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# Medium improvement for higher growth and longer stationary phase of *Dunaliella*

Suong Nguyen<sup>1</sup>, Duc Tran<sup>1,\*</sup>, Sixto Portilla<sup>2</sup>, Trung Vo<sup>1</sup>

<sup>1</sup>School of Biotechnology, International University-VNU, Vietnam

<sup>2</sup>Center for Environmental Research and Coastal Oceans Monitoring, Molloy College, 100 Hempstead Avenue, Rockville Centre, NY

**Email address:**

tnduc@hcmiu.edu.vn (D. Tran)

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**Abstract:** Beta-carotene is a valuable carotenoid in high demand as a natural food coloring agent, provitamin A, additive to cosmetics, and health food. It can be accumulated up to more than 10% of cellular dry weight of *Dunaliella salina* under carotenogenic conditions such as high irradiance, high temperature, high salt concentration and nutrient deficiency. High beta-carotene productivity in *Dunaliella* is best achieved in a two-phase culture system through biomass optimization and beta-carotene induction. A low-cost enriched natural seawater medium (MD4) was previously investigated for biomass optimization (Tran et al., 2014); However, the culture declined rapidly after reaching the stationary phase. Thus the present study is to further improve the effectiveness of this enriched natural seawater medium (MD4) for higher growth and longer stationary phase in order to avoid quick crash phase. Algal culture in MD4 medium used as control medium was subjected to 13 different feeding treatments (TM1 → TM13) using a matrix of concentrations of various compounds (NPK, KNO<sub>3</sub>, KH<sub>2</sub>PO<sub>4</sub>). *Dunaliella* growth was determined based on chlorophyll concentration, cell density. Results revealed the best feeding treatment was TM4 (NPK 0.15g/l) with cell density doubling one week compared with cell density in the control medium, and is recommended for use in the first phase biomass optimization of *Dunaliella*.

**Keywords:** Algae, Carotene, Chlorophyll, Cultivation, *Dunaliella*, Medium

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## 1. Introduction

Unicellular green algae *Dunaliella* belong to the Chlorophytes (Oren 2005, Tran et al 2013). The algae was first described by Dunal in the 1830s (Dunal 1838), but it was not until 1905 that the name *Dunaliella* was given by Teodoresco (Teodoresco 1905). There are currently 23 recognized *Dunaliella* species (Pick 1992, Oren 2005). *Dunaliella salina* TEODORESCO is the type species of the genus, whose vegetative cells are capable of turning red from carotenoid production under environmental stress such as high irradiance, high salinity, or low nutrient concentrations (Lamers et al. 2010, Tran et al. 2014).

*Dunaliella* can be found on all continents and in oceans, salterns and most hyper saline lakes all over the world. Temperature, salinity and nutrients are limiting factors on the growth and development of *Dunaliella* (Polle et al. 2009, Tran et al 2013). *Dunaliella* were found in the Great Salt Lake (Post 1977), the Dead Sea (Oren 2005), and from Antarctic salt lakes to salt lakes in Africa, America, Asia, Australia, and Europe (Ginzburg 1987, Borowitzka &

Borowitzka 1988, Tran et al 2013). Vietnam has a long coastal seawater resource of 3600km (Tran et al. 2005) with the existence of different strains of *Dunaliella salina* (Tran et al., 2013) which is a great potential for beta-carotene production. Commercial exploitation in this respect would contribute to regional economic and environmental stability.

Various artificial and natural seawater media have been devised for *Dunaliella* cultivation (Ben-Amotz 1995, Dipak and Lee 2005, García-González et. al 2003, Fazeli et al. 2006, Ana Prieto et al. 2011). Recently a new low cost enriched natural sea water medium (MD4) was investigated for biomass optimization of *Dunaliella* (Tran et al. 2014). However, there was a premature onset of stationary phase which prompted further efforts to improve the medium to sustain growth. In this study, cultures grown in MD4 medium (as control) were subjected to 13 different feeding treatments (TM1 → TM13) before entering the stationary phase using a matrix of concentrations of various mineral salts. Results revealed the best feeding treatment, TM4 (NPK 0.15g/l), doubled cell density one week compared

with cell density in the control medium, which is recommended for use in first phase biomass optimization.

## 2. Materials & Methods

### 2.1. *Dunaliella Salina* DCCBC15 Growth Conditions

*Dunaliella salina* DCCBC15 was kindly provided by Dr. E.W. Polle, Department of Biology, Brooklyn College of CUNY Brooklyn, NY (USA). The alga was grown and maintained in the low cost modified natural seawater medium 1.5M (MD4) according to Tran et al. (2014). Briefly, the medium contained natural seawater, and was added with NPK 0.1g/l, MgSO<sub>4</sub> 1.86g/l, EDTA 0.00876g/l, FeCl<sub>3</sub> 0.00049g/l, MnCl<sub>2</sub> 0.00189g/l, NaHCO<sub>3</sub> 50mM, pH = 7.5. The medium was sterile-filtered and used as control (treatment 1: TM1), which had no feeding treatment. Algal cultures were subjected to 12 additional feeding treatments of various salts with different concentrations as shown in Table 1.

**Table 1.** Thirteen treatments with different concentrations of various mineral salts

Treatments	NPK* (g/l)	KNO <sub>3</sub> (g/l)	KH <sub>2</sub> PO <sub>4</sub> (g/l)
1	0.00	0.00	0.0000
2	0.05		
3	0.10		
4	0.15		
5		0.25	
6		0.50	
7		0.75	
8			0.0135
9			0.0270
10			0.0405
11		0.25	0.0135
12		0.50	0.0270
13		0.75	0.0405

(\*) N-P-K (30-15-10): 30% N, 15% P<sub>2</sub>O<sub>5</sub>, 10% K<sub>2</sub>O, 0.05% Mg, 0.05% Ca, 0.01% B, 0.05% Zn, 0.05% Cu, 0.05% Fe, 0.025% Mn, 0.005% Mo. (Tran et al 2013)

Cultures were grown in 50 mL flasks containing 25 ml of medium, in triplicate. They were maintained at 25°C with a photon flux density of 50  $\mu\text{moles m}^{-2}\text{s}^{-1}$  (PAR) provided by white fluorescence lamps. Nutrients were fed on the 8<sup>th</sup> day of culture before entering the stationary phase.

### 2.2. Growth Determination

Cell density was estimated by optical density, cell number, and chlorophyll every two days. Briefly optical

density (OD) was measured at 750 nm by Microplate Reader (Biotek) and cell number was counted using a light microscope with 0.1mm deep counting chamber (Neubauer Haemocytometer). Lugol solution (5% iodine and 10% potassium iodide in distilled water) was used to stop cell movement. Cell number was calculated by following formula:

Number of cells/ml = total cells counted  $\times 10^4 \times$  dilution factor.

Chlorophyll extraction was carried out according to Dipak S. P. and Lele S. S. (2005). One ml aliquots of *Dunaliella salina* cultures were centrifuged at 8,000 rpm for 20 min. The pellets were washed with distilled water, suspended in an 80% acetone solution, thoroughly vortexed and centrifuged to extract pigments until the pellets turned clear/white. Absorbance of relevant pigments was measured using Microplate Reader (Biotek). Chlorophylls (Chl a+ Chl b) were estimated according to Lichtenthaler, H. K., Wellburn, A. R. (1985):

$$\text{Chl } a \text{ (}\mu\text{g/ml)} = 11.75 (A_{662}) - 2.35 (A_{645})$$

$$\text{Chl } b \text{ (}\mu\text{g/ml)} = 18.61 (A_{645}) - 3.96 (A_{662})$$

$$\text{Cx+c (}\mu\text{g/ml)} = (100A_{470} - 2.270 \text{ Chl } a - 81.4 \text{ Chl } b)/198$$

Where: Chl *a* is chlorophyll a, Chl *b* is chlorophyll b, Cx+c: Total carotene.

Specific growth rate (G: divisions/day) and cell growth productivity (P: cells/ml/day) were determined using equations according to Levasseur et al (1993):

$$G = \ln(N_t/N_0)/t; P = (N_t - N_0)/t$$

Where:  $N_t$  and  $N_0$  are cell density at time *t* and time 0 respectively.

### 2.3. Data Analysis

Data was tested by one-way ANOVA analysis using SPSS software. All significant levels were set at  $p < 0.05$ .

## 3. Result and Discussion

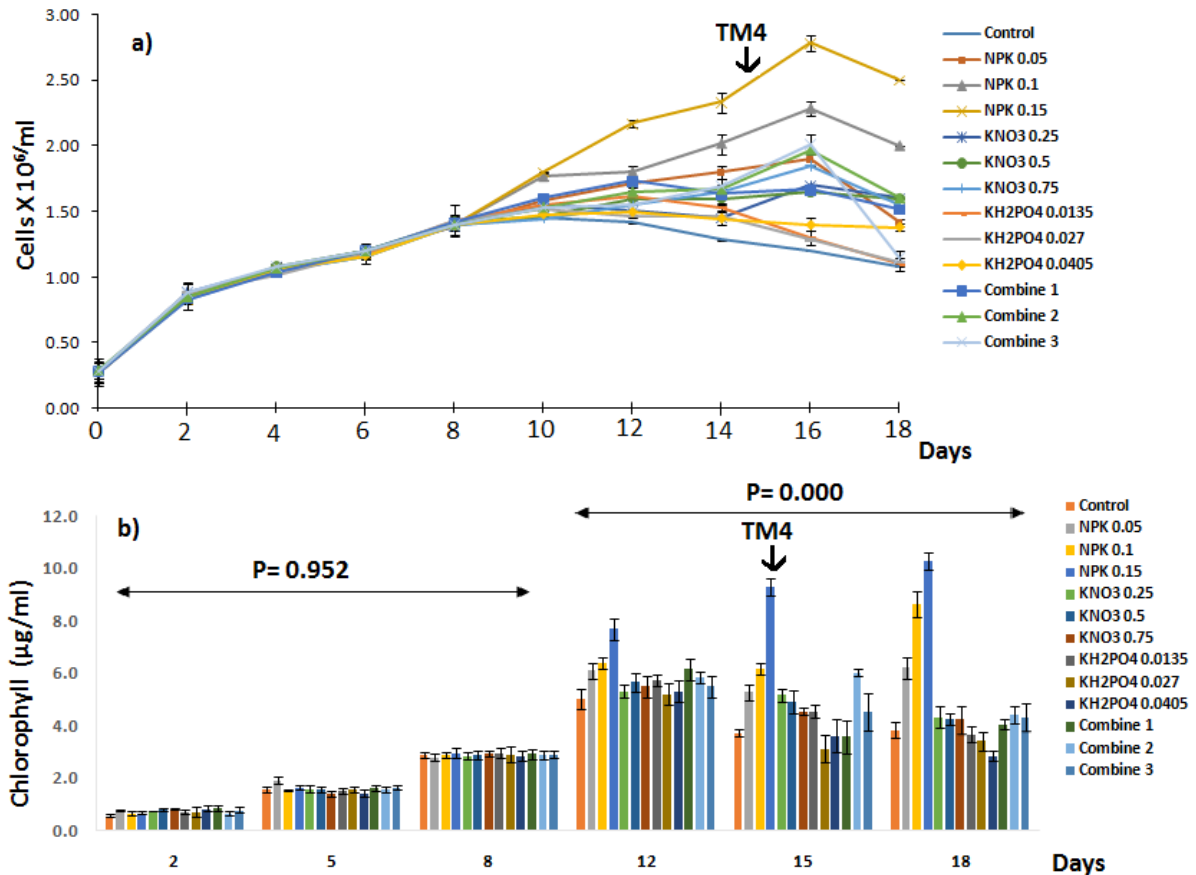
An overview of *Dunaliella salina* growth in 13 different treatments by cell count, optical density and chlorophyll *a* and *b* during the 18 day experiment is shown in Figure 1. The cultures were started with initial cell concentrations of  $0.24 \times 10^6 \text{ cells.ml}^{-1}$  and OD value of 0.07, and were then subjected to feeding treatments after 8 days of culture, which were about 2 days before the projected onset of stationary phase, at average cell concentration of  $1.4 \times 10^6 \text{ cells.ml}^{-1}$  and OD value of 0.30 (Figure 1). The cell counts, chlorophylls and growth rates revealed no differences among the cultures before feeding treatments (Figure 1 and 2), but diverged significantly after feeding treatments ( $P < 0.05$ ) (Figure 1 and 3)

Feedings with NPK (TM2, TM3 and TM4,

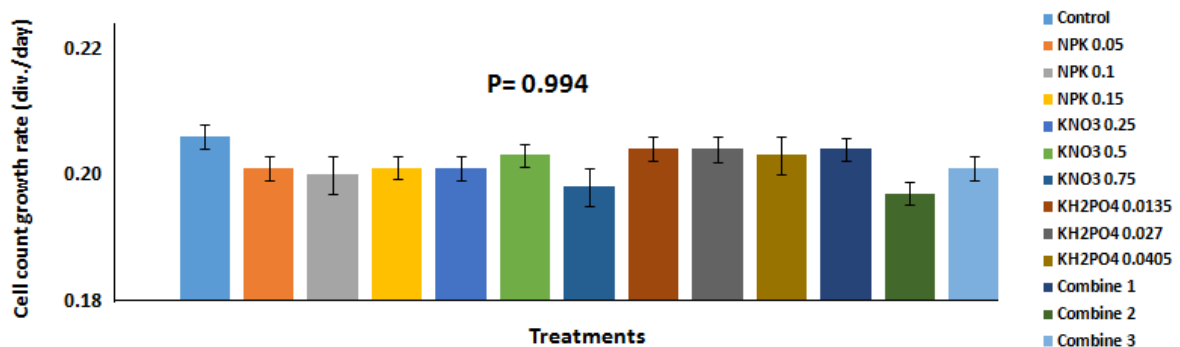
corresponding to NPK 0.05g/l, 0.1g/l and 0.15g/l respectively) supported good overall growth (Figure 1, 3), but TM4 (NPK 0.15g/l) supported the the highest growth up to  $2.78 \times 10^6$  cells. $\text{ml}^{-1}$  after 16 days, whereas TM2 (NPK 0.5g/l), TM3 (NPK 0.1g/l), and TM1 (control) were  $2.28 \times 10^6$  cells. $\text{ml}^{-1}$ ,  $1.9 \times 10^6$  cells. $\text{ml}^{-1}$   $1.28 \times 10^6$  cells. $\text{ml}^{-1}$ , respectively. In addition, the NPK feedings maintained the cultures longer, up to 1 week before starting the death phase (Figure 1). The efficacy of NPK on *Dunaliella* growth was in agreement with a prior work (Tran et al. 2014).

Our data showed that treatments with KNO<sub>3</sub> (TM5, TM6 and TM7), Combined KNO<sub>3</sub> & KH<sub>2</sub>PO<sub>4</sub> (TM11, TM12, TM13), and KH<sub>2</sub>PO<sub>4</sub> (TM8, TM9 and TM10) also supported better growth than the control (TM1), but the efficiency was lower than the NPK feedings (Figure 1, 2, 3). However KNO<sub>3</sub>, an important salt for algal growth, supported the algal cultures better than KH<sub>2</sub>PO<sub>4</sub>.

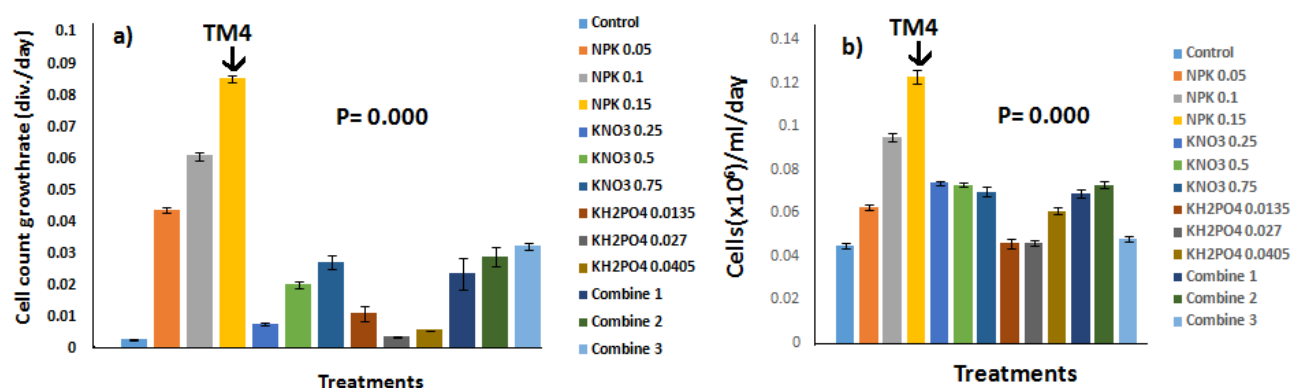
Our study focuses on the first stage for growth improvement. Yet total carotenes of treatments were also analyzed, which were different after feeding but not statistically significant (Figure 4).



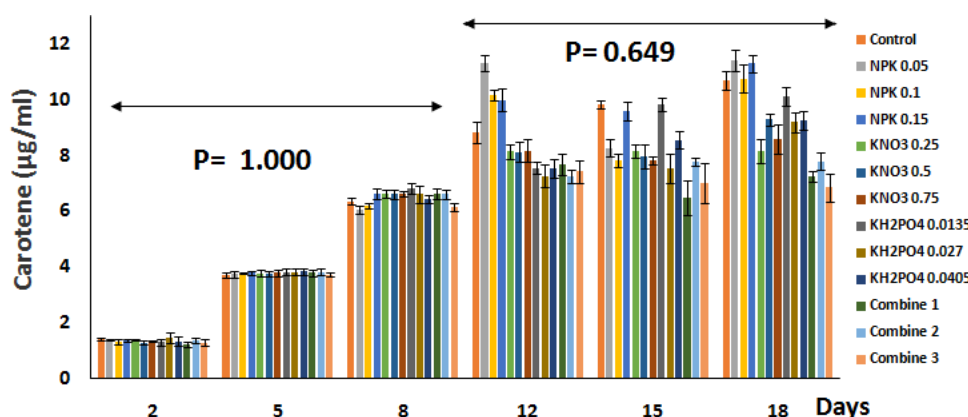
**Figure 1.** Growth curve of *Dunaliella salina* in 13 feeding treatments based on cell count (a) and chlorophyll (b). TM4 provided the best effect on growth among treatments. Numbers in the legend indicate amount (g/l) of mineral salts used for feeding. P values on the graph were statistical values



**Figure 2.** Growth rate of *Dunaliella salina* before feeding treatment based on cell count. The numbers in the legend indicate amount (g/l) of mineral salts used for feeding. P values on the graph were statistical values



**Figure 3.** Growth rate (a) and productivity (b) of *Dunaliella salina* after feeding treatments. The numbers in the legend indicate the amount (g/l) of mineral salts used for feeding. *P* values on the graph were statistical values



**Figure 4.** Total carotenoids of different treatments. The numbers in the legend indicate the amount (g/l) of mineral salts used for feeding. *P* values on the graph were statistical values

## 4. Conclusion

Growth of *Dunaliella* in the MD4 medium (Tran et al. 2014) used in this investigation was significantly improved by applying TM4 (NPK 0.15g/l) before the projected stationary phase. Culture density doubled within a week. This new improved medium is recommended for use in the first phase biomass optimization. Other treatments (TM5, TM6, TM7 and TM11, TM12, TM13) also produced longer lasting and more stable cultures.

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## References

- [1] Ana Prieto J., Canavate P., García-González M., 2011. Assessment of carotenoid production by *Dunaliella salina* in different culture systems and operation regimes. *Journal of Biotechnology*. Vol. 151: 180-185
- [2] Ben-Amotz A., Avron M., 1983. On the factors which determine massive  $\beta$ -carotene accumulation in the halotolerant alga *Dunaliella bardawil*. *Plant physiol.* Vol 72: 593-597.
- [3] Ben-Amotz A., Avron M., 1989. The biotechnology of mass culturing *Dunaliella* for products of commercial interest. In: Cresswell, R.C., Rees, T.A., and Shah, N., (Eds). *Algal and Cyanobacterial Biotechnology*. Longman Scientific & Technical, Harlow, U.K.: 91-114.
- [4] Ben-Amotz A., Shaish A., 1992.  $\beta$ -Carotene bio-synthesis. In: Avron, M., Ben-Amotz, A. (Eds.), *Dunaliella: Physiology, Biochemistry and Biotechnology*. CRC Press, Boca Raton, FL: 206-216.
- [5] Borowitzka L.J., Kessly D.S., Brown A.D., 1977. The salt relations of *Dunaliella*. Further observations on glycerol production and its regulation. *Archives of Microbiology*. Vol. 113: 131-138.
- [6] Borowitzka M.A., Borowitzka L.J., 1988. *Dunaliella*. In: Borowitzka M.A., Borowitzka L.J (Eds), *Micro-algal Biotechnology*. Cambridge University Press, Cambridge, UK: 27-58.
- [7] Borowitzka M.A., 1995. The biotechnology of microalgal carotenoid production. In: Aubert J.P. & Martin P.M.V. (eds.), *Microbes, Environment, Biotechnology. Colloques Internationaux de L'annee Pasteur*. Institut Louis Malarde: Papeete, Tahiti: 149-151.
- [8] Borowitzka M.A., 1999. Economic evaluation of microalgal processes and products. In: Cohen, Z. (Ed). *Chemicals from Microalgae*. Taylor & Francis, London: 377-409.

- [9] Borowitzka M.A., Siva C. J., 2007. The taxonomy of the genus *Dunaliella* (Chlorophyta, Dunaliellales) with emphasis on the marine and halophilic species. *Journal of Applied Phycology*. Vol.19:567–590.
- [10] Burtan G.W., Ingold K.U. , 1984.  $\beta$ -carotene an unusual type of lipid antioxidant. *Science*. Vol. 224: 569–574.
- [11] Chitlaru E., Pick U., 1989. Selection and Characterization of *Dunaliella salina* Mutants Defective in Haloadaptation. *Plant Physiol*. Vol.91, 788-794.
- [12] Dipak S. P. and Lele S. S. (2005), Carotenoid production from microalga, *Dunaliella salina*. *Indian J of Biotech*. Vol.4: 476-483
- [13] García-González M., Moreno J., Cañavate J.P., Anguis V., Prieto A., Manzano C., Florencio F.J., Guerrero M.G , 2003. Conditions for open-air culture of *Dunaliella salina* in Southern Spain. *Journal of Applied Phycology*. Vol.15:177-184.
- [14] García-González M., Moreno J., Carlos Manzano F., Florencio J., Guerrero M. G, 2005. Production of *Dunaliella salina* biomass rich in 9-cis- $\beta$ -carotene and lutein in a closed tubular photobioreactor. *Journal of Biotechnology*. Vol. 115:81–90.
- [15] Ginzburg M., 1987. *Dunaliella*: a green alga adapted to salt. *Advances in Botanical research*. Vol.14: 93-183.
- [16] Grobbelaar J. U., 1995. Influence of areal density on  $\beta$ -carotene production by *Dunaliella salina*. *Journal of Applied Phycology*. Vol.7: 69-73
- [17] Lamers P.P., Van de Laak C.C., Kaasenbrood P.S., Lorier J., Janssen M., DeVos R.C., Bino R.J., Wijffels R.H. (2010), Carotenoid and fatty acid metabolism in light –stressed *Dunaliella salina*. *Biotechnol Bioeng*. Vol.106 (4), 48-63
- [18] Levasseur, M., P.A. Thompson, and P.J. Harrison. 1993. Physiological acclimation of marine phytoplankton to different nitrogen sources. *J. Phycol*. Vol.29:87-595.
- [19] Lichtenthaler H. K., Wellburn A. R., 1985. Determination of total carotenoids and chlorophylls A and B of leaf in different solvents. *Biol. Soc. Trans*. Vol. 11, 591-592
- [20] Moulton T.P., Borowitzka L.J., Vincent D.J., 1987. The mass culture of *Dunaliella salina* for  $\beta$ -carotene: From pilot plant to production plant. *Hydrobiologia*. Vol.41: 99-105.
- [21] Oren A., 2005. A hundred years of *Dunaliella* research: 1905-2005. *Saline Systems*. Vol. 1(2). doi: 10.1186/1746-1448-1-2
- [22] Pick, U., 1998. *Dunaliella*-A model extremophilic alga. *Israel Journal of plant sciences*. Vol.46: 131-139.
- [23] Polle J. E. W, Tran D., Ben-Amotz A., 2009. Chapter 1: History, Distribution, and Habitats of Algae of the Genus *Dunaliella* TEODORESCO (Chlorophyceae). *The Alga Dunaliella: Biodiversity, Physiology, Genomics & Biotechnology*. ISBN 13: 9781578085453 ISBN 10: 1578085454
- [24] Psal D. S., Lele S. S., 2005. Carotenoid production from microalga, *Dunaliella salina*. *Indian Journal of Biotechnology*. Vol.4: 476-483.
- [25] Tawfiq S., Abu-Rezq Al-Hooti, S., Jacob, D.A., 2010. Optimum culture conditions required for the locally isolated *Dunaliella salina*. *Journal of Algal Biomass Utilization*. Vol. 1(2): 12-19.
- [26] Tran A.T., Le H.V, Le N.Q., 2005. Ministry of natural resources and environment of Vietnam. Technical report on the identification and assessment of technology needs for GHG emission reduction and climate change adaptation in Viet Nam.
- [27] Tran D., Vo T., Portilla S., Louime C., Doan N., Mai T., Tran D. and Ho T., 2013. Phylogenetic study of some strains of *Dunaliella*. *American Journal of Environmental Science*. Vol.9 (4): 317-321
- [28] Tran D., Doan N., Louime C., Giordano M., Portilla S., 2014. Growth, antioxidant capacity and total carotene of *Dunaliella salina* DCCBC15 in a low cost enriched natural seawater medium. *World Journal of Microbiology and Biotechnology*. Vol.30(1): 317-322. DOI 10.1007/s11274-013-1413-2