Journal of **A**pplied **B**iotechnology **R**eports

Original Article

Biochemical and Physiological Characterization of Tree *Microalgae* spp. as Candidates for Food Supplement

Neda Soltani[1], Ali mohammad Latifi[2*], Naba Alnajar[1], Mehrouz Dezfulian[3], Shadman Shokarvi[4], Mohammad Heydari1, Ali Choopani[2,5]

Abstract

With increasing world populations, production of cost-effective and proper nourishment sources that can rapidly produce large amounts of nutritional value are needed. Microalgae are publicly used as nutrient supplement. In this research a screening of endemic potent microalgae was carried out. *Chlorella*, *Scenedesmus* and *Spirulina* sp. were isolated and purified and cultivated in liquid proper medium. Regarding to this, amino acid and fatty acid profiles, biochemical characters, antioxidant and antimicrobial and anticancer properties of experimented microalgae were evaluated by HPLC, GC, spectrophotometry, DPPH, MIC and MTT Assay respectively. The results showed highest content of total protein in *Spirulina* sp.1 (46.08 ppm) and total carbohydrates in *Chlorella* sp. (48.01 ppm). Antioxidant content was detected in mentioned microalgae. Cytotoxic effect of aqueousextract on L929 cells showed 10 mg/mL had highest effect on these cells. According to the results, *Chlorella* spp. and *Spirulina* spp.1 are better candidates for food supplement.

1. Department of Petroleum Microbiology, Research Institute of Applied Science, ACECR, Tehran, Iran
2. Applied Biotechnology Research Center, Baqiyatallah University of Medical Sciences, Tehran, Iran
3. Department of Microbiology, Karaj Branch, Islamic Azad University, Karaj, Iran
4. Department of Biology, Gorgan Branch, Islamic Azad University, Gorgan, Iran
5. Department of Biology, Payamnoor University, Tehran, Iran

* **Corresponding Author**
Ali Mohammad Latifi
Applied Biotechnology Research Center, Baqiyatallah University of Medical Sciences, Tehran, Iran
E-mail: amlatifi290@gmail.com

Keywords: Bioactive Metabolites, Carbohydrate, Microalgae, Nourishment, Protein.

Submission Date: 10/2/2015
Accepted Date: 1/25/2016

Introduction

Modern food industry leads to an increase of cheaper, healthier and more convenient products. The use of natural ingredients, like polyunsaturated fatty acids (PUFA's) and antioxidant pigments, exhibiting high impact on functional properties is important to reduce chronic diseases incidence, which are strongly considered of capital importance. The impact of natural substances introduced in the diet via "usual" foods is proved to be efficient at long term. Algae are a diverse group of autotrophic organisms that have the ability to grow rapidly, efficiently use light energy, fix atmospheric CO_2, and produce more biomass per acre than vascular plants [1]. Algae have been used as a food source and for treatment of various ailments for over two thousand years [2, 3]. Microalgae are an enormous biological resource, representing one of the most promising sources for new products and applications [4]. Microalgae are able to enhance the nutritional content of conventional food and feed preparation and hence to positively affect humans and animal health due to their original chemical composition. Different types of algae, specifically microalgae, could become more prevalent in food supplements and nutraceuticals [5]; like *Dunaliella*, *Botryococcus*, *Chlorella*, *Chlamydomonas*, *Scenedesmus*, and *Porphyridium* etc. *Chlorella* and *Scenedesmus* has been used as an alternative medicine in the Far East since ancient times and it is known as a traditional food in the Orient. It is widely produced and marketed as a food supplement in many countries, including China, Japan, Europe and the US, despite not possessing GRAS status. *Chlorella* is being considered as a potential source of a wide spectrum of nutrients (e.g. carotenoids, vitamins, minerals) being widely used in the healthy food market as well as for animal feed and aquaculture. *Chlorella* is important as a health promoting factor on many kinds of disorders such as gastric ulcers, wounds, constipation, anemia, hypertension, diabetes infant malnutrition and neurosis [6]. It is also attributed a preventive action against atherosclerosis and hypercholesterolemia by glycolipidsand phospholipids, and antitumor actions by glycoproteins, peptides and nucleotides [6]. However the most important substance in *Chlorella* seems to be a beta-1, 3-glucan, which is an active immunostimulator, a free-radical scavenger and a reducer of blood lipids [7].

Scenedesmu spp. has a lysine level higher than the required for the Food and Agriculture Organization of the United Nations (FAO) and proteins levels between 25 and 35%, becoming a rich protein source. *Spirulina* grows profusely in certain alkaline lakes in Mexico and Africa and has been used as food by local populations since ancient times [6].

It is extensively produced around the world (3000 tons/year) and broadly used in food and feed supplements, due of its high protein content and its excellent nutritive value, such as high g-linoleic acid level [8, 9]. In addition, this microalga has various possible health promoting effects: the alleviation of hyperlipidemia, suppression of hypertension, protection against renal failure, growth promotion of intestinal Lactobacillus, suppression of elevated serum glucose level [7], anti-carcinogenic effect and have hypocholesterolemia properties [10]. *Spirulina* is also the main source of natural phycocyanin, used as a natural food and cosmetic coloring (blue color extract) and as biochemical tracer in immunoassays, among other uses [8, 9, 11].The aim of this chapter is to screen endemic microalgae *Scenedesmus*, *Chlorella* and *Spirulina* spp. as candidate for nutritional supplement.

Material and Methods

Chlorella, *Spirulina* and *Scenedesmus* were isolated from water and soil of Northern provinces of Iran by agar plate method. *Chlorella* and *Scenedesmus* were cultured in N8 and BBM media. *Spirulina* was cultured in Zarrok medium [12]. After purification, axenic algae were transferred to same liquid media. Cultures were bubbled and illuminated continuously. Light was prepared via three fluorescent lamps (2000 lux for *Spirulina* spp. and 4000 lux for *Chlorella* spp. and *Scenedesmus* spp.) according to Soltani et al., [13]. Temperature was regulated on 30 ± 2 ºC and pH was adjusted at 7 for *Chlorella* spp. and *Scenedesmus* spp. and 8.5 for Spirulina spp. The algae were transferred to semi-large scale media for enhancement of biomass. Growth was estimated by dry weight and biochemical composition was evaluated by standard methods [14]. The analysis for fatty-acid-based biofuels was performed on a Varian 4000 gas chromatography with FID detector. A capillary column CBP1-M25-025 (Having highly pure silica inner surface) with dimension of 25 m × 0.22 mm, 0.25 mm film thickness was used for the separation of fatty-acid-based biofuels. The oven temperature was initially maintained at 180ºC for 1 min, increased at 4°C/min to 240°C, (held for 15 min). The split ratio was 1:10, and helium was used as carrier gas at a flow rate of 1.0 mL/min in the constant flow mode. The injector and detector temperatures were both 250°C. The mass spectrometer was operated in the electron impact (EI) mode at 70 eV in the scan range of 50–650 m/z. The injection sample volume was 2.0 µl [15]. The components were identified by comparing their retention time and fragmentation patterns with those for standards seven fatty acids (C14:0, C16:0, C16:1, C18:0, C18:1, C18:2, C18:3) were used as standard mate. Amino acids were quantified using HPLC system. 100 mg of freeze-dried biomass was suspended in 1 mL ethanol 80%. Sample'sshacked in 80°C for 1 hour and then was centrifuge (5 min, g 14000, 4°C). Solutions were dried at freeze-drier and filtered. 250 µl sample filtered was added 200 µl borate buffer, 100 µl OPA and 50 µl of HCl 0.5 M. Probes were evaporated, dissolved in a sample solution buffer and injected on action separation column (4.6×150 mm, HALO C18; Knauer, Germany) and were detected at 330 nm and at 450 nm. The analyses were run under the following conditions: analysis cycle time 25 min; flow rates 1.1 ml/min for buffer. The amino acid content is given as the summed content of alanine, asparagine, aspartic acid, arginine, citrulline, glutamic acid, glutamine, glycine, histidine, isoleucine, leucine, taurine, methionine, phenylalanine, proline, serine, threonine, tyrosine, α-amino butyric acid, tryptophan and valine. Cytotoxicity was evaluated by MTT assay. The algal cells in the logarithmic phase were sampled and kept at 50°C for 24 h. Methanol was poured on dried and weighed samples and then vortexed for 10 min. Suspension was placed in ice for 2 h. Then, the falcons were centrifuged at 16000 g turn at the temperature of 4°C for 15 min. L929and HeLa cell lines were obtained from Tehran Pasteur Institute.

The cells were cultured in RPMI 1640. Then the cells were transferred to a 96 wells flask. The prepared aqueous and methanol extracts were added to the wells at different. Three wells containing 100 µl cell and 100 µl RPMI+FBS 10% culture medium were selected as control groups. Then the plate was placed in incubator of CO_2 for 24 hours in order the desired material to have its effect on cells. After the incubation, MTT test was used to measure the cell toxicity. Data are the means and standard deviation of at least three replicates. Statistical differences were examined by ANOVA test using software SPSS ver. 19.

Results and Discussion

Total lipid, protein and carbohydrate contents are critical indicators to decide whether the screened algal strain is a promising producer with high production ability. Table 1 shows the typical common components of experimented microalgae. As is shown total protein is higher in experimented cyanobacteria in comparison to green algae (16.37 & 46.08 ppm). Among cyanobacteria *Spirulina* spp. 1 has the highest content (46.08 ppm). Same result is in the case of total lipid. *Spirulina* spp. have the higher content but the maximum content belongs to *Spirulina* spp. 2 (40.01 ppm). Taking account to carbohydrate, it can be seen that *Chlorella* spp. has the highest content (48.1 ppm).

Table 1. Typical Common Components of Experimented Microalgae.

Component	Synechococcus	Chlorella	Scenedesmus	Spirulina-1	Spirulina-2
Total protein*	16.37	9.16	2.76	46.08	10.23
Total Carbohydrate*	33.08	48.1	7.53	23.03	17.13
Total Lipid*	20.36	11.79	12.65	22.32	40.01

* ppm

Typical amino acid analysis of experimented microalgae is shown in Table 2. Data in Table 2 indicated the presence of essential amino acids in the profile of screened microalgae. Essential amino acids cannot be made by the body. As a result, they must come from food.From the nine essential amino acids, seven are found in the profile of experimented algae: Histidine, Leucine, Methionine, Phenylalanine, Threonine, Tryptophan, and Valine. Most of them are found in higher content in *Spirulina* spp. 2.

Table 2. Typical amino acid analysis of experimented microalgae.

Amino acid (µM/grDw)	*Synechococcus*	*Chlorella*	*Scenedesmus*	*Spirulina-M*	*Spirulina-A*
	mM/gFw				
Aspartic acid	0.407	637.15	609.7	8711.7	9243.11
Glutamic acid	0.782	4310.3	5017.33	33128.47	35127.97
Asparagine	0.194	138.64	215.32	2196.45	2228.26
Serine	1.435	2064.28	3439.29	15993.46	16016.07
Glutamine	0.249	810.78	16091.59	9502.68	8763.09
Glycine	0.184	3165.33	2880.26	7663.34	17849.17
Histidine	1.348	1326.99	2474.11	20502.56	27510.39
Threonine	0.249	7760.09	8065.91	3235.71	12003.30
Citrulline	0.043	168.1	2763.01	2768.32	8021.66
Arginine	0.075	583.99	630.58	40319.16	59768.62
Taurine	0.025	8137.73	11575.39	16202.58	19303.75
Alanine	3.158	1759.59	1860.22	4173.71	9341.51
Tyrosine	0.050	157.28	2992.17	1989.24	1753.51
α-Amino butric acid	0.119	1064.39	374.82	858.20	29563.77
Tryptophane	3.496	1256.06	1795.87	25615.58	2410.37
Methionine	0.056	132.29	200.76	703.85	1035.76
Valine	1.163	935.84	1304.60	14941.23	12486.11
Phenylalanine	0.292	4741.82	9134.05	18130.23	31670.53
Leucine	0.540	1676.17	2152.37	19479.85	25016.58

Although two different methods, total capacity of antioxidant (DPPH) and capacity of reducing Fe^{2+} (FRAP), were used to determinate the antioxidant capacity of screened microalgae, the results were in agreement with each other. Accordingly, the results indicate the higher antioxidant capacity of *Chlorella* spp. and *Spirilina* spp.1 respectively (Figures 1 & 2).

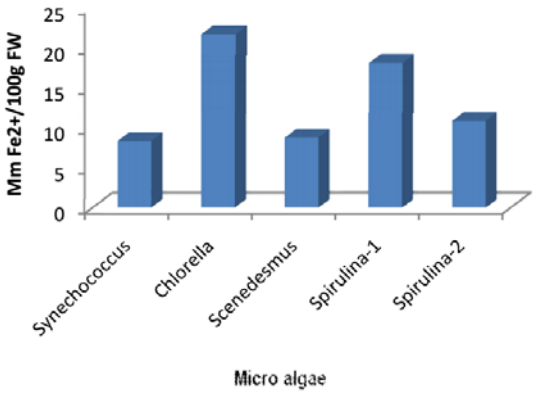

Figure 1. Antioxidant capacity of experimented microalgae (FRAP).

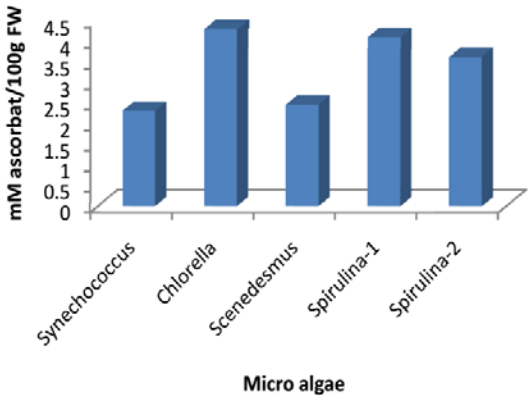

Figure 2. Antioxidant capacity of experimented microalgae (DPPH).

The obtained results of the cytotoxicity testing of aqueous extracts of the samples on Helaand L929 cell lines showed that the highest cytotoxicity concerns with *Chlorella* spp. at the dose of 10 mg /mL (Figure 3). These results also indicated that the higher viability of L929 is obtained with the aqueous extract of *Spirulina* and *Chlorella* respectively (figure 4) at the dose of 10 mg/mL.

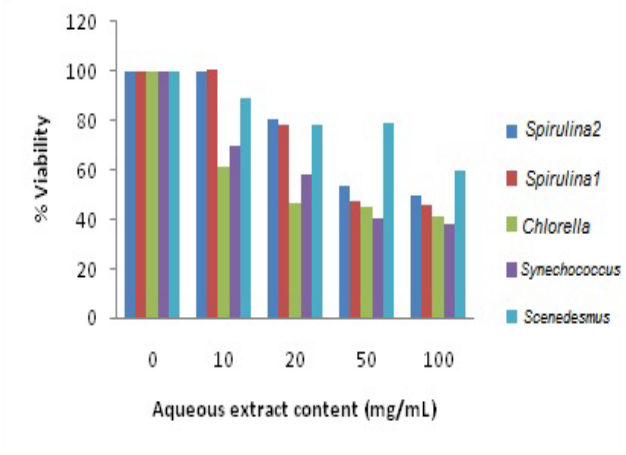

Figure 3. The effect of experimented microalgae on HeLa cells.

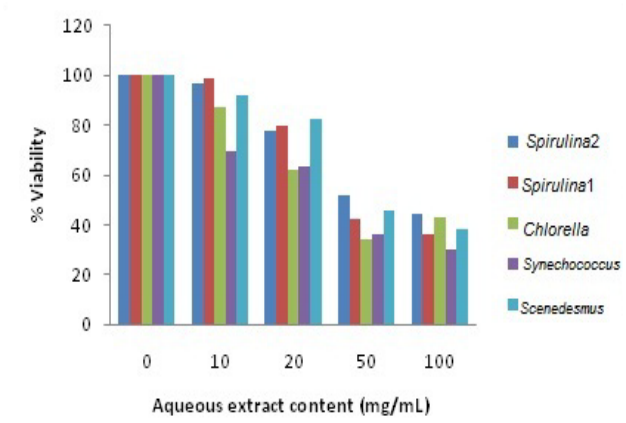

Figure 4. The effect of experimented microalgae on L929 cells.

As mentioned above, microalgae are potent bio sources for nourishment supplement. With this respect, five microalgae were screened to choose best candidate and so critical parameters are described. The high protein content of various microalgae species is one of the main reasons to consider them as an unconventional source of protein [16], well-illustrated by the great interest in microalgae as single cell protein (SCP) during the 1950s. In addition, the amino acid pattern of almost all algae compares favorably with that of other food proteins. Since the cells are capable of synthesize all amino acids, they can provide the essential ones to humans and animals [17]. As other bioactive compounds synthesized by microalgae, amino acids composition, especially the free amino acids, varies greatly between species as well as with growth conditions and growth phase [18]. The amino acid profile of experimented microalgae showed well pattern including essential amino acids which cannot be produced by human body. These are needed to absorb by external sources. Our results indicated that *Spirulina* spp. 1 has most of these amino acids. One of the other parameters was total lipid, which content is an indicator to decide whether the screened algal strain was a promising producer with high production ability. Some microalgae synthesize fatty acids with particular interest, namely g-linoleic acid (GLA, 18:3w6) (*Spirulina*), arachidonic acid (AA, 20:4w6) (*Porphyridium*),eicosapentaenoic acid (EPA, 20:5w3) (*Nannochloropsis*, *Phaeodactylum*, *Nitzschia*, *Isochrysis*, *Diacronema*) and docosahexaenoic acid (DHA, 22:6w3) (*Crypthecodinium*, *Schizochytrim*) [7, 19, 20]. These long chain polyunsaturated fatty acids (morethan 18 carbons) cannot be synthesized by higher plants and animals, only by microalgae which supply whole food chains with [4]. Among our screened microalgae the highest level of total lipid belonged to *Spirulina* spp. 2. The fatty acid profile indicated essential fatty acid (Data not shown). This statement confirms the importance of the inclusion of these long chain fatty acids in daily diet. The evidence of a dietary deficiency in long-chain omega3 fatty acids is firmly linked to increased morbidity and mortality from coronary heart disease. Fish oils as the main sources of LC-PUFA's, has unpleasant taste and poor oxidative stability [21]. The production of LC-PUFA from microalgae biotechnology is an alternative approach [4, 7]. Polysaccharides are widely used in the food industry primarily as gelling and/orthickening agents. Many commercially used polysaccharides like agar, alginates and carrageenan's are extracted from microalgae (e.g. Laminaria, Gracilaria, Macrocystis) [18]. Nevertheless, most microalgae produce polysaccharides and some of them could have industrial and commercial applications, considering the fast growth rates and the possibility to control the environmental conditions regulating its growth. Our results showed that among tested microalgae *Chlorella* ssp. is a good resource of carbohydrate with a high growth rate. Other research reports some other microalgae such as *Porphyridium cruentum* and *Chlamydomonas mexicana*. Following this, we evaluated the antioxidant potential of our five micro algal strains. The highest ability was indicated in *Chlorella* spp. and *Spirulina* spp.1. Microalgae are photoautotrophic organisms that are exposed to high oxygen and radical stresses, and consequently have developed several efficient protective systems against reactive oxygen species and free radicals. Hence, there is increasing interesting in using microalgae as natural antioxidants source for cosmetics (e.g. sun protecting) and functional food/nutraceuticals. Natrah et al., [22] reported a stronger antioxidant activity exhibited by methanolicmicro algal crude extracts.

Isochrysis galbana, *Chlorella*, *vulgaris*, *Nannochloropsis oculata*, *Tetraselmis tetrathele*, *Chaetoceros calcitrans*) when compared with a-tocopherol, but lower than the synthetic antioxidant BHT. The microalgae represent a very large, relatively unexploited reservoir of novel compounds, many of which are likely to show biological activity, presenting unique and interesting structures and functions [6]. We investigated the anticancer ability of five screened microalgae by MTT assay. Our results showed higher anticancer ability in *Spirulina* spp. 1 and *Chlorella* spp. The reported biological activities comprise cytotoxic, antitumor, antibiotic, antimicrobial (antibacterial, antifungal, antiprotozoal), antiviral (e.g. anti-HIV) activities as well as bio modulatory effects like immunosuppressive and anti-inflammatory [23, 24]. The cytotoxicity activity, important for anticancer drugs development, is likely related to

defense strategies in the highly competitive marine environment, since usually only those organisms lacking an immune system are prolific producers of secondary metabolites such as toxins [25, 26]. Results showed that among experimented samples, microalgae *Chlorella* and *Spirulina*-1 had highest nutrient values and should be physiologically optimized in next steps in order to making mass culture and public consumption [27]. The combination of the exceptional nutritional value of microalgae with coloring and therapeutically properties, associated with an increase demand of natural products, make microalgae worth exploring for utilization in the future in feed, food, cosmetic and pharmaceutical industries, with recognized advantages comparing with the traditional ingredients. In the actual scenario with multiple pharmacological treatments, many believe that simple dietary interventions or nutritional supplements may be more natural, acceptable and feasible method of providing benefits.

Conclusion

According to the results, *Chlorella* spp. and *Spirulina* spp.1 are better candidates for food supplement.

Acknowledgments

This study was supported by Applied Biotechnology Research Center. Authors also thank Research Institute of Applied Science, ACECR.

References

1. Shay, E.G., Diesel fuel from vegetable oils: status and opportunities. *Biomass Bioenerg*, 1993, Vol. 4, pp. 227-242.
2. Richmond, A., Handbook of microalgal mass culture. Vol. 528. 1986: CRC press Boca Raton, FL.
3. Gao, K., Chinese studies on the edible blue-green alga, Nostoc flagelliforme: a review. *J Appl Phycol*, 1998, Vol. 10, pp. 37-49.
4. Pulz, O., Gross, W., Valuable products from biotechnology of microalgae. *Appl Microbiol Biotechnol*, 2004, Vol. 65, pp. 635-648.
5. West, M., Evaluation of microalgae for use as nutraceuticals and nutritional supplements. *J Nutr Food Sci*, 2012, Vol. 2, pp. 147-152.
6. Yamaguchi, K., Recent advances in microalgal bioscience in Japan, with special reference to utilization of biomass and metabolites: a review. *J Appl Phycol*, 1996, Vol. 8, pp. 487-502.
7. Spolaore, P., Joannis-Cassan, C., Duran, E., Isambert, A., Commercial applications of microalgae. *J Biosci Bioeng*, 2006, Vol. 101, pp. 87-96.
8. Ötle , S., Pire, R.e., Fatty acid composition of Chlorella and Spirulina microalgae species. *J AOAC Int*, 2001, Vol. 84, pp. 1708-1714.
9. Shimamatsu, H., Mass production of Spirulina, an edible microalga, in Asian Pacific Phycology in the 21st Century: Prospects and Challenges. 2004, Springer. p. 39-44.
10. Reinehr, C.O., Costa, J.A.V., Repeated batch cultivation of the microalga Spirulina platensis. *World J Microbiol Biotechnol*, 2006, Vol. 22, pp. 937-943.
11. Kato, T., Blue pigment from Spirulina. *New Food Indust*, 1994, Vol. 29, pp. 17-21.12. Andersen, R.A., Algal culturing techniques. 2005: Academic press.
13. Soltani, N., Baftechi, L., Dezfulian, M., Shokravi, S., Alnajar, N., Molecular and morphological characterization of oil polluted microalgae. *Int J Environ Res*, 2012, Vol. 6, pp. 481-492.
14. Federation, W.E., Association, A.P.H., Standard methods for the examination of water and wastewater. American Public Health Association (APHA): Washington, DC, USA, 2005, Vol. pp.
15. Guan, W., Zhao, H., Lu, X., Wang, C., Yang, M., Bai, F., Quantitative analysis of fatty-acid-based biofuels produced by wild-type and genetically engineered cyanobacteria by gas chromatography–mass spectrometry. *Journal Chromatogr*, 2011, Vol. 1218, pp. 8289-8293.
16. Soletto, D., Binaghi, L., Lodi, A., Carvalho, J., Converti, A., Batch and fed-batch cultivations of Spirulina platensis using ammonium sulphate and urea as nitrogen sources. Aquaculture, 2005, Vol. 243, pp. 217-224.
17. Guil-Guerrero, J., Navarro-Juárez, R., López-Mart nez, J., Campra-Madrid, P., Rebolloso-Fuentes, M., Functional properties of the biomass of three microalgal species. *J Food Eng*, 2004, Vol. 65, pp. 511-517.
18. MA, B., Vitamins and fine chemicals from Microalgae, 1988, Borowitzka, MA and Borowitzka, LJ, Cambridge University Press.
19. Badarpa, N.M., Pereira, P.A., Batista, I., Vilela, M.H., fatty acids, sterols and tocopherol in isochrysis galbana. *J Food Lip*, 2003, Vol. 10, pp. 25-34.
20. Donato, M., Vilela, M.H., Bandarra, N.M., Fatty acids, Strols, Tocopherol and Total Carotenoids Composition of Diacronema Vlkianum. *J Food Lip*, 2003, Vol. 10, pp. 267-276.
21. Luiten, E.E., Akkerman, I., Koulman, A., Kamermans, P., Reith, H., Barbosa, M.J., Sipkema, D., Wijffels, R.H., Realizing the promises of marine biotechnology. *Biomol Eng*, 2003, Vol. 20, pp. 429-439.
22. Natrah, F., Yusoff, F., Shariff, M., Abas, F., Mariana, N., Screening of Malaysian indigenous microalgae for antioxidant properties and nutritional value. *J Appl Phycol*, 2007, Vol. 19, pp. 711-718.
23. Fedorov, S.N., Ermakova, S.P., Zvyagintseva, T.N., Stonik, V.A., Anticancer and cancer preventive properties of marine polysaccharides: Some results and prospects. *Marine drugs*, 2013, Vol. 11, pp. 4876-4901.
24. Santoyo, S., Jaime, L., Plaza, M., Herrero, M., Rodriguez-Meizoso, I., Ibañez, E., Reglero, G., Antiviral compounds obtained from microalgae commonly used as carotenoid sources. *J Appl Phycol*, 2012, Vol. 24, pp. 731-741.
25. Guedes, É.A., Silva, T.G.d., Aguiar, J.S., Barros, L.D.d., Pinotti, L.M., Sant'Ana, A.E., Cytotoxic activity of marine algae against cancerous cells. Revista Brasileira de Farmacognosia, 2013, Vol. 23, pp. 668-673.
26. Papadopoulos, K.N., Food chemistry research developments. 2008: Nova Publishers.
27. Beheshtipour, H., Mortazavian, A., Mohammadi, R., Sohrabvandi, S., Khosravi Darani, K., Supplementation of Spirulina platensis and Chlorella vulgaris algae into probiotic fermented milks. *Compr Rev Food Sci Food Saf*, 2013, Vol. 12, pp. 144-154.