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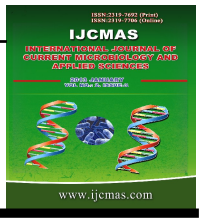

Original Research Article

Optimization of β - Carotene production by Marine Microalga - *Dunaliella salina*

D. Selvi Dhanam[1] and K. Dhandayuthapani[2*]

[1]Research and Development centre, Bharathiar University,
Coimbatore – 641 046, Tamil Nadu, India
[2]Department of Plant Biology and Biotechnology, Arignar Anna Govt. Arts College,
Cheyyar - 604 407, Tamil Nadu, India.
**Corresponding Author: kdpani_bio@yahoo.co.in*

A B S T R A C T

Keywords

Ammonium chloride; β-carotene; *Dunaliella salina;* Light intensity; Sodium Phosphate.

Halotolerant microalga *Dunaliella* sp. is the best commercial source of natural β-carotene in the world. In the present investigation, effect of different concentrations of ammonium chloride, sodium phosphate and light intensities on growth and β-carotene accumulation by *Dunaliella salina* were studied. The maximum cell division rate (0.896 day^{-1}) and β-carotene accumulation (18µg/10^6cells) was found to be at 1.0mM/L ammonium chloride. When grown in the different concentrations sodium phosphate the maximum cell division rate (0.947day^{-1}) and β-carotene production (24µg/10^6cells) was noticed at 0.75mM/L. The light intensity 1000µmol/m^{-2}/s^{-1} with low temperature showed the maximum division rate (0.875 day^{-1}) and β-carotene production (21µg/10^6cells).

Introduction

In 21st century, the usage of synthetic pigments is being more than 50% in the total industrial production. The usage of such pigments is really a bane to society because as it causes many cancerous and chronic diseases in humans. To overcome these problems, natural pigments which are obtained from the natural resource can be preferred. Hence the emerging field of Algal Biotechnology which leads to the major industrial production of natural pigments in low cost and less time consumption. These natural pigments can be employed in various fields like medicine, cosmetics, feed stock and food industry.

The biotechnological potential of algal biomass as a source of industrially valuable compounds has been exploited (Leon-Banares *et al.*, 2004; Del Campo *et al.*, 2007). β- Carotene is the one among the industrially valuable compound which is obtained from the halotolerant microalga *Dunaliella salina.* The β-carotene is applied in the food, cosmetic, and pharmaceutical industries as a colorant, antioxidant, and

anti-cancer agent since it is a lipophilic high-value compound and known as pro-vitamin A (Leach, *et al*., 1998).

The eukaryotic green alga *D. salina* one of the species subjected to considerable mass culture in several countries, since it can accumulate high amounts (more than 10% of algal dry weight) of β-carotene when maintained under growth limiting conditions, namely high salinity, high temperature, high irradiance and/or limiting nutrients (*Raja et al.,* 2007). *D. salina* is occurring naturally in a number of locations worldwide. In the marine environment, *D. salina* appears green, however, in conditions of high salinity and light intensity, the microalgae turns red due to the production of protective carotenoids in the cells. *Dunaliella* have some advantages such as disruption of cells is much easier than that in other algae because of its cell wall-less nature, continuous culture in laboratory is easy and the growth rate is relatively high and resistance to various environmental conditions is higher than in other algae.

Main aim of the present work was to study the influence of different concentrations of ammonium chloride, sodium phosphate and light intensities on β-carotene production by *Dunaliella salina*.

Materials and Methods

Organism and media used

Dunaliella salina used in this study and was cultivated in Dewarln's medium (Orset and Young, 1999). Media composition is as follows: NaCl- 2.14M; $FeCl_3.6H_2O$-4.81µM, H_2BO_3-0.54mM, Na_2EDTA-0.12mM, $NaH_2PO_4.2H_2O$-0.13mM; $NaNO_3$-1.18mM;$ZnCl_2.4H_2O$-0.10µM; $CaCl_2.6H_2O$-0.08µM;$(NH_4)_6Mo_7O_{24}.4H_2O$-.28nm; $CuSO_4$. $5H_2$ O -0.08µM; -1000 ml of sea water and media pH was adjusted into 8.0.

Media optimization

The media optimization was carried out in 250ml conical flask contain 100ml Dewarln's medium. The media $NaH_2PO_4.2H_2O$ and $NaNO_3$ was replaced with various concentrations of ammonium chloride (0.1 to 2 mM) and sodium phosphate (0.1 to 1.0mM). The media was sterilized and cooled at room temperature. 10 ml of fresh culture was inoculated and incubated in the growth chamber at 25 ± 1^0C with continuous cool white fluorescent lamps (Philips, 40 w) at an intensity of 2000 lux in a 12:12 light dark regime but the influence of light intensity was studied by incubated the cultures in the growth chamber at range of light intensity 100 to 2000µmol/m^{-2}/s^{-1}.

Growth rate estimation

On the 6th day samples were withdrawn and growth was estimated microscopically by using a counting chamber (haemocytometer). Since the alga is motile, one drop of HCl was added to arrest the motility. The specific growth rate (µ) (Division/day) was arrived at using the following formula.

$$\mu_{max} = \frac{\log N - \log No}{\log 2 \, X \, t}$$

Where, log N- final, log N_0 - initial and t-time

Extraction and estimation of β- carotene

β- carotene was determined spectrophotometrically. 10 mL sample was centrifuged at 4000 rpm for 5 min,

discarding supernatant and the pellet was dissolved with 10 mL acetone solution, shaking and stewing till separated. Repeating above processes until the extract turned to white, then adding 60% KOH of 1/10 volume at 49°C, transferring the supernatant without chlorophyll and lipid to 10 mL volumetric flask and added acetone to the volume. After determining the A_{453} value, the concentration (CA_{453}) of the β-carotene solution could be found from the standard curve. Standard curve was prepared by dissolved different concentrations of standard β-carotene in pure acetone and measured at 453 nm. Graph was drawn by plotted the β-carotene concentrations on X-axis and OD values on Y-axis.

Statistical Analysis

Correlations analysis (Karl Pearson) was performed to find the degree of relationship between the variables. This was done by Software - MINITAM Release 12.2.

Result and Discussion

Influence of Ammonium chloride on growth and β-carotene production of *Dunaliella salina*

Nitrate and ammonia are the most common forms of nitrogen in aquatic systems. Nitrogen can be an important factor controlling algal growth than other nutrients. Ammonium is the inorganic nitrogenous form of easier assimilation, since nitrate and nitrite first have to be reduced to ammonium before assimilation. In addition to ammonia removal, microalgae can also produce biomass which can be used in several ways to improve the economic efficiency of aquaculture systems (Muller-Feuga *et al*., 2003; Lubzens and Zmora, 2003; Plaza *et al*., 2010; Rodríguez-Meizoso *et al*., 2010). In the present study exponentially grown, N-depleted culture of *Dunaliella salina* was inoculated into N-free Dewarln's media amended with different concentrations of NH_4Cl ranging from 0.1mM to 2.5mM. The cultures were incubated for six days and estimated the growth rate and β-carotene.

The maximum growth rate ($0.896 day^{-1}$) and β-carotene accumulation ($18\mu g/10^6$cells) was found to be at 1.0mM/L NH_4Cl Present media (Table 1; Fig.1). Nutrient concentration/ growth rate (S/μ) plotted against substrate concentration (S) was a straight line conforming that the growth is related to nutrients confirms to Michaeils - Menten kinetics. μ_{max} and K^g_s value of NH_4Cl grown culture was 134 and 36.42 respectively. *D. salina* growth and β-carotene are interdependent since they showed very high degree of positive correlation (γ = +0.913). Phytoplankton accumulates intracellular nitrogen in the form of a variety of compounds, such as nitrate, ammonium, amino-acids, proteins, RNA and pigments. These compounds serve as a long-term nitrogen storage reservoir (Dortch *et al*., 1984). Von Rückert and Giani (2004) reported that the microalga *Microcystis viridis* grew faster with ammonium (μ = 0.393 day^{-1}) than with nitrate (μ = 0.263 day^{-1}) and ammonium + nitrate (μ = 0.325 day^{-1}).

Influence of Phosphate on growth and β-carotene production of *Dunaliella salina*

PO_4^- depleted actively grown *D. salina* was inoculated into Dewarln's media amended with different concentrations of sodium phosphate ranging from 0.1 to 1.0mM. The cultures were incubated for six days and estimated the growth rate and β-carotene.

Based on division rates the μ_{max} and K^g_s values were calculated and given inTable.2. The maximum growth rate (0.947 day^{-1}) and β-carotene accumulation (24µg/10^6cells) was found to be at 0.75mM/L PO_4^- Present media (Fig.2). Correlation analysis showed the fairly high degree of positive correlation (γ = +0.780) so the alga growth and β-carotene production are interdependent. Microalgal or cyanobacterial cells have been widely used to remove the excessive nutrients and other contaminants because they have a high capacity of uptaking inorganic nutrients in tertiary waste water treatment, while producing potentially valuable biomass (Martínez *et al*., 2000; Chevalier *et al*., 2000). Zhang et al., (2012) reported that the immobilized *Chlorella* sp. has great potentialities in nutrient removal and they removed the phosphate with the efficiency of 77.4% after 1.25 h and 100% after 4 h.

Influence of light intensity on growth and β-carotene production of *Dunaliella salina*

The accumulation of β-carotene and its isomeric ratio are strongly dependent on the light intensity and the quality of light used (Senger *et al*., 1993). Trabelsi *et al.*, (2009) reported that the optimum light intensity was higher than 180 µmol photons m^{-2} s^{-1} for *Arthrospira platensis* growth and production of extracellular polymeric substances (EPS). In this study *D. salina* was inoculated into Dewarln's media and incubated the cultures in the growth chamber at range of light intensity 100 to 2000µmol/m^{-2}/s^{-1}. The maximum division rate (0.875day^{-1}) and β-carotene production (21µg/10^6cells) was found to be at the light intensity 1000µmol/m^{-2}/s^{-1} with low temperature (Fig.3). The μ_{max} and K^g_s values are given in Table. 2. *D. salina* growth and β-carotene are interdependent since they showed very high degree of positive correlation (γ = +0.906). The accumulation of β-carotene depends on the integral light intensity to which *Dunaliella* is exposed during a division cycle (Lers *et al*., 1990). This ratio is promoted at low range from 20 to 50 µmol/ photons/m^{-2}/s^{-1} rather than high irradiances from 200 to 1250 µmol/photons/m^{-2}/s^{-1} (Orset and Young, 2000). When *Spirulina maxima* grown at 5 Klux light intensity its protein and Chlorophyll a content was 64.2% and 9.5mg/gm respectively (Ogbonda *et al*., 2007; Jai Prakash Pandey and Amit Tiwari, 2010).

D. salina has demonstrated an ability to produce large percentage content of β-carotene when grown in the optimum culture conditions. Micro algal productivity in marine ecosystem is also often limited by the availability of nutrient and light. In the present investigation the growth kinetic results may suggest the optimum concentration of ammonium chloride, sodium phosphate and light intensity to enhance the biomass production of *D. salina* to obtain the maximum production of β – carotene.

Figure.1 Influence of different concentration of ammonium chloride on β-carotene production of *Dunaliella salina*

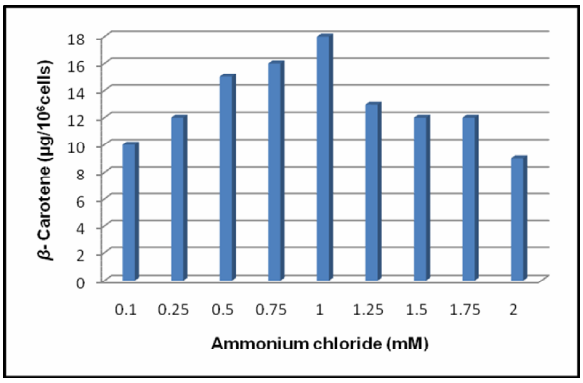

Figure.2 Influence of different concentration of phosphate on β-carotene production of *Dunaliella salina*

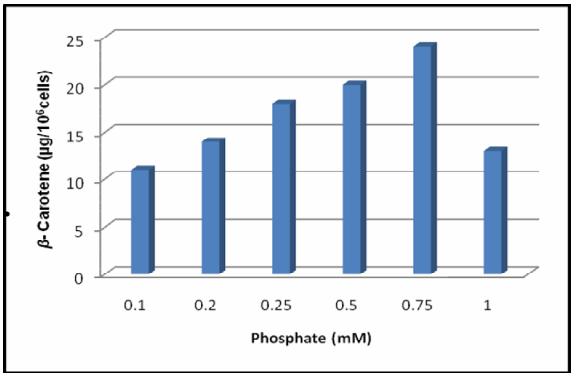

Figure.3 Influence of different light intensity on β-carotene production of *Dunaliella salina*

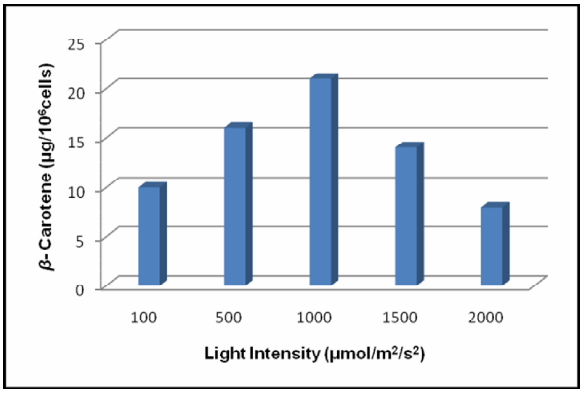

Table.1 Growth rate of *D. salina* in different concentration of NH_4Cl, PO_4 and Light Intensity

NH_4Cl		**PO_4**		**Light Intensity**	
Conc. (mM)	**Division rate(µ)**	**Conc. (mM)**	**Division rate(µ)**	**Intensity (µmol/m^2/s^2)**	**Division rate(µ)**
0.1	0.71	0.1	0.806	100	0.66
0.25	0.735	0.2	0.839	500	0.712
0.5	0.796	0.25	0.883	1000	0.875
0.75	0.815	0.5	0.908	1500	0.802
1	0.896	0.75	0.947	2000	0.566
1.25	0.815	1	0.663		
1.5	0.802				
1.75	0.745				
2	0.657				

Table.2 μ_{max} and K^g_s values of D. *salina* grown in different concentration of NH_4Cl, PO_4 and Light Intensity

Parameters	**μ_{max}**	**K^g_s**
NH_4Cl	134	36.42
PO_4	36.94	31.48
Light Intensity	15.39	2.99

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