

THE POTENTIAL OF HETEROTROPHIC MICROALGAE (*Schizochytrium* sp.) AS A SOURCE OF DHA

Potensi Mikroalga Heterotroph (*Schizochytrium* sp.) sebagai Sumber DHA

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ABSTRACT

Docosahexanoic acid (DHA) is commercially obtained from marine fish. With an increasing human population, the supplies of DHA are still not sufficient to meet the world's need of DHA as food supplement. The objective of this review is to discuss *Schizochytrium* sp., one of microalgae which is rich in DHA, as one of the best candidate as producer of sustainable and affordable DHA. Heterotrophic microalgae, especially genus *Schizochytrium*, produces omega-3 fatty acids up to 40% of total unsaturated fatty acids. Cultivation of the microalgae is easy as it does not require sunlight as source of energy. Previous publication reported that several local strains of *Schizochytrium* have been isolated from mangrove area in Indonesia. We expect that those strains can be cultivated in mass production as producer of DHA.

Keywords: docosahexanoic acids, fatty acids, *Schizochytrium* sp.

ABSTRAK

Docosahexanoic acid (DHA) komersial sebagian besar diperoleh dari ikan laut. Dengan meningkatnya populasi penduduk dunia, sumber ini tidak akan mencukupi permintaan dari masyarakat terhadap DHA sebagai salah satu makanan kesehatan. Tujuan dari review ini adalah untuk mendiskusikan *Schizochytrium* sebagai salah satu mikroalga yang kaya DHA, sebagai kandidat mikroalga yang mampu memproduksi DHA secara berkelanjutan dan terjangkau. Jenis mikroalga yang bersifat heterotrof terutama genus *Schizochytrium* mampu menghasilkan asam lemak omega 3 mencapai 40% dari total asam lemak tak jenuhnya. Kultivasi mikroalga ini juga lebih mudah karena tidak membutuhkan sinar matahari sebagai sumber energinya. Publikasi sebelumnya menunjukkan bahwa beberapa strain lokal telah di isolasi dari daerah mangrove di Indonesia. Kami berharap strain tersebut bisa dikembangkan ke produksi DHA secara masal.

Kata Kunci: asam docosaheksanoat, asam lemak, *Schizochytrium* sp.

INTRODUCTION

Docosahexanoic acid (DHA; 22:6(n-3)) and *Eicosapentanoic acid* (EPA; 20:5(n-3)) are long chain polyunsaturated fatty acids that is referred to omega 3. Both of these fatty acids have beneficial effect to the human body such as maintaining cardiovascular health, preventing or treating arteriosclerosis and certain cancer (Nettleton, 1995; Ward & Singh, 2005).

Marine fish such as salmon, mackerel, and tuna are sources of omega-3. On the other hand, over fishing, pollution, and global climate change caused scarcity in the sustainability of omega 3 sources (Lewis *et al.*, 1999). Moreover, the application of fish oil as food additive is limited due to problems associated with its smell characteristic, unpleasant

taste, and poor oxidative stability (Spolaore *et al.*, 2006). As consequence, various studies should be conducted continuously in order to find better quality and sustainable sources of omega-3 (De Swaaf *et al.*, 2003).

Some marine microalgae such as dinoflagellates and species in the Heterokonta phylum contain high DHA (Barclay *et al.*, 1994; Apt & Behrens, 1999; De Swaaf *et al.*, 2003; Wu & Lin, 2003; Wu *et al.*, 2005). However, majority of those microalgae are photoautotrophic that dependent on light as energy source and on weather conditions. Heterotrophic microalgae are able to take energy from simple organic substances without requiring light (Apt & Behrens, 1999). One of heterotrophic microalgae is *Schizochytrium* sp. which can be utilized as an

alternative to replace of fish oils due to its rapid growth rate; its weather condition independent and its DHA content which reach up to 48.95% of its total fat (Ren *et al.*, 2010). This paper will discuss the potential of heterotrophic microalgae (*Schizochytrium* sp.) as a source of DHA fatty acids.

Recently, one of food supplement industry, Martek, produced omega-3 oil from a different strain of *Schizochytrium* sp., named Algal Oil which containing approximately 37% of DHA and 16% of EPA (w/w). Algal Oil, as a source of DHA and EPA is intended for food ingredient use and dietary supplement (Fedorova *et al.*, 2011a). A number of studies including sub chronic feeding to rodents (Hammond *et al.*, 2001) and non-rodents (Abril *et al.*, 2003), developmental toxicity in rodent and non rodent species (Hammond *et al.*, 2001b), reproduction (Hammond *et al.*, 2001c), and *in vitro* mutagenicity and genotoxicity (Hammond *et al.*, 2002) confirmed the safety of DHA-rich dried *Schizochytrium* sp. Recently, DHA algal oil derived from *Schizochytrium* sp. is generally recognize as safe (GRAS) and available for food use and for dietary supplements (FDA, 2004).

DHA AND BENEFITS

DHA (Docosahexaenoic acids) is a type of polyunsaturated fatty acids that has chemical structure of C22: 6n-3, which is also called as derived omega 3 fatty acids. This fatty acids become important since it was part of essential fatty acids that humans must ingeste because the body requires them for good health but cannot synthesize them. One rich source of DHA is fish oil. The high amount of DHA in fish oil is originated from photosynthetic and heterotrophic microalgae consumed by the fish (Anon., 2009). Currently, DHA is also commercially manufactured from microalgae i.e. *Cryptocodinium cohnii* and other microalgae from the genus of *Schizochytrium*.

As shown in Fig. 1, the chemical structure of DHA consist of 22 carbon atoms and 6 double bonds is the longest and the most unsaturated, and therefore perhaps the most influential. Among the member of the omega-3 group of polyunsaturated fatty acids (PUFA), DHA is the longest and the most unsaturated, therefore perhaps DHA is the most influential PUFA (Stillwell *et al.*, 2005). DHA has a tremendous benefit

to the human body, especially as the prevention of several diseases such as cancer (Carrol, 1991; Zerouga *et al.*, 1996). DHA is compiler of phospholipids membrane in the human brain and retina. Therefore, pregnant women and infants need to have sufficient DHA intake, especially when pregnancy reaches the age of third trimester in which the average stage development of the brain and retina is the fastest. Infant could fulfill DHA diet by breast milk; however the breast milk is influenced by the mother's diet (Brenna *et al.*, 2007). According to Hoffman *et al.* (2009) fortification of milk with DHA 0.15-0.36% from the total fatty acids in the milk improves nutritional quality of infant milk.

TAXONOMY AND MORPHOLOGY OF *Schizochytrium*

Schizochytrium is a heterotrophic microalgae belongs to the family of Thraustochytriaceae (Luying *et al.*, 2008). *Schizochytrium* is a spherical unicellular microorganism. This microorganism has been favored for study as it is much easier to cultivate than other (Wu *et al.*, 2005). According to Yokoyama *et al.* (2007), morphological characteristics of *Schizochytrium* under the microscope would show a ectoplasmic nets, a formation of zoospores, aplanospores, and amoeboid cells, and the size between 10-20 μm . The taxonomy detail of *Schizochytrium* is as follows:

Kingdom	: Chromista (Stramenopilia)
Phylum	: Heterokonta
Class	: Thraustochytridae
Order	: Thraustochytriales
Family	: Thraustochytriaceae
Genus	: <i>Schizochytrium</i>
Species	: <i>Schizochytrium</i> sp.

(Source: Leipe *et al.*, 1994)

Schizochytrium produces biflagellate zoospore and the mature cells divided by repeated binary division to form diads, tetrads and clusters (Fig. 2). Each *Schizochytrium* cell could develop into a sporangium that produces several zoospores (Kamlangdee & Fan, 2003).

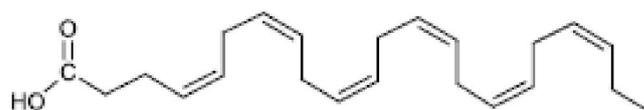


Figure 1. Structure of *Docosahexaenoic acid* /DHA, 22:6^Å4, 7, 10, 13, 16, 19 (Source: Stillwell *et al.*, 2005).

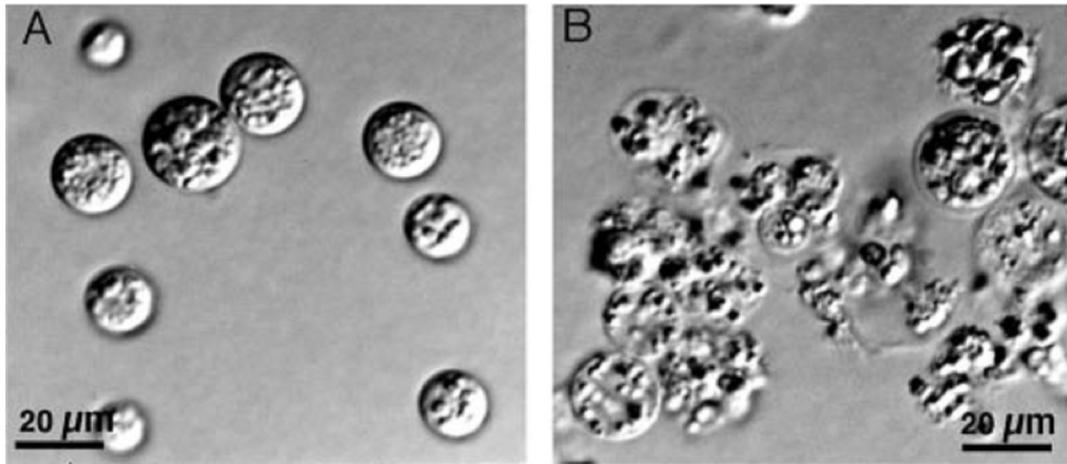


Figure 2. Morphology of *Schizochytrium* cell (Source: Takao *et al.*, 2005).

ISOLATION OF *Schizochytrium*

According to Yang *et al.* (2010) *Schizochytrium* could be isolated from macroalgae, fallen leaves of mangrove trees and also mud from mangrove ecosystem. *Schizochytrium* grows in mangrove and coastal ecosystem. Several studies showed that *Schizochytrium* is symbiosis with mud degrading bacteria, so that these microalgae also could be isolated from soil or mud around mangroves.

Isolation techniques of *Schizochytrium* can be initiated by taking samples from those places. The samples were stored in sterile plastic bags and sent to the laboratory within 1 day for algal cell isolation. Microalgal cells attached to samples were washed down using sterile seawater, passed through a 60- μm . plankton net to remove zooplankton, then collected using a 10- μm plankton net. The cells were rinsed several times over a filter using sterile seawater to remove as many bacteria as possible, then transferred by loop, and streaked onto agar plates. The 0.8% agar medium was prepared using full strength seawater, containing 1 g/L peptone, 2 g/L yeast extract, 4 g/L glucose, and antibiotics including ampicillin (sodium form), streptomycin sulfate, and kanamycin sulfate (100 mg/L each). After inoculation, the plates were wrapped then stored at 26°C for 2–5 days. Single colonies composed of spherical of a typical cell were picked and carefully transferred to a new plate. After becoming established, these algal strains were identified according to their 18S rRNA gene sequences.

Rangkumar (1992) explained different method of isolation of *Schizochytrium*. Samples were taken from decaying leaves of the mangrove, and were suspended in sterile seawater and baited by piece of pine pollen and then shaken for 2-3 days. Then the leaves-suspension was then streaked on agar plates, and

incubated for 4-5 days. Single colony grown in the agar then was inoculated in to fresh agar plate. Inoculation was done repeatedly until strains which have characteristics of *Schizochytrium* has been obtained.

For obtaining DNA sequences of 18S rRNA gene from one strain, a single colony of the strain grown on an agar plate was carefully transferred to a 50 mL tube with 10 mL liquid medium containing 1 g/L peptone, 2 g/L yeast extract, and 4 g/L glucose prepared with seawater. The culture was then cultivated at 26°C for 1 week with continuous shaking (150 rpm). The algal cells were collected by centrifugation (3.000 rpm for 5 min), rinsed with 5 mL deionized water, and lyophilized before DNA sequencing (Yang *et al.*, 2010). The resulting 18S rRNA gene sequences were then aligned and compared to the nucleotide sequences of some known microorganisms in GenBank database of the National Center for Biotechnology Information by using Basic Local Alignment Search Tool (BLAST) (Tamura *et al.*, 2007).

The other method to compared to the nucleotide sequences was using multiple alignment program CLUSTAL W to construct a neighbor-joining (NJ) tree. The bootstrap values were obtained from 1,000 replications of NJ analyses (Kuo *et al.*, 2005; Burja *et al.*, 2006).

PRODUCTION OF *Schizochytrium*

Cultivation of pure culture was conducted in liquid medium to yield *Schizochytrium* biomass for the extraction of fatty acid content (DHA). Many factors may effect this process such as medium composition, salinity, acidity and temperature. Many research have been conducted to determine the type of carbon and nitrogen that important for the *Schizochytrium* growth

Table 1. Effect of carbon sources on biomass, lipid and DHA production



Source: Wu *et al.* (2005).

Table 2. Effect of nitrogen sources on biomass, lipid, and DHA production



Source: Wu *et al.* (2005).

and the production of its polyunsaturated fatty acids, especially DHA. According to Wu *et al.* (2005), type of carbon sources which could be used to grow *Schizochytrium* were glucose, fructose, lactose, maltose and sucrose. While the nitrogen sources needed to grow *Schizochytrium* was including yeast extract, peptone, tryptone, urea, monosodium glutamate, sodium nitrate, ammonium chloride. Each medium has different effects on the biomass and DHA production as shown in Table1 and 2.

Meanwhile according to Yokochi *et al.* (1998), glycerol and monosaccharide (glucose and fructose) could be used to grow *Schizochytrium* cell (Table 3).

In addition to type of media, initial degree of acidity (pH) also affects on *Schizochytrium* growth. The initial

pH value of medium affects cell membrane functions, the uptake of nutrients and product biosynthesis (Kim *et al.*, 2005). Therefore, the pH of medium influences to the cell growth and metabolites obtained. Maximum biomass (14.99 g/L) could be obtained in medium pH 4.0 whereas maximum DHA (77 g/L) was obtained at pH 7.0 (Luying *et al.*, 2008).

Schizochytrium is microalgae that lives in mangrove environment, therefore to obtain the optimum culture conditions, salinity conditions in culture media must be adapted to its natural environment. Salinity affects the growth of the microorganism by controlling the cytoplasmic ion gradient and the activity of enzymes involved in cell wall expansion (Ho & Chou, 2001). *Schizochytrium* is high tolerance to salinity and could

Table 3. Fatty acid composition of *Schizochytrium* grown on various medium sources

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Source: Yokochi *et al.* (1998).

grow over a wide range of salinity 5-35 ppt (Kamlangdee & Fan, 2003). Moreover *Thraustochytrium aureum* and *Schizochytrium aggregatum* could grow even with salinity as low as 1 ppt, but no growth was observed in a medium prepared from distilled water because of absence of major ions of Na, Ca, K and Mg which are essential for the growth of microalgae (Bahnweg, 1979). Influence of salinity to the biomass and DHA production is showed in Fig. 4.

Temperature is an important factor for the growth of microorganism includes *Schizochytrium*. However, Nakahara *et al.* (1996) found that 28°C was the optimal temperature for DHA production from *Schizochytrium*

limacinum. Meanwhile, Luying *et al.* (2008) explained that *Schizochytrium limacinum* could grow in temperatures ranging from 16 to 37°C, while the optimum temperature of DHA production was obtained at 23 °C.

Schizochytrium is expected to be one of alternative DHA sources. Several researchers showed that this microorganism could produce higher DHA. In addition to that, Schizochytrium is irrespective to the weather condition resulting in easier propagation.

Kamlangdee & Fan (2003) isolated 4 strains of *Schizochytrium* (N-1, N-2, N-5, and N-9) from fallen, senescent leaves of mangrove tree. These strains were cultivated in glucose yeast extract medium containing

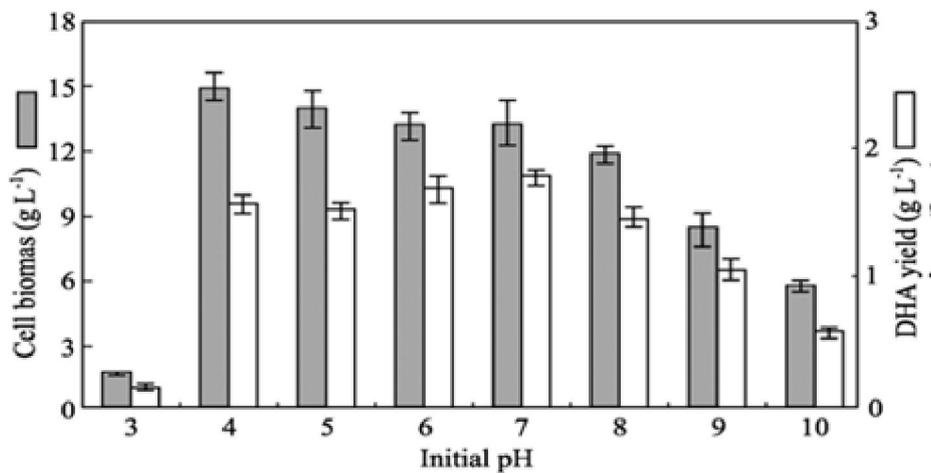


Figure 3. Effects of initial pH on growth of *Schizochytrium* and DHA production (Source:Luying *et al.*, 2008).

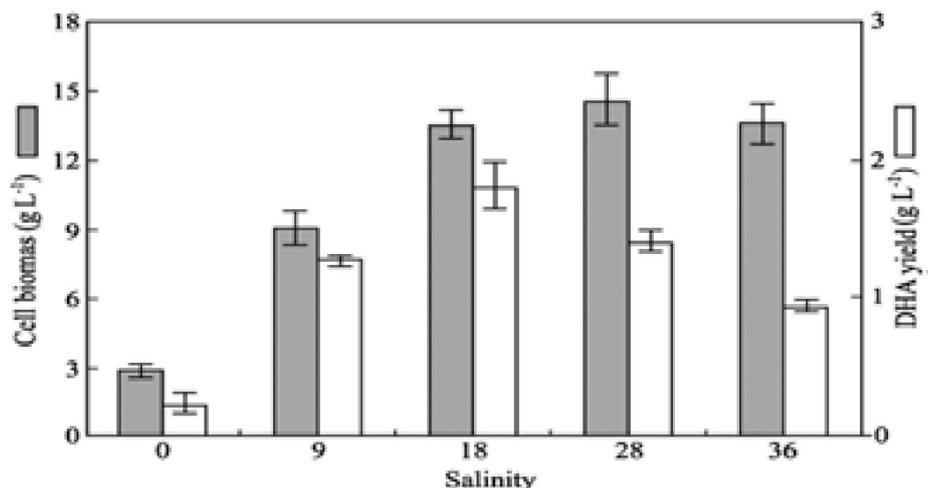


Figure 4. Effects of salinity on growth of *Schizochytrium* and DHA production (Source: Luying *et al.*, 2008).

60 g of glucose, 10 g of yeast extract and 1 L of 15‰ artificial seawater, initial pH 6.0, with shaking for 52 h at 25°C. Biomass of 5 isolates ranged from 10.8 to 13.2 g/L. The contents of DHA in biomass varied from 157.9–203.6 mg/g of dried-biomass. The result showed that *Schizochytrium* produced higher DHA level than other bacteria.

Yano *et al.* (1994) found that five bacterial strains (*Vibrio* sp.) from the intestine of deep-sea fish produced DHA, and one of them produced 0.8 mg/L DHA within 6 day. Meanwhile, Bajpai *et al.* (1991) reported his research about the production of DHA from *Thraustochytrids*. The cell productivity of 3.8 g/L and DHA productivity of 270 mg/L for 6 days were obtained on glucose and glutamate. Later, they obtained DHA productivity of 510 mg/L for 40 hours on soluble starch as carbon source. Furthermore Nakahara *et al.* (1996) successfully produced DHA and DPA (*Docosapentaenoic*) from *Schizochytrium* cell isolated from the coral reef area of the Yap Islands Japan. Cultivation was conducted in a medium that containing 60 g glucose, 0.7 g corn steep liquor, 2 g (NH₄)₂SO₄, 3 g KH₂PO₄ in 1 L of a half salt concentration of artificial sea water at temperature 28 °C. These productivities were equivalent to 9.0 g/L dry cells, 2.0 g/L DHA and 0.44 g/L DPA per day.

Cultivation techniques to increase the biomass of *Schizochytrium* have been carried out by increasing the concentrations of carbon and nitrogen in the growth medium, or by modifying the culture system. Ganuza *et al.* (2008) conducted research on the optimization of cell growth of *Schizochytrium* sp. by employing pH-auxostat system (pH-auxostat fermentation), and cultivation medium containing glucosa (150 g/L) and ammonia (2.4 g/L). pH-auxostat fermentation was initiated in which NH₄OH was added to control the pH

(7). The ammonia concentration within the fermenter was maintained between 300 mg/L and 400 mg/L by this strategy. Thus, *Schizochytrium* sp. could be grown under non-nitrogen limiting conditions to a high biomass density (60 g/L) in 2 days. In this period, it was obtained biomass *Schizochytrium* cell 60 (g/L) which contains fatty acids 25% (w/w) and DHA reaching 40% of total fatty acids.

Furthermore, Ren *et al.* (2010) have developed the production technique of DHA from *Schizochytrium* in 1,500 L bioreactor. Cultivation was conducted stepwise. The single colony in agar medium was inoculated into a 250-mL flask with 50 mL medium and cultivated for 24 h. After three generations of cultivation, the preculture was inoculated into a 150-L seed fermentor with an inoculum size of 1% (v/v) and cultivated for 24 h. The seed culture (10%, v/v) was then transferred to a 1500-L fermentor with a working volume of 1,000 L for 96 h. Biomass were achieved (71 g/L), and contained high lipid content (35.75 g/L), and high DHA percentage (48.95%).

Compared with other *photoautotroph* microorganism, *Schizochytrium* cell produced higher DHA (Table 4).

POTENTIAL UTILIZATION

Schizochytrium is underdeveloped in Indonesia. Up to date, only 7 species were found from several mangrove areas in Indonesia (Basuki, 2011); this is likely caused by the lack of information and research about this microalgae. Many factors such as natural environment and medium growth may affects the production of *Schizochytrium*.

Indonesia has a very large mangrove areas, which is a tremendously potential as a source of isolate local

Table 4. Profile of fatty acid produced by various microorganisms

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Source: Anon., 2002.

Table 5. Result of chemical and physical test of *Schizochytrium* oil

Chemical Test	Specification	Test Method
Acid value	≤ 0.5 mg KOH/g	AOCS Method Cd 3d 63
Peroxide Value	≤ 5.0 meq/kg oil	AOCS Method Cd 8-53
Moisture and volatiles	≤ 0.05%	AOCS Method Ca 2d-25
Unsaponifiables	≤ 4.5%	AOCS Method Ca 2d-25
Trans-fatty acids	≤ 2%	AOCS Method Ca 6b-53
DHA	≥ 32,0%	AOCS Method Cd 14-61
Hexane	≤ 10 mg/kg	AOCS Method Ca 3b-87

Source: Baldwin, 1997.

strains of *Schizochytrium*. Growth media of *Schizochytrium* are easy to find. Moreover, sugar cane molasses which is very abundance in Indonesia can be used to substitute the glucose in the *Schizochytrium* growth media.

The fact that the alga is capable to utilize glycerol as a carbon source causes the exploration leads to the feasibility of using crude glycerol from biodiesel for growing *Schizochytrium*. Currently, the market is flooded with an abundance of crude glycerol from

Table 6. Utilization of *Schizochytrium* oil in food products

Source: Commission Decisions 2003/427/EC and 2009/778/EC in Howlett (2011).

biodiesel, the use of glycerol as carbon sources to produce PUFA algae provide an alternative utilization of glycerol while economically helping the growth of the biodiesel industry in Indonesia (Basuki, 2011).

Changes in utilization trend of unsaturated fatty acids especially DHA resources in recent years leads to marine microbial as one alternative sources of DHA. The easy, sustainable continuously technology to produce yield high biomass of *Schizochytrium* has been developed in several industries. *Schizochytrium* cell (10-20 μm) aggregates each other, making it easier in the harvest and filtration process. *Schizochytrium* biomass could be further used directly as food for shrimp larvae, and the extracted fatty acid can be used as food fortification. Fatty acid extraction from *Schizochytrium* can be done using organic solvents (hexane). Prior extraction, cell of *Schizochytrium* must be dried to obtain dried *Schizochytrium* with 10-15% moisture content. Then the dried *Schizochytrium* was milled into smaller size. The mixture of crude oil and organic solvent chilled and then filtered to remove solids. Addition of acid or base was conducted to remove the organic solvent which mixed in oil. The crude oil obtained was then centrifuged to remove the remnants solid that remain. The oil chilled again. If it is intended to be used in a long time, antioxidant should be added into the oil (Anon., 2002; Baldwin, 1997). The chemical test result of *Schizochytrium* oil can be seen at Table 5.

Various type of oils from *Schizochytrium* can be added to foods to increase the content of DHA. Some food product have been fortified using *Schizochytrium*'s oil (Table 6).

CONCLUSION

Consumers around the world are moving toward functional foods as a way to prevent and to fight

diseases, to increase their energy and wellness, and to help them live longer, healthier, more productive lives. They are beginning to search for not only low-fat and low-sugar products, but also for foods considered as natural or with ingredients taken from natural sources as opposed to synthetically produced ingredients. With that in mind, food scientist's found microalgae as a potential source for functional food and bioactive ingredients. In the last few decades microalgae have been produced and marketed as nutraceuticals and food supplements. Genera *Schizochytrium*, have become popular microalgal as sources of protein-rich biomass and compounds, especially carotenoids, pigments, antioxidant extracts, and essential fatty acids. The potential of heterotrophic microalgae as a source of omega 3, especially DHA have not been yet widely utilized. The ease of production process, heterotrophic high characteristic level of biomass and DHA produced by *Schizochytrium* make them more advantages compared to other types of photoautotroph microalgae. The extract oil from *Schizochytrium* also can be utilized to increase the DHA content in some food products.

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