Available Online at http://www.journalajst.com

ASIAN JOURNAL OF SCIENCE AND TECHNOLOGY

ISSN: 0976-3376

Asian Journal of Science and Technology
Vol. 09, Issue, 02, pp.7610-7615, February, 2018

RESEARCH ARTICLE

FTIR ANALYSIS OF B CAROTENE PRODUCED FROM *CHLORELLA VULGARIS* AS-3 STRAIN, A FRESH WATER MICRO ALGAE

Shakeel Ahmed Adhoni, Megha. R Shinge and *Shivasharana, C. T.

P. G. Department of Biotechnology and Microbiology, Karnatak University, Dharwad, India

ARTICLE INFO

Article History:
Received 15th November, 2017
Received in revised form
18th December, 2017
Accepted 12th January, 2018
Published online 28th February, 2018

Key words:
Environmental education,
Consumerism,
Ideological Epistemology,
Knowledge and power.

ABSTRACT

The investigation deals with the production and characterisation of β carotene using microalgae *Chlorella vulgaris* AS-3 strain isolated from fresh water lake. High light intensity and nutrient starvation results in synthesis of beta carotene through several interdependent stages. *Chlorella vulgaris* AS-3 was isolated and selected for higher yield of carotenoids and wide range of growth in phototrophic conditions (200 lux) in BG-11 medium supplemented with NPK (nitrogen, phosphours and potassium) for 14 days and the pigment production was facilitated by adding 1% NaCl. When the cell count reached at 500,000 cells/ml centrifuged and pigment was extracted with 80% acetone. The amount of dry weight was 4g/L of which 3-6% was β carotene, using optimised media. The biomass can be increased by supplementation of pure CO_2. Both paper and TLC were conducted for separation of pigments. Isolated Pigments were then analysed by FTIR. IR spectrum resulted showed positive qualitative identification of β carotene and confirmations of its amines and aromatic compounds. Pilot scale investigations has to be carried out for the production and commercialisation of nutraceuticals and their supplements.

INTRODUCTION

Microalgae are green, unicellular, eukaryotic, photosynthetic microorganisms dwelling in fresh as well as marine water ecosystems, rich in essential amino acids, proteins essential antioxidants, amino acids, vitamins, fatty acids and other bioactive molecules used in pharmaceuticals, nutraceuticals, cosmetics and biofuel industry (Pulz and Gross, 2004; Oncel, 2013). They got wide range of valuable components including carbohydrates, essential fatty acids and diversified pigments such as chlorophylls, carotenoids and phyco-bili- proteins (Valdes *et al.,* 2016). Chlorophyceae are biotechnologically most relevant and widely commercialized for nutritional supplements (Khan *et al.,* 2005). Genus *Chlorella vulgaris* are characterized by their ease of cultivation rate and product yield such as protein, chlorophyll, lutein and other important essential micro-nutrients (Buono *et al.,* 2014; Jeon *et al.,* 2012). Recently, numerous epidemiological studies in relation to health point of view, especially foods have proven that the increased intake of green and yellow vegetable and fruits are associated significantly with decreased risks such as affecting chronic diseases (Heiner *et al.,* 2012). These finds reveals Phytochemicals, carotenoids and other antioxidants are found to be the efficient substances among others. Against this background, media optimization and FTIR studies conducted reveals changes in cellular components during the course of cell cycling in obtaining the synchronous culture.

***Corresponding author:* Shivasharana, C. T.**
P. G. Department of Biotechnology and Microbiology, Karnatak University, Dharwad, India

Another objective was to standardize the methods of producing phytochemical rich organisms using phototrophic synchronous culture (PSC) at higher cell density levels. Finally, the productivity and the quality of the cells obtained in the PSC systems are also discussed.

MATERIALS AND METHODS

Microalgae cultivation: A fresh water microalgae *Chlorella vulgaris* AS-3 was previously isolated, characterized and maintained in our laboratory using BG-11 media (Shakeel *et al.,* 2015). In the present investigation, we aimed to synthesize carotenoid pigments from the isolated algae. Phototrophic cultivation was carried out using BG-11 media, at temperature 25 ºC, pH 6.8, light intensity 2000 lux, then incubated to obtain biomass.

Media optimization and biomass harvesting: Media optimization was aimed for selection of potent *Chlorella vulgaris* AS-3 strain producing high concentration of carotenoids using cost effective media components under phototrophic conditions. The culture media was supplemented with NPK as nutrients, 1% NaCl was added to the culture media after 14 days of incubation and continued to be added for every 5 days' time interval for 21 days. After 21 days biomass was harvested by flocculation, filtration and centrifugation methods. Centrifuged biomass was shade dried and used for further work. Culture density and dry weight were estimated to quantify carotenoids obtained.

Pigment extraction: Pigments were extracted using an organic solvent chilled 80 % acetone, followed by 10 minutes centrifugation at 1000 rpm. Thus extracted pigments were analyzed using both Paper as well as TLC methods.

TLC (Thin Layer Chromatography): TLC was performed using silica gel glass plates, as stationary phase. Extracted sample was loaded on the gel allowed to move through stationary phase using petroleum ether: acetone mixture (7:1) as mobile phase. After progression of the sample chromatographic plate was sun dried and sprayed Ninhydrine to observe the pigments. Pigments separated on silica gel plate were eluted in 70 % methanol and used for further analysis.

FTIR analysis : Thus eluted pigments from TLC were further analyzed and characterized using Fourier transfer infrared spectroscopy [NICOLET 6700 (USA 2013)] in USIC, Karnatak University, Dharwad. In this test, extract was converted to gaseous state and numbers of ions are counted.

RESULTS AND DISCUSSION

Vitamin A deficiency is the leading cause of blindness in children and rises the risk of diseases and death from severe infections in pregnant women. Vitamin A deficiency causes night blindness and may increase the risk of maternal mortality. In order to meet the demand of vitamin A, apart from administration, vitamin A palmitate is a supplement of carotenoids.

In general β carotene is particularly used widely. In this concern carotenoids are gaining more and more attention in the direction of searching newer sources and renewable production system. Biotechnology is the tool to enrich carotenoid production and is receiving attention as evident from the interest generated in golden rice. Several studies have demonstrated that seaweeds are an excellent source of components such as polysaccharides, tannins, flavonoids, phenolic acids, bromophenols and carotenoids have exhibited different biological activities (Rodriguez *et al.,* 2010). Microalgae are also rich source of carotenoids and are known to accumulate high quality of β carotene under certain parts of the world (Shinichi Takaichi, 2011). Recently, microalgae have attracted increased attention in the field of biomass production and biosynthesis of feed stock components or high valuable products like polyunsaturated fatty acids, antioxidants, pigments like carotenoids which can be used for food colorants, etc. (Wagner *et al.,* 2014). The most challenging approach to increase the productivity is not only to develop photo-bioreactors which offer optimal conditions for algae growth, but also to increase the yield of a special product (Olivieri *et al.,* 2014). Present investigation revealed that *Chlorella vulgaris* AS-3, a fast growing freshwater microalgal strain, which was previously isolated, characterised and maintained in our laboratory (Shakeel *et al.,* 2015). Phototrophic cultivation, which is a promising strategy for obtaining valuable natural products in cost effective way, was adopted and the growth studies revealed that *Chlorella vulgaris* showed increased β carotene production in BG-11 media supplemented with NPK and 1% NaCl in presence of light of 2000 lux and aeration supplemented externally through air pump (Fig 1). Culture conditions are optimized for large scale production and also aimed for biomass production. After 21 days, harvested biomass was characterized for presence of β carotene using chromatography technique where samples were run on silica gel along with standard β carotene. Orange colored pigment was separated on TLC plate. Carotenoid pigments along with chlorophylls were separated based on TLC (Fig 4). Downstream process plays a crucial role in effective extraction of carotenoids from cell and storage of the biomass extracts without degrading them, as they are sensitive to the factors like light, oxygen and temperature to the extent (Woutersen *et al.,* 1999). Attention was also focused on identifying the carotenoids. Eluted samples were taken for FTIR analysis along with standard β carotene as a reference for comparison (Fig 5 and 6). Seyfabadi *et al.,* (2011) studied the effects of irradiance and photoperiod on growth rate, chlorophyll a, β carotene, total protein and fatty acid content of *C. vulgaris.* Our results show the amount of dry weight was 4 g/L of which 3-6% was β carotene. Media optimization facilitated the production of β carotene. Process parameters

Table 1. Paper and TLC analysis

Method	Sample fractioned	Standard	Sample	Rf value
Paper chromatography	Solvent moved	5.5 cm	5.0 cm	4.8÷5.0=0.96 cm
	β carotene	3.7 cm	4.8 cm	
Thin layer	Solvent moved	5.7 cm	7.6 cm	6.2÷7.6=0.81 cm
chromatography	β carotene	5.6 cm	6.2 cm	5.4÷7.6=0.71 cm
	Chlorophyll	5.1cm	5.4 cm	

Table 2. Peak description by FTIR of standard β Carotene

Frequency/cm	Bond	Functional group	Bond Strength
3436.84	O-H stretch, H bonded	Alcohols, phenols	Strong, Broad
2966.80	C-H stretch	Alkanes	Strong
2924.71	C-H stretch	Alkanes	Strong
2854.58	C=N stretch	Nitriles	Weak
1748.16	C=O stretch	Esters, saturated aliphatic	Strong
1618.51	N-H bend	1* amines	Medium
1459.69	C-H bend	Alkanes	Medium
1409.30	C-C stretch (in ring)	Aromatics	Medium
1330.17	N-O symmetric stretch	Nitro compounds	Medium
1259.21	C-N stretch	Aromatic amines	Strong
1112.35	C-N stretch	Aliphatic amines	Medium
1023.66	C-N stretch	Aliphatic amines	Medium
603.27	C-Cl stretch	Alkyl halides	Medium
592.44	C-Br stretch	Alkyl halides	Medium

were optimized, which are suitable for large scale production of β carotene. FTIR analysis of both standard carotene and β carotene *Chlorella vulgaris* samples confirmed the presence of functional groups like Alkanes, amides, alkyl halides, alcohols, phenols, aromatic amines, aliphatic amines. Amines at CH bond stretch at 1646.87 and aromatics C-C stretch at 1411.87 in standard β carotene, in sample CH bond stretch at 1618.51 and aromatics C-C stretch at 145.60 confirmations of the functional groups (Fig 5).

Llewellyn, 1983). Chilled acetone was found to be the best solvent for pigment extraction, similar findings were also reported by Strickland and Parsons (1972) in which they extracted chlorophyll a and carotenoids in cold 90% acetone, in darkness, at -20°C for 4 h. Latała and Misiewicz, (2000) also determined photosynthetic pigments in cyanobacterium *G. Amphibium* by spectrophotometric and chromatographic methods. Wright *et al.,* (1991) determined these pigments in *Dunaliella tertiolecta*-CCMP.

Table 3. Peak description of FTIR analysis of Algal β Carotene

Frequency/cm	Bond	Functional group	Bond nature
3428.31	O-H stretch ,H-bonded	Alcohol, phenol	Strong, Broad
2923.95	C-H stretch	Alkanes	Strong
2854.95	C-H stretch	Alkanes	Medium
1646.47	N-H bond	1* amines	Medium
1542.11	N-O symmetric stretch	Nitro compounds	Strong
1459.69	C-H bend	Alkanes	Medium
1411.87	C-C stretch (in ring)	Aromatics	Medium
1260.40	N-O symmetric stretch	Nitro compounds	Medium
1162.39	C-H wag (-CH2X)	Alkyl halide	Medium
1024.18	C-N stretch	Aliphatic amines	Strong
559.87	C-Cl stretch	Alkyl halides	Medium

Fig. 1. *Chlorella vulgaris* AS-3 strain cultivated in Phototrophic conditions

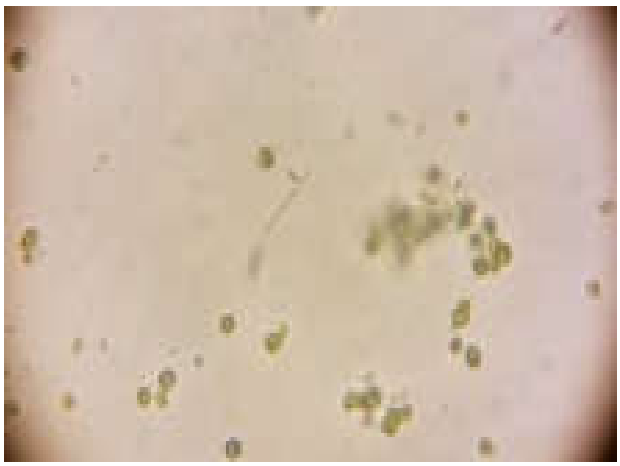

Fig. 2. Micro algal ce lls of *Chlorella vulgaris*

Fig. 3. β Carotene dried powder

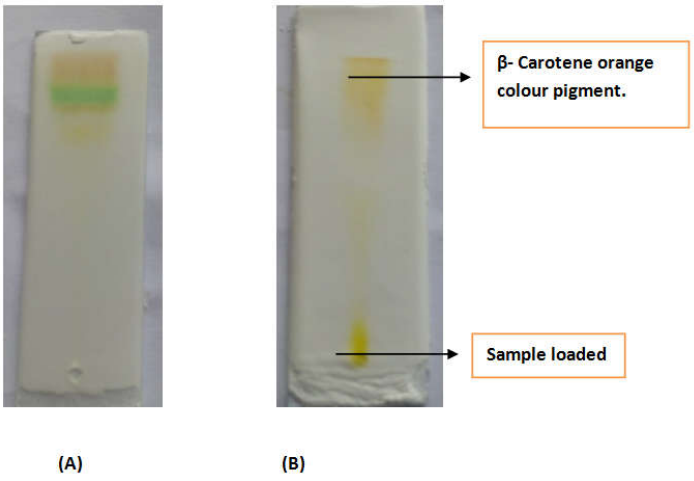

Fig. 4. Standard on TLC plate, β-carotene separation extracted from *Chlorella vulgaris* AS-3 on TLC plate

It was reported that spectrophotometric method enables to estimate the only total and approximate amount of carotenoids, but not the amounts of individual carotenoids (Mantoura and

Similar finding were observed during the culture of *H. pluvialis* where high light levels accelerated the growth process, increasing the rate of nutrient depletion and providing

more energy for astaxanthin biosynthesis (Fabregas *et al.,* 1998). The same effect was observed in β carotene and phycocyanin production by *Dunalliella salina* and *Spirulina platensis* respectively. In some studies upon increasing light exposure the synthesis of these pigments also increased (Hejazi *et al.,* 2004; Walter *et al.,* 2011).

another factor which has the great influence on the stability of carotenoids (Chen *et al.,* 1996; Hii *et al.,* 2010; Mercadante, 2008a; Rodriguez-Amaya, 2001; Ye & Eitenmiller 2006). Carotenoids undergo photodegradation and isomerization when exposed to light (Chen *et al.,* 1996; Mercadante 2008b; Vásquez-Caicedo *et al.,* 2007).

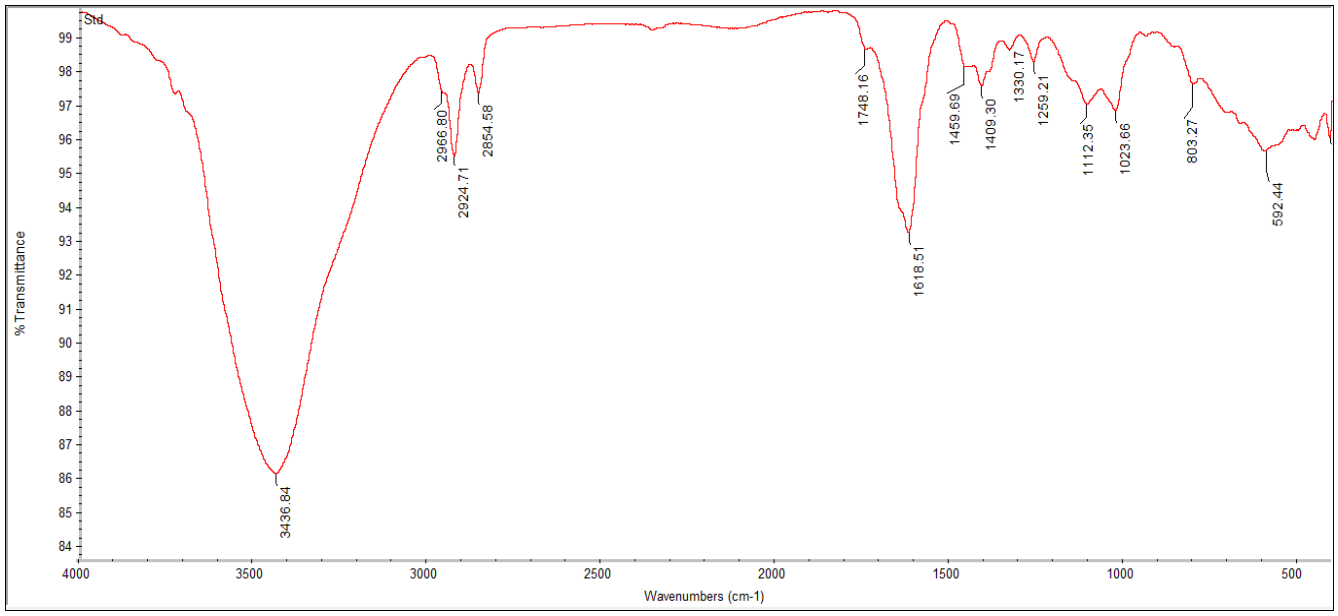

Fig. 5. FTIR analysis of Standard carotene sample

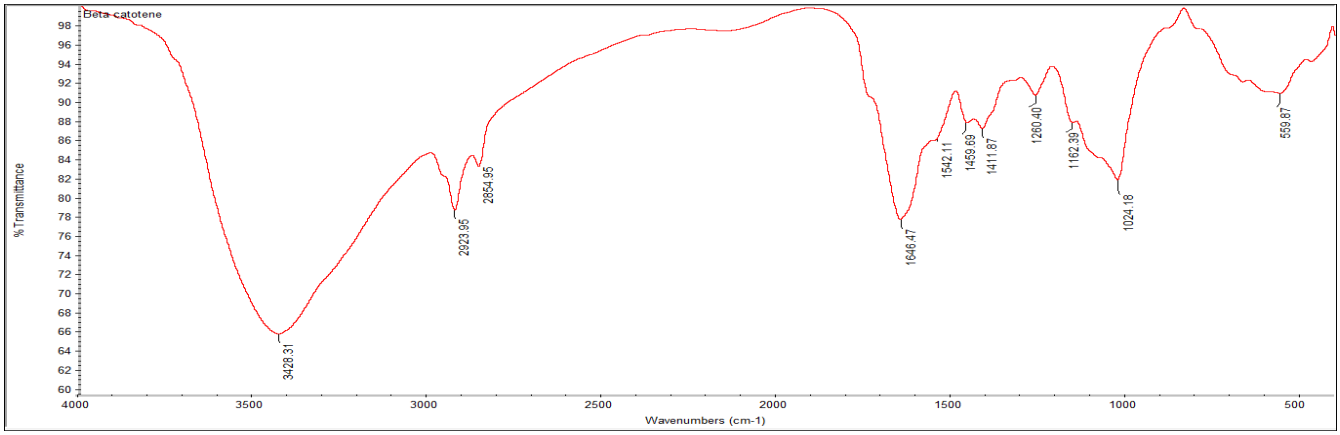

Fig. 6. FTIR analysis of β Carotene

The major pigment of brown seaweeds is fucoxanthin, which is one of the most abundant carotenoids in nature (10% estimated total production of carotenoids) (Pangestuti & Kim, 2011). It is an orange-coloured pigment, found in brown seaweeds along with chlorophyll, to give a brown or olive-green colour (Chandini *et al.,* 2008; Hosokawa *et al.,* 2009). The wavelength range of 350 -750 nm was chosen as most carotenoids absorb lights in the region between 400 and 500 nm and chlorophylls absorb light at 500 to 700 nm at room temperature (Dere *et al.,* 1998, Mercadante 2008a and Marquez & Sinnecker 2008). According to Chandini *et al.,* (2008) and Hosokawa *et al.,* (2009), fucoxanthin is the major carotenoid found in brown seaweeds. According to Papagiannakis *et al.,* (2005), the absorbance of fucoxanthin ranges from 420-470 nm. Carotenoids are relatively stable at alkaline condition. A study conducted by Chen *et al.,* (1996) reported that the rate of degradation of carotenoids were significantly slower at alkaline pH than at acidic pH. Light is

High temperature may cause the breakage of double bonds in the carotenoid molecule and caused pigment degradation (Boon *et al.,* 2010; Mercadante, 2008b). Therefore, fucoxanthin-rich extract is more suitable to be applied into food products that are kept at room temperature or lower. Carotenoid concentrations are often used to describe the composition of phytoplankton in water and to estimate their abundance in mixed populations (Wright *et al.,* 1991; Millie *et al.,* 1993; Andersen *et al.,* 1996; Stoń and Kosakowska, 2002; Buchaca *et al.,* 2005; Llewellyn *et al.,* 2005; Wulff *et al.,* 2005; Schagerl and Kunzl, 2007). However, estimation of the ratio of carotenoids to chlorophyll a is useful to understand the reaction of organism to changing environmental light condition (Jodłowska and Latała, 2010).

Conclusion

Marine algae are the valuable sources of marine ecosystem. Marine algae act as the source of bioactive metabolites that

produce a wide range of antimicrobial potentials. The phytochemical analysis of seaweeds evaluated the presence of various secondary metabolites such as phenols, terpenes, saponins, proteins, steroids, carbohydrates etc. The presence of various bioactive metabolites was detected by using UV, FTIR and HPLC analysis. From the study it is confirmed that the *Valonopsis pachynema* and *Sargassum swartzii* algae could be used for the development of drugs. Since carotenoids have beneficial function and values in food and pharmaceutical industries, hence further analysis in this research was focused on the carotenoid.

REFERENCES

Andersen, R.A., R.R. Bidigare, M.D. Keller and M. Latasa. 1996. A comparison of HPLC pigment signatures and electron microscopic observations for oligotrophic waters of the North Atlantic and Pacific Ocean. *Deep Sea. Res. Topical Stud. Oceanography,* 43: 517-537. DOI: 10.1016/0967-0645(95)00095-X.

Boon, C.S., McClements, D.J., Weiss, J. & Decker, E.A. 2010. Factors Influencing the chemical stability of carotenoids in foods. *Critical Reviews in Food Science and Nutrition* 50: 515-532.

Buchaca, T., M. Felip and J. Catalan. 2005. A comparison of HPLC pigment analyses and biovolume estimates of phytoplankton groups in an oligotrophic lake. *J. Plankton Res*., 27: 91-101. DOI: 10.1093/plankt/fbh154pap.

Buono S, Langellotti AL, Martello A, Rinna F, Fogliano V. 2014. Functional ingredients from microalgae. *Food Funct*. Aug; 5(8):1669-85.

Chandini, S.K., Ganesan, P., Suresh, P.V. & Bhaskar, N. 2008. Seaweeds as a source of nutritionally beneficial compounds - A review. *Journal of Food Science and Technology* 45(1): 1-13.

Chen, H.E., Peng, H.Y. & Chen, B.H. 1996. Stability of carotenoids and vitamin A during storage of carrot juice. *Food Chemistry*. 57(4): 497-503.

Dere, S., Gűnes, T. & Sivaci, R. 1998. Spectrophotometric determination of chlorophyll - A, B and total carotenoid contents of some algae species using different solvents. *Turkish Journal of Botany* 22: 13-17.

Fábregas J., A. Domínguez, D. García-Álvarez, T. Lamela, A. 1998. Otero, Induction of astaxanthin accumulation by nitrogen and magnesium deficiencies in *Haematococcus pluvialis*, *Biotechnol. Lett.* 20, 623–626.

Heiko Wagner [1]*, Anne Jungandreas [1,2], Andrea Fanesi [1] and Christian Wilhelm [1] Surveillance of C-Allocation in Microalgal Cells. *Metabolites* 2014, *4*, 453-464; doi:10.3390/metabo4020453

Heiner Boeing., Angela Bechthold., Achim Bub., Sabine Ellinger., Dirk Haller., Anja Kroke., Eva Leschik-Bonnet., Manfred J. Mu¨ller., Helmut Oberritter., Matthias Schulze., Peter Stehle., Bernhard Watzl. 2012. Critical review: vegetables and fruit in the prevention of chronic diseases. *Eur J Nutr.* 51:637–663.

Hejazi M.A., E. Holwerda, R.H. Wijffels. 2004. Milking microalga Dunaliella salina for β carotene production in two-phase bioreactors, *Biotechnol. Bioeng*. 85.

Hii, S.W., Choong, P.Y., Woo, K.K. & Wong, C.L. 2010. Stability studies of fucoxanthin from *Sargassum binderi. Australian Journal of Basic and Applied Sciences* 4(10): 4580-4584.

Hosokawa, M., Wanezaki, S., Miyauchi, K., Kurihara, H., Kohno, H. & Kawabata, J. 1999. Apoptosis-inducing effect of fucoxanthin on human leukemia cell line HL-60. *Food Science and Technology Research* 5: 243-246.

Jeon JY, Kim KE, Im HJ, Oh ST, Lim SU. 2012. The production of lutein-enriched eggs with dietary Chlorella. *Korean J Food Sci Anim Resour*. 32:13–17. doi: 10.5851/kosfa.2012.32.1.13.

Jodłowska, S. and A, Latała. 2010. Photoacclimation strategies in the toxic cyanobacterium *Nodularia spumigena* (Nostocales, Cyanobacteria). *Phycologia*, 49: 203-211. DOI: 10.2216/PH08-14.1.

Khan Z, Bhadouria P, Bisen PS. 2005. Nutritional and therapeutic potential of Spirulina. *Curr Pharm Biotechnol*. Oct; 6(5):373-9.

Latała, A. and S. Misiewicz. 2000. Effects of light, temperature and salinity on the growth and chlorophyll a content of Baltic cyanobacterium *Phormidium amphibium*. *Arch. Hydrobiol. Supplement band, Algological Stud.*, 136: 157-180.

Llewellyn, C.A., J.R. Fishwick and J.C. Blackford. 2005. Phytoplankton community assemblage in the English Channel: a comparison using chlorophyll a derived from HPLC-CHEMTAX and carbon derived from microscopy cell counts. *J. Plankt. Res*., 27: 103-119. DOI: 10.1093/plankt/fbh158.

Mantoura, R.F.C. and C.A. Llewellyn. 1983. The rapid determination of algal chlorophyll and carotenoid pigments and their breakdown products in natural waters by reverse-phase high-performance liquid chromatography. *Anal. Chem. Acta*, 151: 297-314. DOI: 10.1016/S0003-2670(00)80092-6.

Marquez, U.M.L. & Sinnecker, P. 2008. Analysis of chlorophylls. In *Food Colorants: Chemical and Functional Properties*, edited by Socaciu, C. New York: CRC Press. pp. 429-446.

Mercadante, A.Z. 2008a. Analysis of Carotenoids. In *Food Colorants: Chemical and Functional Properties*, edited by Socaciu, C. New York: CRC Press. pp. 447-478.

Mercadante, A.Z. 2008b. Carotenoids in foods: Sources and stability during processing and storage. In *Food Colorants: Chemical and Functional Properties*, edited by Socaciu, C. New York: CRC Press. pp. 213-240.

Millie, D.F., H.W. Paerl and J.P. Hurley, 1993. Microalgal pigment assessments using high performance liquid chromatography: A synopsis of organismal and ecological applications. *Can. J.Fish Aquat. Sci*., 50: 2513-2527. DOI:10.1139/f93-275

Olivieri, G., Salatino P., Marzocchella, A. 2014. Advances in photobioreactors for intensive microalgal production: Configurations, operating strategies and applications. *J. Chem. Technol. Biotechnol.* 89, 178–195.

Oncel S. 2013. Microalgae for a macro energy. *World, Renew. Sust. Energ. Rev*. 26. 241–264.

Pangestuti, R. and Kim, S.K. 2011. Biological activities and health benefit effects of natural pigments derived from marine algae. *Journal of Functional Foods* 3: 255-266.

Pulz O., and W. Gross. 2004. Valuable products from biotechnology of microalgae, *Appl.Microbiol. Biotechnol*. 65, 635–648.

Rodriguez Amaya, D.B. 2001. *A Guide to Carotenoid Analysis in Foods*. USA: ILSI Press.

Schagerl, M. and G. Kunzl. 2007. Chlorophyll a extraction from freshwater algae-a reevaluation. *Biologia*, 62: 270-275. DOI: 10.2478/s11756-007-0048-x.

Seyfabadi J., Z. Ramezanpou, Z.A. Khoeyi. 2011. Protein, fatty acid, and pigment content of *Chlorella vulgaris* under different light regimes, *J. Appl. Phycol*. 23. 721–726.

Shakeel AA, Shivasharan CT and Kaliwal BB (2015) Physicochemical studies of freshwater lakes in Dharwad and screening of algal species for bio fuel production, European Journal of Experimental Biology, 5(4):60-67.

Shinichi Takaichi. 2011. Carotenoids in Algae: Distributions, Biosyntheses and Functions. *Mar. Drugs*, 9, 1101-1118; doi:10.3390/md9061101.

Stoń, J. and A. Kosakowska. 2002. Phytoplankton pigments designation-an application of RP-HPLC in qualitative and quantitative analysis. *J. Applied Phycol*., 14: 205-210. DOI:10.1023/A:1019928411436

Strickland, I.D.H. and T.R. Parsons. 1972. A Practical Handbook of Seawater Analysis. 2nd Edn., Fisheries Research Board of Canada, Ottawa, pp:310.

Vásquez-Caicedo, A.L., Schilling, S., Carle, R. & Neidhart, S. 2007. Impact of packaging and storage conditions on colour and β-carotene retention of pasteurised mango puree. *European Food Research and Technology*. 224: 581-590.

Walter A., J.C. Carvalho, V.T. Soccol, A.B. Bisinella De Faria, F. Ghiggi, C.R. Soccol. 2011. Study of phycocyanin production from Spirulina platensis under different light spectra, *Braz. Arch. Biol. Technol*. 54, 675–682.

Woutersen RA, Wolterbeek AP, Appel MJ, van den Berg H, Goldbohm RA, Feron VJ. 1999. Safety evaluation of synthetic β carotene. *Crit Rev Toxicol.* 29:515–42.

Wright, S.W., S.W. Jeffrey, R.F.C. Mantoura, C.A. Llewellyn and T. Bjornl. 1991. Improved HPLC method for the analysis of chlorophylls and carotenoids from marine phytoplankton. *Mar. Ecol. Prog. Ser*. 77: 183-196.

Wu Hon Yip, Lim Seng Joe, Wan Aida Wan Mustapha, Mohamad Yusof Maskat & Mamot Said. 2014. Characterisation and Stability of Pigments Extracted from *Sargassum binderi* obtained from Semporna, Sabah. *Sains Malaysiana.* 43(9): 1345–1354.

Wulff, A., S. Vilbaste and J. Truu. 2005. Depth distribution of photosynthetic pigments and diatoms in the sediments of a microtidal fjord. *Hydrobiology*. 534: 117-130. DOI:10.1007/s10750-004-1417-x.

Ye, L. & Eitenmiller, R.R. 2006. Fat-soluble vitamins. In *Handbook of Food Science, Technology, and Engineering*, edited by Hui, Y.H. USA: CRC Press. pp. 212-241.

****