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Modeling Growth of Microalgae *Dunaliella Salina* under Different Nutritional Conditions

Sergio Santos Jesus and Rubens Maciel Filho
Department of Chemical Process, Laboratory of Optimization,
Design and Advanced Control, School of Chemical Engineering,
University of Campinas, Unicamp Cidade Universitária Zeferino Vaz,
CP 6066, Campinas, SP, Brazil

Abstract: Problem statement: The aim of this study was to find the empirical model that describes the growth kinetics of *Dunaliella salina*, with low production cost and to estimate parameters of this model. **Approach:** In this study the strain of *D. salina* UTEX 200 was cultivated in seawater (0.5 M NaCl) at room temperature with agitation of 150 rpm and luminous intensity of 60 mmols.m-2.s-1. The synthetic medium AS100 (0.2 M NaCl) was used in this study for comparison purposes and in order to determine the optimal growth of the microalgae. Kinetics of growth and β-carotene production was determined in a period of 15 days. **Results:** After the analysis of the behavior graphic, an unstructured model was used for describing the cell growth (logistic model). It was observed that the model was well adjusted to experimental data for the two conditions of analysis. It was observed that alga produces carotenoids under conditions of stress, in which the cell division are retarded. In the case of cells grown in seawater (higher salt concentration), the cell growth was lower but the concentration of β-carotene was higher. **Conclusion:** In general, these results suggest that *D. salina* presents higher potential for β-carotene accumulation and that high salinity decreases cellular concentration (measured by the parameter Xm of the kinetic model proposed); however there is an increase in β-carotene production.

Key words: β-carotene, culture medium, *Dunaliella salina*, kinetic model, initial concentration, biological functions, natural pigments, salinity decreases, cellular concentration, synergistic effect, microalgal species

INTRODUCTION

Carotenoids are natural pigments with much diversified structure-yet similar in their general chemical structure-and widely spread in nature, where they fulfill their essential biological functions. Carotenoids, some of which are provitamin A, have a range of diverse biological functions and actions, especially regarding human health. The carotene's pigment is of international importance because of its dual function as vitamin and colorant in the food industry to pigment salmon, trout and poultry meat, or to intensify the color of egg yolk (Martelli *et al*., 1992; Johnson and Schroeder, 1995). The β-carotene also present other physiological effects as inhibitor of cancer and some diseases concerning photosensible skin. It also increases immunological response to certain types of infection (Mayne, 1996).

Dunaliella salina, unicellular green microalgae, halophylic with free wall, is scientifically interesting for its capacity of producing β-carotene and glycerol. *D. salina* can accumulate a very high concentration, (up to 14%) of cell dry weight of β-carotene under conditions of high light, high salinity and nutrient deprivation and it is recognized as the best biological source of this carotenoid (Borowitzka and Borowitzka, 1990; Markovits *et al*., 1993; Lee, 2001). Severe conditions such as high salinity, low nutrient levels, high irradiance and high temperature have been claimed to induce β-carotene production in the cell but, at the same time, decrease the number of cells per culture volume by affecting cell division (Tafreshi and Shariati, 2006). Ben-Amotz and Avron (1983) showed that the salinity and irradiance have an additive or synergistic effect on carotenogensis in *D. salina*.

Corresponding Author: Sergio S. Jesus, Department of Chemical Process, Laboratory of Optimization, Design and Advanced Control, School of Chemical Engineering, University of Campinas, Unicamp Cidade Universitária Zeferino Vaz, CP 6066, Campinas, SP, Brazil Tel: +55 19 3521 3958 Fax: +55 19 3521 3965

Among the factors that influence the productivity of biomass and β-carotene content in *D. salina*, salinity is a very important of them. Some studies have shown a direct relationship between β-carotene production and growth-limiting conditions such as increasing salinity. Therefore, several strategies have been used for introducing β-carotene in *Dunaliella* mass cultures by changing salt concentration (Borowitzka and Borowitzka, 1990; Tafreshi and Shariati, 2006). Other authors commented that the salinity does not have a clear effect on β-carotene accumulation per cell (Gomez *et al*., 2003). It seems that the effect of salinity, like other inducing actors on β-carotene production in *D. salina,* is strain-dependent and that only a few strains of *Dunaliella* have the potential to produce up to 10% β-carotene (Ben-Amotz and Avron, 1983).

The world market for carotenoids was estimated at $887 million in 2004 and $1 billion in 2009 and 80% of this market is attributed to carotenoids that can be produced by using the invention process (Vermeglio, 2010). Today, *D. salina* is one of the most used microalgal species for mass culture due to its ability to accumulate b-carotene, a molecule which in this alga can represent up to 95% of total carotenoids (Jimenez and Pick, 1994). The global yield of *Dunaliella* is estimated at about 1,200 ton per year (Pulz and Gross, 2004). It was relevant to establish a mathematical model capable of describing *D. salina* growth and to study the maximum production of b-carotene. Mathematical models have an important role in the optimization of the fermentation process. The most widely used unstructured models for describing cell growth are the Monod kinetic model, the logistic equation and the Haldane model. The logistic equation is a substrate independent model. It can finely describe the inhibition of biomass on growth, which exists in many batch fermentations (Mitchell *et al*., 2004; Younesi *et al*., 2006).

The aim of this study was to find this empirical model that describes the growth kinetics of *D. salina*, consideres the effect of increasing the salinity in the two different culture mediums and estimates this model's parameters. Moreover, this study aims to assess the production of β-carotene using these two mediums.

MATERIALS AND METHODS

Algal material and growth condition: *Dunaliella salina* UTEX 200 was cultivated in seawater (Copacabana beach, Rio de Janeiro, Brazil) (Table 1) at room temperature (25 ± 2°C) with agitation of 150 rpm and continuously illuminated by fluorescent lamps providing 60 mmols.m-2.s-1 at the surface of the flasks.

Table 1: Chemical composition of seawater

Salts	Concentration (g L^{-1})
NaCl	27.6
MgCl	25.4
MgSO	46.9
CaCl	21.4
KCl	0.6
NaHCO	30.2

The pH was controlled to stay at pH 7.6 by injecting carbon dioxide. For comparison, synthetic medium AS100 (Vonshak, 1986) modified with the addition of 0.2 M NaCl was also used to determine the microalgae optimal growth. The initial concentration of seawater was determined by Mohr method (Sheen and Kahler, 1938), which uses chromate ions as an indicator in the titration of chloride ions with silver nitrate standard solution.

Biomass concentration: Cell counts were carried out using a 0.1 mm deep Neubauer haemocytomer.

Determination of β-carotene content: An aliquot (5.0 mL) of *D. salina* cell suspension was centrifuged at 3000xg for 15 min. The pellet obtained was washed with distilled water and, after water removal by centrifugation, suspended in acetone. The cell membrane is ruptured because of water (osmotic shock), thus extracting β-carotene along with chlorophyll. The extracting separates from cell debris by centrifuging it at 3000 xg for 10 min. β-Carotene was assessed according to Ben-Amotz and Avron (1983). The β-carotene content of *Dunaliella* cells was given by pigment content per cell (pg.$cell^{-1}$).

Statistical analysis: The representation and estimation of the model's parameters were performed using *Statistica* 7.0 software.

RESULTS

Kinetics: *Dunaliella salina* was cultivated at laboratory scale to increase β-carotene content of the biomass by using two different salinity conditions. It was possible to observe that cell density increased from 1.2×10-5 to 4.5×10-5 cell.mL^{-1} in a period of 3 days when cultivated in seawater, with an initial concentration of 0.5 M. However, when AS100 (0.2 M) culture medium was used for the same period that the previous experiment, cell concentration increased from 1.2×10-5 to 5.6×10-5 cell.mL^{-1} (Fig. 1). This may be because when a microalga is grown in saline environment, osmosis plays a defining role. If very high salinity is used in the medium, the external environment of the cell contains a hypertonic solution, i.e., higher concentration of solute (NaCl) and lower concentration of water, than that present inside the cell.

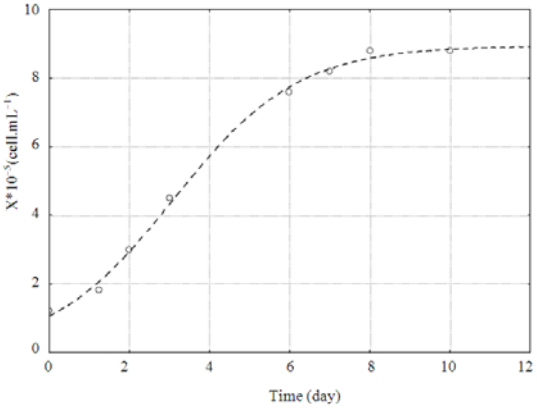

Fig. 1: Comparison of experimental data and calculated values for the proposed models, where: (o) experimental dates, (--) model, (a) seawater medium and (b) AS100 (0.2 M NaCl) medium

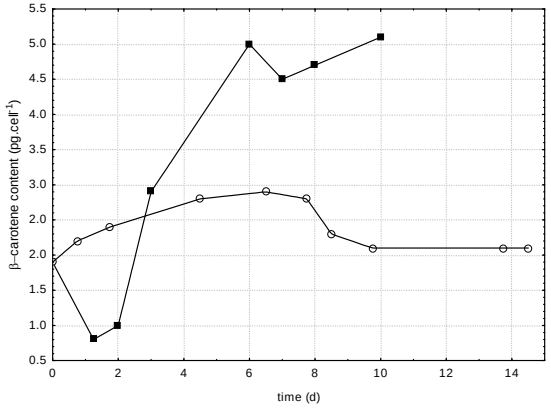

Fig. 2: Kinetics of β-carotene accumulation by cells of *D. salina*, where: (■) seawater medium and (o) S100 (0.2 M NaCl) medium

In such conditions, there is a net flux of water molecules leaving the cell. This results in cell shrinkage and subsequent destruction of the cell and cell components (plasmolysis) (Boussiba and Vonshank, 1992).

β-carotene concentration: The β-carotene content of *D. salina* cells during the 15 days (AS100 medium) and 10 days (seawater medium) following salt stress were determined and expressed on β-carotene per cell (Fig. 2).

DISCUSSION

Growth kinetics was determined as a maximum period of 15 days. After behavior graphic analysis, a kinetic model was developed to fit experimental data (Fig. 1).

Empirical equations that have been proposed to describe these curves (Fig. 1) are shown in Eq. 1. The equation does not include the effect of nutrient concentration on growth. To do so, it would require the modeling of intraparticle diffusion processes. The empirical equations are simply fitted to experimental growth profiles by non-linear regression.

The logistic equations have been used for quite a while. The causes of the growth deceleration are not specified in the simple empirical growth kinetic equations.

The kinetic model is given by the following equation:

$$X = \frac{X_m}{1 + \left[\left(\frac{X_m}{X_0}\right) - 1\right] e^{-\mu t}} \quad (1)$$

Where:

X = The biomass concentration (dependent variable)
T = The time (independent variable)
X_m [cell.mL^{-1}] = The maximum possible microbial biomass
X_0 [cell.mL^{-1}] = The initial biomass
m [t-1] = The specific growth rate constant

The parameters X_m and m were estimated by the least square method. The numerical method quasi-Newton was used to solve the system of nonlinear equations, obtained from the least square method. The parameters estimated for two cultures mediums are shown in Table 2.

It was verified that the proposed model fit very well the experimental data obtained for both the growth conditions analyzed, especially when the microalgae were cultivated in seawater. It was observed that high salinity decrease the growth in this alga.

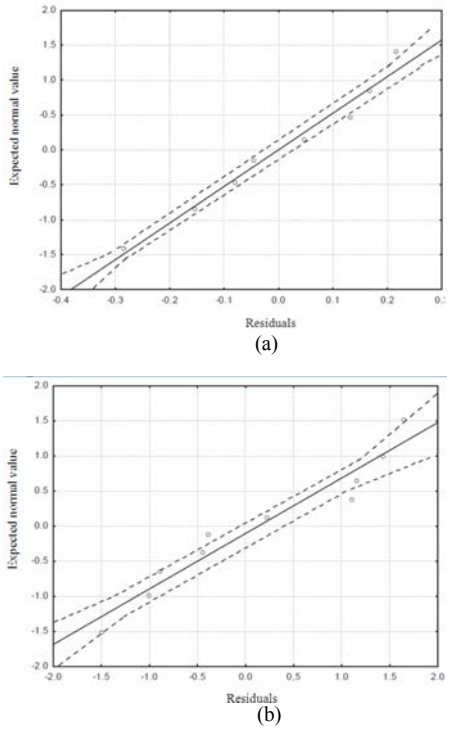

Fig. 3: Normal probability plot of residuals, where: (a) seawater medium and (b) AS100 (0.2 M NaCl) medium

Table 2: Parameters and determination coefficients obtained from kinetic model

Medium	Parameters	Coefficient of determination (r^2)
Seawater	X_m = 8.95±0.41	
	μ = 0.64±0.09	0.998
AS100 (0.2 M NaCl)	X_m = 38.31±1.38	
	μ = 0.43±0.04	0.996

where: Xm (cell.mL^{-1}) and m (d^{-1})

The parameter X_m (value of maximum biomass concentration) was higher when the microalgae was grown in AS100 (0.2 M NaCl) medium, where the highest cell concentration was experimentally observed. However, higher concentration of β-carotene was observed when seawater was used. According to Borowitzka *et al*. (1990), salinity is a major inducing factor of carotenogenesis in some species of halotolerant green algae *Dunaliella. D. salina* could survive in mediums containing a wide range of NaCl concentrations ranging from about 0.05 M to saturation (around 5.5 M) (Chen *et al*., 2009).

The model fit was verified by standard deviations of the parameters, normal probability plot of residuals and coefficient of determination. The model generated from the experimental data of seawater medium provided a better linear fit of the data than the model generated from AS100 (0.2 M NaCl) medium (Fig. 1). The correlation coefficient was higher for seawater medium (0.998) than for AS100 (0.2 M NaCl) (0.996), as shown in Table 2. Fig. 3 shows the normal probability plot, which presented normal distribution, as required by least square method. A residual plot allows visual assessment of the distance of each observation from the fitted line (Kasperski and Schneider, 1997). In addition, residuals from seawater and AS100 (0.2 M NaCl) were normally distributed (Fig. 3). The residuals should be randomly scattered in a constant width band about the zero line, if the prior assumption of constant variance is met. Runs of residuals above or below the zero line may indicate a non-linear relationship. In general, the results for both seawater and AS100 (0.2 M NaCl) mediums present all points close to the line and contained within the limits of 95%.

It was observed that alga produces carotenoids under conditions of stress, in which cell division are retarded. When cells were grown in seawater (higher salt concentration) cell growth was lower, but the concentration of β-carotene was higher (Fig. 2). Indeed, the higher the salinity-and, as a result, the lower the growth rate of the alga-the higher the amount of light absorbed by the cell during a division cycle. This situation can lead to higher accumulation of β-carotene per cell (Tafreshi and Shariati, 2006).

In contrast to other green algae, *Dunaliella* do not contain rigid cell wall (Borowitzka *et al*., 1990). As a result, cells respond rapidly to the changes in osmotic pressure. β-carotene accumulation in *D. salina* at two different salinities confirmed that the stress caused by increases in salt concentration increases the concentration of β-carotene in the cell.

β-carotene is a secondary metabolite and the cells under conditions of stress as a protection mechanism produce these molecules. When salinity is increased, it behaves like stress, which enhances β-carotene production.

In general, these results suggest that *D. salina* presents higher potential of β-carotene accumulation and that high salinity decreases biomass concentration (measured by the parameter Xm the kinetic model proposed); however, there is an increase in b-carotene production. This model can be used to verify the maximum rate of cell growth under a constant light intensity, in order to maximize the production of b-carotene.

CONCLUSION

It was observed that the model was well adjusted to experimental data for the two conditions of analysis. Above all, it showed good result when microalgae were

cultivated in seawater. The maximum value of biomass concentration, in both cases, seems conveniently described by the parameter X_m.

High salinity decreases the biomass concentration (measured by parameter X_m); however, there is an increased of β-carotene production.

Therefore, it is recommended that adjusting salinity properly is one of the best strategies to achieve optimal β-carotene production in mass cultures of *D. salina*.

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