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Research article

Assay for Antioxidant Activity of Algal, Yeast and Fungal Culture Extracts

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Abstract

We had conducted study to discover whether or not there was any antioxidant compound in extracts of algal (*Scenedesmus dimorphus* and *Spirulina fusiformis*) and yeast (*Phaffia rhodozyma*) or fungus (*Monascus purpureus*). The purpose of this study was to determine activity of respective culture extracts as sources of natural antioxidant through their inhibition activities on bleaching of β-carotene against free radicals derived from oxidation of linoleic acid. Analysis of chemical constituents of the respective cultures was performed using GC-MS and the results showed that extract of *S. dimorphus* and *S. fusiformis* contained terpenoids (phytol), and benzofuranon that might be potential as antioxidant compound. The assay for antioxidant activity indicated that extracts of algal, yeasts and fungal cultures demonstrated considerably high activity to retain the bleaching of β-carotene, however, they were lower than that of exhibited by butylated hydroxy toluene (BHT) as positive control for a synthetic antioxidant.

Key words: Culture extract, Antioxidant activity, β–carotene, linoleic acid.

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1. Introduction

Nowadays, free radicals and antioxidants are important concerns in the medical world, since free radical is suspected can cause cell damage and underlie a wide variety of pathological conditions, such as coronary heart disease, diabetes mellitus, heart disease, and is also suspected to play a role in the aging process, whereas the benefits and usefulness of antioxidant is ward off the free radicals [31]. Free radicals are considered dangerous since it will be very reactive in attempt to get a pair of electrons resulting in formation of new free radicals. As a result of these chain reactions, it may cause damage to various parts of cells and organs of body, and plays a major role in development of a wide range of degenerative diseases [11]. During this time, butylated hydroxy toluene (BHT) and butylated hydroxy anisole (BHA) are commonly used as synthetic antioxidants. However, their use are increasingly avoided by community, since some studies have shown a toxic and

carcinogenic effects against human body. Therefore lately, more attention is drawn to natural antioxidants, such as bioactive compounds derived from plants and microbes, since they are better than synthetic antioxidants when viewed in terms of food safety, security, and health [21]. Potential microbial secondary metabolites that may be used as a source of natural antioxidants are necessarily to be studied furthermore. Search for new antioxidant compounds that are more effective and safer is consequentally encouraged. Microbial resources that may be empowered as a source of natural antioxidants can be obtained from yeasts, fungi and algae including *P. rhodozyma*, *M. purpureus*, *S. dimorphus* and *S. fusiformis* [27]. The purpose of study was to determine the bioactive compounds derived from algal culture of *S. dimorphus* and *S. fusiformis*, yeast culture of *P. rhodozyma* and fungal culture of *M. purpureus* as source of microbial antioxidants, which was assayed regarding with preventing on bleaching of β-carotene caused by oxidizing of free radicals derived from linoleic acid.

2. Methodology

Proliferation of algal culture

Proliferation of algal culture of *S. fusiformis* applied for this study had been carried out on Zarrouk media using 2500 lux light intensity at 25° C, whereas culture of *S. dimorphus* was grown on PHM media with 2500 lux of light intensity at 25°C [9].

Preparation of PHM media

The media consisted of 1g KNO_3 and 0.2g $MgSO_4.7H_2O$ was dissolved into distilled water (1000 ml), and then added with 1mL trace metal solution and 1 mL soil extract and a 150 ml of each solution was poured onto three conical flask (300 ml) and autoclaved at 121°C for 20 min. After cooling down, medium was added with 0,15 mL Fe and 0,15 mL K_2HPO_4 solution, and culture of *S. dimorphus* was then inoculated and incubated onto these three growth media and stirred continuously at 25°C and 2500 lux light intensity.

Preparation of Zarrouk Media

The media containing 3g KNO_3, 1g NaCl, 1g K_2SO_4, and 16.8g $NaHCO_3$ was dissolved into distilled water (1000 ml) and poured onto two conical flask (500 ml), and added with 0.25mL H_3PO_4, and sterilized by autoclaving at 121°C for 20 minutes. After cooling down, medium was then added with 0.005 g $FeSO_4.7H_2O$, 0.5 mL trace elements of Za and and Zb. The culture of *S. fusiformis* was then inoculated and incubated onto these two growth media and stirred continuously at 25°C and 2500 lux light intensity.

Preparation of Yeast Malt Borth Media

The media containing 0.9 g yeast extract, 0.9g malt extract, 3g glucose, 1.5g peptone was dissolved into acetate buffer (pH 5.5) in a 500 mL conical flask, and then autoclaved at 121°C for 15 min. After cooling down, medium was inoculated with culture of *P. rhodozyma* and then incubated at 22°C for 5 days by continuously shaking on a rotary shaker at 120 rpm.

Making Media Potato Dextro Borth (PDB)

Media containing 100 ml potato extract and 10 g glucose was dissolved into acetate buffer (pH 5.5) in a 500 mL conical flask, and then autoclaved at 121°C for 15 min. After cooling down, medium was inoculated with culture of *M. purpureus* and then incubated at 30°C for 5 days by continuously shaking on the rotary shaker at 120 rpm.

GC-MS Analysis of Chemical Compounds

The culture of *S. dimorphus* and *S. fusiformis* were extracted by using centrifuge and their supernatants were then analyzed with GC-MS to determine composition of organic compounds that contained in the respective extract of cultures. One mg of sample was dissolved into 1.0ml methanol, and filtered with a 0.22 µm filter membrane. A 1µl of samples was injected onto GC-MS using column of HP-5ms (∅ 0,25mm and film 0,25µl) eluted with 5% diphenyl and 95% methyl polysiloxane as stationary phase at 300^{o}C of injection and interface temperature. The oven was set at initial temperature of 100°C for 2 min at a rate of temperature rise of 15^{o}C/min and then stabilized at 300^{o}C for 20 min with helium as a mobile phase was set at a constant rate of 1 ml/min. Mass spectrometry was set at ion source temperature of 250^{o}C and quadrupole temperatures at 50^{o}C, energy 70ev and Mass Setting Range from 30 to 1050 amu.

Measurement of Cells Biomass

Biomass cells was measured using gravimetric method by measuring 1.0mL of respective culture in eppendorf tubes where its dry weight of cells had been determined. The cells were centrifuged for 15 min at 4000 rpm. Supernatant was discarded and obtaining pellets were dried using oven at 80^{o}C for 36 h and weighed. The dry weight of cells was calculated by deducting the obtaining total weight with the weight of eppendorf tubes [30].

Assay for Proteolytic Activity

Media containing skim milk (2g) was dissolved into 150 ml Na-phosphate buffer pH 7 and heated for 60 min. Simultaneously, 0.5g peptone and 2.2g agar were dissolved into 150 ml Na-phosphate buffer pH 7 and sterilized by autoclaving and then mixed and poured onto sterilized petridish and allow to solidify. The cultures were grown on the solidified media and incubated for observation regarding with a clear zone formation [8].

Assay for Amylolytic Activity

Media containing yeast extract (0.2g), 0.5g peptone, 0.3g KH_2PO_4, 0.05g $MgSO_4.7H_2O$, 0.01g $CaCl_2$, and 2.0g soluble starch in conical flask (300 ml), diluted with 50 ml Na-phosphate buffer pH 7. Simultaneously, agar (2.2g) in 100 mL conical flask was dissolved into 50 ml Na-phosphate buffer pH 7 and homogenized using microwave and then mix them all and sterilized by autoclaving. The cultures were grown on the solidified media and incubated for observation regarding with a clear zone formation [7].

Assay for Lipolytic Activity

Media containing 0.5g beef extract, 1g peptone, 0.5g NaCl, 0.3g yeast extract, 0.1g polyvinyl alcohol and 2.2g agar was dissolved into 100 mL Na-phosphate buffer pH 7 and then poured into a large test tube (15 ml) and sterilized by autoclaving. After sterilizing 0.3mL tributyrin was added into petridish then poured with sterilized media. The cultures were grown on the solidified media and incubated for observation regarding with clear zone formation [20].

Assay for Antioxidant Activity

Antioxidant activity of respective culture extracts were tested using β-carotene as their substrates and linoleic acid as oxidator by dissolving 2.0mg β-carotene into 10 mL chloroform. Of the solution was pipetted as amount of 1 mL into 100 mL conical flask and then evaporated upto dry at 40°C. Furthermore, it was successively added into the flask with 20

mL linoleic acid, 184mL Tween 80 as emulsifiers and 50 ml distilled water which had been aerated, and then stirred to form emulsion. As amount of 5.0 ml of emulsion was pipetted into each tubes which had contained antioxidants at various concentrations. Absorbances were observed at time-0 and at each 30 min interval time after incubation at 50^{o}C and λ 470 nm. Antioxidant activity was expressed as a protective factor (Fp) could be calculated based on ratio of absorbance of sample and absorbance of control at 30 min [1].

3. Results and Discussion

Assay for enzymatic activity of cultures

Figure 1 showed a visual observation dealing with enzymatic activities of yeast and fungal cultures by performance of clear zone surrounding their colonies. The result showed that culture of *P. rhodozyma* exhibited lipolytic activity on medium containing tributyrin, but it did not show amylolytic and proteolytic activities in media containing starch and skim milk. While the culture of *M. purpureus* showed lipolytic, amylolytic and proteolytic activities as it could perform some clear zones surrounding colonies on media containing tributyrin, soluble starch and skim milk.

Analysis of algal components

Figure 2 showed the results of analysis on biochemical constituents of algal culture extracts using GC-MS. The results showed a presumption on existence of compounds that containing vitamins such as terpenoid (phytol), benzofuranon and fatty acids. High content of phycocyanin pigment may become main attraction in cultivation of algae, since it exhibited antioxidant characteristics [24, 25], and therefore it is considered to have a potential market in the field of healthcare related industry. The antioxidant activity of these compounds are mainly attributed to scavenging activity against superoxide and hydroxyl radicals, chelating ability, quenching singlet and triplet oxygen [3, 26]. It is important to develop, identify and utilize new source of safe and effective antioxidants of natural origin [17, 22].

There are several synthetic antioxidants such as butylated hydroxyanisole (BHA), butylated hydroxytoluene (BHT), Propylgallate (PG) and butylated hydroxyquinone (TBHQ) are available commercially and currently used. However, the use of these synthetic antioxidants for food or medicine components has been stricted by the toxicity and safety that can lead to the problems of the potential health in human. Due to the reasons, many researchers have tried to find the more effective oxidation inhibitors that may be used as antioxidants for food or medicine compositions without the side effects for the past several years. They have paid attention to many kinds of natural antioxidants that can be used without toxicity in human.

Algae are known to contain reactive antioxidant molecules, such as ascorbate and glutathione (GSH) when fresh, as well as secondary metabolites, including carotenoids (α- and β-carotene, fucoxanthin, astaxanthin), mycosporine-like amino acids (mycosporine-glycine) and catechins (e.g., catechin, epigallocatechin), gallate, phlorotannins (e.g., phloroglucinol), tocopherols (α, χ, δ-tocopherols) [29].

Among algal natural antioxidants, terpenoids, phlorotannins, polyphenols, phenolic acids, anthocyanins, hydroxycinnamic acid derivatives, and flavonoids are important [4]. Recently, researchers have isolated various types of antioxidant compounds from different

algal species, including fucoxanthin in *Hijikia fusiformis* [28]; phycocyanin and phycocyanobilin in *Spirulina platensis* [5, 6, 13], phenolic acids, tocopherols, and β-carotene in *Spirulina maxima* [18], fatty acids, polyphenols, and phlorotannin in *Sargassum siliquastrum, Rhodomela confervoides, Symphjocladia latiuscula* and *Kappaphycus alvarezzi* [10, 14], lutein in *Botryococcus braunii* [23], and carotenoids in *Dunaliella salina* [12, 15].

Antioxidant activity of culture extracts

Assay for antioxidant activity of algal culture extracts had been carried out using reaction mixture containing β-carotene and linoleic acid. The bleaching time towards changing color of β-carotene indicated level of antioxidant activity exhibited by these culture extracts. The longer the time required for bleaching, the better the quality of the antioxidants possessed. [19]. Linoleic acid was utilized as a substrate that played a role on oxidizing free radicals which resulted in bleaching the color of β-carotene. Existence of antioxidant which has activity as free radical scavengers are potentially protecting β-carotene from the effect of free radicals thus its bleaching time will be longer. The antioxidant activity expressed as a protective factor (Fp) which is ratio of absorbance value of samples towards absorbance value of control in 30 min of incubation. Figure 4 showed the Fp value of respective samples of algal culture extracts at concentration of 100 ppm compared to BHT as the synthetic antioxidant. The result showed that the antioxidant activity of the algal culture extract of *S. fusiformis* showed higher antioxidant activity than the culture extract of *S. dimorphus*. The test results showed that the extract of *S. fusiformis* chemically protective factors was higher (3.85) compared to extracts of *S. dimorphus* (1.78) after 30 min incubation, however lower than that of BHT synthetic antioxidant (4.22).

Figure 5 showed the Fp value of respective yeast and fungal culture extracts at concentration of 100 ppm compared to BHT as the synthetic antioxidant. The result showed that the antioxidant activity of the algal culture extract of *P. rhodozyma* showed higher antioxidant activity than the culture extract of *M. purpureus*. The results showed that the extract of *P. rhodozyma* chemically protective factors was higher (2.66) compared to extracts of *M. purpureus* (1.43) after 30 min incubation, however lower than that of BHT synthetic antioxidant (3.48).

Butylated hydroxytoluene (BHT) is a material that is often used as synthetic antioxidant that has the highest protective factor compared to the natural antioxidants contained in microbial culture extracts including algal culture of *Scenedesmus*, *Spirulina* those were cultivated on media of Zarrouk and PHM. It showed that the BHT is the most powerful antioxidative compound that has activity in capturing free radicals that cause damage in various products containing oils and fats. Although some synthetic antioxidant, including BHT is commonly used in processed foods, which have some adverse effects [2].

There is also concern that BHT may convert to other substances that may be harmful for human body since it is carcinogenic. It was reported that a conversion product of BHT had been shown to disrupt chemcial signals that are sent from cell to cell [16], and therefore the use of BHT as antioxidant dealing with food preservation has become obsolete. Hence the use of algal culture extracts of *S. dimorphus* and *S. fusiformis*, as well as yeast and fungal culture extracts of *P. rhodozyma* and *M. purpureus* are potentially to be applied as a source of

natural antioxidants, since there is no report indicating that these microbial culture extracts are carcinogenic or cause harmful side effects.

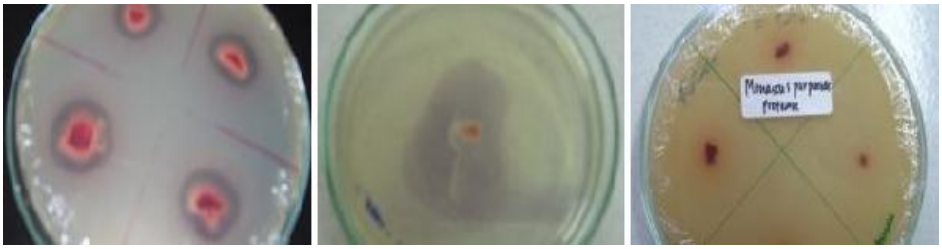

Figure 1. Performance of clear zone surrounding colonies of *P. rhodozyma* and *M. purpureus*.

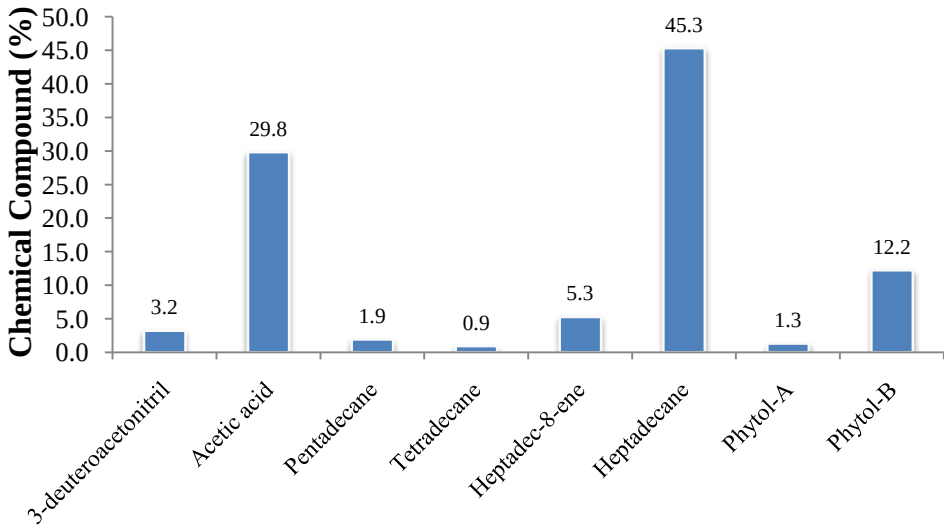

Figure 2. Composition of culture of *S. fusiformis.*

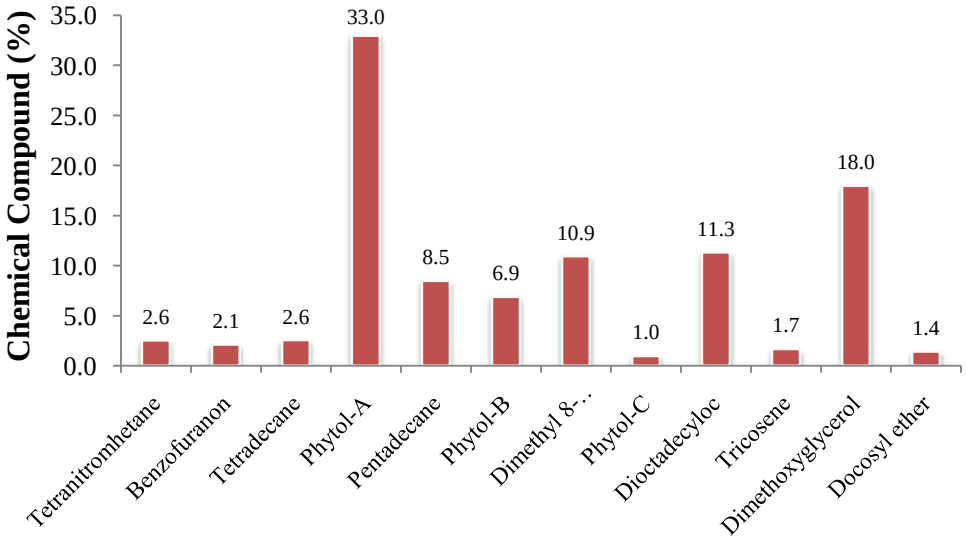

Figure 3. Composition of culture of *S. dimorphus*.

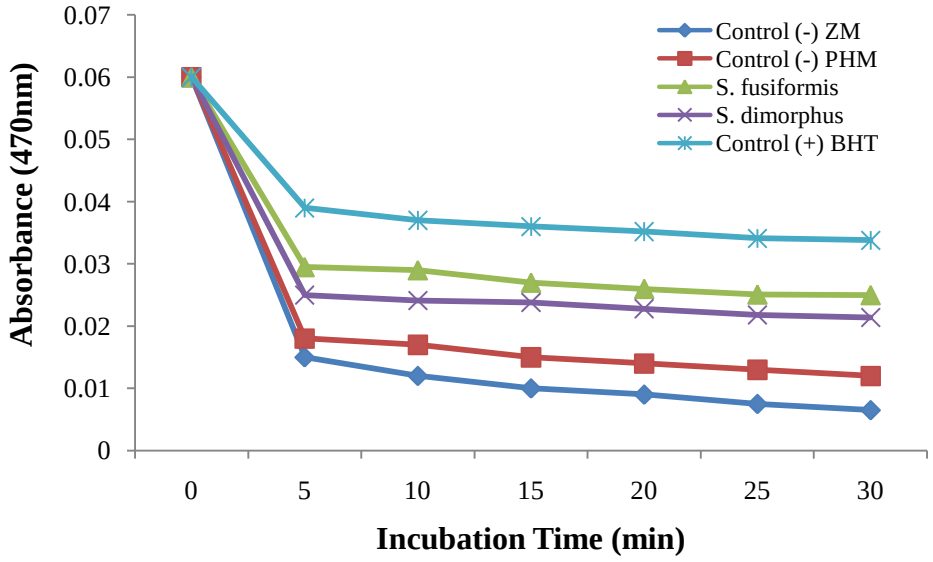

Figure 4. Assay graph for antioxidant activity of algal culture extracts.

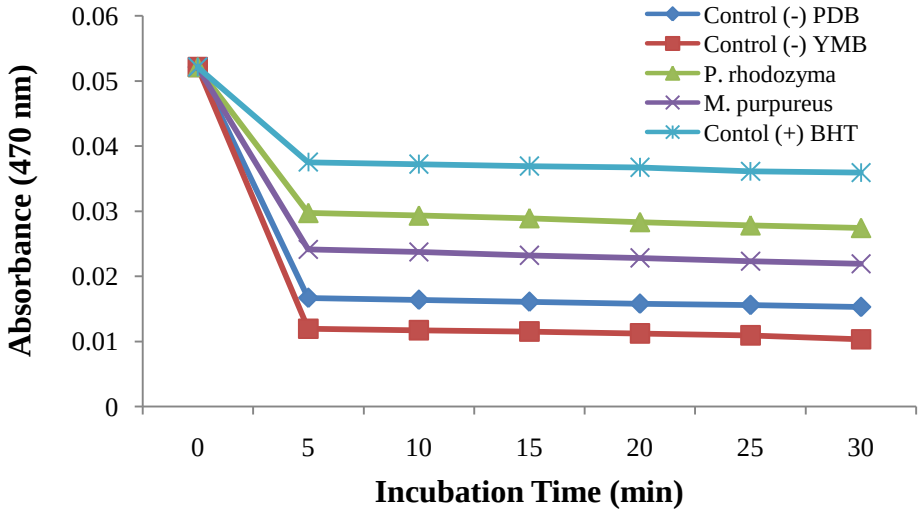

Figure 5. Assay graph for antioxidant activity of yeast and fungal culture extracts.

Conclusion

From the results of this study indicated that culture extracts of *S. fusiformis*, *S. dimorphus*, *P. rhodozyma* and *M. purpureus* showed Fp value of antioxidant activity on inhibiting the bleaching of β-carotene against free radicals derived from oxidation of linoleic acid those were at 3.85, 1.78, 2.66 and 1.43, respectively, although they all showed lower than that of antioxidant activity showed by BHT (3.48 to 4.22) as the synthetic antioxidant. These algal, yeast and fungal culture may be considered to have an important role since they experimentally exhibited antioxidant activity, and therefore they are potentially useful for healthcare industrial market.

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References

1. Andarwulan, N., Fardiaz, D., Wattimena G.A. and Shetty, K. Antioxidant Activity Associated with lipid and phenolic Mobilization During Seed Germination of Pangium Edule Reinw, J. Agric. Food Chem. 1999, 47:3158-3163.
2. Aruoma, O.I., Spencer, J.P.E., Rossi, R., Aeschbach, R., Khan, A., Mahmood, N., Mijnoz, A., Murcia, A., Butler J. and Halliwell, B. An Evaluation of the antioxidant and antiviral action of extracts of rosemary and provencal herbs. Food Chem. Toxicol. 1996, 34:449-456.
3. Athukorala Y., Nam, K and Jeo, Y. Anti proliferative and antioxidant properties of an enzymatic hydrolysate from brown alga. Food Chem. Toxicol. 2006, 44:1065-1074.
4. Bandoniene, D and Murkovic, M. On-line HPLC–DPPH screening method for evaluation of radical scavenging phenols extracted Malusdomestica L. J. Agric. Food Chem. 2002, 50:2482-2487.
5. Bhat, V.B. and Madyastha, K.M. Scavenging of peroxynitrite by phycocyanin and phycocyanobilin from Spirulina platensis: protection against oxidative damage to DNA. Biochem. Biophys. Res. Commun. 2001, 285:262-266.
6. Benedetti, S, Benvenuti, F., Pagliarani, S., Francogli, S., Scoglio, S. and Canestrari, F. Antioxidant properties of a novel phycocyanin extract from the blue-green alga Aphanizomenon flosaquae. Life Sci. 2004, 75:2353-2362.
7. Bernfeld, P. Amylases α and β. In Methods in Enzymology, Vol.1 (Colowick, P. and Kaplan , N.O., eds), 1951, pp:149-157. New York: Academic Press.
8. Cappucino, J.G. Mikrobiologi. A Laboratory Manual, Addison-Wesley, USA. 1983.
9. Chrismadha, T. and Nofdianto, Pengaruh Konsentrasi Nutrien Terhadap Pertumbuhan dan Produktivitas Chlorella sp. pada Sistem Kultur Kontinyu, Limnotek. 1994, 2 (1) 33-43.
10. Fayaz, M., Namitha, K.K., Murthy, K.N.C., Swamy, M.M., Sarada, R., Salma, K., Subbarao, P.V. and Ravishankar, G.A. Chemical composition, iron bioavailability, and antioxidant activity of Kappaphycus alvarezzi (Doty). J. Agric. Food. Chem. 2005, 53:792-797.
11. Halliwel, B. and Guttridge, J.M.C. Free Radical in Biology and Madicine. Claderon press. Oxford, 1990, 412-438.
12. Herrero, M., Jaime, L., Martı´n-A´ lvarez, P.J., Cifuentes, A., and Iba´n˜ez, E. Optimization of the extraction of antioxidants from Dunaliella salina microalga by pressurized liquids. J. Agric. Food.Chem. 2006, 54:5597-5603.
13. Hirata, T., Tanaka, M., Ookie, M., Tsunomura, T. and Sakaguchi, M. Antioxidant activities of phycocyanobilin prepared from Spirulina platensis. J. Appl. Phycol. 2000, 12:435-9.
14. Huang H.L and Wang, B.G. Antioxidant capacity and lipophilic content of seaweeds collected from the Qingdao coastline. J. Agric. Food. Chem. 2004, 52:4993-4997.
15. Levy, Y., Haya-Zaltsberg, H., Ben-Amotz, A., Kanter, Y. and Aviram, M. Dietary supplementation of a natural isomer mixture ofbeta-carotene inhibits oxidation of LDL derived from patients with diabetes mellitus. Ann. Nutr. Metab. 2000, 44:54-60.
16. Levin, H.E.B., Staying healthy with nutrition: the complete guide to diet and nutritional medicine. Celestial Arts, 2nd edition, 2006.
17. Li, A.H., Cheng, K., Wong, C., King-Wai, F., Feng C. and Yue, J., Evaluation of antioxidant capacity and total phenolic content of different fractions of selected microalgae. Food Chem; 2007, 102: 771-776.

18. Miranda, M.S., R.G. Cintra, S.B. Barros and J.M Filho., Antioxidant activity of the microalga Spirulina maxima. Braz. J. Med. Biol. Res. 1998, 31:1075-1079.
19. Miyashita, K., M. Yasuda, T. Ota and T. Suzuki. Antioxidant activity of a cathodic solution producedby the electrolysis of a dilute NaCl solution, Biosci. Biotechnol. Biochem. 1999, 63 (2): 421-423.
20. Mourey A. and Kilbertus, G. Simple Media Containing Stabilized Tributyrin for demonstrating Lipolitic Bacteria in Foods and Soils. J. Appl. Bact. 1976, 40:47-51.
21. Osawa T., Katsuzaki H., Hagiwara Y., Hagiwara H. and Shibamoto T. A novel antioxidant isolated from young green barley leaves. J. Agric. Food Chem. 1992, 40, 1135-1138.
22. Qi, H., T. Zhao, Q. Zhang, Z. Li, Z. Zhao and R. Xing. Antioxidant activityof different molecular weight sulfated polysaccharides from Ulva pertusa Kjellm (Chlorophyta), Appl. Phycol; 2005, 17: 527-534.
23. Rao, A.R., R. Sarada, V. Baskaran and G.A. Ravishankar. Antioxidant activity of Botryococcus braunii extract elucidated invitro models. J. Agric. Food. Chem. 2006, 54:4593-99.
24. Reddy, C.M., V.B. Bhat, G. Kinarmay, M.N. Redding and K.M.R. Pand-Mediastla, Selective inhibition of cyclooxygenase-2 by c-phycocianin from Spirulina platentis. Biochem. Biophys. Res. Com. 2000 277: 597-603.
25. Romay, C., J. Armesto, D. Ramirez, R. Gonzalez, N. Ledon and I. Garcia. Antioxidant and anti-inflammatory properties of c-phycocyanin from blue-green algae. Inflammatory Research. 1998, 47(1): 36-41.
26. Ruberto, G., M.T. Baratta, D.M. Biondi and V. Amico. Antioxidant activity of extracts of the marine algal genus Cystoseira in a micellar model system. J. Appl. Phycol. 2001, 13: 403-407.
27. Sulistyo, J., R.D. Rahayu, and S. Purwaningsih. Assay for natural antioxidant activity of algae and microbial cultures. Seminar Nasional Kimia Terapan Indonesia. Chemical Research Center, Solo, Center of Java, Indonesia, May 23 2013. (Unpublished presented paper).
28. Yan, X.J., Y. Chuda, M. Suzuki and T. Nagata. Fucoxanthin as the major antioxidant in Hijikia fusiformis, a common edible seaweed. Biosci. Biotechnol. Biochem. 1999, 63:605-607.
29. Yuan Y.V., D.E. Bone and M.F. Carrington. Antioxidant activity of dulse (Palmaria palmata) extract evaluated in vitro. Food Chemistry. 2005, 91:485-494.
30. Vasquez, M., V. Santos and J.C. Parojo, Fed Batch Culture of Phaffia rhodozhyma in xylose Containing Media Made from Wood Hydrolisates. Food Biotechnology. 1998, 12:43-55.
31. Wijaya, A., Free Radicals and Antioxidant Status Parameter. Forum Diagnosticum, Prodia Diagnostic Educational Services. 1996, 1:1-12.