UNIVERSITY OF SÃO PAULO
FACULTY OF PHARMACEUTICAL SCIENCES
Postgraduate Program in Biochemical-Pharmaceutical Technology
Fermentation Technology Area

Evaluation of *Dunaliella salina* growth and corresponding β-carotene production in tubular photobioreactor

Eleane de Almeida Cezare Gomes

Thesis to obtain the degree of Doctor

Advisor: Prof. Dr. João Carlos Monteiro de Carvalho
Co-advisor: Prof. Dr. Anil Kumar Singh

São Paulo
2018

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Corrected Version

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" A educação não transforma o mundo. Educação muda as pessoas. As pessoas transformam o mundo"

Paulo Freire

" Somos todos anjos de uma só asa, e só podemos voar quando nos abraçamos uns aos outros"

Fernando Pessoa

"Dizem que a vida é para quem sabe viver, mas ninguém nasce pronto. A vida é para quem é corajoso o suficiente para se arriscar e humilde o bastante para aprender"

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LIST OF FIGURES

CHAPTER I

Fig. 1. Percentage of total microalgae species in relation to different habitats. Data collected from the database of the website algaebase.org, dated on: 10/23/2017 (Business Communications Company, 2015) 22

Fig.2 Structures of some common carotenoids. Adapted from Chandi et al. (Chandi & Gill, 2011) 25

Fig.3 Possible pathway of carotenoids production in most green microalgae. The enzymes involved are phytoene synthase (PSY), phytoene desaturase (PDS), ζ-carotene desaturase, lycopene β-cyclase (LCY-B), β-carotene hydroxylase (BHY), lycopene ε-cyclase (LCY-E), ε-carotene hydroxylase (EHY), β-caroteneketolase (BKT). Adapted from: Eonseon et al. (Eonseon, Lee, & Polle, 2006) and Ye et al. (Ye, Jiang, & Wu, 2008) 26

Fig. 4 27
PART A: Carotenogenesis of *Haematococcus pluvialis* - A. Initial phase of astaxanthin acumulation and B. Red cells – carotenogenesis. Strain from of Culture Collection of Algae and Protozoa (CCAP) 34/8.
PART B: Carotenogenesis of *Dunaliella salina* - A. Green phase cells; B. Orange cells – carotenogenesis (β-carotene accumulation). Strain from of Culture Collection of Algae and Protozoa (CCAP) 19/18

CHAPTER II

Fig. 1 Chemical Structure of β-carotene. Adapted from Guedes et al. (Guedes et al., 2011) 46

Fig. 2 Representative chromatograms of β-carotene from the commercial β-carotene capsules (minor), reference standard β-carotene (50.0 µg.mL-1) and extracted from *D. salina* using modified DLLME method 53

Fig. 3 Response surface graphics for solvents utilized in the DoE experiments. A combined graphic utilizing polarity parameter was also plotted 54

Fig. 4 Chromatogram of β-carotene obtained from standard sample subjected to environmental conditions (day light, with air) 56

Fig. 5 Absorbance spectrum of all trans and cis β-carotene from *D. salina* sample in methanol:water (95:5 v/v) 57

CHAPTER III

Fig. 1 Schematic diagram of the photobioreactor in the green phase and of carotenogenesis 64

Fig. 2. Experiments carried out with 1N1P. A) Replacement of 20%. Experiment 1a: first cycle (10-13 days); second cycle (13- 19 days); third cycle (19 – 25 days), and carotenogenesis (25 – 28 days). Experiment 1b: first cycle (10-13 days); second cycle (13- 19 days); third cycle (19 – 25 days), and carotenogenesis (25 – 28 days). B) Replacement of 80%. Experiment 2a: first cycle (10-16 days); second cycle (16- 26 days); third cycle (26 – 38 days), and carotenogenesis (38 – 41 days). Experiment 2b: first cycle (10-23 days); second cycle (23- 32 days); third cycle (32 – 46 days), and carotenogenesis (46 – 49 days). 70

Fig. 3. Experiments carried out with 1.5N1.5P. A) Replacement of 20%. Experiment 3a: first cycle (9-12 days); second cycle (12- 15 days); third cycle (15 – 18 days), and carotenogenesis (18 – 21 days). Experiment 3b: first cycle (9-12 days); second cycle (12- 15 days); third cycle (15 – 18 days), and carotenogenesis (18 – 21 days). B) Replacement of 80%. Experiment 4a: first cycle (11-21 days); second cycle (21- 32 days); third cycle (32 – 43 days), and carotenogenesis (43 – 46 days). Experiment 4b: first cycle (11-21 days); second cycle (21- 32 days); third cycle (32 – 43 days), and carotenogenesis (43 – 46 days). 71

Fig. 4. Experiments carried out with 3N3P, where 3:3 corresponds to 225 mg L^{-1} of $NaNO_3$ and 16.95 mg L^{-1} of $NaH_2PO_4.2H_2O$ 75

Fig. 5. Figure 5. Experiments carried out with replacement of 20% (A), and replacement of 80% (B) 76

Fig. 6. *D. salina* green cells (CCAP 19/18) in 1N1P condition with 80% fresh medium replenishment (cells from 42nd day, after 10 days of the third cycle) 77

LIST OF TABLES

CHAPTER I

Table 1 Carotenoids from microalgae 29

Table 2 Lutein concentrations in some products 31

CHAPTER II

Table 1 - The design of experiment (DoE) layout for each solvent 50

Table 2. The results obtained in the assessment of precision and adequateness of proposed method 55

CHAPTER III

Table 1 Cell concentration of *Dunaliella salina*, and β-carotene content in carotenogenesis 69

Table 2 Results of semi-continuous cultivation of *Dunaliella salina* 73

CEZARE-GOMES, E.A. Avaliação do crescimento da *Dunaliella salina* e correspondente produção de β-caroteno em fotobiorreator tubular. 2018. 108 p. Tese (Doutorado) – Faculdade de Ciências Farmacêuticas, Universidade de São Paulo, São Paulo, 2018.

RESUMO

As microalgas, micro-organismos fotossintetizantes, são ricas em lipídios, ácidos graxos poli-insaturados, carboidratos, proteínas, vitaminas, além de carotenoides que são antioxidantes com potencial de proteger o organismo humano de várias doenças incluindo a obesidade, doenças cardiovasculares, doenças relacionadas à visão como a degeneração macular e certos tipos de câncer, entre outras. Esses pigmentos naturais têm aplicações em indústrias farmacêuticas (nutracêuticos), alimentícias (colorantes, alimentos funcionais e suplementos) e de cosméticos (exemplo: filtro solar) e na aquacultura (ração animal). A microalga *Dunaliella salina* é capaz de sintetizar, sob alta intensidade luminosa e limitação de nutrientes como fontes de fósforo e nitrogênio, dentre outras condições de estresse, 10 % do peso seco em β-caroteno (pigmento laranja com atividade pró-vitamina A). Assim, neste trabalho, numa primeira etapa, foi feita uma revisão da literatura abordando a produção de carotenoides por microalgas, bem como sua aplicação. Nesse levantamento bibliográfico abordou-se, dentre outros assuntos, as vantagens do cultivo de microalgas em relação as fontes tradicionais (plantas superiores), assim como uma discussão dos diferentes sistemas de cultivos e sua importância no crescimento celular. Esse review apresentou uma análise crítica dos principais regimes operacionais como batch, fed-batch, semicontínuo e contínuo. Apresentou-se também informações relevantes sobre os mais importantes produtores mundiais de carotenoides de microalgas. Numa segunda etapa, foi desenvolvido um método modificado de microextração líquido-líquido dispersivo modificado (DLLME) para a rápida extração de β-caroteno de *Dunaliella salina* cultivada em fotobiorreatores tubulares, com subsequente desenvolvimento de método cromatográfico em uma coluna C4 para a separação do isômero geométrico de β-caroteno. A extração ótima de β-caroteno foi obtida com benzeno como solvente extrator e água com 50% de acetona como dispersante. Empregando uma fase móvel composta por metanol e água (95:5, v/v) em HPLC, foi possível a detecção/quantificação de β-caroteno com 14 minutos de tempo de retenção. Além dos tempos curtos de análises (<20 min), pela extração em volume reduzido (< 10 mL resíduos orgânicos) este método obedece aos princípios da química verde.

Sabe-se que nitrogênio, fósforo, assim como carbono e vitaminas são elementos vitais para o crescimento das microalgas e também exercem influência na composição bioquímica da biomassa. Assim, na terceira etapa deste trabalho, estudou-se a influência das quantidades de

nitrato de sódio (75 mg L^{-1}, denominado 1N; 112,5 mg L^{-1}, denominado 1,5N; 225 mg L^{-1}, denominado 3N) e de fosfato monobásico dihidratado (5,65 mg L^{-1}, denominado 1P; 8,47 mg L^{-1}, denominado 1,5P; 16,95 mg L^{-1}, denominado 3P) em meio f/2, que tem como base a água do mar, no crescimento e na síntese de β-caroteno da *Dunaliella salina* por processo semicontínuo, com uso de frações de corte (R) de 20% e 80%. Foram obtidas produtividades celulares mais elevadas em processos semicontínuos do que em processo descontínuo, com produtividades médias de até 6,7 x 10^4 células mL^{-1} d^{-1} (meio 1N:1P; R =20%). A máxima concentração celular (Xm) obtida neste trabalho não foi dependente de R. Os melhores resultados de Xm foram obtidos quando se usou meio 1,5N:1,5P em vez de meio, com 1N:1P, com valores médios de até 5,6 x 10^5 células mL^{-1} (R =80%). O conteúdo de β-caroteno nas células, de maneira geral, foi maior nas células cultivadas em meio 1N:1P do que no meio 1,5N:1,5P, com valores até 57,5 mg g^{-1} (R =80%). O cultivo de *D. salina* com o meio 3N:3P levou a uma longa fase lag, seguida por uma diminuição na concentração celular e sua lise. O cultivo de células em um fotobiorreator tubular contribuiu para um crescimento celular sem contaminação por protozoários. O cultivo de *Dunaliella salina* em fotobiorreator tubular com o uso de fotoperíodo 12:12 foi apropriado, assim como induzir a carotenogênese, no segundo estágio, por meio do aumento da intensidade luminosa e ausência de controle de pH.

Palavras-chaves: *Dunaliella salina*, fotobiorreator tubular, cultivo semicontínuo, β- caroteno, carotenoides, microextração líquido-líquido dispersiva*;* cromatografia

CEZARE-GOMES, E.A. Evaluation of *Dunaliella salina* growth and corresponding β-carotene production in tubular photobioreactor. 2018. 108 p. Thesis (Ph.D.) – Faculty of Pharmaceutical Sciences, University of São Paulo, 2018.

ABSTRACT

Microalgae, photosynthetic microorganisms, are rich in lipids, polyunsaturated fatty acids, carbohydrates, proteins, vitamins, as well as carotenoids, which are antioxidants that may protect human body from various diseases including obesity, cardiovascular disease, vision-related diseases such as macular degeneration and certain types of cancer. These natural pigments have applications in the pharmaceutical (nutraceutical), food (coloring, functional food, and supplements), and cosmetics industries (e.g. sunscreen), as well as in aquaculture (animal feed). The *Dunaliella salina* microalga can synthesize 10% of dry weight in β-carotene (orange pigment, pro-vitamin A activity) under high light intensity and nitrogen and phosphorus limitation, among other stress conditions. The first chapter of this thesis presents a review focused on microalgae carotenoids: culture systems, mode of operation, and applications. In this bibliographic survey, the advantages of microalgae cultivation in relation to traditional sources (higher plants) were discussed, as well as a discussion of the main cultivation systems and their importance in cell growth. This review presented a critical analysis of the different operational regimes like batch, fed-batch, semi-continuous and continuous. Relevant information on the most important world producers of microalgae carotenoids were presented. Chapter II presents the development of a modified method of dispersive liquid-liquid microextraction (DLLME) for rapid extraction of β-carotene from *Dunaliella salina* cultivated in tubular photobioreactor, with subsequent development of a rapid chromatographic screening method using a C4 column for separation of geometric isomer of β-carotene. The use of benzene as extraction solvent and water with 50% acetone as dispersant provided the best condition for the extraction of this carotenoid. In HPLC (High Performance Liquid Chromatography), employing mobile phase composed of methanol and water (95:5, v/v), it was possible to detect/quantify β-carotene at 14 min (retention time). Besides the short analysis time (<20 min), by the miniaturized extraction (< 10 mL organic waste) this method abide by green chemistry analytical principles.

It is known that nitrogen, phosphorus, as well as carbon and vitamins are vital elements for the growth of microalgae, also determining the biochemical composition of biomass. In this sense, Chapter III presents the study of the influence of different amounts of sodium nitrate (1N = 75

mg L^{-1}; 1.5N = 112.5 mg L^{-1}, and 3N = 225 mg L^{-1}) and phosphate monobasic dehydrate (1P = 5.65 mg L^{-1}, 1.5P = 8.47 mg L^{-1}, and 3P = 16.95 mg L^{-1}) in seawater-based f/2 medium on the growth of *Dunaliella salina* and β-carotene biosynthesis, by continuous process with different replenishment proportions (R = 20% and 80%). Best results of cell productivity were obtained by semicontinuous process (mean values of Px up to 6.7 x 10^4 cells mL^{-1} d^{-1} with medium 1N:1P; R =20%) in comparison with batch process cultivation. Maximum cell density (Xm) obtained in this work was not dependent of R, but the best results were obtained when using medium 1.5N:1.5P (mean values up to 5.6 x 10^5 cells mL^{-1} with R =80%) instead of 1N:1P. The content of β-carotene in the cells, in general, was higher in cells grown in medium 1N:1P (mean yield values up to 57.5 mg g^{-1} with R =80%) in comparison with medium 1.5N:1.5P. The cultivation of *D. salina* with media 3N:3P led to a long lag phase, followed by decrease in cell density and cell lysis. The use of a tubular photobioreactor contributed to successfully cultivate this microalga without contamination by protozoa. The cultivation of *Dunaliella salina* in tubular photobioreactor with the use of 12:12 photoperiod was appropriate, as well as to induce carotenogenesis, in the second stage, by increasing the light intensity and absence of pH control.

Keywords: *Dunaliella salina*, tubular photobioreator, semi-continuous cultivation, β-carotene, carotenoids; dispersive liquid-liquid microextraction; chromatographic analysis

CONTENTS

GENERAL INTRODUCTION 16
GENERAL AND SPECIFIC OBJECTIVES 17

CHAPTER I - Review - Potential of Microalgae Carotenoids for Industrial Application 18
1. Introduction 19
2. Microalgae 20
2.1.Importance of microalgae in terrestrial ecosystem 21
3. Carotenoids 22
3.1. Applications of carotenoids 24
3.2. The Global Market for carotenoids 24
3.3. Traditional sources 28
3.4. Carotenoids from microalgae 32
3.4.1. β-carotene from *Dunaliella salina* 32
3.4.2. Astaxanthin from *Haematococcus pluvialis* 33
3.4.3. Carotenoids from *Chlorella* sp. 34
3.4.3.1. Lutein from *Chlorella protothecoides* 34
3.4.3.2. Canthaxanthin from *Chlorella zofingiensis* 34
3.4.3.3. Zeaxanthin from *Chlorella ellipsoidea* 36
3.4.4. Other carotenoids 37
3.4.4.1. Lycopene 37
3.4.4.2. Echinenone 37
4. Microalgal culture systems 37
4.1. Open system 38
4.2. Closed systems 38
5. Operation regime 40
6. Conclusions 44

CHAPTER II - Miniaturized Extraction and Rapid analysis of β-carotene in cultivated *Dunaliella salina* 45
1. Introduction 46

2. Experimental 47
2.1.Chemicals and Reagents 47
2.1.1. Reference Substance 47
2.1.2. Cultivation of *Dunaliella salina* in tubular photobioreactor 47
2.1.3. Gelatinized capsules 48
2.1.4. Solvents and reagents 48
2.2. Instrumentation and analytical conditions 49
2.3. Preparation of standard solution 49
2.4. Design of experiment (DoE) applied to extraction of β-carotene from *D. salina* 49
2.4.1. Optimization of modified DLLME procedure 50
2.5. Optimum conditions for extraction of β-carotene from *D. salina* samples 50
2.6. Extraction of β-carotene from commercial capsules 51
2.7. Validated chromatographic method applied to analysis of β-carotene 51
2.8. Stability of β-carotene solutions 52
3. Results 52
3.1. System suitability 52
3.2. Modified DLLME applied to β-carotene extraction 52
3.3. Chromatographic method validation parameters 53
3.3.1. Specificity of proposed method 53
3.3.2. Precision of proposed method 54
3.3.3. Accuracy of proposed method 54
3.3.4. Linearity of proposed method 55
3.3.5. Stability of β-carotene 55
4. Discussion and conclusions 56

CHAPTER III - Influence of nutrients in the β-carotene production on semi-continuous culture of *Dunaliella salina* in Tubular Photobioreactor 59
1. Introduction 60
2. Materials and methods 61
2.1. Microalga strain, cell maintenance and inoculum production 61

2.2. Experimental 62

2.2.1. Tubular photobioreactor and growth conditions 62

2.2.2. Semi-continuous mode of photobioreactor operation 63

2.2.3. Carotenogenesis 63

2.2.4. Determination of cell density 65

2.2.5. Analysis in cell-free supernatant 65

2.2.5.1. Residual Nitrate concentration 65

2.2.5.2. Residual Phosphorus concentration 65

2.2.6. Determination of β – carotene concentration in wet biomass 66

2.3. Kinetic Parameter 66

2.4 Statistical analysis 67

3. Results and discussion 67

FINAL CONCLUSION 79

REFERENCES 80

APPENDIX A – Nitrate Calibration Curve 106

APPENDIX B – Phosphorus Calibration Curve 107

ATTACHMENT A – Statement for exemption of Ethics Committee approval 108

GENERAL INTRODUCTION

Microalgae are promising sources of carotenoids, hydrocarbons divided into two groups: carotenes containing only carbon and hydrogen (e.g., β-carotene and lycopene) and xanthophylls containing carbon, hydrogen, and oxygen (e.g., lutein, zeaxanthin and astaxanthin). These natural pigments have applications in the food, pharmaceutical, and cosmetics industries, besides in aquaculture. In the medical field can combat diseases such as obesity, cardiovascular disease, and eye diseases (e.g., age-related macular degeneration). It is a strong and growing market and countries like Australia, USA, and Japan are examples of producer countries. The green microalga *Dunaliella salina* together with other microalgae such as *Haematococcus pluvialis* and *Chlorella protothecoides* are important producers of β-carotene (pro-vitamin A), astaxanthin (powerful antioxidant), and lutein (eye health) respectively. *Dunaliella salina* can accumulate 10% dry weight of this orange pigment under stress conditions, such as high light intensity and nitrogen and phosphorus limitation. These nutrients are essential for the cell growth and may affect carotenoid accumulation. The cultivation of these microorganisms can be done in open system (raceway for example) or in closed system, that allows an optimization of parameters such as pH, light intensity, and temperature, providing higher biomass yields. Many types of closed system for microalgae cultivation have been developed such as flat plate, column, cylindrical, and tubular. The model tubular is the most suitable type of growing cells in outdoor environments, providing good biomass productivities and, their construction is relatively cheap. Also, the cultivations can be performed by different operation regimes, such as semi-continuous, with advantage of operating the bioreactor for long periods without the need to prepare a new inoculum, and the productivity may be greater in comparison with batch process. Given this context, the present thesis was divided into chapters written in the form of articles. Chapter I addresses a review on carotenoids produced by microalgae, including details like recent global market, comparisons between traditional carotenoid sources and microalgae, applications, and a critical evaluation of the best microalgae systems to feasibly produce carotenoids in commercial scale. In Chapter II a methodology was developed for rapid extraction of β-carotene from *Duanaliella salina*. Chapter III deals with the semi-continuous cultivation of *D. salina* for the production of β-carotene, evaluating different replenishments proportion of medium and nitrogen and phosphorus concentrations.

GENERAL AND SPECIFIC OBJECTIVES

The main objective of this thesis is the evaluation of influence of sodium nitrate and monobasic phosphate amounts on the growth of *Dunaliella salina* and β-carotene biosynthesis by semi-continuous process.

In this sense, the following specific objectives were established:

1. Review the potential of microalgae carotenoids for industrial application, which include the production of β-carotene by *Dunaliella salina;*

2. To develop a method to extract and analyze β-carotene from *Dunaliella salina* by HPLC (High performance liquid chromatography);

3. To study the growth of *D. salina* in tubular photobiorreactor and β-carotene production in semi-continuous system with different replenishments proportion of the culture medium (20 and 80%) using different concentrations of sodium nitrate (75 mg L^{-1} and 112.5 mg L^{-1}) and monobasic phosphate (5.65 mg L^{-1} and 8.5 mg L^{-1}) in the medium;

CHAPTER I[1]

Review
Potential of Microalgae Carotenoids for Industrial Application

Eleane A Cezare-Gomes[a], Lauris del Carmen Mejia-da-Silva[a], Lina S Pérez-Mora[a], Marcelo C Matsudo[b], Lívia S Ferreira-Camargo[c], Anil Kumar Singh[d], João Carlos Monteiro Carvalho[a*]

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ABSTRACT

Microalgae cultivation, when compared to the growth of higher plants, presents many advantages such as faster growth, higher biomass productivity, and lesser requirement of land area for cultivation. For this reason, microalgae are an alternative platform for carotenoids production when compared to the traditional sources. Currently, commercial microalgae production is not well developed but, fortunately, there are several studies aiming to make the large-scale production feasible by, for example, employing different cultivation systems. This review focus on the main carotenoids from microalgae, comparing them to the traditional sources, as well as a critical analysis about different microalgae cultivation regimes that are currently available and applicable for carotenoids accumulation. Throughout this review paper, we present relevant information about the main commercial microalgae carotenoids producers; comparison between carotenoids content from food, vegetables, fruits, and microalgae; and the great importance and impact of these molecules applications, such as in food (nutraceuticals and functional foods), cosmetics and pharmaceutical industries, feed (colorants and additives), and in healthcare area. Lastly, the different operating systems applied to these photosynthetic cultivations are critically discussed, as well as conclusions and perspectives are made concerning the best operating system for achieving high cell densities and, consequently, high carotenoids accumulation.

Keywords: carotenoids; carotenoids sources; microalgae; microalgae application; cultivation process

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1. Introduction

Microalgae are photosynthetic microorganisms that convert carbon dioxide CO_2 into bioactive compounds as pigments, lipids, polyunsaturated fatty acids, carbohydrates, proteins, and vitamins (Walker, Purton, Becker, & Collet, 2005; L. Xu, Weathers, Xiong, & Liu, 2009). They present some advantages over higher plants, as faster growth, easier harvesting, lower water consumption and high CO_2 fixation and O_2 production (Gouveia & Oliveira, 2009) which results in an important role for the terrestrial ecosystem balance (Gupta, Lee, & Choi, 2015). Microalgae can also be considered as the main sustainable source of pigments, phycobilin, eicosapentaenoic acid (EPA), and docosahexaenoic acid (DHA). The natural pigments from microalgae may present several interesting properties, such as antivirals, antimicrobials, anti-inflammatory, anti-oxidant, pro-vitamin A, immunomodulatory activity, and anticancer (Chiu et al., 2017; Fimbres-Olivarria et al., 2017), and many species are considered GRAS (Generally Recognized as Safe – www.fda.gov), according to the US Food and Drug Administration (Gantar & Svirčev, 2008; Rosenberg, Oyler, Wilkinson, & Betenbaugh, 2008). Natural pigments such as carotenes (β-carotene and lycopene), and the xanthophylls (astaxanthin, canthaxanthin, lutein and zeaxanthin) have applications in nutraceutical, cosmetics and pharmaceutical industries, including food, feed and in healthcare area (Berman et al., 2015). For obtaining these bio-products, cultivation of microalgae may take place in open ponds, raceway ponds (most used), or closed-system. Raceway ponds construction and maintenance are cheaper, but this photobioreactor configuration carries some disadvantages like uneven light distribution and the high risk of contamination by other microalgae and bacteria (Singh & Sharma, 2012). On the other hand, the closed system is efficient for light-harvesting, the water loss and risk of contamination are lower, and biomass density is higher than in open ponds, among other advantages (Del Campo, García-González, & Guerrero, 2007). Flat and tubular bioreactors are the most commonly used closed systems for microalgae production. The choice of photobioreactor configuration also depends on the product of interest and local environmental conditions. The adopted operation regime for photosynthetic microorganisms production can be batch, which is the most used one; fed-batch, which is widely used for heterotrophic microalgae cultivation; and alternative modes as the semi-continuous or continuous processes (J.C.M. Carvalho, Bezerra, Matsudo, & Sato, 2013; Fernandes, Mota, Teixeira, & Vicente, 2015; Morales-Sánchez, Martinez-Rodriguez, & Martinez, 2017; Moreno-garcia, Adjallé, Barnabé, & Raghavan, 2017). Many foods, fruits and vegetables contain carotenoids, such as β-carotene, α-carotene, β-cryptoxanthin, lycopene, lutein and zeaxanthin,

which are considered important in human health, associated with eye healthcare, for instance. Some vegetables and fruits are rich in these natural pigments. For example, the spinach *Spinacia oleracea* contains 775.8 μg/g dry weight of lutein (Saini, Nile, & Park, 2015), one of the main natural pigments, while the microalga *Chlorella protothecoides* produces a biomass containing seven times more lutein (5.35 mg/g) (Shi, Jiang, & Chen, 2002). Moreover, some microalgae can also contain astaxanthin, a powerful antioxidant (higher than β-carotene and vitamin E) such as microalga *Haematococcus pluvialis*, between 1.5 % - 3.0% of the biomass dry weight, amount is much greater than krill oil (about 0.15%) for example (Lorenz & Cysewski, 2000). Thus, it is possible to assert that the pigment accumulation in microalgal biomass is an advantage over the higher plants (Gong & Bassi, 2016). Indeed, many companies around the world (Australia, EUA, Israel, Japan, China, India, and more) produce carotenoids from microalgae, and this is a growing market (3.9% per year) (Business Communications Company, 2015).

In this review, we considered general aspects related to the production of microalgae as alternative and sustainable source for obtaining carotenoids. In this regard, not only health benefits provided by these biomolecules but also economical aspects and microorganism characteristics (including pigments content) are pointed out. Lastly, detailed discussion about different culture systems and operation regime is presented.

2. Microalgae

Microalgae are eukaryotes and photoautotrophic unicellular organisms, with simple structure, filamentous or colonial, requiring for their growth and reproduction: light, water, carbon dioxide and inorganic nutrients. Photosynthetic microorganisms exhibit commercial interest because they are efficient at converting carbon dioxide in biomass from where natural bioactive compounds (L. Xu et al., 2009) such as pigments, lipids, polyunsaturated fatty acids, carbohydrates, proteins, and vitamins can be extracted (Gong & Bassi, 2016). They present advantages over higher plants (soybean, corn, and jatropha), which are traditional sources of these compounds, such as rapid growth or short cultivation time, high biomass productivity, high CO_2 fixation and O_2 production, and low water consumption. Also, considering that microalgae are cultivated in suspension, which is easy to handle, the production may be performed in large climatic variations, on non-fertile soil (e.g., desert and seacoast) and in non-potable water or even in wastewater. Finally, the production is not seasonal and the culture can be daily harvested (Gong & Bassi, 2016; Gouveia & Oliveira, 2009). Many species are

considered GRAS (Generally Recognized as Safe – www.fda.gov), status to the US Food and Drug Administration (Gantar & Svirčev, 2008). Regarding food safety, *Haematococcus pluvialis* has been approved in several European countries for human consumption as a dietary-supplement ingredient (Lorenz & Cysewski, 2000). In addition, *Dunaliella* sp. and *Chlorella* sp. that do not produce toxic substances are classified as safe food, and they are some of the main species for human and animal consumption (Walker et al., 2005). *Chlamydomonas reinhardtii* has also been acknowledged as GRAS for production of therapeutic proteins (Rasala & Mayfield, 2015). Therefore, microalgae can be applied in nutritional and biomedical areas, and are associated with therapeutic properties such as antioxidants, anti-inflammatories, anticancers, anti-obesity (Gammone & D'Orazio, 2015; Peng, Yuan, Wu, & Wang, 2011), immunostimulants, and antivirals (Yaakob, Ali, Zainal, Mohamad, & Takriff, 2014).

2.1. Importance of microalgae in terrestrial ecosystem

Microalgae, as well as cyanobacteria, are the first constituents of terrestrial microflora, and have a fundamental importance to the environment's primary production, decomposition, nutrient cycling, and energy (McCann & Cullimore, 1979). They are responsible for about half of the earth's oxygen production, fixing 100 million tons of carbon dioxide per day. They are so-called "biological pump", since it is estimated that 1 kg of dry algal biomass utilizes about 1.83 kg of CO_2 (Chisti, 2007). Even with all the benefits, since 1950 the amount of phytoplankton on the planet has decreased to 40% of total biomass, due to anthropogenic activities, which has been resulted in the global climate change (Boyce, Lewis, & Worm, 2010). Microalgae are mainly found in aquatic environments, 49.78% in freshwater and 44.48% in seawater (Fig. 1), and they can be adaptable to contaminated and extreme environments (García-Balboa et al., 2013) such as thermal and glacial lakes, and in wastewater. Species of the genera *Galdieria* and *Cyanidium* (unicellular red algae), for example, grow in waters with temperatures between 45° and 57 °C (Brock, Science, Series, & Feb, 2014).

Some microalgae are able to reproduce on the glacial surface of Antarctica, even in low light conditions and low temperatures (from -1.8 °C to 6 °C); they often tend to accumulate large amounts of intracellular lipids (Sakshaug & Slagstad, 1991). Microalgae (e.g. *Chlorella* sp., *Chlamydomonas* sp., among others) were found in the High Arctic, where the soil crusts are considered to be one of the most extreme habitat types on earth. They are responsible for fixing

atmospheric nitrogen, reducing erosion and water runoff, while secreting polysaccharides (Pushkareva, Johansen, & Elster, 2016).

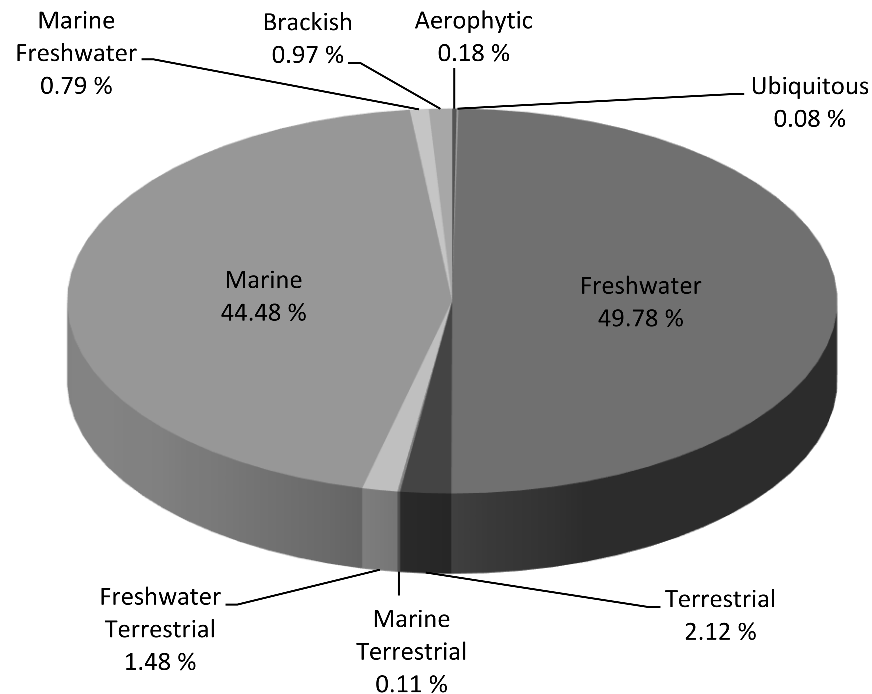


Fig. 1. Percentage of total microalgae species in relation to different habitats. Data collected from the database of the website algaebase.org, dated on: 10/23/2017 (Business Communications Company, 2015).

Other species may grow in extremely polluted lagoons of wastewater with high concentrations of metals, such as uranium, and low pH (1.7 - 2.5), as in the case of the microalgae found in Tinto River, located in Spain. Microalgae are also able to adapt to environments containing antibiotics, herbicides, xenobiotics, toxic spills and even in volcanic effluents (García-Balboa et al., 2013).

3. Carotenoids

Carotenoids are terpenoid pigments derived from tetraterpenes (C_{40}). They are responsible for the yellow, orange and red colorations of fruits, flowers, and vegetables. In addition, they provide the coloration to animals like flamingos and some aquatic organisms, as crustaceans' shells, and fish skin as in salmon (Negro & Garrido-Fernández, 2000). These hydrocarbons are

divided into two groups: first, carotenes, which present only carbon and hydrogen atoms (e.g., α-carotene, β-carotene, γ-carotene, phytoene and lycopene), and a second group, xanthophylls, which present carbon, hydrogen and oxygen on their molecule structure. Oxygen may be present in the form of hydroxyl (e.g., lutein and zeaxanthin), as oxy group (e.g., canthaxanthin and echinenone) or a combination of both in the case of astaxanthin and violaxanthin. The xanthophylls, lutein and zeaxanthin are structurally similar, and they are important for eye healthcare, helping to prevent age-related macular degeneration (Fig.2) (Bhosale & Bernstein, 2005). Only plants, algae, and some species of fungi and bacteria synthesize carotenoids that must be provided in the diet of animals, since they are important substances on vertebrates' health and development. More than 600 carotenoids have been identified (Negro & Garrido-Fernández, 2000). Commercially, the most important carotenoids are astaxanthin, canthaxanthin, lutein, zeaxanthin, β-carotene and lycopene (Berman et al., 2015). The steps for carotenoid formation begin within the chloroplast, with GGPP (geranylgeranyl pyrophosphate). An example of chemical pathway for carotenoid formation is shown in figure 3. It can be observed that, for example, β-carotene is converted to zeaxanthin via β-cryptoxanthin by enzyme β-carotene hydroxylase (BHY).

Carotenoids can be stored inside or outside the chloroplast. The primary pigments are important for the photosynthesis, and they are located in the chloroplast, the secondary pigments are located outside the chloroplast, and are not essential for the photosynthetic mechanism. There is a hypothesis that secondary carotenoids are transported from the chloroplast to the lipid vesicles, playing a role on the communication between the two biosynthesis that happens in the chloroplast and in the cytosol (Grünewald, Eckert, Hirschberg, & Hagen, 2000). However, Collins and collaborators (Collins et al., 2011) reported that the chlorophyll, in the microalgae *H. pluvialis*, was found only in the chloroplast while the secondary carotenoid astaxanthin was located in the cytoplasm. Considering that β-carotene is a precursor of astaxanthin and that β-carotene was found in the chloroplast and in the cytosol, it is possible to say that the synthesis of astaxanthin occurs outside the chloroplast. Some green microalgae, such as *Chlorella zofingiensis, Dunaliella salina*, and *Haematococcus pluvialis,* accumulate secondary carotenoids in lipid bodies under stressful environments, such as light excess, increased salinity, low or high temperature, UV-B irradiation, and nutritional stress (e.g., nitrogen deficiency). These extreme and harsh conditions makes the cell protect itself by storing energy and carbon. The microalga *Haematococcus pluvialis* accumulates secondary keto-carotenoids (canthaxanthin and astaxanthin) in vesicles in the cytosol, when it is subjected to stress. In this condition, cell morphological modifications may occur, e.g., the flagellated green cell may turn

into large red cysts (Eonseon, Lee, & Polle, 2006; Lemoine & Schoefs, 2010). *Haematococcus pluvialis* and *Dunaliella salina'* carotenogenesis can be observed in figures 4A and 4B, respectively. For example, the orange *Dunaliella salina* cells are significantly larger than the green *D. salina* cells, in this figure, about 30.51%.

3.1. Applications of Carotenoids

Many microalgae species have been identified, between 40,000 to 70,000 from nine different phyla (Ghosh et al., 2016). However, researchers believe that about 10,000 microalgae species have not been identified yet (Rosello Sastre & Posten, 2010). Only a few of them are commercially exploited, such as *Chlorella* sp. as a protein source for human and animal consumption, or *Dunaliella salina* and *Haematococcus pluvialis* for the extraction of β-carotene and astaxanthin, respectively. The interest in algal biotechnology has increased in recent years. These microorganisms produce biomolecules with several applications in many areas: pharmaceutical, cosmetic, and food industries (nutraceuticals and functional foods), healthcare sector, aquaculture, agriculture (bio-fertilizers), wastewater treatment, as well as an alternative source of fatty acids for biofuel production (Borowitzka, 1992, 2013).

3.2. The Global Market for carotenoids

The production of pigments is mostly performed by chemical synthesis, although consumers prefer pigments of biological origin. In 2014, carotenoids industry produced US$ 1.5 billion globally, and about US$ 1.8 billion are expected for 2019 (Business Communications Company, 2015), including all sources of carotenoids (synthetic and natural). All carotenoids (β-carotene, lutein, and astaxanthin) market share was about 60% in 2010. From this market, β-carotene production stands out, representing approximately US$ 270 million, of which US$ 68 million are natural β-carotene. The average market price is close to US$ 300 – 1,500 kg^{-1}. Astaxanthin is also an important carotenoid from microalgae. About US$ 230 million was produced in 2010 with an average market price of US$ 2,500 kg^{-1} (value of 2004) (Borowitzka, 2013). In 2014, this value fell to about US$ 1,900 kg^{-1} (Yaakob et al., 2014). More than 95% of the commercially available astaxanthin has a synthetic origin, due to the low cost (US$ 1,000 kg^{-1}), whereas natural astaxanthin, from algae, represents only 1% of the market.

CHEMICAL STRUCTURE OF SOME MICROBIAL CAROTENOIDS

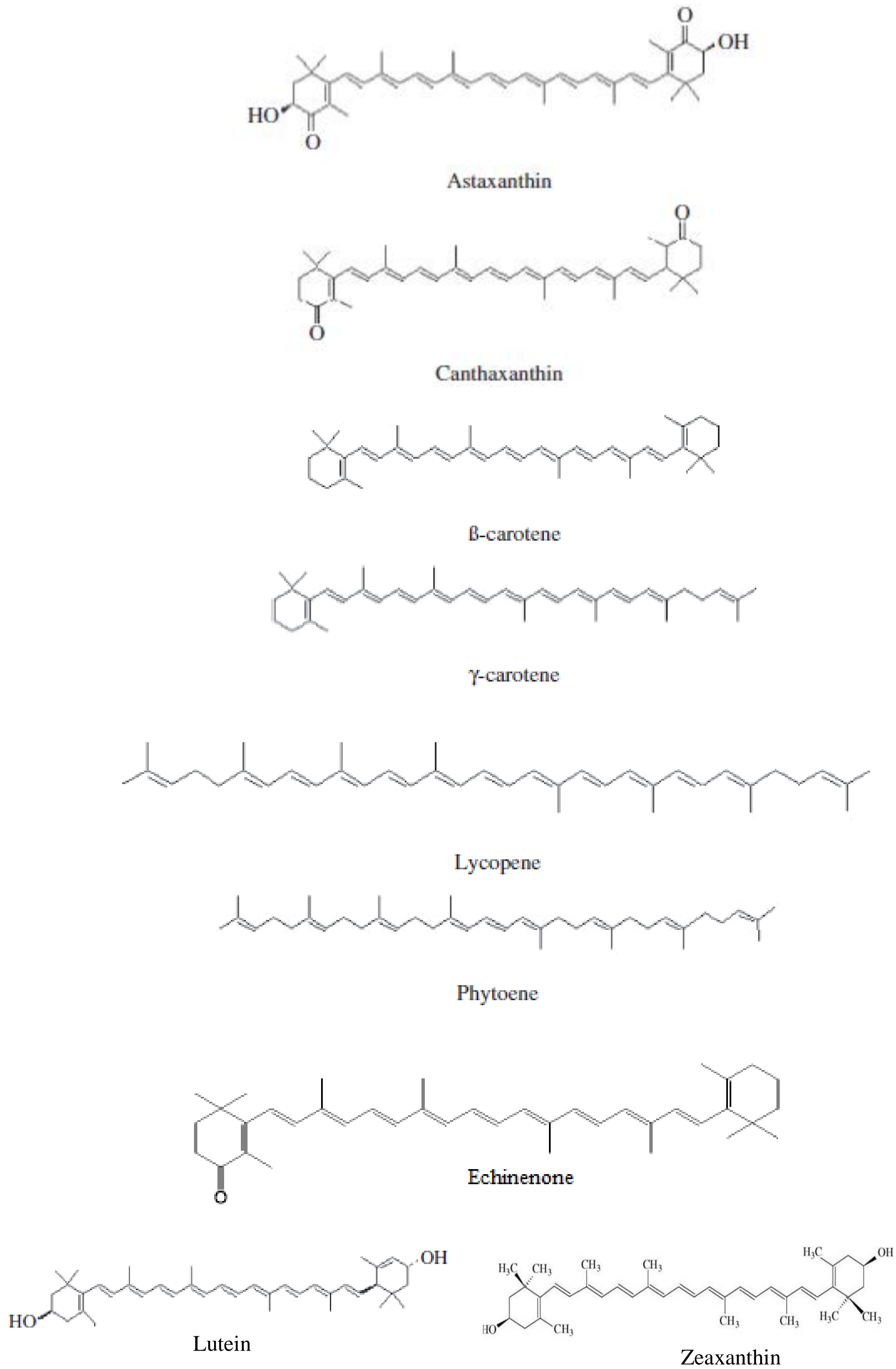


Fig.2 Structures of some common carotenoids. Adapted from Chandi et al. (Chandi & Gill, 2011)

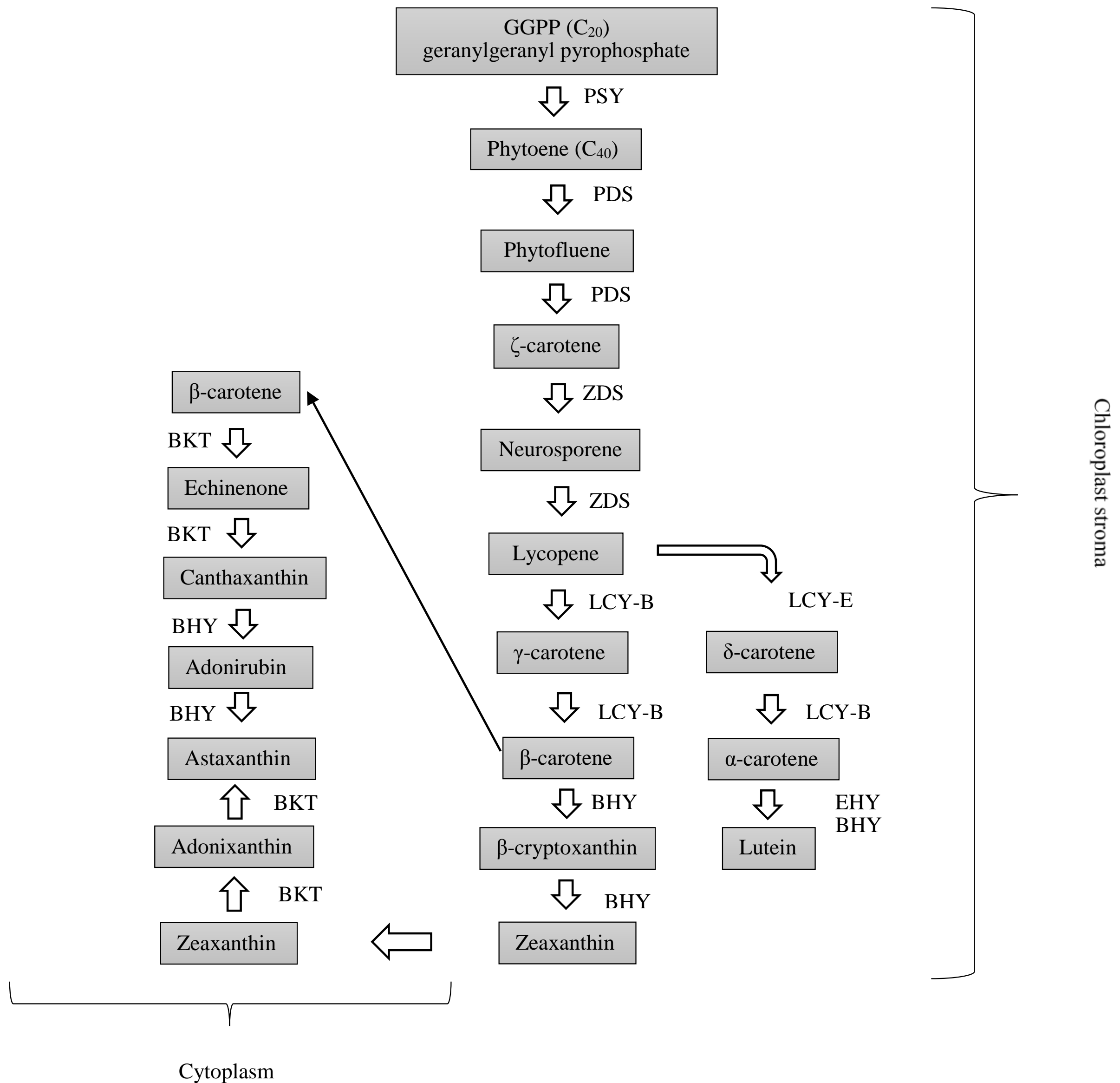


Fig.3 Possible pathway of carotenoids production in most green microalgae. The enzymes involved are phytoene synthase (PSY), phytoene desaturase (PDS), ζ-carotene desaturase, lycopene β-cyclase (LCY-B), β-carotene hydroxylase (BHY), lycopene ε-cyclase (LCY-E), ε-carotene hydroxylase (EHY), β-caroteneketolase (BKT). Adapted from Eonseon et al. (Eonseon et al., 2006) and Ye et al. (Ye, Jiang, & Wu, 2008)

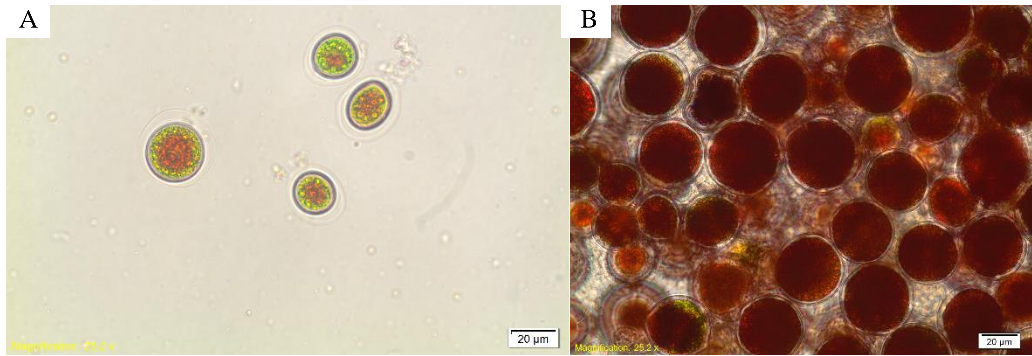


Fig. 4- PART A: Carotenogenesis of *Haematococcus pluvialis* - A. Initial phase of astaxanthin acumulation and B. Red cells – carotenogenesis. Strain from of Culture Collection of Algae and Protozoa (CCAP) 34/8.

Cell suspensions

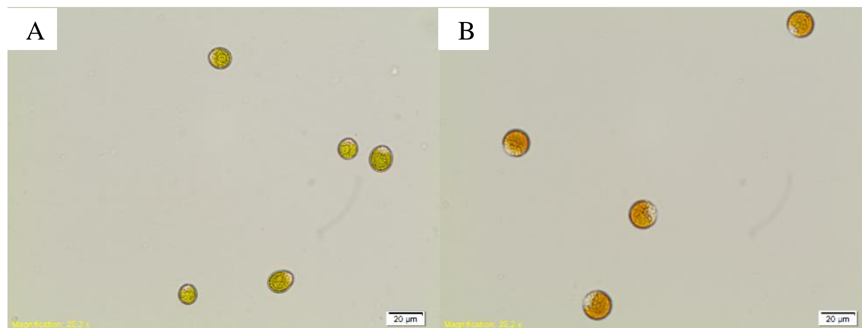
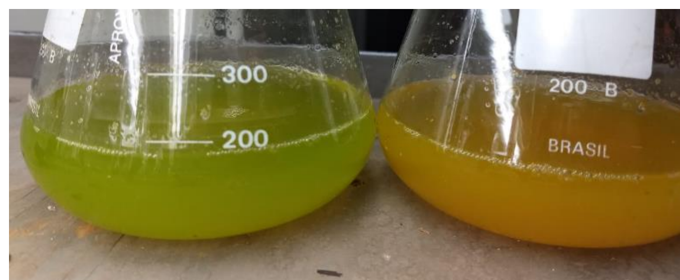


Fig. 4 - PART B: Carotenogenesis of *Dunaliella salina* - A. Green phase cells; B. Orange cells – carotenogenesis (β-carotene accumulation). Strain from of Culture Collection of Algae and Protozoa (CCAP) 19/18.



Cell suspensions

Petrochemical industries produce synthetic astaxanthin, raising questions related to toxicity of the final product, environmental pollution, and unsustainable product. In fact, synthetic astaxanthin cannot be directly used for human consumption in food or supplements, only as fish feed additives (Li, Zhu, Niu, Shen, & Wang, 2011).

Since nowadays society prefers natural products, thus the microalgae astaxanthin has a great potential in the market (Nguyen, 2013). The pigment zeaxanthin, together with lutein, surpassed US$ 250 million per year. They were approved by the EU as E161h (zeaxanthin) and E161b (lutein), as food additives and colorants, to the eye healthcare (Lin, Lee, & Chang, 2015). Lutein market is expected to grow up to US$ 308 million by 2018, with an annual growth rate of 3.6% (Business Communications Company, 2015). The market of zeaxanthin is still incipient (Lin et al., 2015), and the market of canthaxanthin is currently growing (Grama, Chader, Khelifi, Agathos, & Jeffryes, 2014). In the late 1970s, this xanthophyll was marketed in Canada and Europe, but it was discontinued when it was observed that it causes retinal crystallization (Boudreault et al., 1983). Xanthophyll is an important and promising biomolecule, so the study of new biological sources and/or productivity efficiency will increase the prospect of success in industrial production. The microalgal carotenoids' producer industries and applications can be observed in Table 1.

3.3.Traditional sources

Below there are some examples of traditional carotenoids sources found in nature. Canthaxanthin can be found in flamingos (*Phoenicopterus chilensis*, *Phoenicopterus ruber*, and *Phoenicopterus roseus*) and other red-feathered birds, in fish such as carp, golden mullet, sea-bream, and thrush wrasse. The edible mushroom *Cantharellus cinnabarinus*, in which carotenoid was isolated for the first time, also contains this red-orange pigment. It is added in the animal feed to intensify the color of some foods such as egg yolks, broilers, salmon, and trout, and it is mainly accumulated in fatty tissues, which became a rich source of this pigment (Esatbeyoglu & Rimbach, 2017). The astaxanthin, red pigment, is a xanthophyll from *Haematococcus pluvialis*, which can accumulate this pigment in a larger amount than salmon, trout, and shrimp. In the marine environment, these aquatic animals (traditional sources) consume microalgae or phytoplankton, which biosynthesized astaxanthin.

Table 1 Carotenoids from microalgae

Microalgae	Carotenoid/Producers	Applications	References
Dunaliella salina (Chlorophyta) Unicellular, green algae, halophilic, biflagellate, wall-less and they can reproduce asexually by longitudinal division or sexually by fusion of two motile cells to form a zygote and they take various forms. Biomass rich in glycerol, protein, and carotenoids.	β-carotene: much as 10% of dry weight (orange pigment, primary carotenoids – in this species may also be considered as a secondary carotenoid, since it the location of pigment is extrathylakoidal accumulated in lipid bodies) Cognis Nutrition and Health – BASF (Australia), Aquacarotene (Australia) Cyanotech (Kona, Hawaii, EUA) Nature Beta Technology (Israel) Nikken Sohonsha Corporation (Japan) Tianjin Lantai Biotechnology and Inner Mongolia Biological Eng. (China) Parry´s Pharmaceuticals (India) and more	Pigment (food and feed), pro-vitamin A (retinol), potent antioxidant (additive to cosmetics and health food), anti-inflammatory, immunomodulatory activity, hepatoprotective agent, and multivitamin preparations. Anticancer effect of *Dunaliella salina* (carotenoids)	(L. J. Borowitzka, Borowitzka, & Moulton, 1984; Borowitzka, 2013; Chiu et al., 2017; Hsu et al., 2008; Hu, Lin, Lu, Chou, & Yang, 2008; Lamers, Janssen, De Vos, Bino, & Wijffels, 2008)
Haematococcus pluvialis (Chlorophyta) Unicellular, green algae and freshwater. The complex life cycle, divided into two phases: motile (thin wall and two flagella – cell growth) and non-motile (thick wall, no flagellum – astaxanthin accumulation).	Astaxanthin: up to 3% dry weight (secondary carotenoids, red pigment found in the cytosol of *Haematococcus* cells) Algatechnologies, Ltd (Israel) Cyanotech Inc. (Kona, Hawaii, EUA) Mera Pharmaceuticals Inc. (Kailua-Kona, Hawaii, EUA) and Bioreal (Kihei, EUA) Biogenic Inc. (Japan) and Parry´s Pharmaceuticals (India) and more	Colorant for aquaculture (salmon and trout) and poultry industries, applications in the nutraceutical, pharmaceutical, cosmetics, and food industries (dietary supplements). Potent antioxidant (prevention of certain cancers), and age-related macular degeneration and Alzheimer and Parkinson diseases. Biomass high in vitamin E	(Cardozo et al., 2007; Del Campo et al., 2007; Guerin, Huntley, & Olaizola, 2003; Kang, Lee, Park, & Sim, 2005; Lorenz & Cysewski, 2000; Sarada, Vidhyavathi, Usha, & Ravishankar, 2006; Zhang, Wang, Wang, & Liu, 2014; Zhang, Pan, Wei, Gao, & Liu, 2007)
Chlorella protothecoides (Chlorophyta) Unicellular, green algae, non-motile, spherical form, freshwater, and asexually reproduces. Can grow photoautotrophically or heterotrophically under different culture conditions. - Other green algae (*Muriellopsis* sp., 4.3 mg/g and *Scenedesmus almerienses*, 4.5 mg/g)	Lutein: 5.4 mg/g (primary carotenoids, yellow pigment, located within the chloroplast) Carotenoid found within the retina of the eyes, in the peripheral retina Producer countries of lutein from Marigold Flowers: China, India and Mexico	Pigmentation of fish and poultry. Coloration of foods, drugs, cosmetics, and functional additive in infant food. Effective in the prevention of macular degeneration and certains cancers.	(Basu, Del Vecchio, Flider, & Orthoefer, 2001; Del Campo et al., 2001, 2007; Lin et al., 2015; Shi & Chen, 2002)
Chlorella zofingiensis (Chlorophyta) Green algae, freshwater microalga, can grow well photoautotrophically as well as heterotrophically - Other green algae (e.g., *Chlorella emersonii*, *and Coelastrella striolata* variant multistriata, about 4.8%)	Canthaxanthin: 8.5 mg/g (secondary carotenoids, dark red pigment, accumulated in lipid bodies located outside the chloroplast). Commercial production is dominated by chemical	Pigment: aquaculture, poultry - for coloring chicken skin and egg yolks, food, cosmetics (tanning agent), medicine, nutraceuticals and pharmaceuticals. Strong antioxidant and anticancer activities	(Abe, Hattori, & Hirano, 2007; Bhosale & Bernstein, 2005; Gong & Bassi, 2016)
Chlorella ellipsoidea Green algae, marine species, used in Japan and Korea to feed rotifers and shrimps *Dunaliella salina (mutant)* (6 mg zeaxanthin per g dry weight, higher than the wild type – 0.2 mg/g)	Zeaxanthin: 4.26 mg/g, more than nine times that of red pepper, a plant source of zeaxanthin (primary carotenoids, yellow - orange pigment, located in the chloroplast thylakoids) Carotenoid found within the retina of the eyes, in the central macula. Market young Synthetic zeaxanthin, manufactured by DSM Ltd. (produced before by F. Hoffmann-La Roche Ltd.)	Potent antioxidant and a high- value bio product, natural colorant and additive in the cosmetic and food industries. Reducing age-related macular Degeneration. Recommended for the prevention of cardiovascular disease, and some types of cancer.	(Beatty, Boulton, Henson, Koh, & Murray, 1999; Bone, Landrum, Guerra, & Ruiz, 2003; Ferruzzi & Blakeslee, 2007; Jin, Feth, & Melis, 2003; Kim et al., 2017; Koo, Cha, Song, Chung, & Pan, 2012)

This microalga biomass contains between 1.5 - 3% dry weight in astaxanthin (Lorenz & Cysewski, 2000). Another important natural pigment is the lutein, that commonly coexists with its stereoisomer zeaxanthin. They are found in fruits, green vegetables, some seeds (e.g. corn) (Sommerburg, Keunen, Bird, & van Kuijk, 1998), and flowers, as the marigold flowers (*Tagetes erecta*), the unique industrial source of lutein with 0.3 mg/g of this carotenoid (Piccaglia, Marotti, & Grandi, 1998).

Collards, turnip greens, broccoli, carrots, kale, spinach, green lettuce, peas, olives, zucchini squash, pumpkin, green pepper, Japanese persimmons, peaches, kiwi, red seedless grapes, eggs yolk, chicken (broilers), and cheese are also important sources of lutein (Holden et al., 1999). In Table 2 it is possible to observe some lutein rich foods. The microalga *Chlorella protothecoides* synthesizes between 4.65 – 5.35 mg g^{-1} and yield around 200 mg L^{-1} (Shi et al., 2002) of lutein and the productivity is 379.1 kg ha^{-1} $year^{-1}$, about 100 times greater than the productivity of lutein from marigold flower, which is 3.6 kg ha^{-1} $year^{-1}$. Productivity calculation was based on microalgae production in raceway (Lin et al., 2015) and percentage of lutein in marigold flower of 0.3 mg/g (Gong & Bassi, 2016).

Foods that contain the highest levels of zeaxanthin are wolfberries, collards, *Capsicum annuum*, yellow corn, orange pepper, orange juice, honeydew, mango, chicken egg yolk, cold-pressed marionberry, boysenberry, red raspberry, blueberry seed oils, spinach, apricots, peaches, avocado, cantaloupe, and a variety of pink grapefruit. Corn (drained, sweet, yellow, canned, whole kernel) contains the highest amount of zeaxanthin (528 µg 100 g^{-1}), followed by raw spinach with 331 µg 100 g^{-1}, butternut (280 µg 100 g^{-1}), collards (drained, cooked, boiled, without salt) (266 µg 100 g^{-1}), and other sources (Sajilata, Singhal, & Kamat, 2008). It is known that the minimum quantity of carotenoids (lutein and zeaxanthin) recommended to have a good eye health is 6 mg day^{-1} (Seddon, Ajani, & Sperduto, 1994). *Chlorella ellipsoidea* contains 4.26 mg g^{-1} (Koo et al., 2012) and *Dunaliella salina (mutant)* 6 mg zeaxanthin per g of dry weight (Jin et al., 2003).

The yellow-orange fruits like mandarin, orange, papaya, apricot, cantaloupe, mango, peach, watermelon, and nectarine are sources of β-carotene. In green leafy vegetables, β-carotene is the second pigment present in a range of 25 – 30 % of total carotenoids. Vegetables as coriander, spinach, squash, drumstick leave, pumpkin, fenugreek, and parsley contain higher quantity of β-carotene than other vegetables (Perry, Rasmussen, & Johnson, 2009; Saini et al., 2015).

Table 2
Lutein concentrations in some products

Sources of lutein	Botanical name	Quantity (mg 100 g $^{-1}$)	References
Kale (lutein + zeaxanthin)	*Brassica oleracea*	3.04 – 39.55	(Holden et al., 1999)
Spinach	*Spinacia oleracea*	6.49 – 12.98	(Kopsell et al., 2006)
Broccoli raw (lutein + zeaxanthin)	*Brassica oleracea*	2.44	(Holden et al., 1999)
Avocado	*Persea americana*	0.293	(Lu et al., 2005)
Kiwi *	*Actinidia deliciosa*	0.352	(Nishiyama, Fukuda, & Oota, 2007)
Kiwi	*Actinidia arguta*	0.50 – 1.51	(Nishiyama et al., 2007)
Corn (yellow dent)	*Zea mays*	1.57	(Moros, Darnoko, Cheryan, Perkins, & Jerrell, 2002)
Egg (yolk + white), cooked	_____	0.273	(Perry et al., 2009)
Egg yolk cooked	_____	0.744	(Perry et al., 2009)

*the species of kiwi *Actinidia deliciosa* is most commonly commercialized, the values in mg 100 g^{-1} may vary depending on the species

Dunaliella salina has a biomass rich in β-carotene, more than 10% of the biomass dry weight (Ye et al., 2008), and the productivity is 7.02 x 10 ^{3} kg ha^{-1} year^{-1}, while the productivity of carrot (traditional source) is 1.38 x 10^{-1} kg ha^{-1} year^{-1}. Productivities were calculated based on literature data (Favacho, Lima, Neto, Silva, & Barros, 2017; Lin, Durance, & Scaman, 1998). Finally, the lycopene, a red color pigment has been found in large quantity in tomatoes and products made from tomatoes such as tomato juice, paste, puree, ketchup, and sauce. The concentration of lycopene in fresh tomato fruit is 0.72 – 20 mg 100 g $^{-1}$. Tomato is considered as a healthy food, because it contains lycopene (major carotenoid, about 80 – 90%), β-carotene (7 – 10%), vitamins A and C, and potassium. In addition, it is cholesterol free and has low calorie, fiber, and protein (Frusciante et al., 2007; Shi & Maguer, 2000). Other sources of lycopene are (Shi & Maguer, 2000):

Watermelon	2.30 – 7.20 mg 100 g $^{-1}$
Guava (pink)	5.23 – 5.50 mg 100 g $^{-1}$
Pink grapefruit	0.35 – 3.36 mg 100 g $^{-1}$
Papaya	0.11 – 5.30 mg 100 g $^{-1}$
Rosehip puree	0.68 – 0.71 mg 100 g $^{-1}$
Carrot	0.65 – 0.78 mg 100 g $^{-1}$
Pumpkin	0.38 – 0.46 mg 100 g $^{-1}$
Sweet potato	0.02 – 0.11 mg 100 g $^{-1}$
Apple pulp	0.11 – 0.18 mg 100 g $^{-1}$
Apricot	0.01 – 0.05 mg 100 g $^{-1}$

Marine algae *Chlorella marina* can synthesize lycopene (Renju, Kurup, & Kumari, 2013; Renju, Muraleedhara Kurup, & Bandugula, 2014).

3.4.Carotenoids from microalgae

3.4.1. β- carotene from *Dunaliella salina*

In Table 1, several applications of microalgal carotenoids are listed. *Dunaliella salina* can produce up to 10.0% dry weight of β-carotene. In nature, this microalga is considered the largest source of this carotenoid that present the following properties: antioxidant, anti-inflammatory, immunomodulatory, hepatoprotective, retinoprotective, and dermoprotective. This natural pigment can be used as food colorant, in vitamin preparations, as pro-vitamin A (retinol), in cosmetic industries (e.g., sunscreen formulation) (Chiu et al., 2017; Edge, McGarvey, & Truscott, 1997; Saini & Keum, 2018), and as anticancer. Many epidemiological and oncological studies point out that *Dunaliella salina* can be efficient in the treatment of various types of cancer (breast, prostate, colon, lung, liver, skin cancer, among others) (Chuang, Ho, Liao, & Lu, 2014; Jayappriyan, Rajkumar, Venkatakrishnan, Nagaraj, & Rengasamy, 2013; Sheu et al., 2008).

Research with *Dunaliella salina* as adjunctive therapy oral cancer along with traditional treatment showed that this microalga biomass can be antiproliferative, anti-inflammatory, and proapoptotic on human oral squamous carcinoma cells (Chiu et al., 2017).

3.4.2. Astaxanthin from *Haematococcus pluvialis*

Astaxanthin is found in nature as with one (monoester) or two esterified fatty acids (diester). Different astaxanthin isomers may be formed based on the configuration of the two hydroxyl groups in the molecule (carbon at the 3-position and carbon at the 3'-position of the benzenoid rings): 3R and 3'R; 3R and 3'S (meso); and 3S and 3'S. The main form of the pigment found in *Haematococcus pluvialis* is the stereoisomer 3S and 3´S. The arrangement of synthetic astaxanthin is 1:2:1 (3S, 3'S), (3R, 3'S), (3R, 3'R) (Grung, D'Souza, Borowitzka, & Liaaen-Jensen, 1992; Yaakob et al., 2014). The astaxanthin from *H. pluvialis* is predominantly esterified and constituted of 70% monoesters, 15-20% diesters, and 4-5% free forms (Rao et al., 2013). This microalga is recognized as the major source of astaxanthin, and it can accumulate this carotenoid up to 3% of dry biomass, which represents a quantity higher than those found in plants, bacteria (e.g., *Agrobacterium aurantiacum*), and few fungi (e.g., red yeast *Phaffia rhodozyma*). Other microalgae such as *Chlorella zofingiensis* and *Chlorococcum* sp., and marine animals (salmonids, shrimp, lobsters, and crayfish) (Guerin et al., 2003) contain this pigment. This carotenoid is synthesized by phytoplankton (are the primary producers in the marine environment) and, then the microalgae are consumed by zooplankton and, in turn are ingested by fish (Lorenz & Cysewski, 2000). It has been considered as an excellent antioxidant (activity greater than vitamin E and β-carotene) being the major dietary supplement for vertebrates that can be benefited with several clinical and cosmetics properties. Health benefits such as anti-inflammatory effect with no side have been reported. This pigment protects neurons from the retina, brain and spinal cord and may exert an anti-obese activity (Guerin et al., 2003). It has been reported, in diabetic rats, that the consumption of astaxanthin can soften diabetes effects (Otton et al., 2010). As hepatoprotector, it can prevent the development of liver fibrosis, in cardiovascular diseases it can inhibit the oxidation of LDL (in hypertensive rats it has proven that an astaxanthin-rich diet reduces arterial blood pressure) (Monroy-Ruiz, Sevilla, Carrón, & Montero, 2011), and it also can inhibit carcinogenesis of many cancer types. Astaxanthin (mono and diester) was used in rats with skin cancer and the results showed higher anticancer potency than in animals not supplemented with astaxanthin (Rao et al., 2013). This red pigment can also be used as colorant in aquaculture feed and food industries (Guerin et al., 2003).

3.4.3. Carotenoids from *Chlorella spp.*

Chlorella sp. produce lutein, canthaxanthin and zeaxanthin. These green microalgae can grow both photo- and heterotrophically.

*3.4.3.1.*Lutein from *Chlorella protothecoides*

Currently, the main source of lutein is from marigold flowers, and there is no commercial production of lutein from microalgae, although the microalgae production rate may be 3-6 times higher or even more, depending on the genus. Lutein is used to intensify the yellow color of poultry egg yolk and feather, through feed addition, as well as food colorant for human consumption (Lin et al., 2015). This molecule is also used in drugs and cosmetics, aquaculture, and as functional additive in infant food (Kläui, 1982). Lutein can bring health benefits as reduced risk of cardiovascular disease. Its supplementation protects the body against the development of early artherosclerosis (Dwyer et al., 2001), it lowers the risk of lung cancer (Marchand et al., 1993; Ziegler et al., 1996), and age-related macular degeneration (AMD) (Chiu & Taylor, 2007). Lutein, as well as zeaxanthin, are found in high concentrations in human retina. Many studies have shown that a diet supplemented with lutein can result in the increase of this macular pigment amount and, consequently, protects against AMD (Schnebelen-Berthier et al., 2015). In fact, low levels of lutein and zeaxanthin are observed in people with cataract (Olmedilla, Granado, Blanco, Vaquero, & Cajigal, 2001). An interesting alternative to replace lutein is the production of the green microalga *Chlorella protothecoides*, because of their relatively high growth rate under heterotrophic conditions and high productivity of lutein (Wei et al., 2008). The lutein quantity in this microalga is in the range of 4.6 – 5.4 mg g^{-1} and yield around 200 mg L^{-1} (Shi et al., 2002). Other microalgae that also present high levels of lutein in their biomass are *Dunaliella salina* (6.6 ± 0.9 mg g^{-1}), *Scenedesmus almeriensis* (5.3 mg g^{-1}), and the red microalga *Galdieria sulphuraria* (0.4 ± 0.1 mg g^{-1}) (Sun, Li, Zhou, & Jiang, 2015). It is also known that the microalgae *Muriellopsis* sp. can produce lutein (Del Campo et al., 2007).

*3.4.3.2.*Canthaxanthin from *Chlorella zofingiensis*

The canthaxanthin, along with astaxanthin, both secondary carotenoids, can be produced by microalgae, and they are considered as more powerful antioxidants than primary carotenoids, such as β-carotene. Canthaxanthin is a dark red pigment, derived from β-carotene (see Fig. 3),

used as colorant in food industries and as additive in animal feed to poultry and fish (salmonid and crustacean pigmentation, as well as rainbow trout). It is a natural and healthy colorant that improves fish skin colors and it is approved by the Food and Drug Administration (FDA) (Bhosale & Bernstein, 2005). It does not present pro-vitamin A activity like β-carotene, but it is suggested that this biomolecule is able to reduce the risk of cancer due to its high antioxidant power in animal models. This carotenoid, in association with β-carotene, is effective in the treatment of light polymorphic eruptions, the most common and frequent type of idiopathic photodermatosis (Suhonen, 1981). In 1990, Gensler and Holladay (Gensler and Holladay, 1990), reported the prevention of photocarcinogenesis in mice and also the stimulation and maintenance of the immune system with dietary supplementation of canthaxanthin and retinyl palmitate.

Although many microorganisms, such as non-photosynthetic bacteria and algae, have the potential to produce canthaxanthin, the industrial production occurs mainly by chemical synthesis (Ernst, 2002). Among the algal species, it is possible to mention: *Chlorella emersonii,* whose cultivation was carried out under high irradiance and low concentration of nitrogen (Malis, Cohen, & Ben Amotz, 1993); *Scenedesmus komarekii*, cultivated under high light intensity and nitrogen limitation (Hanagata & Dubinsky, 1999); and *Chlorella zofingiensis* (Pelah, Sintov, & Cohen, 2004). The green microalga *Chlorella zofingiensis* under stress conditions, such as high-intensity irradiation and nitrogen limitation, can accumulate keto-carotenoids as canthaxanthin, and it has the advantage of rapidly accumulating the secondary carotenoid when exposed to this binomial stress (Bar, Rise, Vishkautsan, & Arad, 1995). Studies showed that the *C. zofingiensis,* exposed to low irradiance and high salinity together with nitrogen limitation, produced higher concentrations of secondary carotenoids such as astaxanthin and canthaxanthin. In addition, under these conditions, *C. zofingiensis* accumulates more canthaxanthin than astaxanthin (Pelah et al., 2004). *C. zofingiensis* can be a potential producer of astaxanthin, since its growth rate is fast, and its cultivation and scaling up are easier. This microalga can be cultivated under different conditions: autotrophically, heterotrophically, and mixotrophically. Moreover, it can reach higher cell density than the microalga *H. pluvialis*, thus being a good alternative for the industrial production of astaxanthin (Chen, Liu, & Wei, 2017; Liu et al., 2014).

3.4.3.3. Zeaxanthin from *Chlorella ellipsoidea*

Zeaxanthin (yellow-orange pigment) is used as a dye in food industries and as antioxidants in cosmetic industries (Bhosale & Bernstein, 2005). It is known that the consumption of carotenoids, lutein and zeaxanthin, is associated with low rates of cancer incidence, such as lung and pancreatic cancer in diabetic pacients (Chew, Clemons, San Giovanni, Danis, Ferris, Elman, et al., 2013; Jansen et al., 2014), besides of also acting efficiently in cardiovascular diseases (Trumbo & Ellwood, 2018). Zeaxanthin, as well as lutein, are naturally occurring carotenoids in the retina of the eye, more specifically located in the central part of the macula (Bone, Landrum, Hime, & Cains, 1993). Many species of *Chlorella* sp. are potential producers of this carotenoid that can promote eye health by primarily acting on macular degeneration, which can result in blindness with aging (Bernstein et al., 2015). A promising source of natural production of zeaxanthin is the marine microalga *Chlorella ellipsoidea*. In addition to accumulating a concentration of approximately nine times greater (4.26 mg g -1) than in red pepper (source rich in zeaxanthin), this is a free form of this carotenoid (Koo et al., 2012). It is known that the xanthophylls found in *C. ellipsoidea* biomass, strongly induced apoptosis in neuroblastoma cells (Maccarrone, Bari, Gasperi, & Demmig-Adams, 2005) and these bioactive carotenoids can also be effective for human cancer prevention (Kwang, Song, & Lee, 2008).
One of the zeaxanthin production pathways occurs by violaxanthin reversible reaction, through the enzyme VDE (violaxanthin de-epoxidase), and it is regulated by the irradiance excess on the photosynthetic apparatus. In plants, under low light intensities, violaxanthin is efficient as an accessory pigment for photosynthesis. On the other hand, under high light intensities, zeaxanthin plays an important role in the cellular protection (Othman, Mohd Zaifuddin, & Hassan, 2014). Another alternative source of zeaxanthin is *Dunaliella salina*. A study of a *D. salina* mutant, showed that it could accumulate about 6 mg of zeaxanthin per g of dry weight, a concentration much higher than in the wild type (0.2 mg g^{-1}), under low light intensity (100 µmol photons m^{-2} s^{-1}). On the other hand, the growth under high light intensity (2000 µmol photons m^{-2} s^{-1}) provided a concentration of 2.62 mg g^{-1} for the wild type and 4.18 mg g^{-1} for the mutant one (Jin et al., 2003). In another study, the maximal yield was 8 mg L^{-1} of zeaxanthin of the mutant *Dunaliella tertiolecta*, produced by fed-batch culture. With this result, the mutant strain becomes interesting for industrial production and commercial applications of one of the best carotenoids for degenerative diseases prevention and treatment. There are two main advantages of producing carotenoids from *Dunaliella* sp.: first, there is a low risk of culture contamination by other microalgae, since the microalgae culture medium contains high salt

concentrations, and second, these microalgae cell wall is not rigid, facilitating the extraction of pigments (Kim et al., 2017).

3.4.4. Other carotenoids

3.4.4.1.Lycopene

Lycopene carotenoid (red pigment) is an antioxidant and pro-vitamin A. The consumption of foods rich in this pigment, such as tomatoes and other red/orange fruits and vegetables, may reduce the risk of chronic diseases (Barton-Duell, 1995), some types of cancer (prostate, lung and stomach) (Giovannucci, 1999) and macular degeneration that can lead to blindness (Seddon et al., 1994). Due to its antioxidant property, this carotenoid is marketed as a nutraceutical product in the form of capsules, tablets and gels. Recent studies showed the possibility of using lycopene from *Chlorella marina* for prostate cancer treatment (Renju et al., 2014) and as an anti-inflammatory (Renju et al., 2013).

3.4.4.2. Echinenone

The green microalga *Botryococcus braunii* synthesizes and accumulates a variety of lipids, including high amounts of hydrocarbons (30 – 70 % of dry biomass)(Wolf, Nonomura, & Bassham, 1985), and ether lipids, for example, the carotenoid echinenone (Metzger & Largeau, 2005). Microalgae are well known as a green and sustainable alternative for biofuels production (Bwapwa, Anandraj, & Trois, 2017), but under stress conditions such as nitrogen limitation and high luminous intensity to accumulate the orange pigment - echinenone. This pigment (see chemical structure fig.2), presents antioxidant power and pro-vitamin A activity, being usually obtained as a by-product of astaxanthin production (Matsuura, Watanabe, & Kaya, 2012)

4. **Microalgal culture systems**

Microalgae cultivation can be performed in open ponds - raceway ponds (the most used), or in closed-systems (Ugwu, Aoyagi, & Uchiyama, 2008).

4.1. Open system

The construction and operation cost in open systems are cheaper than in closed ones and the process is also simpler. The raceway type lagoon is the most commonly used for algae cultures, in which the circulation of the culture medium occurs by means of paddles, that are responsible for keeping cells in suspension. The shallowness in these tanks grants the efficiency in the light penetration. The system is generally continuous, that is, nutrient (nitrogen, phosphorus and inorganic salts) is continuously fed, and cells are simultaneously out flowed (J.C.M. Carvalho et al., 2013; J.C.M. Carvalho, Matsudo, Bezerra, Ferreira-Camargo, & Sato, 2014).This system is ideal for microorganisms that grow under extreme conditions such as the cyanobacteria *Spirulina* sp. (high alkalinity), and the microalga *Dunaliella salina* (high salinity) and *Chlorella* sp. (nutrient-rich media). It is known that all industrial *Dunaliella* sp. production use the open pond technology (Borowitzka, 2013). However, microalgae that do not grow in selective environments such as *Skeletonema* sp., *Chaetoceros* sp., *Thalassiosira* sp., *Tetraselmis* sp., *Isochrysis* sp., and *Crypthecodinium* sp., should not be cultivated in open systems (Borowitzka, 1999). Some disadvantages are observed in this type of system, since it is not possible to control the environment around the tank: lack of temperature and light control, and high risk of contamination by other microalgae, bacteria and protozoa, that turns this system vulnerable. Other critical factors, such as large losses of water through evaporation, diffusion of CO_2 to the atmosphere, the need for large areas of land, and the inefficiency of mass transfer causing low cell growth can be pointed out (Brennan & Owende, 2010; Ugwu et al., 2008).

An alternative to minimize these problems associated with the open tank system, is to close the ponds. The cost is higher than the open pond system but considerably lower than other closed photobioreactor configurations. The control of the environment becomes more efficient providing, through a cover, better CO_2 assimilation, and consequently greater biomass productivity (Singh & Sharma, 2012).

4.2.Closed systems

Closed systems were designed to minimize some problems found in open systems for microalgae cultivation. The low risk of contamination is an important advantage, providing the production of biomolecules of high commercial value with applications in the pharmaceutical and cosmetic industries, called ‘clean’ algal culture. In addition, it is possible to mention better control of parameters like pH, light intensity, temperature, efficient CO_2 capture, reduction of

water loss through evaporation, and considerably higher biomass productivities. On the other hand, the construction cost is higher than for open system, and large scale cultivations are usually more expensive and complex. Some types of closed photobioreactors (PBRs) are designed for algae cultivation (Borowitzka, 1999; Brennan & Owende, 2010; Carvalho et al., 2014; Singh & Sharma, 2012; Ugwu et al., 2008), among them:

Tubular: is the most suitable closed type of bioreactor for growing cells in outdoor environments, providing satisfactory biomass productivities. It is usually built with glass or plastic tubes (construction is relatively cheap), the culture homogenization is usually done by means of air pump, and it has large surface area for illumination. Some limitations are observed as, fouling, pH variation, dissolved oxygen and CO_2 heterogeneity in the system. There are several configurations: horizontal / serpentine, vertical, near horizontal, and inclined (J.C.M. Carvalho et al., 2014). There are several studies showing the suitability of using this kind of photobioreactor for high quality cyanobacteria (J.C.M. Carvalho et al., 2013; Ferreira, Rodrigues, Converti, Sato, & Carvalho, 2012; Matsudo, Bezerra, Converti, Sato, & Carvalho, 2011) or microalgae (J.C.M. Carvalho et al., 2014; Pérez-Mora, Matsudo, Cezare-Gomes, & Carvalho, 2016) biomasses production.

Flat plate: has a large surface area exposed to light and high cell productivity, usually higher than in tubular photobioreactor. The system is built with transparent material to optimize the capture of light energy, easy to sterilize, suitable for outdoor cultures, ideal for cell immobilization, and relatively inexpensive. It also has a short light path, and the dissolved oxygen is not easily accumulated inside the photobioreactor. Difficulty in controlling the culture temperature and the presence of some encrustation are some observed limitations (Eze, Ogbonna, Ogbonna, & Aoyagi, 2017; Ugwu et al., 2008). Various designs and scales of flat-plate photobioreactors have been tested for microalgae cultivation, for example, *Chlorella vulgaris* for the lipid accumulation (Liao et al., 2017); *Phaeodactylum tricornutum* (marine diatom) for lipid, eicosapentaenoic acid, fucoxanthin, and chrysolaminarin production (Gao, Chen, Zhang, Li, & Zhang, 2017); the green microalgae *Scenedesmus obtusiusculus* (Koller, Löwe, Schmid, Mundt, & Weuster-Botz, 2017) and *Scenedesmus ovalternus* (Koller, Wolf, & Weuster-Botz, 2017); and *Nannochloropsis sp* for the fatty acids and protein production (Hulatt, Wijffels, Bolla, & Kiron, 2017).

Column: is compact, easy to operate, easy to sterilize, and easy to scale-up. It allows high mass transfer, low energy consumption, good mixing with low shear stress, reduced photoinhibition and photo-oxidation, and high surface area to volume ratio. There are some critical points like: small illumination area, sophisticated construction, possibility of shear stress, and the light intensity inside the bioreactor decreases with the scale-up. Many studies with microalgae cultivation in column photobioreactors have been carried out, as the study of shear-sensitive dinoflagellate microalga *Karlodinium veneficum* cultivation in bubble column photobioreactor (López-Rosales, García-Camacho, Sánchez-Mirón, Contreras-Gómez, & Molina-Grima, 2017). Another study investigated the nitrous oxide (N_2O) emissions during cultivation of two microalgae, *Chorella vulgaris* and *Neochloris* sp., and one cyanobacterium, *Arthrospira platensis* (Plouviez, Shilton, Packer, & Guieysse, 2017). The marine microalgae *Tetraselmis* sp. were cultivated in bubble column photobioreactor for evaluating biomass and fatty acid productivities (Kim, Park, Ryu, & Lee, 2017).

Hybrid systems may also be used for large-scale production of marine microalgae. Huntley et al. (Huntley et al., 2015) cultivated the diatom *Staurosina* sp. and the chlorophyte *Desmodesmus* sp. in a hybrid system with closed photobioreactor ($25m^3$) and open pond (400 m^2). In this combination, the closed photobioreactor continuously ensured a source of consistent inoculum (low risk of contamination) for short-time batch cultivation in open ponds, in which large-scale biomass production takes place, with lower cost and easy operation.

In the production of astaxanthin from the green microalga *Haematococcus pluvialis,* Cyanotech Corporation is an example of companies that use a closed photobioreactor for the inoculation stage and green phase but an open system (ponds) for the carotenoid accumulation (red phase) (Cyanotech Corporation, 2018).

5. Operation regime

The cultivations can be performed by different operation regimes: batch, fed-batch, semi-continuous and continuous (J.C.M. Carvalho et al., 2013, 2014).

Batch

Is an operation regime in which all the nutrients together with the inoculum are added at the initial time, and there is no addition of nutrients during the process. It is frequently used for bench scale process to study variables that influence the cultivation. Considering the

carotenoids production, besides the traditional parameters that affect the cell growth and production of metabolites, cultivation medium salinity (Fu et al., 2014), nutrients addition (Cuaresma, Casal, Forján, & Vílchez, 2011), and light intensity (Castro-Puyana et al., 2017; Fu et al., 2014; Ma et al., 2018) influence carotenoids production. These pigments production are usually induced by stress conditions that can result in high carotenoid concentration, as studied by Pan et al (Pan-utai, Parakulsuksatid, & Phomkaivon, 2017), who cultivated *Haematococcus pluvialis* in bench scale glass column photobioreactor. Some great advantages of this process are reported as ease of setting up, ease of operation, efficient nutrient removal, and low risk of contamination, such process was evaluated for lutein production by *Botryococcus* sp*.,* and new species growth in outdoor raceway ponds (Dayananda & Kumudha, 2010). It is important to notice that there are also disadvantages in the application of this system type, such as low biomass productivity, expensive scale-up, and high harvesting costs due to low cell density (Fernandes et al., 2015; Zhu, 2015); as well as longer "dead time", i.e. time of loading, discharge, washing, and sterilization of the bioreactor (Zhu, 2015), which explains the need to understand and choose the most appropriate process operation mode.

Fed-batch

The difference between batch and fed-batch processes is that one or more nutrients are added to the bioreactor during cultivation time in the second one. The products are discharged only at the end of the cycle, the flow rate may be constant or vary with time, and the addition of the nutrients may be continuous or intermittent. The volume of the bioreactor can vary throughout the cultivation, and it will depend on the rate of evaporation and the substrate concentration. This operation mode is important for promoting adequate concentration of a specific substrate that may be toxic in higher concentrations (J.C.M. Carvalho et al., 2013), as well as in cases when the nutrient deviate the metabolism to an unwanted metabolic pathway, or in processes that require lack of some nutrient to activate a specific metabolic pathway, as it happens with the lack of phosphorus or nitrogen in the cultivation medium for carotenoids production. *Scenedesmus incrassatulus*, microalga that accumulates carotenoids, was cultivated under different nitrogen concentrations in airlift photobioreactors and, in fact, the results showed biomass values approximately 3 times higher (4.05 ± 0.04 g L^{-1}, which corresponds to $8.24 \times$ 107 cell mL^{-1}) than those obtained in batch operation mode (García-cañedo et al., 2016). It is also being reported the benefits from organic carbon sources feeding on the growth of microorganisms that produce carotenoids in dark cultivation. Fed-batch mode has been applied to evaluate microalgae growth in heterotrophic conditions when using glucose as carbon source

to produce astaxanthin by *Chlorella zofingiensis* (Sun, Wang, Li, Huang, & Chen, 2008), or lutein by *Chlorella sorokiniana* (Chen et al., 2018). Regarding heterotrophic conditions for production of any photosynthetic microorganism, it is important to give special attention to contamination, also because this is an important parameter to be evaluated in pilot and industrial scale. One last application of this operation mode for carotenoids production is the perfusion, in order to remove excess of ions, sodium for example, and inhibitors. When cultivating *Haematococcus pluvialis* increases of 14.3% and 37.8% on, respectively, cell productivity and astaxanthin concentration were obtained for fed-batch process using perfusion if compared to the one in which perfusion was not used (Park, Choi, Hong, & Sim, 2014).

Semi-continuous

It begins with the addition of the culture medium and the inoculum to the bioreactor. After the end of the process, part of the culture medium is removed, being replaced by the same volume of fresh medium. It presents the advantage of operating the bioreactor for long periods without the need to prepare a new inoculum, and the productivity may be greater than the batch process as a consequence of working with cells adapted to the cultivation medium throughout the time. Semi-continuous process can lead to adequate nutrient deprivation, depending on the combination of dilution rate (D) and nutrient concentrations. Indeed, when *Chlorella zofingiensis* was cultivated by semi-continuous process using limited nitrogen concentration of 10 $mg.L^{-1}$ and D of 0.5 d^{-1}, high astaxanthin productivities were reached when compared with higher or lower D value (Liu, Mao, Zhou, & Guarnieri, 2016). Such findings, evidence the importance of choosing the best combination of D and nitrogen concentration to optimize the production of carotenoids. The nutrient limitation was also applied for lutein production by *Chlorella minutissima*, and the results of lutein productivity, photosynthetic efficiency and CO_2 sequestration rate were further increased by 19%, 41% and 34% respectively, when 20% of the cultivation medium was replenished (Dineshkumar, Subramanian, Dash, & Sen, 2016). It must be pointed out that in semi-continuous cultivations there are authors who prefer to express the fraction of medium replenishment as a dilution rate, which is expressed at a certain time, daily, for example. Other authors prefer to express the medium replenishment as a fraction of total volume of the cultivation removing the time parameter in such information. In the former idea the unit is expressed as $time^{-1}$ and in the later the unit is expressed as percentage. Although the commercial cultivation of *Dunaliella salina* occurs in open-air cultures (Prieto, Pedro Cañavate, & García-González, 2011), the use of closed tubular photobioreactor configuration has been proposed to this production associated with semi-continuous mode of operation

(García-González, Moreno, Manzano, Florencio, & Guerrero, 2005; Prieto et al., 2011). Taking into account that closed photobioreactors generally leads to higher cell concentrations than open ponds and less problems with contamination, the semi-continuous process applied to closed photobioreactors promises to be a feasible process to produce carotenoids.

Continuous

In this operation mode, culture medium is continuously fed (flow rate is constant) with continuous withdrawal of culture medium, maintaining the reactor in a constant working volume. Along the time, the cell specific growth rate became the same as the dilution rate (D), which, consequently, results in the reach of a steady state condition (Fernandes et al., 2015), where the cell concentration does not change over time. This system can be operated with or without cell recycle. The advantages of the continuous process comparing to the batch process are: increased productivity, maintenance of the cells in the same physiological state, process automation, etc. There are not so many reports about the continuous operation mode for carotenoids production by microalgae, mainly because the highest cell productivities occur at higher values of D and in such conditions, when there are more nutrients to the cells, the production of carotenoids is not activated. For this condition, it would be necessary to work with lower values of dilution rates, which could lead to higher death growth rates with consequent higher risks of contamination. Thus, such process would be better applied in cultivations with more extreme conditions, as salinity or alkalinity for example. Besides, on the literature is possible to find some research applying chemostat systems, which are frequently used for specific studies, because the medium condition does not vary during the time. It is also noticed on the literature the influence of light on astaxanthin accumulation by *Haematococcus pluvialis* (Chaumont Daniel, 1995), and β-carotene by *Dunaliella tertiolecta* (Barbosa et al., 2005). As mentioned earlier, under nutrient deficiency, microalgae accumulates carotenoids. Thus, continuous operation mode was applied to study the influence of nitrate concentration in the feeding cultivation medium of *Haematococcus pluvialis* cultivation. Under adequate nitrogen conditions (adequate for cell growth), when nitrate concentration was higher than 4.7 mM in the feeding cultivation medium, the astaxanthin percentage was lower than 0.1% of dry weight. Under limiting nitrogen concentration, lower than 4.7 mM in the feeding cultivation medium, astaxanthin accumulation was increased, reaching maximum percentages of 0.6% of dry weight (Cerón et al., 2007).

6. Conclusions

Many microalgae, such as *Dunaliella salina*, *Haematococcus pluvialis, and Chlorella,* are considered healthy and sustainable sources of carotenoids and other biomolecules. There are several studies with the goal of making feasible the large-scale production of microalgae, employing different types of cultivation systems and operation regimes. Closed systems seem to be the best option for large-scale microalgae production, mainly because of the high productivity, lower risk of contamination and better control of the parameters that influence cell growth. On the other hand, if extreme conditions are applied (e.g. high salinity or alkalinity), open ponds may be employed considering the low cost and ease of construction and operation. The semi-continuous operating system can be an alternative for achieving high cell densities, with, consequently higher carotenoids accumulation.

CHAPTER II[1]

Modified dispersive liquid-liquid microextraction method applied to high-throughput extraction and analysis of *all-trans* β-carotene from *Dunaliella salina* cultivated in tubular photobioreactors

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Abstract

Dunaliella salina is a halophytic microalga and known for its remarkable ability to produce carotenoids. From a chemical standpoint, the carotenoids are poly-isoprenoid compounds (8 units, 40 carbon atoms), liposoluble pigments, responsible for distinct coloration of various organisms. The β-carotene is the main carotenoid produced by *Dunaliella salina*, with antioxidant activity and is widely used in cosmetics and food industries. A modified dispersive liquid-liquid microextraction (DLLME) method was developed for rapid extraction of β-carotene from *Dunaliella salina* cultivated in tubular photobioreactors. The dispersion and extraction conditions were optimized by design of experiment approach (DoE). The optimum extraction of β-carotene was achieved with benzene (P=2.7) as extractor phase and water with 50% acetone as dispersant. Besides, a tailor-made rapid-screening chromatographic method was developed on a C4 column for separation of geometric isomer of β-carotene, subjected to stress conditions. The proposed method was successfully applied in reliable and efficient analysis of *all-trans* β-carotene from *Dunaliella salina* cultivated in tubular photobioreactors and commercial nutraceutical. The miniaturized extraction (< 10 mL organic waste) and analytical method with short analysis time (<20 min), abide by green chemistry analytical principles.

Keywords: *Dunaliella salina*; Cultivation; Beta-carotene; Dispersive liquid-liquid microextraction; chromatographic analysis

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1. Introduction

The green microalga *Dunaliella salina*, included in the class Chlorophyceae, is unicellular, takes many forms depending on the external environment, halophilic and biflagellate. The *D. salina* can accumulate up to 10% of dry weight for β-carotene under adverse growth conditions such as light, pH, culture medium (high salinity and nutrient limitation) and extreme temperatures (A. Ben-Amotz, Lers, & Avron, 1988; Guedes, Amaro, & Malcata, 2011; Lamers, Janssen, De Vos, Bino, & Wijffels, 2012; Loeblich, 1982). The production of β-carotene is often associated to algal protective capacity against light induced cellular injury. Typically, *D. salina* accumulates glycerol in the cytosol to create osmotic resistance against invasions (Ami Ben-Amotz, Sussman, & Avron, 1982).

The poly-isoprenoid chemical structure of β-carotene is presented in Figure 1, with 40 carbon atoms.

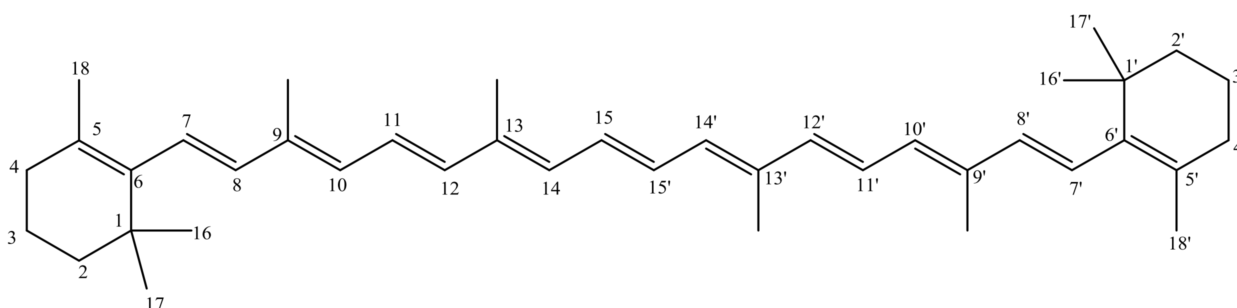

Figure 1. Chemical Structure of β-carotene. Adapted from Guedes et al. (Guedes et al., 2011)

The β-carotene is a powerful antioxidant and is often used as an additive in multivitamin preparations. Its application and use is wide spread, from pharmaceutical, food, feed industries to cosmeceutical and health foods market (nutraceuticals and functional foods) (Ariede et al., 2017; Bendich & Langseth, 1989; Borowitzka, 1988; Raja, Hemaiswarya, & Rengasamy, 2007; Saini & Keum, 2018; Villarruel-López, Ascencio, & Nuño, 2017). The global market for carotenoid was estimated at US$ 1.5 billion in 2014, with β-carotene occupying the major part of production (about 23% in 2010) (Borowitzka, 2013). With an annual growth of 3.9%, it is expected to reach US$ 1.8 billion in 2019 (Ulrich Marz, 2015).

The classical sample preparation techniques for extraction of carotenoids especially in plant and algal samples generates considerable amount of organic wastes. The miniaturized extraction technique such as Dispersive Liquid-Liquid Microextraction (DLLME) abide by the green chemistry principles. Originally introduced by Rezaee and coworkers (Rezaee et al.,

2006), DLLME make use of a dispersing solvent (few milliliters), normally miscible in the extractive organic phase (few microliters) as well as in the aqueous sample matrix (few milliliters). Though highly efficient, DLLME is restricted to the extraction of analytes in biological matrixes. Focusing on lyophilized *D. salina* samples, it was learned that the rehydration of microalgal cells is of great importance to promote effectual contact between extractor phase and carotenoid therein.

Few chromatographic methods were found in the literature that were used in the analysis of β-carotene in microalgae. All separations were made in reverse phase or polar organic mode using octadecylsilane (C18) stationary phases (Anila, Simon, Chandrashekar, Ravishankar, & Sarada, 2015; H. F. Chiu et al., 2017b; Chou et al., 2010; Fazeli, Tofighi, Samadi, & Jamalifar, 2006; Srinivasan, Babu, & Gothandam, 2017). The C30 chain stationary phase are addressed for the separation of a series of carotenoids, including geometric isomers (Britton, Liaaen Jensen, & Pfander, 2009). Although, the natural biosynthetic pathway generates mainly *all-trans* carotenoids, they can isomerized to *cis* carotenoids under determined conditions (Jian-guo, Chao-yuan, Nian-hong, Yu-jun, & Li-dong, 1996). The C18 and C30 chromatographic phases require long run times and stronger mobile phase modifiers, which, sometimes, are inadequate especially when the purpose is to quantify the major carotenoid only.

The objective of this study was to develop a dispersive liquid-liquid microextraction (DLLME) method for rapid extraction of β-carotene from *Dunaliella salina* cultivated in fed-batch tubular photobioreactors, and to minimize isomerization during extraction and analysis of β-carotene by a tailor-made high-throughput chromatographic method.

2. Experimental

2.1. Chemical and reagents

2.1.1. Reference substance

The β-carotene reference substance was obtained from Sigma-Aldrich (Brazil) with declaimed purity of >97%.

2.1.2. Cultivation of Dunaliella salina in tubular photobioreactor

Dunaliella salina 19/18, was obtained from the Culture Collection of Algae and Protozoa – CCAP (Scotland, UK) and cultivated in tubular photobioreactor in which the culture was circulated by an airlift pump with aeration rate of 30.85 L h^{-1}. The cells were grown in f/2

medium (Robert R. L. Guillard, 1975). The model used was based on the photobioreactor described by Carvalho and collaborators (João C. M. Carvalho, Matsudo, Bezerra, Ferreira-Camargo, & Sato, 2014) with working volume of 3.2 L (illuminated volume - 2.0 L). The homogeneity was achieved by using a magnetic stirrer, with two nylon sphere 8 mm, in degasser flask (Pérez-Mora et al., 2016).

The fed-batch cultivation of the *D. salina* was carried out under controlled pH (7.0 ± 0.2) by CO_2 injection, temperature at 24 ± 1 °C, and with a light intensity of 50 μmol photons m^{-2} s^{-1} (illuminated by two fluorescent lamps of 20W) under photoperiod of 12:12 light/dark. In such fed-batch process, the nitrogen and phosphorus consumed by cell growth, estimated by percentage of these elements in dry cell, were restored at the times corresponding to the measurement of cell concentration, by addition of sodium nitrate and sodium monobasic phosphate. The cell concentration was determined by absorbance and correlated with both dry cell concentration and number of cell per unit of volume. The maximum cell concentration was of 6.93 x 10^5 cells mL^{-1} at 25th day of cultivation and after this time it was ceased the dark period and the light intensity was raised at 100 μmol photonsm^{-2} s^{-1}. Additionally, the fed of nutrients was interrupted. At the 30th day of cultivation, the cell concentration dropped to 5.19 x 10^5 cells mL^{-1} and the cells, whose coloration became orange, were harvested and centrifuged at 3419 g, at 10° C for 20 min. The cell pellets were washed twice with ammonium formate (1.5 M) to remove salts (Fachet, Hermsdorf, Rihko-Struckmann, & Sundmacher, 2016). The lyophilized cells were stored at -18 °C and were used in the dispersive liquid-liquid microextraction of β-carotene.

2.1.3. Gelatinized capsules

The gelatinized capsule of β-carotene, with declared 3.6 mg/capsule (6000 IU), were obtained from local nutritional supplements store. The pooled content of ten capsules was used for extraction and analysis.

2.1.4. Solvents and reagents

Acetonitrile and methanol were of HPLC grade (J. T. BAKER®, PA, USA) and ultra-pure water was obtained from a Milli-Q® Plus (Millipore, MA, USA) water purification system.

The reagent grade solvents such as acetone, benzene, chloroform, ethyl acetate, hexane and dichloromethane were used in the DLLME steps (Millipore, MA, USA and Merck, Darmstadt, Germany).

2.2. Instrumentation and analytical conditions

Chromatographic separations were obtained using a Shimadzu liquid chromatographic system consisting of a solvent delivery pump system model SCL-10Avp, an online degasification system model DGU-14A, an auto-injector model SIL-10AD-vp (fitted with a 20 µL loop), a UV detector model SPD-M10Avp. The output signal was monitored and integrated using CLASS VP® V6.14 software (Shimadzu® Corporation, Japan). Chromatographic separations were obtained with a YMC-Pack C4 (Butyl) analytical column (250x4.6mm, 5 µm). The signal was monitored by a PDA detector from 190 to 800 nm and for quantitative analyses of β-carotene, the wavelength was set at 450 nm.

The mobile phase was composed of methanol and water (95:5, v/v). The mobile phase was prepared at the beginning of each day and degassed by sonication for 20 min. All samples were filtered through a 0.45 µm Millex® HV membrane. The mobile phase flow rate was set at 1.0 $mL.min^{-1}$, column temperature set at 40 ± 1°C. A 5 µL aliquots of standard and sample solutions were injected into the system.

2.3. *Preparation of standard solutions*

A mass equivalent to 10.0 mg of β-carotene reference substance was accurately weighed and transferred to a 50mL volumetric flask. The volume was completed with ethyl acetate to obtain a stock solution containing 200 $\mu g.mL^{-1}$ of β-carotene.

2.4. Design of experiment (DoE) applied to extraction of β-carotene from D. salina

A design of experiment (DoE) approach was used to optimize extraction of β-carotene from cultivated *D. Salina*. The modified DLLME conditions were evaluated using a response surface methodology (RSM). The factors analyzed were, solvent polarity parameter (R. M. Smith, 1995), solvent volume and sonication time. The solvents were selected in-order-to cover a wide range of polarity index (P) and prevent rapid degradation of β-carotene. The selected solvents, in ascending polarity index (P) were, hexane (P=0.01), benzene (P=2.7), dichloromethane (P=3.1), chloroform (P=4.1), ethyl acetate (P=4.4). The DoE was based on a two (2) level factorial, with a central point, for each solvent, totalizing 25 extractions, as shown in Table 1.

2.4.1. Optimization of modified DLLME procedure

Initially, 10.0 mg of lyophilized *D. salina* biomass was weighed into a 15 ml ampule. An aliquot of 6.0 mL of a 15% solution of acetone in water was added and stirred vigorously to promote dispersion and rehydration of solid material. The ampoule was then subjected to an ultrasonic bath (20 min) to efficiently lysate the cell and plastid membranes. The resulting contents in the ampoule were transferred, in equal parts of 1.0 ml each, to five labelled ampoules (Table 1).

Table 1 - The design of experiment (DoE) layout for each solvent

	Vial 1	**Vial 2**	**Vial 3**	**Vial 4**	**Vial 5**
Solvent Volume	3 mL	6 mL	3 mL	6 mL	4.5 mL
Ultrasonic bath	5 min	5 min	15 min	15 min	10 min

From vial 1 to 4, extractor solvent equivalent to 3.0 or 6.0 mL was added, stirred vigorously and sonicated for 5 to 15 minutes. The fifth ampoule was used as the central point of factorial design. In this case, the extracting solvent volume and ultrasound time were 4.5 mL and 10 minutes, respectively. For extraction, solvents with wide range of polarity were used (described in previous section). After dispersion and extraction step, the ampules were centrifuged for 10 minutes at 2000 rpm, at room temperature. The acceptor phase was transferred to fresh ampoules and subjected to dryness under nitrogen flow. The dried material was reconstituted in 1.0 mL 50:50 (v / v) chloroform/methanol. An aliquot equivalent to 5µL was injected into chromatographic system. All procedures were performed in duplicate, including chromatographic analysis.

2.5. Optimum conditions for extraction of β-carotene from D. salina samples

A mass equivalent to 10.0 mg of lyophillized *Dunaliella salina* was weighed in screw top vials. A 12-mL aliquot of cold acetone with water (50%) was transferred to the vial, along with 5 glass beads that helped to homogenize the sample. The vial was closed and submitted to an ultrasonic bath for 20 minutes. An aliquot of 1.0 mL of the above liquid was transferred to a clean vial, followed by 2.0 mL of water (dispersant) and 6.0 mL of benzene (extractor). The

new vial was closed and vigorously shaken, then put in ultrasonic bath for additional 5 min. These conditions were obtained from the previously optimized conditions (section 2.4). The phase separation was accelerated by centrifugation (2000 rpm, 5 min). An aliquot of 5.0 mL of the extractor organic phase was collected and dried under nitrogen flow. The dried material was resuspended in 3.0 mL of ethyl acetate, filtered through 0.45 μm PVDF® membrane (Millipore, MA, USA) and 5μL aliquot was injected into the chromatographic system.

2.6. Extraction of β-carotene from commercial capsules

Ten gelatinized capsules were weighed, individually, and the mean content of one capsule, equivalent to 108.9 mg, was transferred to a screw top amber vial. The sample solutions were prepared following procedure described in section 2.5.

2.7. *Validated chromatographic method applied to analysis of β-carotene*

The validation of an analytical method is integral part of method development and are carried out to prove that the method can be used reliably for its intended purpose. To assess adequateness of the proposed method for a defined analysis, following parameters were evaluated: specificity/selectivity, linearity, precision, accuracy, detection and quantitation limits (International Conference on Harmonization, 1996; Ludwing Hubeer, 2001; Pal, Dan, & Das, 2014)

The specificity and selectivity of the proposed method was evaluated by spectra purity of β-carotene peak obtained in the analysis of *D. salina* and commercial capsules. Besides, visible absorption spectra of β-carotene extracted from sample solutions were compared with the β-carotene reference substance. The repeatability and intermediate precision were determined by repeated analysis of multiple sampling of the same homogeneous sample, on two consecutive days and are expressed as relative standard deviation (RSD).

The linearity was studied by duplicate injection of standard solutions within concentrations ranging from 13.125 to 93.125 $\mu g.mL^{-1}$. The calibration curve was constructed by linear regression of responses with least mean square method. The detection and quantitation limits were assessed based on system signal/noise ratio. The estimated values were confirmed by actual analyses of β-carotene sample solutions. The accuracy of the proposed method was estimated based on recovery tests, carried out at three different concentrations, within the linear dynamic range of calibration curve. For that sample matrixes were spiked with known

concentration of β-carotene standard and submitted to extraction process. The accuracy was accessed based on the recovery of β-carotene standard from processed sample matrix.

2.8. *Stability of β-carotene solutions*

The stability of β-carotene was assessed by exposing the standard solutions of β-carotene to light, acidic and oxidative conditions. The standard substance solutions (5.5 μg.mL^{-1}) were exposed to day light, 0.1N HCl and 0.3% of hydrogen peroxide solution, for 2 hours period, in separate vails. The sample solutions were processed as described in section 2.5 and were kept in dark, except in case of day light. After 2 hours of exposure, an aliquot equivalent to 5μL was injected into chromatographic system, in each case.

3. Results

The chromatographic separations were obtained in reverse phase mode, using a mixture of methanol:water (95:5 v/v) as a mobile phase. The β-carotene can be eluted rapidly from column by increasing methanol content in the mobile phase, however several carotenoids, of miner expression, coelute with β-carotene peak. The polar organic mode, with methanol as mobile phase, might be useful for analysis of total carotenoid content in *D. salina*. Since objective if this study was to develop rapid screening method for β-carotene only (*cis* and *trans*), the methanol content was optimized to 95%. The representative chromatogram of β-carotene from the commercial capsules (minor), β-carotene reference standard (50.0 μg.mL^{-1}) and β-carotene extracted from *D. salina* using modified DLLME method can be observed in Figure 2.

3.1. System suitability

The system suitability parameters, an integral part of the liquid chromatographic method, were satisfactory under described analytical conditions (The United States Pharmacopeial Convention, 2017). The total analysis time was 20 minutes with capacity factor (K'=3.6) for *all trans* β-carotene peak. The main peak resolution was 1.6 in relation to nearest eluting peak (*cis* β-carotene), mean peak asymmetry was 1.2, with mean tailing factor less than 1.2. These parameters demonstrate the adequateness of the proposed method for its intended purpose.

3.2. Modified DLLME applied to β-carotene extraction

A design of experiment (DoE) approach was successfully applied in the optimization of extraction conditions for β-carotene. The extraction conditions and extraction efficiency were evaluated using a response surface graphs and results are presented in Figure 3.

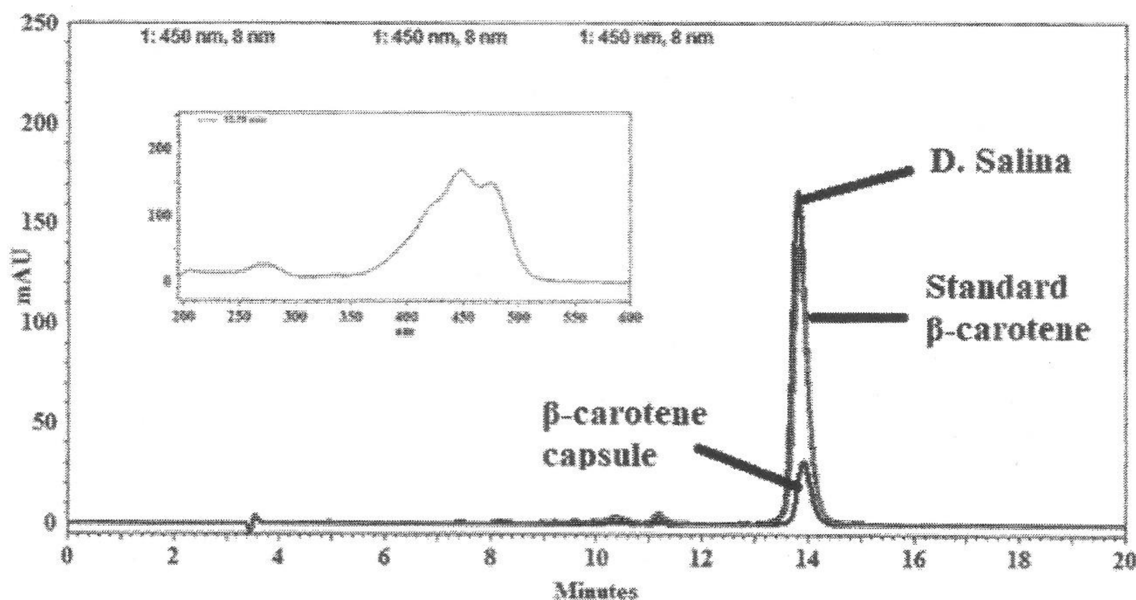

Figure 2. Representative chromatograms of β-carotene from the commercial β-carotene capsules (minor), reference standard β-carotene (50.0 $\mu g.mL^{-1}$) and extracted from *D. salina* using modified DLLME method.

Amongst all solvents, most efficient extraction was obtained with benzene. Optimum extraction of β-carotene was obtained with 5 minutes ultrasonic bath, with 6.0 mL of benzene as acceptor phase.

3.3. Chromatographic method validation parameters

3.3.1. Specificity of proposed method

The peak purity index was evaluated within the wavelength range of 300 to 550nm for β-carotene peak. The peak purity index was better than 0.9999 for both the gelatinized capsule and the *D. salina* samples. Thus, the proposed chromatographic method could be considered specific towards *all-trans* β-carotene.

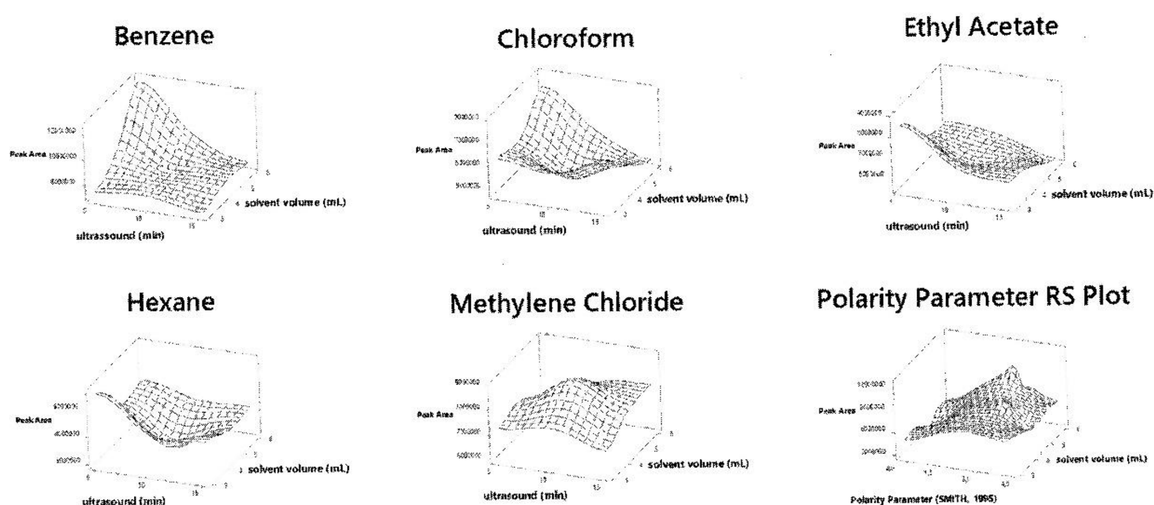

Figure 3. Response surface graphics for solvents utilized in the DoE experiments. A combined graphic utilizing polarity parameter was also plotted.

3.3.2. Precision of proposed method

The repeatability of the method was assessed by analyzing a single sample in replicate (n=10). The mean relative standard deviation (RSD) amongst the responses was 3.19%. The reproducibility of method was assessed by comparing the results of repeatability on two consecutive days. The mean RSD was less than 2.57%. For complex samples such as biological fluids, plants and microalgae, relative standard deviation below 5% is acceptable (BRASIL, 2017; ICH, 2005). Table 2 presents the results obtained for the repeatability assessment with mean values with RSD for areas (n=10), retention time (Rt), theoretical plates (N), selectivity (α) and peak asymmetry (As).

3.3.3. Accuracy of proposed method

The mean recovery of the standard β-carotene from the sample metrics, at three concentration levels were better than 98.38%. A near total recovery of the standard from sample matrix proves accuracy of the proposed method.

Table 2. The results obtained in the assessment of precision and adequateness of proposed method

Mean area*	Retention time* (Rt)	Theoretical plates* (N)	Selectivity* (α)	Peak asymmetry* (As)
1704005 (day 1)	14.01	11576	2.95	1.18
RSD= 2.05	RSD=2.20	RSD=5.56	RSD=3.02	RSD=1.41
1746903 (day 2)	13.80	11752	2.95	1.18
RSD= 3.19	RSD=0.08	RSD=7.72	RSD=0.14	RSD=1.39

* Mean of ten injections

3.3.4. Linearity of proposed method

The method linearity can be described by the equation y= 57802x-345745, with coefficient of correlation better than 0.9966. The residues of standard deviations graph show that there are no trends, i.e. the points are uniformly distributed above and below linearity curve. Based on the linear regression data and unbiased residual standard deviation, it is possible to confirm that the method is linear in a concentration range from 13.125 and 93.125 µg.mL^{-1}. The detection and quantitation limits estimated based on signal/noise ratio were 0.24 and 0.72 µg.mL^{-1}, respectively.

The estimated amount of β-carotene in cultivated *D. salina* was about 10% of the dry mass (~100mg/g of dry mass). The results are in congruence with those found in the literature (C.-C. Hu, Lin, Lu, Chou, & Yang, 2008; Shen et al., 2012). The β-carotene content in commercial capsule was 85.08% of the labeled value.

3.4. Stability of β-carotene

To evaluate the stability of β-carotene, reference standard solutions were prepared in methanol and were subjected to predefined conditions, followed by chromatographic analysis. The obtained results were compared with those obtained with freshly prepared sample solutions. After 2 hours storage period, exposed to day light, there was a slight reduction in the peak area (12.5%). Besides, a peak characteristic of *cis* β-carotene appeared immediately before mean peak (Figure 4).

The *cis* β-carotene peak was identified based on its retention time and characteristic spectral fine structure (Figure 5). According to literature, β-carotene *cis* isomer present additional absorption band at 340 nm. Further, a *cis*-isomer of β-carotene has λ_{max} slightly lower than those of *trans* β-carotene (λ_{max} 450nm) (Azevedo-Meleiro & Rodriguez-Amaya, 2004; Rodriguez-Amaya & Kimura, 2004).

There were no significant differences in the β-carotene content when stored in tightly closed container, at room temperature (24 °C), protected from light. The sample solutions subjected to acidic hydrolyses (0.1N HCl) and harsh oxidation (0.3% H_2O_2) presented rapid and total degradation after 2 hours.

4. Discussion and conclusions

The proposed modified dispersive liquid-liquid microextraction method was helpful in removing cell debris from the injection material and ensure chromatographic system suitability.

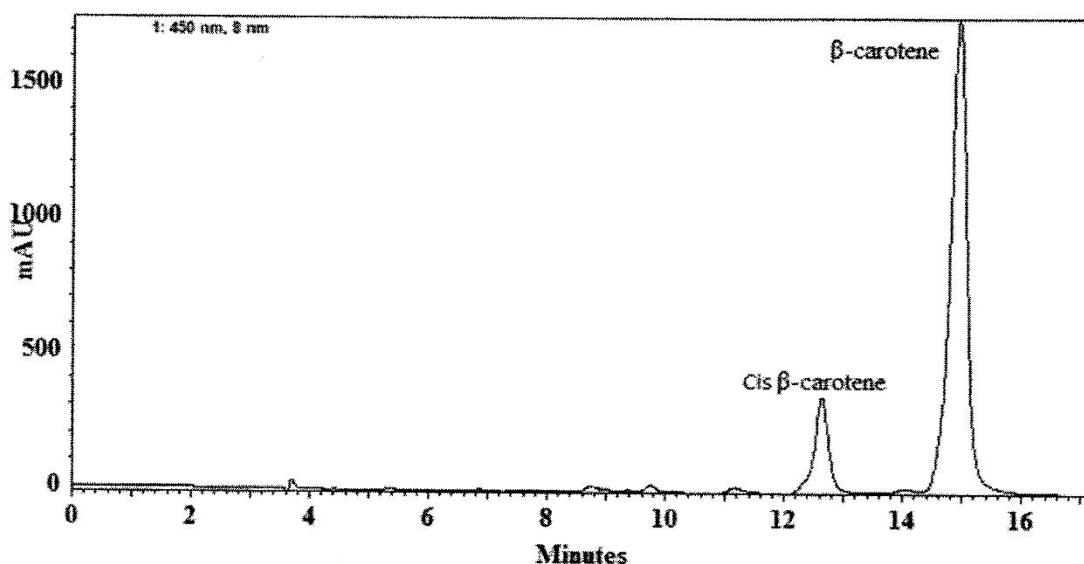

Figure 4. Chromatogram of β-carotene obtained from standard sample subjected to environmental conditions (day light, with air).

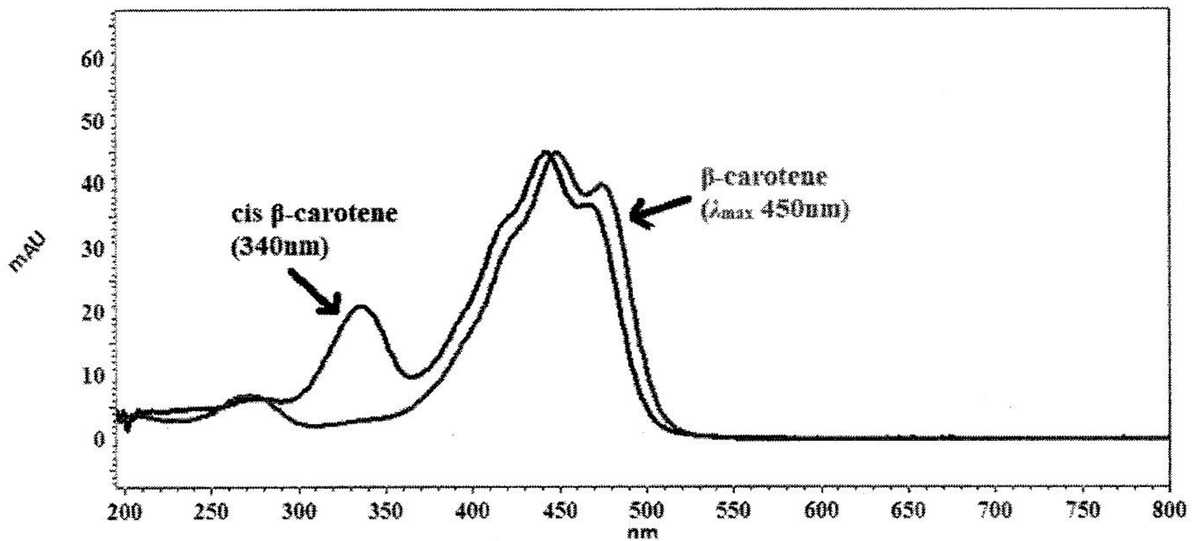

Figure 5. Absorbance spectrum of *all trans* and *cis* β-carotene from *D. salina* sample in methanol:water (95:5 v/v).

The volume of extractor phase was increased from few microliters to milliliters scale to handle cultivated *D. salina* samples with cell debris and accumulated hydrophobic material from harvested cells.

Though the antioxidant properties of β-carotene are interesting for biological purposes, for the analyst it is challenging. During sample preparation and extraction steps, care must be taken to minimize exposure of carotenoids, specifically, β-carotene to air, light and acidic media. Crafts and coworkers suggested tetrahydrofuran (THF) as a diluent in chromatographic analysis of β-carotene (Craft & Soares, 1992). However, the THF needs to be stabilized with antioxidants such as BHT. The samples prepared in THF, with or without BHT presented considerable degradation and variations in the peak area of β-carotene, during 8 hours analysis period.

The β-carotene presented excellent solubility in ethyl acetate as well as stability during 8 hour analysis period, thus final dilutions were made in this solvent, before injection.

The chlorinated solvents (such as chloroform and methylene chloride) presented satisfactory extraction efficiency, as observed in preliminary studies. The benzene was selected as an extracting solvent instead to avoid HCl residuals from chlorinated solvents. The HCl can adversely affect the stability of carotenoids and is widely reported in the literature (Britton et al., 2009; Rodriguez-Amaya & Kimura, 2004; Rossberg et al., 2011).

Unexpectedly, the hexane which is a most lipophilic solvent (P=0.01) of the series, provided lower extraction efficiency for β-carotene. Probably, the hexane had limited access to β-carotene, that was "encapsulated" within the hydrophilic sample matrix (raptured cell wall, amino acids etc).

The use of ultrasonic bath for longer periods (20 min) adversely affected the extraction of β-carotene. This could be attributed to heat generated during sonication process, which enhance degradation kinetics. The use of ultrasonic bath was restricted to minimum, just sufficient to promote droplet formation (extractant), sample homogenization and mass transfer.

The DLLME technique make use of few microliters of extractor phase to extract drugs and metabolites from biological matrixes. The similar volumes were infeasible in case of lyophilized cultivated microalgae due to inherent physical state (dry powder). The modified DLLME method and a tailor-made rapid screening chromatographic method was successfully applied in the analysis of β-carotene extracted from *Dunaliella salina*, cultivated in tubular photobioreactors. The fully validated method is suitable for quantitative and selective determination of β-carotene in gelatinized capsules, as well.

CHAPTER III

Influence of nutrients in the β-carotene production on semi-continuous culture of *Dunaliella salina* in Tubular Photobioreactor

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Abstract

Natural pigments from microalgae have applications in the nutraceutical, food and cosmetics industries, as well as in aquaculture. *Dunaliella salina* has been used to commercially produce β-carotene. It was evaluated the semicontinuous cultivation of *D. salina* under different fraction of medium replenishment (R), concentration of sodium nitrate ((75 mg L^{-1}, named 1N; 112.5 mg L^{-1}, named 1.5N; 225 mg L^{-1}, named 3N) and monosodium phosphate dihydrate (5.65 mg L^{-1}, named 1P; 8.47 mg L^{-1}, named 1.5P; 16.95 mg L^{-1}, named 3P) using cultivation medium f/2, which has as basis the sea water. They were obtained higher cell productivities in semicontinuous processes than in batch process, with mean productivities up to 6.7 x 10^4 cells mL^{-1} d^{-1} (medium 1N:1P; R =20%). The maximum cell concentration (Xm) obtained in this work was not dependent of R. The best results of Xm were obtained when using medium 1.5N:1.5P instead of medium 1N:1P, with mean value up to 5.6 x 10^5 cells mL^{-1} with R = 80%. The content of β-carotene in the cells, in a general way, was higher in cells grown in medium 1N:1P than in medium 1.5N:1.5P, with maximum mean results up to 53.4 mg g^{-1} and 57.5 mg g^{-1}, with R of 20% and 80%, respectively. The cultivation of the cells with media 3N:3P led to a long lag phase, followed by a both decrease in cell density and their lysis. The cultivation of cells in a tubular photobioreactor contributed to have a cell growth without contamination by protozoa.

Keywords: carotenoids; carotenoids sources; microalgae; microalgae application; cultivation process.

1. Introduction

Fruits and vegetables are the main sources of carotenoids, but microalgae are promising alternative for the production of these natural pigments. Besides carotenoids, these photosynthetic microorganisms are rich in lipids, polyunsaturated fatty acids, carbohydrates, proteins, and vitamins. In addition, microalgae cultivation have advantages over higher plants such as rapid growth, absence of seasonality, and sustainable cultivation among other profitable characteristics (Ahmed et al., 2014; Priyadarshani & Rath, 2012). The carotenoids are hydrocarbons divided into two groups: carotenes containing only carbon and hydrogen (e.g., β-carotene and lycopene) and xanthophylls containing carbon, hydrogen and oxygen (e.g., lutein, zeaxanthin, and astaxanthin) (Saini et al., 2015). It is known that a diet rich in natural pigments and other compounds such as vitamins, fatty acids, and polysaccharides, protects the body from various diseases including obesity, cardiovascular disease, eye diseases (e.g., age-related macular degeneration), and certain cancers, among others (Bhosale & Bernstein, 2005; De Jesus Raposo, De Morais, & De Morais, 2015). This is due to the high antioxidant activity of these bioactive compounds. Carotenoids also have applications in the food, pharmaceutical, and cosmetics industries, besides in aquaculture. In fact, it is possible to see application of it in dyes, functional foods and food supplements, nutraceuticals, sunscreen, and feed additive (e. g., eggs, fish, and shrimp) (De Jesus Raposo et al., 2015; C. C. Hu et al., 2008; Sathasivam & Ki, 2018). Currently, only few microalgae carotenoids are produced and marketed around the world, and countries like Australia, USA, Israel, Japan, China, and India are example of producers of these natural pigments. In this sense, the halophilic microalga *Dunaliella salina* may be applied for the production of β-carotene (pro-vitamin A), the freshwater *Haematococcus pluvialis* for astaxanthin (powerful antioxidant), and *Muriellopsis sp.*, *Chlorella protothecoides,* and *Scenedesmus almeriensis* may be employed for the production of lutein (eye health) (Blanco, Moreno, Del Campo, Rivas, & Guerrero, 2007; M. A. Borowitzka, 2013). Induction of β-carotene synthesis (carotenogenesis) by green microalga *Dunaliella salina*, which can accumulate more than 10% dry weight of this orange pigment, occurs under stress conditions such as high light intensity, high salinity, low-temperature, nutrient deprivation – mainly nitrogen and phosphorus (Guedes et al., 2011; Mendoza, Jiménez Del Río, García Reina, & Ramazanov, 1996; Solovchenko, 2013). It is already known that microalgal growth is influenced mainly by the sources of nitrogen, phosphorus, and carbon. Phosphorus is an essential nutrient and has a key role in the conveyance of metabolic energy, for example, nucleic acid biosynthesis, phospholipid biosynthesis, and membrane formation (Dahmen-Ben Moussa,

Chtourou, Karray, Sayadi, & Dhouib, 2017; Paliwal et al., 2017). Nitrogen is extremely vital to microalgae and it not only plays a role as essential building block of nucleic acids and amino acids, but its availability may affect carotenoid accumulation (J.C.M. Carvalho et al., 2014; Del Campo et al., 2000; Ho et al., 2015). This bioproduct represents a growing market, but it is known that the cost for carotenoids production is high. Growth optimization, genetic engineering, and efficient bioreactor designs and culture system are some items of algal biotechnology that can be improved, making large-scale production economically viable (Matos, 2017; Pires, Alvim-Ferraz, & Martins, 2017). Closed bioreactors (PBR) allow the optimization and control of some parameters such as pH, light intensity, temperature, higher biomass yields, among others (J.C.M. Carvalho et al., 2014). Various studies show that the tubular photobioreactor is the type of culture system most suitable for the production of cyanobacteria (Ferreira et al., 2012; Matsudo et al., 2011) and microalga (Pérez-Mora et al., 2016), and that the semi-continuous operation regime is successful in laboratory scale for lutein (carotenoid) production by microalga *Chlorella minutissima,* (Dineshkumar et al., 2016) and *Scenedesmus obliquus* FSP-3 (Ho et al., 2015). The choice of parameters for the growth of the *Dunaliella salina* microalga were based in the above mentioned citations. This study aimed to improve the performance of *Dunaliella salina* for the production of β carotene carotenoid. For this purpose, different concentrations of nitrate and phosphorus were evaluated on a semi-continuous process, in a tubular photobioreactor.

2. Materials and methods

2.1. Microalga strain, cell maintenance and inoculum production

The microalga used in this study was *Dunaliella salina* 19/18, obtained from the Culture Collection of Algae and Protozoa – CCAP (Scotland, UK), and maintained in test tubes with f/2 medium solidified with 2% agar, in room temperature 25 ± 2 °C. The f/2 medium is composed of (per litre) 0,997 L of sterile natural seawater (sterilized by autoclaving for 20 min at 121°C), 1 mL of stock solution (75 g $NaNO_3$ and 5.65 g $NaH_2PO_4.2H_2O$ per liter of deionized water), 1 mL of trace elements solution and 1 mL of vitamin mix solution. The trace elemental solution (per litre) includes 4.16 g Na_2EDTA, 3.15 g $FeCl_3.6H_2O$, 10 mg $CuSO_4.5H_2O$, 22 mg $ZnSO_4.7H_2O$, 10 mg $CoCl_2.6H_2O$, 0.18 g $MnCl_2.4H_2O$, 6 mg $Na_2MoO_4.2H_2O$. The vitamin mix solution (per litre) includes 100 mg vitamin B1 (thiamine), 0.5 mg vitamin B12 (cyanocobalamin) and 0.5 mg biotin (R.R.L. Guillard, 1975). Cells were grown in gradually

larger flasks up to 1 L Erlenmeyer flasks containing up to 400 mL of f/2 medium. These flasks allowed the cell growth on a rotary shaker (100 rpm), under light intensity of 50 μmol photons m^{-2} s^{-1}, at 25°C. For obtaining biomass enough to achieve initial cell concentration of 2.5 x 10^5 cel/mL, *D. salina* was furtherly cultivated in the same medium in tubular photobioreactor described in item 2.2.1, in the same conditions of light intensity, temperature and with the use of CO_2 for maintaining pH at 7.0, as also described in item 2.2.1.

2.2. Experimental

The cultivations were carried out in two stages. At first, it was carried out in tubular photobioreactor, with the aim of obtaining high cell concentration. When stabilization of cell concentration took place, culture was partially discharged from bioreactor and replaced by same volume of fresh medium, starting a new cycle. In the second stage, cells removed from the photobioreactor (from startup batch process and up to three sequential cycles) were submitted to stress condition by simultaneous lack of nutrients and high pH, in Erlenmeyer flasks. Details of semi-continous process will be given in item 2.2.2.

2.2.1. Tubular photobioreactor and growth conditions

The airlift bioreactor used in this study was developed at the Fermentation Technology Laboratory at the Department of Biochemical and Pharmaceutical Technology of São Paulo University (see details Fig. 1). It was made from 40 transparent glass tubes (1.0 cm internal diameter and 0.5 cm wall thickens) with a slope of 2% (1.15°) to facilitate the flow of culture medium, these tubes were connected with silicone hoses. *Dunaliella salina* was inoculated into the reactor with an initial cell density of 2.5 x 10^5 cells mL^{-1}. The cultivation was submitted to 12 h light/12 h dark cycle (Guermazi, Masmoudi, Boukhris, Ayadi, & Morant-Manceau, 2014), using two fluorescent lamps of 20W with a ligth intensity of 50 μmol photons m^{-2} s^{-1}. The reactor was operated at 24 ± 1 °C, pH 7.0 ± 0.2 by CO_2 injection, through a pH meter (Mettler Toledo – M300) connected to a solenoid valve (Y. Xu, Ibrahim, & Harvey, 2016). Cell suspension was maintained under circulation at a flow rate of 30.85 L h^{-1} through an air lift system and a magnetic stirrer was used to maintain it homogeneous in the degasser flask. Two nylon sphere (8 mm) were incorporated to the system aiming to avoid adhesion of cells to the tubes walls (Pérez-Mora et al., 2016). The total volume of the reactor was 3.2 L and the illuminated volume was 2.0 L.

2.2.2. Semi-continuous mode of photobioreactor operation

D. salina was cultivate by semi-continuous process in tubular photobioreactor. In a startup batch cultivation, when stabilization of cell concentration took place (considering 8.4 % of error in the determination of this parameter), a defined portion of culture was harvested and the same volume of fresh medium was replenished for starting the first cycle. This procedure was performed until the third cycle. Replenishment proportions of 20% and 80% were tested. Also different initial concentrations of $NaNO_3$ (1N = 75 mg L^{-1}; 1.5N = 112.5 mg L^{-1} and 3N = 225 mg L^{-1}) and $NaH_2PO_4 .2H_2O$ (1P = 5.65 mg L^{-1}: 1.5P = 8.47 mg L^{-1} and 3P = 16.95 mg L^{-1}) were evaluated. At the end of each cycle, biomass were harvested according to item 2.2.4.3 for analysis of β-carotene content by HPLC (item 2.2.4.3) and cell-free supernatant was submitted to analysis of nitrogen (item 2.2.4.2.1) and phosphorus (item 2.2.4.2.2).

2.2.3. Carotenogenesis

As commented before, this stage correspond to the stress condition phase in order to get the carotenogenis by the microalgae. Immediately after each culture replacement in the photobioreactor, at the end of startup batch cultivation and each cycle, 600 mL of culture collected from the bioreactor was submitted to carotenogenesis induced by increasing light intensity (150 μmol photons m^{-2} s^{-1}), starvation of nitrogen, and lack of pH control. Carotenogenesis was performed in 3 Erlenmeyer flasks containing 200 mL each in a rotary shaker at 110 rpm and 24 ± 1 °C. Cell morphology and β-carotene accumulation was optically monitored under Olympus microscope (BX 51, Olympus, Tokyo - Japan). Cells were harvested approximately 3 days after the onset of carotenogenesis, when at least 90% of them turned orange, accordind to item 2.2.4. Besides the measurement of pH, at the end of carotenogenesis, cell-free supernatant was submitted to quantification of nitrate and phosphorus according to item 2.2.5.1. and item 2.2.5.2., respectively. Also, at the end of the third cycle, the remaining cells were maintained in the bioreactor and the carotenogenesis took place by continuous illumination, lack of pH control, and lack of fresh medium replenishment.

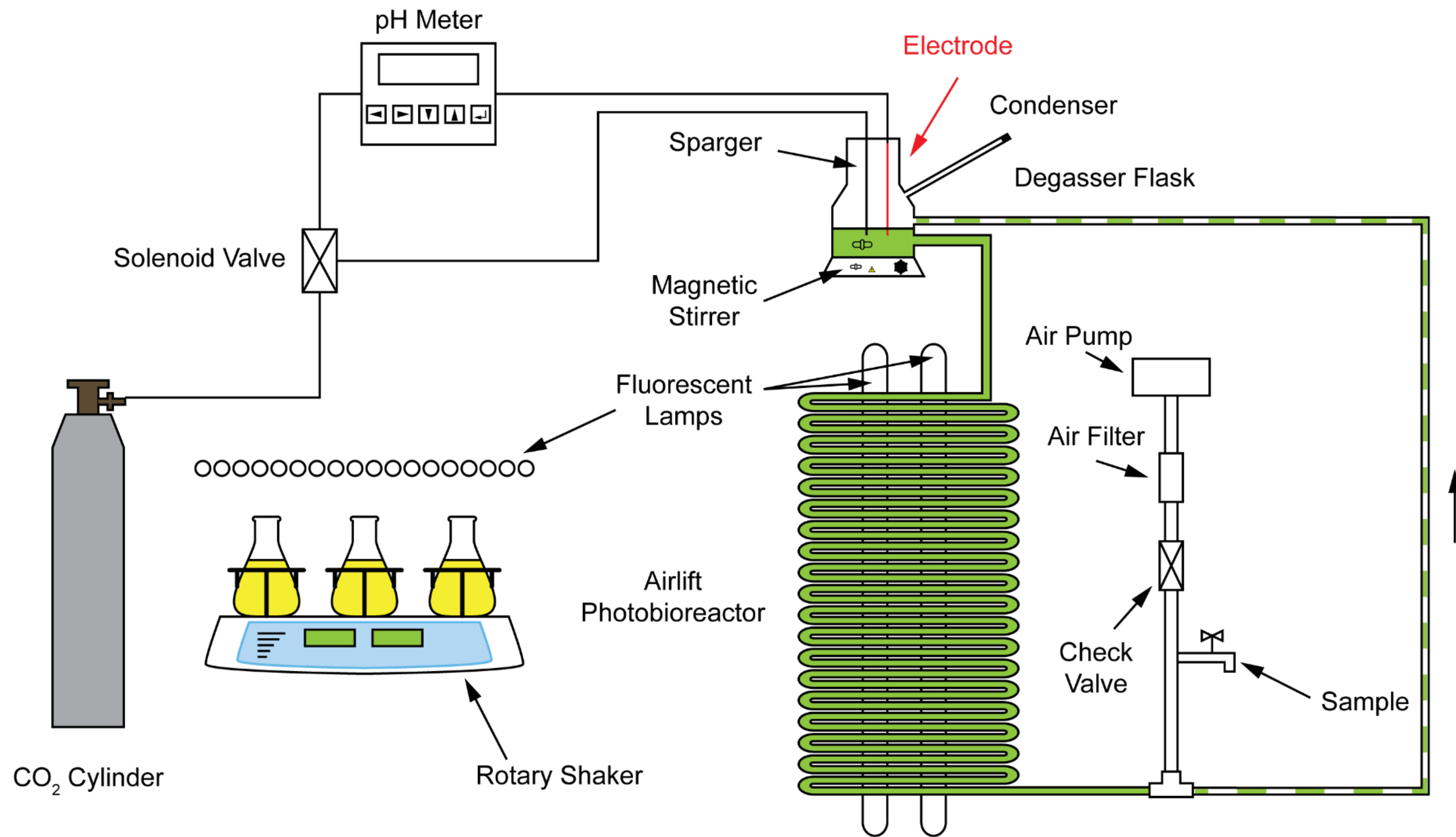

Fig. 1 Schematic diagram of the photobioreactor in the green phase and of carotenogenesis

2.2.4. Determination of cell density

Daily or each 2 days, cell concentration or cell´s number per mL was measured by direct counting the number of cells in a Neubauer chamber using a BX 51 microscope (Olympus, Japan), after fixing the *Dunaliella salina* with Lugol´s iodine solution (ca.0.5%). Before counting cells, the microalgae were observed under the microscope equipped with an Olympus SC-30 digital camera (Olympus - Japan). The microscopic observation also allowed the verification of any contamination and percentage of cells in green phase and in the phase corresponding to accumulation of carotenoid pigments.

2.2.5. Analysis in cell-free supernatant

Samples of 50 mL were centrifuged at 3419 g and 10° C for 20 min and the supernatant was reserved for measurement of nitrate and phosphorus.

2.2.5.1. Residual Nitrate concentration

Concentration of residual nitrate in the culture medium was by indirect colorimetry using salicylic acid, in which nitrate is reduced to nitrite (Cataldo, Maroon, Schrader, & Youngs, 1975). For determination of nitrate, a calibration curve correlating the absorbance (λ = 410 nm) with the sodium nitrate concentration in mM was constructed (Appendix A).

2.2.5.2. Residual Phosphorus concentration

Dissolved inorganic phosphate was used for determination of phosphorus concentration (mg L^{-1}) by the molybdenum blue colorimetric method at λ = 882 nm (CARMOUZE, 1994) using a spectrophotometer, model 700 Plus (Femto) and a calibration curve (Appendix B).

2.2.6. Determination of β – carotene concentration in wet biomass

Samples of the cell suspension were centrifuged at 3419 g, at 10° C for 20 min. Cell pellet were washed twice with ammonium formate 1.5 M to remove salts (Fachet et al., 2016). The wet biomass pellet was stored at – 18 ° C until analysis of β-carotene content. This procedure was carried out at the end of the stress phase in the Erlenmeyer flasks and at the end of the bioreactor culture.

Carotenoid was extracted from 10 mg of wet biomass pellet using 6 mL of 15% acetone followed by 20 min of ultrasonic bath. After that, 1 ml of the lysate was collected, 6 ml of the extractor solvent (benzene) was added and it was maintained in ultrasonic bath for additional 5 min. The organic phase was separated by centrifugation (10 min at 2000 rpm) at room temperature (20 °C), 5 mL of supernatant (organic phase) was collected and evaporated under nitrogen gas, and dry material was resuspended with chloroform and methanol (1:1). Samples were filtered with syringe filter (0.45 μm) before HPLC analysis. In addition, extractions were performed at room temperature (20 °C) under low light conditions.

β-carotene was quantified by High Performance Liquid Chromatography (HPLC), with the use of a Shimadzu HPLC system equipped, and injection volume of 20 μL. The reverse phase chromatographic method was developed using an YMC-Pack Pro C-4 (4.6 mm x 250, 5.0 μm) (YMC, USA) with isocratic elution. The mobile phase consisted of methanol/water (v/v, 95:5) at a flow rate of 1 mL min^{-1}. The β – carotene concentration was determined by comparing the peak area (measured at 450 nm) with that of standard (Sigma Aldrich). Calibration curve was constructed for quantification of carotenoid in sample. For the calculation of β-carotene content, dry weight of all harvested samples was determined.

2.3. Kinetic Parameter

The cell productivity (P_x) was calculated using the equation 1 (Eq. 1):

$$P_x = \frac{X_m - X_i}{t_c} \quad (1)$$

where, X_i e X_m represent the cell density of microalga (number of cells mL^{-1}) at the beginning of each cycle and maximum cell density, respectively, and t_c the time correspondent to this variation of cell density.

2.4. Statistical analysis

All growth experiments in photobiorreactor were performed in duplicate and data were statistically analyzed by one-way analysis of variance (ANOVA), considering them significant when $p < 0.05$ (confidence level of 95%).

3.Results and discussion

Cell growth of *Dunaliella salina* CCAP 19/18 and its β-carotene production were studied using different fresh medium replenishment proportions (20% and 80%) and different concentrations of nitrate and phosphours in the culture medium in tubular photobioreactor. This study was divided into two stage: the first one was the cell growth (green phase) in photobioreactor and the second one was performed with the increase of light intensity under nutrient starvation and lack of pH control for inducing the production of β-carotene in rotary shaker (carotenogenesis). Besides, just after the last cycle of semi-continuous process in photobioreactor, the stress condition was induced by the absence of light/dark cycle and by the lack of pH control as well, since higher pH values favors (8.5 - 9.0) the carotenoids production by *D. salina* (Dhaka & Pal Singh, 2018). Before that, it was not possible to apply such stress, since the photobioreactor had been used to produce cell, being maintained conditions to promote the cell growth inside the photobioreactor.

As pointed out before, in the production of β-carotene by *D. salina,* it is necessary to submit the cell to the stress condition at the end of the cultivation. The procedure applied in this work to lead the cell to a stress phase and consequently production of β-carotene was adequate. In fact, after about 3 days under stress conditions, more than 90% of the cells presented orange color, irrespective of bioreactor condition (photobioreactor or Erlenmeyer flask). Although nutrient starvation, high light intensity, and/or high salinity are the most common stress conditions applied, the lack of pH control is also a good option for this phenomenon, especially when environmental issues are considered. In fact,

the reuse of culture medium is a research field with relatively little information in the literature, and additional studies are required, particularly in *D. salina* medium, which presents high salinity, being its discharge in soil or water bodies harmful to the environment.

In the cultivation of photosynthetic microorganisms, there is a natural increase in the pH as consequence of consumption of inorganic carbon source for cell growth (Moheimani, 2013). This increase in pH values is a consequence of chemical change in the medium due to carbon consumption, negatively affecting carbon assimilation during green phase, since the proportions of CO_2 and bicarbonate forms (assimilated by microalgae cells) decrease and the proportion of carbonate increases (Markou, Vandamme, & Muylaert, 2014). Dissolved CO_2 is the preferred source of carbon in photosynthetic organisms (Moheimani, 2013) but with the use of carbonic anhydrase enzyme, bicarbonate can also be assimilated. This fact justify the addition of CO_2 during the growth phase (green phase) for maintaining pH value at $7{,}0 \pm 0{,}2$.

However, in the second stage, the lack of pH control and consequent pH increase could be useful for promoting the production of carotenoid. In fact, pH increased up to values above 9.0 (Table 1) during carotenogenesis. Concerning environmental aspects, culture medium at the end of carotenogenesis could be submitted to pH adjustment by CO_2 addition, which is much easier than desalinization in the case of carotenogenesis induced by high salinity.

The cell performance under different concentrations of nitrogen and phosphorus in f/2 medium and different replenishment proportions in semi-continuous mode can be observed in Figure 2 and Figure 3. In spite of the fact that cells had previously grown, in the inoculum phase, in photobioreactor using the cultivation medium 1N:1P, a lag phase was observed in runs with the same concentrations of nitrogen and phosphorus (Figures 2A and 2B). On the other hand, it was not observed any lag phase when cells were cultivated in medium 1.5N:1.5P (Figures 3A and 3B). This effect occurred likely because cells were centrifuged for preparing the suspension to inoculate the photobioreactor, and, as a consequence of this unit operation, they may suffered some damage in its system to uptake nutrients, being this deficiency minimized when higher concentration of nutrients (1.5N:1.5P medium) was utilized.

Table 1

Cell concentration of *Dunaliella salina*, and β-carotene content in carotenogenesis

N:P [a]	Replenishment proportion	Carotenogenesis	Inicial cell density	Final cell density	pH	$Y_{p/x}$ [b]
	%		10^5 cells mL $^{-1}$	10^5 cells mL $^{-1}$		mg g $^{-1}$
1:1	20	SB	3.6 ± 0.48	3.7 ± 0.44	8.48 ± 0.12	17.7 ± 1.37
		S1	3.6 ± 1.51	3.6 ± 0.15	8.38 ± 0.02	27.6 ± 9.51
		S2	3.9 ± 0.34	4.1 ± 0.51	8.62 ± 0.02	53.4 ± 18.63
		S3	3.7 ± 0.39	3.3 ± 0.35	8.37 ± 0.09	33.7 ± 5.62
		Biorreactor	3.7 ± 0.39	3.9 ± 0.25	8.75 ± 0.12	38.0 ± 12.14
1.5:1.5	20	SB	4.8 ± 0.21	4.9 ± 1.65	8.75 ± 0.07	21.0 ± 0.96
		S1	4.8 ± 1.11	5.1 ± 2.02	9.03 ± 0.09	23.7 ± 0.20
		S2	5.2 ± 0.58	5.0 ± 1.60	7.97 ± 0.19	26.8 ± 0.51
		S3	4.7 ± 1.50	5.0 ± 1.60	8.05 ± 0.17	19.4 ± 0.14
		Biorreactor	4.7 ± 1.00	5.1 ± 0.67	8.00 ± 0.14	18.3 ± 0.11
1:1	80	SB	3.7 ± 0.87	3.6 ± 1.82	8.70 ± 0.05	33.9 ± 3.01
		S1	4.1 ± 1.56	2.9 ± 1.23	8.77 ± 0.05	11.3 ± 3.73
		S2	2.9 ± 0.19	2.0 ± 0.03	8.48 ± 0.21	33.5 ± 8.47
		S3	3.1 ± 0.07	3.0 ± 0.59	9.00 ± 0.14	57.5 ± 15.18
		Biorreactor	3.1 ± 0.07	3.1 ± 0.58	8.80 ± 0.05	32.2 ± 7.18
1.5:1.5	80	SB	5.6 ± 0.00	5.9 ± 0.72	8.95 ± 0.06	18.3 ± 0.23
		S1	3.8 ± 0.62	5.2 ± 2.46	9.35 ± 0.21	20.0 ± 0.15
		S2	4.2 ± 0.09	5.1 ± 0.38	9.42 ± 0.21	23.4 ± 0.67
		S3	4.2 ± 1.17	4.4 ± 0.78	9.27 ± 0.05	22.8 ± 0.05
		Biorreactor	4.2 ± 1.17	4.9 ± 0.74	8.70 ± 0.000	17.6 ± 0.42

[a] Proportion of N ($NaNO_3$) and P ($NaH_2PO_4.2H_2O$) in relation to f/2 medium, where 1:1 corresponds to 75 mg L^{-1} and 5.65 mg L^{-1} and 1.5:1.5 corresponds to 112.5 mg L^{-1} and 8.47 mg L^{-1}, respectively. Where, SB refers to carotenogenesis in the shaker for batch, S1 refers to carotenogenesis in the shaker for cycle 1, S2 shaker for cycle 2, and S3 shaker for cycle 3.

[b] β-carotene-to-cell conversion yield.

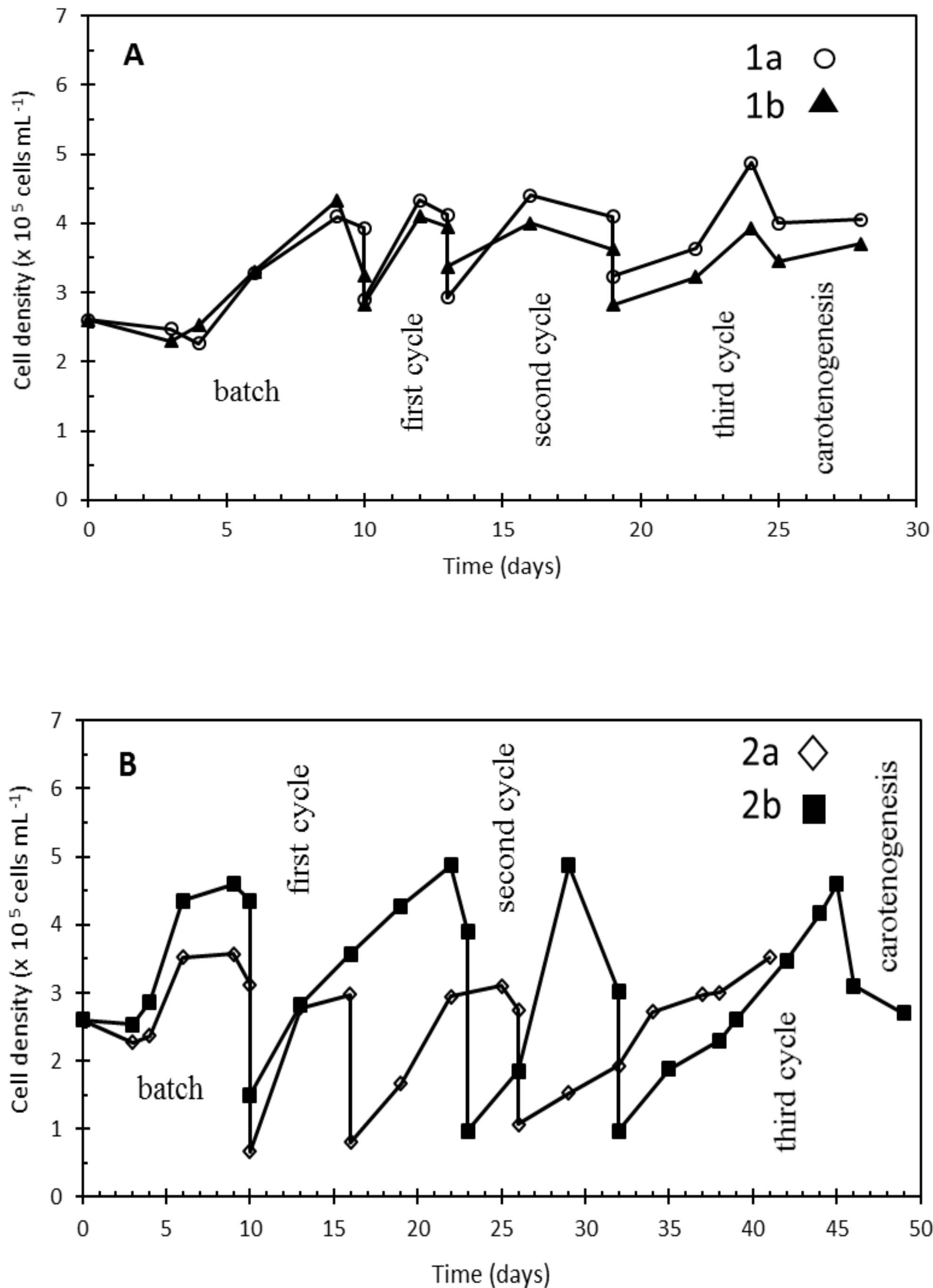

Figure 2. Experiments carried out with 1N1P. A) Replacement of 20%. Experiment 1a: first cycle (10-13 days); second cycle (13- 19 days); third cycle (19 – 25 days), and carotenogenesis (25 – 28 days). Experiment 1b: first cycle (10-13 days); second cycle (13- 19 days); third cycle (19 – 25 days), and carotenogenesis (25 – 28 days). B) Replacement of 80%. Experiment 2a: first cycle (10-16 days); second cycle (16- 26 days); third cycle (26 – 38 days), and carotenogenesis (38 – 41 days). Experiment 2b: first cycle (10-23 days); second cycle (23- 32 days); third cycle (32 – 46 days), and carotenogenesis (46 – 49 days).

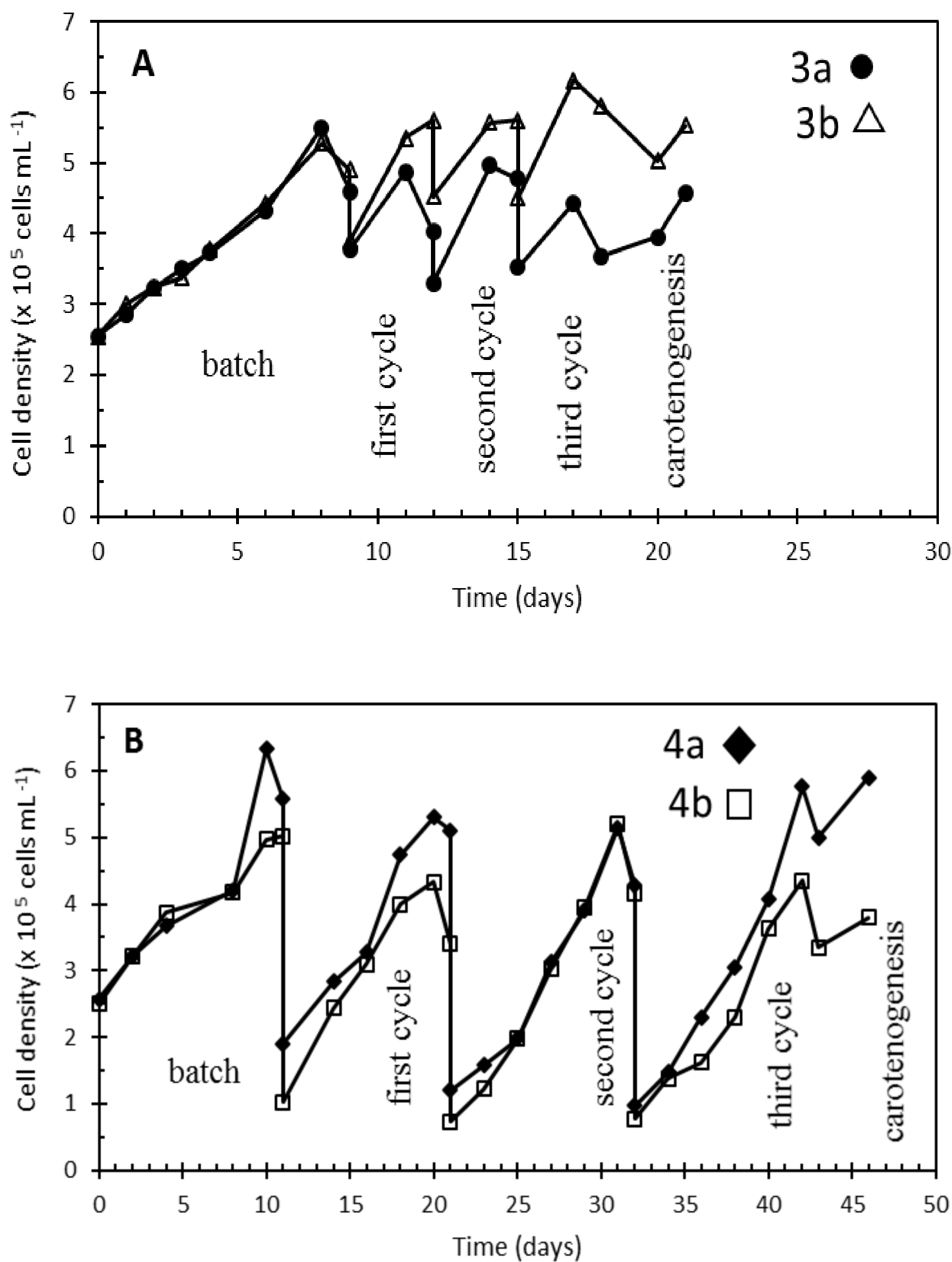

Fig 3. Experiments carried out with 1.5N1.5P. A) Replacement of 20%. Experiment 3a: first cycle (9-12 days); second cycle (12- 15 days); third cycle (15 – 18 days), and carotenogenesis (18 – 21 days). Experiment 3b: first cycle (9-12 days); second cycle (12 - 15 days); third cycle (15 – 18 days), and carotenogenesis (18 – 21 days). B) Replacement of 80%. Experiment 4a: first cycle (11-21 days); second cycle (21- 32 days); third cycle (32 – 43 days), and carotenogenesis (43 – 46 days). Experiment 4b: first cycle (11 - 21 days); second cycle (21- 32 days); third cycle (32 – 43 days), and carotenogenesis (43 – 46 days).

In fact, this microalga has a fragile membrane, what lead to a low resistance to mechanical forces (García-González et al., 2005) and could be affected by centrifugal compaction, as it can occur in bacterial cells (Peterson, Sharma, Van der Mei, & Busscher, 2012). The utilization of the semi-continuous process, by avoiding the centrifugation to use the cells as inoculum for its subsequent cycles of production, has provided the absence of lag phases, regardless of the experimental conditions (Figures 2 and 3).

The maximum cell density (Xm) is an important parameter of the process, because besides influencing cell productivity it also directly affects the cost of biomass harvesting. By the values of this parameter in Table 2 and Figures 2 and 3 it is possible to conclude that there was no difference between results of Xm in startup batch and semicontinuos processes for a given N:P condition (1N:1P or 1.5N:1.5P). Xm values are between 3.8 x 10^5 cells mL^{-1} and 4.4 x 10^5 cells mL^{-1}, in runs with 1N:1P, and between 4.8 x 10^5 cells mL^{-1} and 5.6 x 10^5 cells mL^{-1} when in runs with 1.5N:1.5P (Table 2).

Considering the semicontinuous process, there was no variation on cell density according to the medium replenishment proportion (R) (Table 2), even with a high withdraw of culture (R= 80%) and consequent high variation on cell density at each new cycle of cultivation, if compared with R = 20% (Figures 2A and 2B; Figures 3A and 3B). On the other hand, an increase in the nutrients concentration in the cultivation medium from 1N:1P to 1.5N1.5P, allowed an increase in cell growth. In fact, in experiments carried out with R = 20%, for medium 1N:1P, the mean value of the three cycles was 4.3 ± 0.1 x 10^5 cells mL^{-1}, whereas for medium 1.5N:1.5P the mean value was 5.3 ± 0.1 x 10^5 cells mL^{-1}. The same behavior under different nutrient concentrations was found when medium replenishment proportion of 80% was applied, thus evidencing that the nutrients concentration of 1.5N:1.5P is the best condition for cell growth ($p =$ 0.034). Such results indicate that cultivations carried out with the standard medium (1N:1P) were limited by nutrient. This fact evidence that the standard cultivation media for cultivation of photosynthetic microorganisms, although being properly developed for their growth, may require an increase in some nutrients in order to support the cell growth in tubular photobioreactor. Such additional demand of nutrient is a consequence of the high cell growth that occurs when closed photobioreactor is used for cultivation of these microorganisms (Del Campo et al., 2007).

Table 2
Results of semi-continuous cultivation of *Dunaliella salina*

N:P [a]	Replenishment proportion (R) %	Cycle	Cell density (Xm) [b] 10^5 cells mL^{-1}	P_x [c] 10^4 cells mL^{-1} d^{-1}	Phosphorus [d] mg L^{-1}	Nitrate [e] mM
1:1	20	Batch	4.2 ± 0.16	1.8 ± 0.18	0.285 ± 0.077	0.526 ± 0.026
		1	4.2 ± 0.16	6.7 ± 0.59	0.331 ± 0.029	0.572 ± 0.033
		2	4.2 ± 0.28	3.5 ± 2.01	0.197 ± 0.020	0.593 ± 0.003
		3	4.4 ± 0.67	2.7 ± 0.75	-[f]	0.576 ± 1.152
1.5:1.5	20	Batch	5.4 ± 0.16	3.5 ± 0.20	-[f]	0.556 ± 0.019
		1	5.2 ± 0.51	5.6 ± 0.12	-[f]	0.538 ± 0.023
		2	5.3 ± 0.45	6.0 ± 3.36	-[f]	0.557 ± 0.001
		3	5.3 ± 1.23	6.4 ± 2.68	-[f]	0.529 ± 0.019
1:1	80	Batch	4.1 ± 0.73	1.6 ± 0.81	0.214 ± 0.143	0.513 ± 0.019
		1	3.9 ± 1.34	3.3 ± 0.73	0.220 ± 0.056	0.588 ± 0.005
		2	4.0 ± 1.25	4.5 ± 2.78	-[f]	0.586 ± 0.001
		3	3.8 ± 1.13	2.2 ± 0.84	-[f]	0.730 ± 0.003
1.5:1.5	80	Batch	5.6 ± 0.96	3.0 ± 0.91	0.034 ± 0.040	0.519 ± 0.004
		1	4.8 ± 0.69	3.7 ± 0.08	-[f]	0.510 ± 0.014
		2	5.2 ± 0.04	4.2 ± 0.37	-[f]	0.504 ± 0.017
		3	5.1 ± 1.00	4.2 ± 0.86	-[f]	0.518 ± 0.039

[a] Proportion of N ($NaNO_3$) and P ($NaH_2PO_4.2H_2O$) in relation to f/2 medium, where 1:1 correponds to 75 mg L^{-1} and 5.65 mg L^{-1} and 1.5:1.5 corresponds to 112.5 mg L^{-1} and 8.47 mg L^{-1}, respectively

[b] Maximum cell concentration

[c] Productivity

[d] Phosphorus content of culture medium

[e] Nitrate content of culture medium

[f] below analytical method detection

The demand of carbon, that represents about a half of dry cell mass, although could be partially supplied by salts, as bicarbonate, for example, especially in cultivation with higher pH values in the media, is normally supplied by CO_2, being this approach utilized in this work, as mentioned earlier. Otherwise, nitrogen and phosphorus, that represent about 7% and 1% of dry cells, respectively, need to be added in higher concentrations in the cultivation medium or at the beginning of the cultivation process in experiments carried out under batch or semicontinuos processes.

In this way, aiming to improve the cell growth, experiments were carried out with further addition of nitrogen and phosphorus (medium 3N:3P). In this condition, nitrogen and phosphorus concentrations were equivalent to 2.64 mM and 0.11 mM, respectively . However, no growth was observed in such condition, with a clear drop in cell density after 7-8 days of cultivation (Figure 4). Considering that this microorganism grows in high salinity medium (f/2 medium), whose composition is based on sea water, this decrease in cell growth was not expected, since the increase in salinity would be negligible, ie, would increase the f/2 medium salinity from 42.64 g L^{-1} (Pérez-Mora & Carvalho, 2014) to 42.80 g L^{-1}.

In fact, even microalgae sensible to salinity, as *Botryococcus braunii*, support nitrogen and phosphorus concentrations up to 6.0 mM and 0.30 mM, respectively, without suffering clear inhibition effects (Pérez-Mora et al., 2016) as occurred with *D. salina* in lower concentrations of these nutrients. These findings point out to the need of a restricted control to find the best condition of nitrogen and phosphorus concentrations in the cultivation of *D. salina*. It can be observed in Table 2 that the final concentration of nitrate is in the range of 0.5-0.7 mM, irrespective of cultivation medium used for cell growth. This could indicate that *D. salina* is not able to uptake nitrate from the cultivation medium at concentrations lower than 0.5 mM. When medium 1N:1P is used, this threshold is attained before than in runs with 1.5N:1.5P, thus limiting the cell growth, that, as mentioned, was lower with the use of the former medium. Consequently, the residual quantity of phosphorus in the cultivation medium is higher when 1N:1P is used (Table 2), despite the use of lower concentration of this nutrient, since the growth was limited by nitrogen.

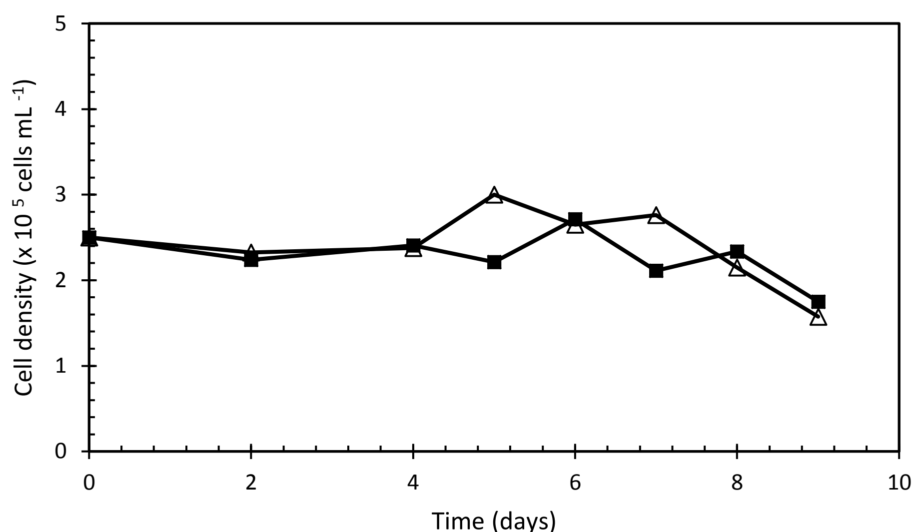

Figure 4. Experiments carried out with 3N3P, where 3:3 corresponds to 225 mg L^{-1} of $NaNO_3$ and 16.95 mg L^{-1} of NaH_2PO_4. $2H_2O$

A well-known advantage of semicontinuous process is the exploitation of the cells adapted to the cultivation medium, thus providing higher productivities that batch process. In fact, as it can be observed in Table 2, irrespective to the medium composition and replenishment proportion, there is a sharp increase in cell productivity with the semicontinous process (p = 0.010), that, in general, is maintained throughout the three cycles of cultivation. An exception is the third cycle of experiments carried out with medium 1N:1P, that, although the maximum cell density had attained about the same value as those obtained in previous cycles, the time to achieve such values was higher. For semicontinuous process, in both cases of medium replenishment proportion (20% and 80%), it was not evident an effect in cell productivity increasing the quantity of nitrogen and phosphorus in cultivation medium, because even obtaining higher cell density in this condition, there was an increment in time to achieve such value. Otherwise, concerning medium replenishment proportion (R), the values obtained with R =20% lead to higher cell productivities. This fact is probably a consequence of the low dilution of cell density at each new cycle of semicontinous cultivation in this condition, what are in agreement with results of (García-González et al., 2003), who observed higher productivities in cultivation of *D. salina* at higher cell densities.

Irrespective of the experimental conditions for cell growth and carotenogenesis induction, *D. salina* was able to produce β-carotene. However, it was possible to observe

differences in the pigment productions between the nutrient conditions studied (Figures 5A and 5B).

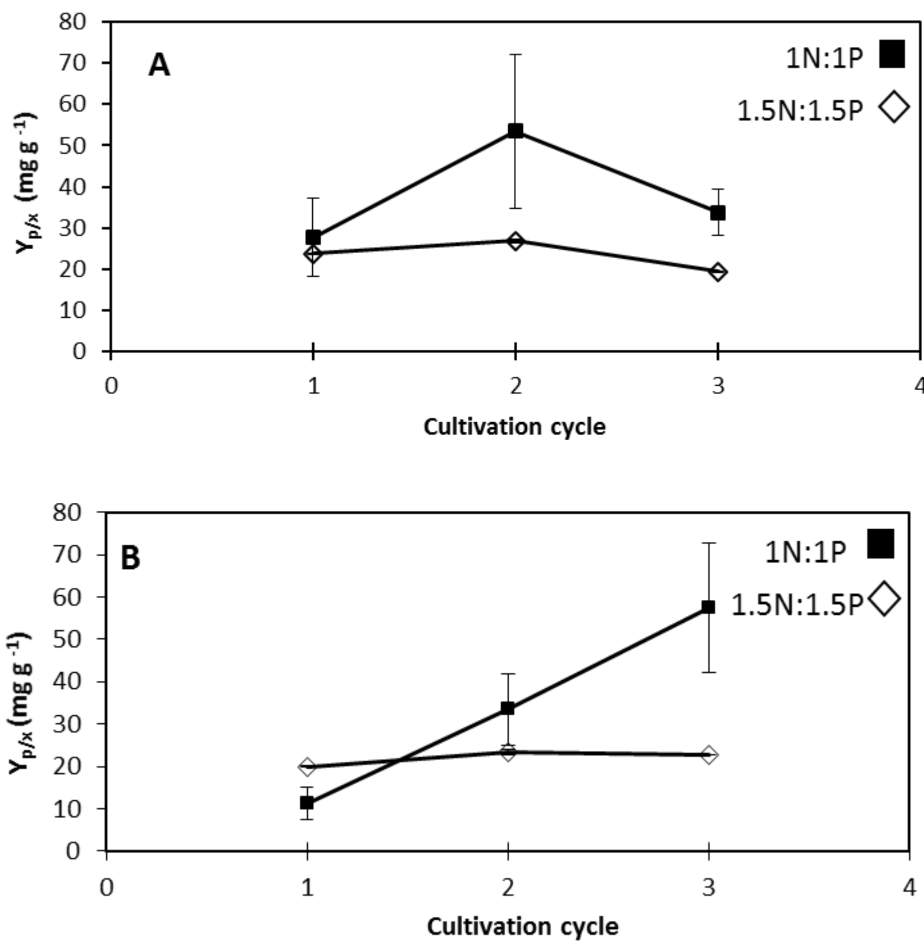

Figure 5. Experiments carried out with replacement of 20% (A), and replacement of 80% (B)

In experiments carried out with medium 1N:1P there was a higher variation in content of β-carotene in dry mass cell ($Y_{p/x}$), having a tendency do increase this yield from 2nd cycle of process. It can also be observed in this Figure that there is a tendency to obtain higher values of $Y_{p/x}$ when medium 1N:1P is used, likely because in such condition the cells have remained longer time under starvation of nitrogen source. These results were obtained in shaker conditions, as stressed in Material and Methods section. Besides, in this work, it was verified the behavior of cells in production of β-carotene in the photobioreactor as well. In general, the results of β-carotene production in shaker conditions and photobioreactor can not be considered different (Table 1, data corresponding to S3 and bioreactor), thus indicating that both conditions were adequate

to promote carotenogenisis. The results of $Y_{p/x}$ obtained in this work are in agreement with results of
(García-González et al., 2003), who had obtained values in the range of 34 to 78 mg g^{-1}, depending on the strain of *D. salina*.
The morphology and behavior of *D. salina* cells, both in the green and orange phases, were examined microscopically. In a general way, most cells in the green phase presented mobility (biflagellate) and ellipsoidal shape, but also it was noticed almost spherical shape (Figure 6).
Similar cell behavior was observed in 1.5N:1.5P in the green phase. However, with the use of 3N:3P medium, the cells, although at the beginning of the culture process did not present morphological differences with those grown in media with lower nutrient concentrations, from 7-8 days of culture started a process of cell lysis.

Figure 6. *D. salina* green cells (CCAP 19/18) in 1N1P condition with 80% fresh medium replenishment (cells from 42nd day, after 10 days of the third cycle)

When the cells were submitted to the conditions that led to carotenogenesis, there was a tendency of the cells to present almost all of their spherical morphology, with loss of mobility, and in some cases there may be an increase in volume. This same behavior was found by Solovchenko et al., 2015. In the carotenogenesis phase, there was a gradual change from green coloration to orange coloration, so that, as commented previously, after 3 days more than 90% had this characteristic.
During green phase in photobioreactor, no contamination by protozoa was observed, likely because closed photobioreactor may help to prevent contamination (Del Campo et al., 2007). As the cells were collected aseptically to be leaded to carotenogenisis

conditions, in experiments carried out in Erlenmeyer flasks, even in this phase there was not observed contamination by protozoa.

As concluding remark, the results from this work evidence the feasibility of cultivating *D. salina* by semicontinuous process under different experimental conditions, that is medium replenishment proportion (R) and concentrations of nitrogen and phosphorus in seawater-based f/2 medium. In comparison with batch process, semicontinuous process allowed the achievement of higher cell productivities (Px up to 6.7 x 10^4 cells mL^{-1} d^{-1}). The maximum cell density (Xm) obtained in this work was not dependent of R, but the best results were obtained when using medium 1.5N:1.5P (mean values up to 5.6 x 10^5 cells mL^{-1}) instead of 1N:1P. The content of β-carotene in the cells, in general, was higher in cells grown in medium 1N:1P (mean yield values up to 57.5 mg g^{-1}) in comparison with medium 1.5N:1.5P. The cultivation of the cells with media 3N:3P led to a long lag phase, followed by decrease in cell density and their lysis. The use of a tubular photobioreactor contributed to successfully cultivate *D. salina* without contamination by protozoa.

FINAL CONCLUSIONS

As stressed in CHAPTER I, microalgae represent a sustainable alternative source of carotenoids for the promising market of this bio-product. The rapid growth rate, possibility of cultivation in wastewater, CO_2 bio-fixation, low water consumption, and the easier daily harvesting are some of the advantages over fruits and other vegetables (traditional sources).

Carotenoids present potential of application in food, cosmetics, and pharmaceutical industries, as well as aquaculture and healthcare area. As source of these bio-molecules, microalgae may be produced by different culture systems and operation regime, and this information might be of utmost importance for obtaining carotenoids by commercial scale microalgal cultivation.

In this context, *Dunaliella salina* was selected for studying cell growth in tubular photobioreactor, carotenogenesis in stress condition, and β-carotene extraction by liquid-liquid microextraction method.

As pointed-out in CHAPTER II, by a modified dispersive liquid-liquid microextraction (DLLME) method β-carotene could be successfully extracted from *Dunaliella salina,* employing benzene as extractor phase and water with 50% acetone as dispersant. A rapid chromatographic screening method was developed on a C4 column for separation of geometric isomer of β-carotene, with retention time of 14 minutes.

Lastly, in CHAPTER III, it was possible to notice the feasibility of cultivating *Dunaliella salina* in tubular photobioreactor by semicontinuous process. Regardless of replenishment proportion, best results of maximum cell density were obtained with 1.5N:1.5P. However, medium 1N:1P allowed best β-carotene yield. Medium 3N:3P was not suitable for cell growth. Tubular photobioreactor with application of ligh-dark cycle is appropriated for the cultivation of *D. salina* without contamination by protozoa. Moreover, high pH and light intensity as stress condition showed to be excellent protocol for inducing carotenogenesis.

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APPENDIX A - Nitrate Calibration Curve

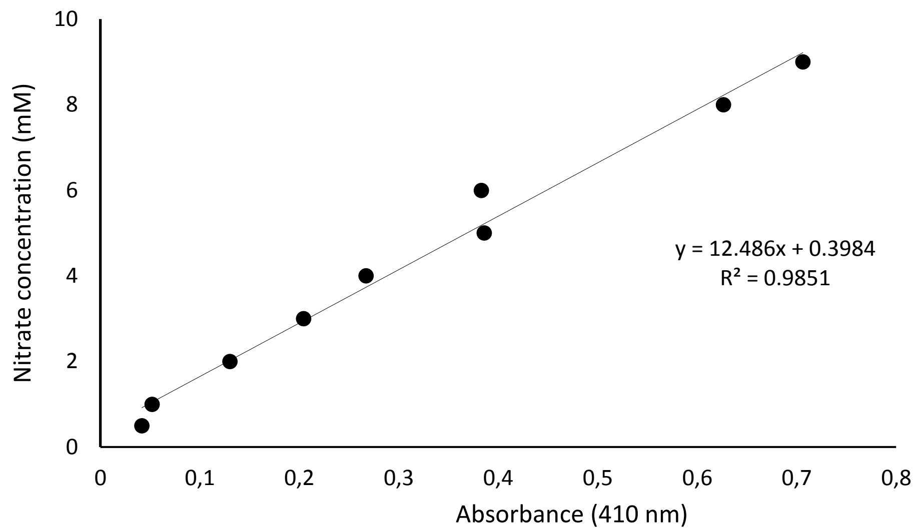

Figure 1: Calibration curve for nitrate quantification

A calibration curve was used for converting absorbance (410 nm) to nitrate concentration (mM). By the graphic observed in Figure 1, the following equation (1) was obtained:

$$[NaNO_3] = 12.486 * Absorbance_{(410\ nm)} + 0.3984 \quad (1)$$

where $[NaNO_3]$ = Nitrate sodium concentration

APPENDIX B - Phosphorus Calibration Curve

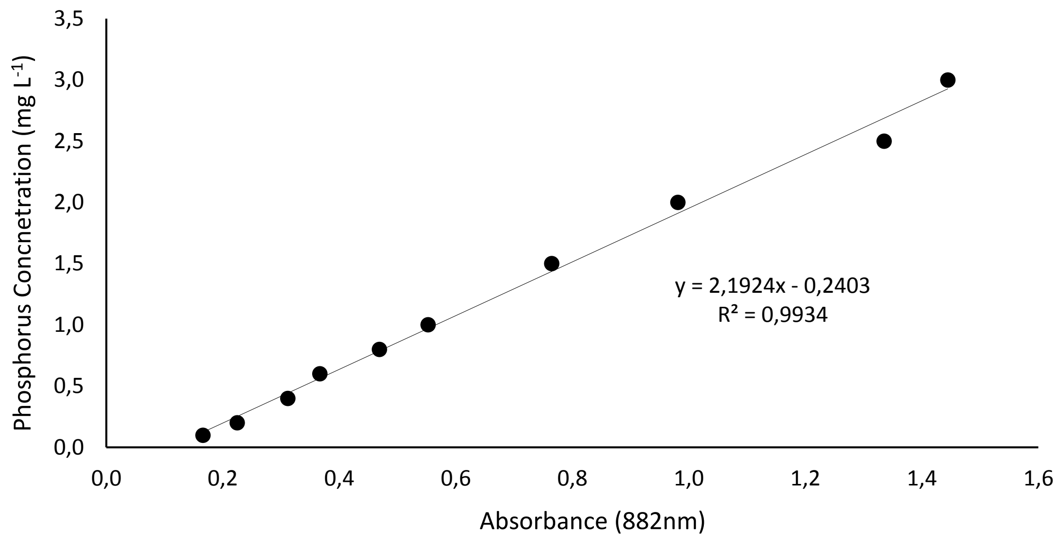

Figure 2: Calibration curva for phosphorus quantification

Figure 2 shows the calibration curve correlating absorbance (882 nm) and phosphorus concentration ($mg.L^{-1}$). The equation (2) was used to convert absorbance values to phosphorus concentration (in the form of PO_4^{3-}).

$$P = 2.1924 * Absorbance_{(882nm)} - 0.2403 \quad (2)$$

where P = concentration of phosphorus in the form of PO_4^{3-}

ATTACHMENT A – Statement for exemption of Ethics Committee approval

UNIVERSIDADE DE SÃO PAULO

Faculdade de Ciências Farmacêuticas
Departamento de Tecnologia Bioquímico-Farmacêutica

DECLARAÇÃO DE DISPENSA DE AUTORIZAÇÃO DE COMITÊ DE ÉTICA

São Paulo, 22 de setembro de 2018

Eu, Eleane de Almeida Cezare Gomes, Número USP 261197, aluna de doutorado do Departamento de Tecnologia Bioquímico-Farmacêutica na área de Tecnologia de Fermentações, sob orientação do Prof. Dr. João Carlos Monteiro de Carvalho, venho por meio desta afirmar que o projeto de pesquisa intitulado **Evaluation of *Dunaliella salina* growth and corresponding β-carotene production in tubular photobioreactor**, dispensa a autorização pelo Comitê de Ética visto que não serão envolvidos animais ou seres humanos para a realização dos experimentos.

Eleane de Almeida Cezare Gomes

Prof. Dr. João Carlos Monteiro de Carvalho