Isolation and identification of carotenoid-producing microalgae from Demak marine waters

H P Kusumaningrum¹ *, A Suprihadi¹, A Budiharjo¹, M Zainuri², I Misbach³, A Maulidiyah³

¹Department of Biology, Faculty of Science and Mathematics, Diponegoro University
²Marine laboratory. Faculty of Fisheries and Marine Science, Diponegoro University
³Faculty of Science and Mathematics, Diponegoro University
E-mail: herminpk@live.undip.ac.id

Abstract. The abundant number of existing species of microalgae in the environment constitutes biodiversity, which supports potential commercial exploitation of many novel products like carotenoid. Microalgae represent a sustainable source of any kinds of natural carotenoid. Aquaculture requires antioxidant supplements such as carotenoids but are unable to synthesize de novo. Carotenoids are needed to improve survival. The production of carotenoids has been one of the activities in microalgae. The objective of the study was to isolate and identify microalgae that can produce carotenoid from Demak marine waters. Production of carotenoid was measured using Spectrophotometer according to AOAC methods. The research found some potentially carotenogenic microalgae member of diatom in the aquatic environment of Demak which is thought to be a species of *Melosira*, *Thalassiotrix*, *Rhizosolenia*, *Navicula*, *Climacodium*, *Achanthes*, *Loxophyllum*, and *Trichodesmium*. These species potentially produce carotenoid and antioxidant properties with variety range.

1. Introduction

Photosynthetic microalgae were sources of bioactive compounds with great importance for the food, cosmetic, and pharmaceutical industries. They have a central role in photosynthesis, both as light harvesting complex as well as photoprotectors using their antioxidant activity. Microalgae are having a distinct pigment composition which was substantially different from plants. There are some of the photoprotective carotenoids consist of β-carotene and the xanthophylls, diatoxanthin, diadinoxanthin, violaxanthin, antheraxanthin, and zeaxanthin, instead of light-harvesting pigments such as chlorophyll a, chlorophyll c, and fucoxanthin. Microalgae and cyanobacteria are photoautotrophic organisms that are exposed to high oxygen and radical stress in the environment. To defend themselves in the environment, they develop an efficient protection system against reactive oxygen species and free radicals in the form of carotenoids. In particular, microalgae carotenoids will counteract the effects of damage caused by excess sunlight and protecting cells from oxidative damage. Carotenoids are also used to adapt to the environment and maintain its survival. Carotenoids are antioxidants against harmful free radicals and other poisons that enter the algae body. The carotenoid were a subfamily of the isoprenoids, are among the most widespread, ancient, diverse, and rich class of all natural products and biomolecules. Microalgae synthesize isoprenoids from isopentenyl diphosphate (IPP) and its isomer dimethylallyl diphosphate (DMAPP) (Paniagua-Michel et al., 2012). Microalgae carotenoids have several advantages over plant carotenoids. Microalgae carotenoids consist of trans-β-carotene.
and 9-cis-β-carotene. 9-cis-β-carotene can absorb light in a wider spectrum ten times more powerful and active than carotenoids in fruits and vegetables [1]. Microalgae carotenoids are easier to decompose than plant carotenoids, so microalgae carotenoids are more easily digested and absorbed than plants [14][15][20]. Microalgae carotenoids are 55% stronger than β-carotene plants in inhibiting the growth of skin cancer cells. Carotenoids are needed to increase nutritional value, disease resistance, increase the degree of pigmentation and precursors of various metabolites. This carotenoid can act as a source of natural nutrition that can be used for aquaculture animal in order to increase their survival against virus and microbial infection [17]. There is a high diversity of beneficial microalga cell components in lipids and pigments, whose amount in the cell may be partially regulated by certain abiotic stresses or genetic modifications of metabolic pathways. Microalgae are a primary source of omega-3 long-chain polyunsaturated fatty acids [(n − 3) LC-PUFA], such as docosahexaenoic acid [DHA; 22:6(n −3)] and eicosapentaenoic acid [EPA; 20:5(n −3)], which are incorporated in marine animals through the diet and also in humans through consumption of seafood. These fatty acids are recognized as essential for survival and growth during the early stages of the life of many marine animals [28][30].

Microalgae were widely found naturally in the aquatic environment. Coastal areas and river estuary like Demak have dynamics complex waters caused by circulation of water mass, mixing, sedimentation and erosion [6]. Several water physical and chemical processes possesses the dynamics of water quality and fluctuations high productivity. These environments have high nutrient elements on their area which was used by phytoplankton to support photosynthesis [23] — the identification and isolation of potential carotenogenic microalgae from Demak marine waters area offering potency to acquire the promises candidate.

2. Materials and methods

2.1 Determination of the point and Sampling

This study uses the sparsus-temporal approach with a time of observation every month. Primary data will be taken at the highest tide and lowest low tide. Phytoplankton sampling in Demak waters was carried out vertically using plankton-net no. Twenty-five with a filter of 100 liters of water at each observation station, the subsequent sampling results are by a 100 ml sample size container, and labeled.

![Figure 1. The location of Phytoplankton collection in Demak, Central Java, Indonesia.](image)

2.2 Preservation of Samples and Identification of potential Carotenogenic microalgae

Sample preservation methods using up to 4% formalin [23], as many as 500 ml. Identification was carried out using a binocular light microscope with a magnification of 40 times, according to the statement (Nontji, 2006) that the magnification of 40 to 100 times is sufficient for phytoplankton analysis, which is assisted by the Opti Lab tool to documentation [23]. Furthermore, the identification results were distinguished by productive phytoplankton types and those classified as HABs using
identification books: *identifying Marine Phytoplankton*, Tomas, Carmelo R (1997); Planktonologi, Sahlan (1982) and *Marine Plankton, a practical guide*, Newel and Newel (1993).

2.3 Microalgae isolation

Microalgae were collected from waters that are rich in photosynthetic microalgae in Demak aquatic environment. Water temperature varied from 26°C to 34°C, and salinity from 32 to 34. One aliquot was preserved in formadehyde (4%) for microscopic analysis and preparation of slides. All cultures were grown in Walne agar medium prepared from filtrated seawater diluted with distilled water to 60%. Diatom cultures were cultured at room temperatures. The cultures were provided with 116 μmol photons m⁻² sec⁻¹ continuous light from fluorescent tubes (Phillips).

2.4 Identification of carotenogenic microalgae

Cell of microalgae was examined at 400x and 1000x magnification using a binocular microscope. The number of microalgae was count by haemocytometer method under microscope.

2.5 Analysis of carotenoid production

Pigment production was carried out on Walne pH 6 medium which was sterilized by autoclaving for 20 minutes at 121°C. Measurement of carotenoid pigments is carried out according to AOAC methods (2005).

3. Result and Discussion

3.1 Isolation and screening of carotenogenic microalgae

Microalgae were isolated from samples collected from coastal, estuarine and rivers water in Demak. The method for the isolation of microalgae was adjusted and developed during this research. The microalgae that were found on Demak waters environment was shown in Table 1.

| No | Demak Sampling Region | Genus         | Species                  |
|----|-----------------------|---------------|--------------------------|
| 1  | Sampling Region I     | Melosira      | *M. Hyperborea*          |
| 2  |                      | Rhizosolenia  | *R. delicatula*         |
| 3  |                      | Thalassiotrix | *T. longissima*         |
| 4  | Sampling Region II    | Achnanthes    | *A. longipes*            |
| 5  |                      | Climacodium   | *C. frauenfeldianum*    |
| 6  |                      | Navicula      | *N. distance*            |
| 7  |                      |               | *N. membranacea*         |
| 8  |                      | Rhizosolenia  | *R. calcar*              |
| 9  |                      | Thalassiotrix | *T. fraenufeldii*        |
| 10 | Sampling Region III   |              | *T. fraenufeldii*        |
| 11 | Sampling Region IV    | Chaetoceros   | *Chaetoceros lorenzianum*|
| 12 |                      |               | *C. messanense*          |
| 13 | Sampling Region V     | Melosira      | *M. jvergensi*           |
| 14 |                      | Thalassiotria | *T. spinulata*           |

The major source of microalgae isolation came from brackish water area. A total of 14 samples have been used for microalgae isolation. Based on microscopic examination on the mix solution, we have eight genera that were potentially produce carotenoid as illustrated in Figure 2. Unfortunately, these isolates were grown slowly or did not grow in the solid media.

3.2 Identification of isolated microalgae

3.2.1 Navicula

*Navicula* sp. was a benthic diatom. According to (Lee *et al.*, 2006), *Navicula* sp. was reported having bioactive compounds navicular, a sulfated polysaccharide with antiviral activities against herpes simplex viruses 1 and 2 and influenza A virus [18]. This microalgae also have a long chain of polyunsaturated fatty acid (PUFA) [7]. Mansour *et al.* (2005) found a member of Omega 3,
Eicosapentaenoic acid (EPA) in *Navicula* with concentration 5.8 mg L$^{-1}$. Some enzymes from *Navicula* sp. exhibited scavenging activity against higher O$_2$ radical. The extracts of *Navicula* sp. contained high phenolic content which correlated with the antioxidant activity [19]. The extracts of *Navicula* sp. also contained high phenolic content of more than 15 mg GAE g$^{-1}$ extract. It shows that there was a strong correlation between the phenolic contents and the antioxidant activity. The effect of antioxidants on DPPH free radical scavenging was considered to be emanating from their hydrogen donating ability. The *Navicula* was exhibit a strong effect of DPPH free radical scavenging about 31.6%. It reached 74.34% of inhibition at 1 mg of extract, which is considered higher than the ethanol extract of some Bacillariophyceae tested at 2 mg of the extract (22.7%, 31.6%, and 76.6% for *Amphora coffeaeformis*, *Navicula* sp., and *Achnanthes longipes*, resp.) [31].

**Figure 2.** *Melosira, Thalassiotrix, Rhizosolenia, Navicula, Climacodium, Acanthus and Thalassiosira.*

### 3.2.2 Rhizosolenia
*Rhizosolenia* was a member of microalgae that produce the biologically active compounds such as antibiotics, enzyme inhibitors, active pharmacology compounds and toxins [19]. The carotenoid obtained from this genera using isoprenoid biosynthesis that resulted not only diterpenoids, triterpenoids, sterols, phytol and sesterterpenoids using mevalonate route [22].

### 3.2.3 Chaetoceros
*Chaetoceros* was the diatoms with potential carotenoid compounds. Volkman *et al.* (1989) found the polyunsaturated fatty acid 20:5(n-3) (eicosapentaenoic acid) in the diatoms *Chaetoceros calcitrans* and *C. gracilis*, was about 4.6–11.1% of the total fatty acids [30].

### 3.2.4 Thalassiothrix
*Thalassiothrix* was the diatoms with potential biological active compounds such as antibiotics, enzyme inhibitors (to treat diseases caused by excessive enzyme activity, e.g. carbohydrate disorders), pharmacology active compounds and toxins [19].

### 3.2.5 Melosira
The presence of *Melosira* sp. in Demak area was important since *Melosira* has high amounts of polyunsaturated fatty acids (PUFA) [7]. Other researcher found that this isolate is also having extracts which can induce leukemia cell death [26].

### 3.2.6 Climacodium,
Climacodium was the genera of marine diatoms that harbor cyanobacterial symbionts. The symbiont of Climacodium frauenfeldianum has been identified as Crocosphaera watsonii [14][4]. Diatoms and cyanobacteria have higher growth rates in symbiosis than apart, and diatom hosts appear to influence the metabolism and growth of the symbionts. Fixed nitrogen is transferred to the host on a timescale of minutes to hours, but the mechanism of transfer and the identity of the product transferred remain unknown. Diatom symbioses make large contributions to the nitrogen budget of the ecosystems in which they are found, particularly under bloom conditions.

3.2.7 Achnanthes,
Lipid peroxidation was inhibited by enzymes from Achnanthes. This genus is also showing antioxidant activity. These data suggest that activity of this microalga might be valuable sources of antioxidant which can be applied in food and pharmaceutical industry. The extracts of A. longipes contained high phenolic content which correlated with the antioxidant activity. It extracts showed high inhibition about 76.6% using 1 mg of extract.

3.2.8 Thalassiosira
Thalassiosira pseudonana was known as microalgae that producing polyunsaturated fatty acid 20:5(n-3) (eicosapentaenoic acid, EPA) about 4.6–11.1% of its total fatty acids. The whole genomes of Thalassiosira pseudonana had been being sequenced and few analogs of the genes related with microalga carotenoid. The microalgae T. pseudonana CCMP1335 is having phytoene dehydrogenase, phytoene desaturase and carotenoid isomerase-like protein partial mRNA (access. Number XM_002295852). Phytoene dehydrogenase (phytoene desaturase) is an enzyme of carotenoid biosynthesis that converts phytoene into zeta-carotene.

3.2.9 Potency of lipid acid and carotenoid from microalgae
Due to the low concentration of microalgae, measurement of carotenoid was performed in the mix culture solution. Lipid acid also measured further as illustrated in Table 2.

| Nutrient     | Concentration (%) |
|--------------|-------------------|
| 1 Beta-karoten  | <0.09             |
| 2 EPA        | <0.00125          |
| 3 Omega 3   | <0.0016           |
| 4 Omega 3 total | 1.0987           |
| 5 Omega 6   | 0.0038            |
| 6 Omega 9   | 0.0118            |
| 7 DHA       | <0.0012           |
| 8 PUFA      | <1.0987           |

Despite the advantages of all microalgae, the growth of all isolates were very poor. Light is the main factor that determines growth, cell survival, and pigment formation. If the available light is excessive, the carotenoids are photo protectors. If the light is insufficient, chlorophyll will be assisted by carotenoid pigments in the absorption of light that cannot be absorbed by chlorophyll. Furthermore, the energy produced will be sent to the chloroplast, and the excitation energy will be trapped by electron transfer. Carotenoids also function as protein structure stabilizers and facilitators of the assembly of protein-pigment complexes, flow regulators and energy exchanges and single electrons and oxygen, and electron donors for pigment-protein complexes. Chlorophyll and carotenoids will be synthesized in a balanced manner in the chloroplasts. When this balance changes due to the increase in carotenoids, the plastide structure will change and as a result, chlorophyll will degrade [8][17]. Pisa and Lele (2005) state that the synthesis of carotenoid will increase in physiological conditions that are less balanced in cells caused by various environmental stress factors to protect cells and maintain growth and adaptation to the environment [27]. The trick is to increase the production of carotenoid to
ward off harmful free radicals and other poisons that enter the body. The results of the overall study showed the potency of microalgae from Demak environment in producing microalgae. The advantages of microalgae have offered the potential to be improved in the future since marine microalgae have become widely recognized as a source of unique bioactive compounds for potential industrial, pharmaceutical, and medical applications such as biofuels, pharmaceuticals, health foods, biomolecules, materials relevant to nanotechnology, and as bioremediation of contaminated water.

4. Conclusion
Isolation and identification of carotenogenic microalga from Demak Marine Water has gained several potential isolates which can be improved in further research

Acknowledgment
This program was funded by Usul Riset Madya Program from Faculty of Science and Mathematics, Diponegoro University, Indonesia year 2018 according to number 316-53/UN7.5.1/PG/2018 date 05 March 2018 which was gratefully acknowledged.

References
[1] Ben-amotz A 1993 Production of β-carotene and vitamins by the halotolerant Alga Dunaliella. In: Marine Biotechnology: Pharmaceutical and Bioactive Natural Products Vol I. Attaway DH. & Zaborsky OR eds., New York: Plenum Publishing Corporation. p. 411
[2] Bergman B, Sandh G, Lin S, Larsson J and Carpenter EJ 2013 FEMS Microbiol Rev 37 286.
[3] Boonyaratpalin M, Thongrod S, Supamattaya K, Britton G, and Schlipalius Le Aquaculture Research 32 182.
[4] Carpenter EJ and Janson S 2000 Journal of Phycology 36 540.
[5] Carpenter EJ and Romans K 1991 Science 254 1356.
[6] Dahuri R, Rais Y, Putra SG, Sitepu, M.J. 2001. Pengelolaan Sumber daya Wilayah Pesisir dan Lautan Secara Terpadu. Jakarta: PT. Pradnya Paramita.
[7] Falk-Petersen S, Sargent J R, Henderson J, Hegseth E N, Hop H, Okolodkov Y B 1998 Polar Biol 20 41.
[8] Frank, H A 2004 Carotenoids in Photosynthesis USA: Department of Chemistry University of Connecticut Storrs CT 06269-3060 p 1-8.
[9] Goodwin T W and Britton G 1988 Distribution and analysis of carotenoids In: Plant Pigments, Goodwin T W ed, London: Academic Press p 75.
[10] Gouveia L and Empis J 2003 Relative stabilities of microalgal carotenoids in microalgal extracts, biomass and fish feed: effect of storage conditions Innovative Food Sci. Emerging Tech. 4 227.
[11] Guedes A C, Amaro H M, Malcata F X, 2011 Marine Drugs 9 625.
[12] Guedes A C, Amaro H M, and Malcata FX 2011 Marine Drugs 9 625.
[13] Henriques M, Silva A and Rocha J 2007 Extraction and quantification of pigments from a marine microalga a simple and reproducible method In A. Méndez-Vilas (Ed.). Communicating Current Research and Educational Topics and Trends in Applied Microbiology Formatex P:586-593.
[14] Johnson, E.A. & Schroeder, W.A. 1996. Advances in Biochemical Engineering /Biotechnology. In: A. Fiechter (ed.) Microbial Carotenoids. Springer-Verlag, Berlin. P:141-145.
[15] Katz, A., C. Jimenez, dan U. Pick. 1995. Isolation and Characterization of a Protein associated with Carotene Globules in the Alga Dunaliella bardawil. Plant Physiol. 108:1657-1664.
[16] Kelman D, Ben-Amotz A, and Berman-Frank I 2013 Environ Microbiol 11 1897.
[17] Kusumaningrum H P and Zainuri M 2014 International Journal of Marine and Aquatic Resource Conservation and Co-existence 1 1.
[18] Lee J B, Hayashi K, Hirata M, Kuroda E, Suzuki E, Kubo Y, Hayashi T 2006 Biol. Pharm. Bull 29 2135
[19] Lincoln R A, Strupinski K, and Walker J M 1990 Diatom Res 5 337

[20] Lichtenthaler, H.K. 1999. The 1-Deoxy-D-Xylulose 5-Fosfate Pathway of Isoprenoid Biosynthesis in Plants. *Annu. Rev. Plant Physiol. Plant. Mol. Biol.* 1(50):47-65.

[21] Lee S H, Karawita R, and Affan A 2009 *Algae* 24 47.

[22] Masse´ G, Belt S T, Rowland S J and Rohmer M 2004 *PNAS* 101 4413

[23] Nontji A, 2006 *Tiada Kehidupan Di Bumi Tanpa Keberadaan Plankton. Lembaga Ilmu Pengetahuan Indonesia* Jakarta: pusat penelitian oceanografi.

[24] Olson JA. 1994. Absorption, transport, and metabolism of carotenoids in humans. *Pure Appl. Chem.* 66(5):1011-1016,

[25] Pisal D S, Lele S S 2005 *Indian Journal of Biotechnology* 4 476.

[26] Prestegard S K, Ofstedal L, Coyne R S, Nygaard K H, Knutsen G, Døskeland S O and Herfindal L 2009 Marine Drugs 7 605.

[27] Pisa D S and Lele S S 2005 *Dunaliella salina. Indian J. Biotechnol* 4 476.

[28] Reitana K I, Rainuzzo J R, GunvorØie, Olsen Y., 1997 *Aquaculture* 155 207.

[29] Boukhris S, Athmouni K, Hamza-Mnif I, Rayda Sial Elleuch R S, Ayadi H, Nasri M Sellami-Kamoun A, Armbrust E V, Berges J A, Bowler C, Green B R, Martinez D, Putnam N H, Zhou S, Allen A E, Apt K E, Bechner M, Brzezinski M A, Chaal B K, Chiovitti A, Davis A K, Demarest M S, Detter J C, Glavina T, Goodstein D, Hadi M Z, Hellsten U, Hildebrand M, Jenkins BD, Jurka J, Kapitonov V V, Kröger N, Lau W W, Lane T W, Larimer F W, Lippmeier JC, Lucas S, Medina M, Montsant A, Obornik M, Parker M S, Palenik B, Pazour GJ, Richardson P M, Rynearson T A, Saito MA , Schwartz D C, Thamatrakoln K, Valentín K, Vardi A, Wilkerson F P, Rokhsar D S 2004 Science 306 79.

[30] Volkman J K, Jeffrey S W, Nichols P D, Rogers G I, and Garland CD 1989 *Journal of Experimental Biology and Ecology* 128 219.

[31] Zainuri M, Kusumaningrum H P, and Kusdiyantini E, 2008a *J. Natur Indonesia*. 10 66.

[32] Zainuri M, Kusumaningrum H P and Kusdiyantini E, 2008b *Ilmu Kelautan* 13 135.

[33] Zainuri M, Endrawati H, Kusumaningrum H P and Kusdiyantini E, 2008c *Ilmu Kelautan* 13 43.