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

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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

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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

GENERAL INTRODUCTION

Microalgae are promising sources of carotenoids. There are two groups of these hydrocarbons: carotenes composed of only C and H (e.g., β-carotene and lycopene) and xanthophylls containing C, H, and O (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 like obesity, cardiovascular disease, and eye diseases (for example, 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 cultivation of *D. salina* by semicontinuous process for the production of β-carotene, evaluating different replenishment proportions of medium containing different concentrations of nitrogen and phosphorus.

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;

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 appropriate for the cultivation of *D. salina* without contamination by protozoa. Moreover, high pH and light intensity as stress condition showed to be an excellent protocol for inducing carotenogenesis.

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