules for pharmaceutical applications (Jönsson et al., 2020).

SEAWEED POLYSACCHARIDES: ADVANCED TECHNOLOGIES IN MASSIVE PRODUCTION

Several seaweed-derived polysaccharide industries around the world have been producing polysaccharides with advanced technology. Dobrinčić and colleagues (2020) reviewed and discussed some recently advanced extraction techniques that can be used for industrial purposes. The techniques are microwave-assisted extraction, ultrasound-assisted extraction, pressurized liquid extraction, and enzymatic assisted extraction. It is

worth mentioning that laboratorial parameters such as temperature, extraction time, power, and sample to solvent ratio should also be optimized to reach improvements on the results. In order to obtain a better understanding of industry and technology used to the seaweed-derived polysaccharide, Table 28 presents some companies around the world that produce and market polysaccharides obtained from seaweeds.

Table 28 - Global companies involved in production of seaweed-derived polysaccharides

Company	Polysaccharide	Seaweed species	Country	Reference
Gelymar	Carrageenan (Kappa II and Lambda carrageenan) and alginate	Red seaweeds and various species of brown seaweeds	Chile and Indonesia	gelymar.com
Iro Alginate Industry	Alginate	Brown seaweeds	China	iroalginate.com
MCPI Corporation	Carrageenan	Red seaweeds	Philippines	mcpicarrageenan.com
Elicityl	Alginate, fucoidan, galactan, ulvan, and xylan	<i>Laminaria japonica</i> , <i>Fucus vesiculosus</i> , <i>Undaria pinnatifida</i> , <i>Codium fragile</i> , <i>Ulva</i> sp., <i>Enteromorpha</i> sp., <i>Palmaria palmata</i>	France	elicityl-oligotech.com
Agargel	Agar and carrageenan	Red seaweeds	Brazil	agargel.com.br/en
Cargill	Carrageenan	Red seaweeds (Rhodophyceaea) from the Gigartinales group	United States	cargill.com
Gather Great Ocean Algae Industry Group	Alginate, carrageenan, and agar	Brown and red seaweeds	China	en.judayang.com
Algaia	Carrageenan and alginate	Red and brown seaweeds	France	algaia.com
Compañía Española de Algas Marinas (CEAMSA)	Carrageenan and alginate	Red and brown seaweeds	Spain	ceamsa.com

TBK: Manufacturing corporation	Carrageenan	<i>Kappaphycus alvarezii</i> * and <i>Eucheuma denticulatum</i> **	Philippines	tbk.com.ph
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* Formerly known as *Eucheuma cottonii*

** Formerly known as *Eucheuma spinosum*

Although there are several studies about seaweed-derived polysaccharides and their biological activities, existing bottlenecks need some attention due to their influence on the economics of the production process in the industrial sector. Issues related to this approach include:

- Novel extraction and purification methods are not optimized for a large-scale seaweed-derived polysaccharide facilities;
- Extraction yields are still low;
- Lack of information about ecological consequences of implementing large-scale offshore seaweed biorefineries;
- The potential of offshore seaweed farming is rare to estimate;
- Difficulty in controlling cultivation parameters;
- The complex structure of glycans;
- The necessity for new strain development by breeding tools;
- Vulnerability to novel diseases;
- The requirement of expensive techniques for polysaccharide characterization.

The use of biotechnological approaches can reinvent the production cycle and decrease the influence of bottlenecks of large-scale seaweed facility. Mathematical models can be applied in industrial processes to identify the potential productivity of the seaweed. Lehahn and colleagues (2016) integrated climatological oceanographic data associated with seaweed metabolism and growth rate to provide a global potential for offshore production of seaweed-derived biomass, proteins, platform chemicals, transportation fuels, and energy. This methodology could be used to calculate the production potential of an area and estimate the profit of the project.

Another feature with a dramatic impact on large-scale seaweed facilities is the possibility to create new strains that present light and thermal tolerance and resistant to disease (Kim et al., 2017). Seaweed breeding programs are standard in countries such as Korea, Japan, and China through a consecutive selection of individuals that present outstanding performance in the cultivation system (Hwang et al., 2019). According to the authors, breeding methodologies as the generation of mutant strains need a careful evaluation since they can disrupt the genetic structure of natural populations.

Moreover, advanced applications such as the production of nanoengineered injectable hydrogels in tissue regeneration therapy (Lokhande et al., 2018) or the use as the polymer matrix in oral extended-release tablets (Li et al., 2014) should increase the use of seaweed polysaccharides on a commercial scale. Although seaweed-derived molecules have been discussed in several studies, the current scenario invites a significant investment due to the necessity of new molecule sources. However, it might pay off with species with greater polysaccharide productivity, and molecules with high added value to be used in the pharmaceutical, medical, and food sectors.

CONCLUSIONS AND FUTURE PERSPECTIVES

Biological and environmental factors associated with the cultivation system influences seaweed productivity and polysaccharide biological activity. It is worth highlighting that, both in lab- and in pilot-scale seaweed farms, it is vital to evaluate growth parameters that affect production. Scientific efforts should be implanted in large-scale facilities to increase data reliability and reproducibility since environmental variables such as sunlight and temperature present variability during cultivation. Also, appropriate breeding strategy and optimization on cultivation systems is a valuable tool to increase production in the cultivation system.

It is important also to consider the role in new drug development to take into account the biological application diversity obtained from seaweed-derived polysaccharides, which include antiviral, antioxidant, anti-tumor, antidiarrheal, renoprotective effect, among other biological properties. It must be emphasized that studies related to seaweed-derived polysaccharides such as extraction procedures,

purification methods, and chemical modifications are critical since they can address new methodologies that can be applied in large-scale production to build a promising future for polysaccharides obtained from seaweeds.

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