Fractionation and Quantitative Analysis of Different Lipid Components of *Schizochytrium* sp. (POL01 Strain)

Monabel May N. Apao[1*] and Jose M. Oclarit[2]
[1]College of Industrial and Information Technology
Mindanao University of Science and Technology
CM Recto Ave., Lapasan, Cagayan de Oro City, 9000 Philippines
**feminine_blossom@yahoo.com*

[2]Matias H. Aznar Memorial College of Medicine
Southwestern University
Cebu City, 6000, Philippines

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Abstract

The culture of DHA (docosahexaenoic acid)-rich thraustochytrids, a common microheterotroph that is taxonomically aligned with heterokont algae, is seen as one alternative source of polyunsaturated fatty acids (PUFA) since there is a decline of fish stock that cannot sustain conventional PUFA extraction. Fatty acid compositions and lipid components of Schizochytrium sp. POL01 were studied. Lipid class composition and distribution of fatty acids of Schizochytrium sp. POL01 harvested were studied, with special emphasis on the distribution of docosahexaenoic acid (C22:6 n-3, DHA). The isolate obtained 4.8 g/L of oven-dried cells in 100-mL medium after 72 hours of incubation. Lipid components were collected into several fractions. Fraction two and three were among the highest in percent proportion and content in DHA content according to gas chromatographic analysis. Fraction two, composed of neutral lipids, has the highest in DHA content and accounted for 47.6% of the total fatty acids which is 16.1 mg DHA/g of dried cell. Neutral lipids were the major lipid constituents in which triacylglyerol (TAG) was the predominant component. Fraction three, composed of glycolipids, mono- and diglyceride, also indicate significant value of DHA, 35.4%, 7.5 mg DHA/g dried cell, followed by fraction four of phospholipids and polar lipids, 25.1%, 1.8 mg DHA/g dried cell as DHA yield, respectively. However, the DHA content of fraction one of n-alkanes, n-alkenes and free fatty acids had the least amount with a value of 3.3%, 1.1 mg DHA/g dried cell. DHA was found to be distributed in all lipid fractions and to be the major polyunsaturated fatty acid.

Keywords: thraustochytrids, PUFA, DHA

1. Introduction

Humans have a necessity for certain essential fatty acids, such as omega-6 fatty acid and omega-3 fatty acid in the diet because they cannot be synthesized from simple precursors in the diet. Both of these fatty acids are 18-carbon polyunsaturated fatty acids differing in the number and position of the double bonds. Polyunsaturated fatty acids (PUFA) are vital for human health as they are associated with membrane function and membrane phospholipids structure especially in the retina and neuronal tissues, pathophysiological conditions and etc. The predominant sources of polyunsaturated fatty acids are vegetable, fish and algal oils.

Docosahexanoic acid (DHA) is a 22-carbon essential PUFA owing to its functions in the brain and the retina. Vertebrate retinas contain a high level of docosahexaenoic acid (DHA, 22:6n-3) and a relatively low level of arachidonic acid (AA, 20:4n-6) (H Chen and RE Anderson, 1993). The current commercial source of DHA is fish and because of the relatively low proportion of DHA in fish oil, problems encountered in purification and have become scarce in the global market, efforts have been made to obtain eukaryotic marine organisms as the alternative sources for omega-3 fatty acid production.

Thraustochytrids are eukaryotic microheterotrophs and have been reported to produce considerable amounts of PUFA, most especially DHA (Burja *et al*., 2006; Huang *et al*., 2001). However, thraustochytrid strains have large variation in biomass, lipid, and DHA yields (Lewis, Nichols and McMeekin, 2000; Swaaf and Sijtsma, 2004; Ward and Singh, 1997). And to date, thraustochytrid strain from Mindanao have not yet been studied thoroughly, isolation of strain from Mindanao, as well as the quantitative analysis of different lipid components and screening of these strain for PUFA and DHA production is necessary.

The main objective of the study is to quantify and analyze the lipid fractions of *Schizochytrium* sp. POL01 for fatty acid components and to identify which lipid fraction has promising DHA levels. This study also addresses the specific objectives which are to isolate *Schizochytrium* sp. from Polo, Dapitan City of Western Mindanao; to separate and obtain lipid fractions by Column Chromatography; to extract and esterify fatty acids from the lipid fractions; to identify and analyze the fatty acids present in the fractions by Gas Chromatography and to measure desired lipid fraction based on DHA content of total fatty acid profile.

The researcher has seen a significant alternative to minimize pressure on fish stocks, carry on utmost aquaculture production at lower cost, and present more food additives for human consumption. Thraustochytrids have been reported to produce extensive amounts of PUFA, most especially DHA. Since, thraustochytrid strains from Mindanao have not yet been studied; isolation of strains from Mindanao, as well as the screening of these strains for PUFA and DHA production is needed.

Fatty acids as the building blocks of fats have been known as one of the most important dietary factor since it regulates the serum cholesterol levels in the blood. This study also provides information concerning quantification and analysis of lipid fractions from the samples thereby providing specific information which lipid fraction (neutral lipids, glycolipids, phospholipids, n-alkanes or n-alkenes) has the most promising DHA levels.

The study was limited to the selected mangrove site of Polo, Dapitan City in the provinces of Western Mindanao. Fallen mangrove leaves of first decay level, likely those with some spots around, were collected aseptically from selected mangrove species found in the chosen site. The YPG media was used all throughout the isolation process of Thraustochytrids. The isolate was mass-produced for biomass determination, separation into lipid fractions, and for fatty acid profiling. Fractionation of the total lipids that was extracted from the dried cell of Thraustochytrid was done by silica gel column chromatography. The presence of fatty acids in the extracted lipid was analyzed using gas chromatography technique.

2. Methodology

2.1 Isolation of Thraustochytrid

Schizochytrium sp. (strain POL01) was the only microorganism used in this research collected from Polo, Dapitan City of Western Mindanao (Figure 1). Fallen senescent mangrove leaves of various decay levels with preference for the floating, yellow-colored and brown-spotted were collected using forceps and were immediately placed in a resealable plastic. Ecological notes were taken and physico-chemical parameters (i.e. water pH, water temperature, and salinity) were determined.

The leaves in resealable plastic were brought to the laboratory for analysis within twenty four hours of sampling. Leaf discs (approximately 2 cm in

Figure 1. Map showing the source (flag) of *Schizochytrium* sp. POL01 isolate

diameter) were aseptically cut from the collected leaves. Both sides of the disc were swabbed onto an YPG (yeast extract-peptone-glucose) plates. YPG plates constituted 2.5 g yeast extract, 0.5 g peptone, 30 g glucose, and 8 g pharmaceutical agar, per 500 mL of diluted seawater (1 part distilled water: 4 parts of filtered natural seawater) with 0.1 mL for every plate of an antibiotic mixture, composed of penicillin and streptomycin, and were applied onto the plates employing the spread-plate technique, so as to minimize the growth of bacteria and fungi. Plate cultures were incubated at 27 ºC for 2-5 days and thraustochytrid colonies belonging to the genus *Schizochytrium*, presumptively identified by colony morphology and verified by microscopic examination, were subcultured on an YPG plate repeatedly until axenic cultures were obtained. Purified cultures were maintained in YPG slants.

2.2 Heterotrophic Growth and Biomass Determination

Four (4) loopfuls of thraustochytrid cells were introduced onto a 100-mL seed culture medium (constituted with 10 g glucose, 1 g yeast extract, and 1 g peptone, in 1 L NSW (40 ppt, 7.96 pH) contained in a 1000-mL culture bottle. Culture bottles were incubated at 25°C for 48 hours on a tissue roller apparatus (Wheaton Modular Cell Production Roller Apparatus) set at 60 rpm, under continuous fluorescent lighting.

A 5% (v/v) inoculum of the 48-hour old culture broth was used as initial inoculum for mass production. The heterotrophic culture of each isolate was grown in a 1000-mL culture bottle that contained 100 ml of glucose yeast extract medium. The medium was composed of 60 g glucose, 1 g yeast

extract, and 1 g peptone, in 1 L NSW (40 ppt, 7.96 pH). For the strain, triplicate bottles were incubated at 25°C for 5 days on a roller apparatus set at 60 rpm, under continuous fluorescent lighting.

For dry weight biomass determination, the entire content of the 1000 ml culture bottle, the thraustochytrid cells, was transferred to pre-weighed 50-mL centrifuge tubes (Falcon, BlueMax) and harvested by centrifugation at 3000xg for 15 minutes at 25°C.

2.3 Total Lipid Extraction

About 4-6 g oven-dried cell pellet in 50-mL centrifuge tube (Falcon, BlueMax) was homogenized with 5 mL chloroform:methanol (2:1 v/v, Merck). After which, the homogenized pellet was filtered using Whatman No. 1 paper and the filtrate was evaporated to dryness while the cells were discarded. After the solvent was evaporated, total lipid was obtained.

The lipid residue on a pre-weighed fluorescence flask was weighed in an analytical balance to get the total lipid extract for column chromatography.

2.4 Column Chromatography

The lipid sample was dissolved in a 5 ml hexane as the first solvent for fractionation and added to the top of the tightly packed silica gel unisil column. About 8 g of silica gel 60 (Merck) with 35 ml of hexane was degassed and packed in the column rinsed with hexane. The hexane was drained until the solvent is just even with the surface of the silica. After the sample was loaded, the solvent was added continuously at the top of the column and the first lipid fraction of n-alkanes, n-alkenes, free fatty acids was collected as eluates at the bottom. About 40 ml of chloroform as another eluting solvent was added at the top to collect the second lipid fraction of neutral lipids. The process is continued with 40 ml of acetone to collect glycolipids, MAG, DAG, followed by 40 ml of methanol to collect the last lipid fraction of polar lipids, phospholipids. The four lipid fractions collected were then subjected to rotary evaporation in a pre-weighed fluorescence flask to get the total lipid fractions.

2.5 Fatty Acyl-Methyl Esterification

About 25-200 mg of four different lipid fractions were added with 2 ml of 5% KOH dissolved in methanol and incubated at 80ºC for twenty minutes. After allowing the sample to cool at room temperature, 2 ml of 20% Borontriflouride-Methanol Complex was then added and incubated at 80ºC for another twenty minutes. The sample was again cooled at room temperature and diluted with 10 ml of distilled water. After which, 10 ml of petroleum ether was added and agitated using a vortex mixer. The upper yellowish layer (FAME – fatty acyl methyl ester – in petroleum ether) was pipetted out, concentrated and added with 1ml acetone for gas chromatography analysis.

2.6 Gas Chromatographic Analysis of FAME

Gas chromatography using a GC-17A (Shimadzu, Kyoto, Japan) was done at the laboratory of Department of Chemistry, MSU-IIT, equipped with a flame ionization detector, and fitted with a 30 m (long) x 0.32 mm (internal diameter) x 0.20 m (film thickness) SPB PUFA column (Supelco, PA, USA) was used to analyze FAME. Nitrogen was used as carrier gas (linear velocity: 25 cm/sec at 185^0C). Injector and detector temperatures were at 250^0C and 260^0C, respectively. The column temperature was programmed to rise from 185^0C to a final temperature of 210^0C at a rate of 3^0C per minute. FAMEs were identified by comparing the relative retention times of the fatty acid samples with that of a known reference standard. Identification of fatty acids was based on the relative retention time (RRT) of peaks and percent content of fatty acids was calculated through area normalization method with respect to heneicosanoic acid (C21) as the internal standard.

3. Results and Discussion

3.1 Heterotrophic Growth

Thraustochytrid can colonize diverse mangrove leaf species once fallen into water (Leaño, 2001) and are able to breakdown several complex organic substrates such as cellulose cell walls (Bremer and Talbot, 1995). The presence of abundant n-3 fatty acids (Findlay *et al.*, 1986, Fan *et al*., 2000) suggest the importance of this protist as food sources from marine

organisms, such as crabs, shrimps and fish that live in mangroves (Fan, 2000). Hence, the role of thraustochytrids in nutrient recycling in mangroves is significant.

Isolate from Polo, Dapitan City of Western Mindanao was presumptively identified as *Schizochytrium* sp. Based on morphological characteristics, the isolate can be classified under genus *Schizochytrium,* which are differentiated to have biflagellate zoospore and the mature cells divide by repeated binary division to form diads, tetrads, octads and clusters (Kamlangdee and Fan, 2003; Honda *et al.*, 1998).

The isolate were classified based on its growth pattern on solid and liquid media. After twenty-four hours from plating of freshly collected fallen mangrove leaf on YPG agar medium, the isolate which have raised colony elevation, with distinct punctiform splatters forming a halo of the growing off-white colony, could be renowned visually with notable colony size – in a range of 1-3 mm diameter. After 48 hrs of incubation, the colonies increased in diameter size ranging from 3-7 mm, of which the halo on the entire margin became more strongly visible. However, subsequent subcultures of the isolates had lost the distinctive halo on the colony margin.

Instead of culture shakers, the study utilized a cell culture roller apparatus, nevertheless, rolling had facilitated oxygen uptake for thraustochytrids. Due to isolated cells, the thraustochytrid strain appeared as dissolved off-white particulates in the rolled bottles in YPG media, with increasing turbidity as time progresses. Some cells clump together forming distinct foamy clusters despite of mechanical disturbance brought by the roller apparatus. The turbidity was an indication that cells are growing and some are clumping together.

In a yeast extract-peptone-glucose medium, *Schizochytrium sp.* POL01 obtained oven-dried biomass of 4.8 g/L, respectively; lower biomass yield compared to those thraustochytrid strains isolated from temperate and subtropical regions of Kamlangdee and Fan (2003). Their results show that subtropical strains have higher biomass than the temperate strains. However, the above result indicated that some thraustochytrid strains from tropical habitats have also lower biomass yield.

3.2 PUFA Profiles of Lipid Components in DHA-Producing Isolate

Based on area normalization of peaks analyzed through gas chromatography,

it was noted that pentadecaenoic acid (C15:0) and palmitic acid in saturated fatty acids (16:0) and docosahexanoic acid (C22:6 ω-3) in polyunsaturated fatty acid were the dominant fatty acids present in all the lipid fractions of *Schizochytrium* sp. POL01 isolate. Fractions two (neutral lipid) and three (glycolipids, MAG, DAG) had the highest DHA yield with a percent proportion to total fatty acid (TFA) of 47.6% and 35.4%, respectively (Table 1 and Figure 2). DHA occurs naturally in the form of triacylglycerols or neutral lipids (Horrocks and Yeo, 1999). A triglyceride, also called neutral fats is a chemical compound formed from one molecule of glycerol and three fatty acids. Hence, neutral lipids can accommodate more fatty acids like DHA compared to glycolipids, mono- and diglycerides. Followed by the fourth lipid fraction revealing polar lipids and phospholipids having 25.1% DHA yield, which is still higher, compared to DHA content in DHA-rich fish animals (Li and Ward, 1993; Ackman *et al.*, 1988). Fraction one of n-alkanes, n-alkenes and free fatty acids had the least amount of DHA yield with a value of 3.3%. A diglyceride, has two fatty acid molecules and exists in the 1,2 form and the 1,3 form depending on how the fatty acids are attached to the glycerol molecule. A monoglyceride, or monoacylglycerol (MAG), has only one fatty acid radical per molecule of glycerol. Hence, the above results indicate that tryglycerides can obtain more fatty acids like DHA than the other lipid components of the other fractions.

Docosahexaenoic acid, an essential omega-3 long-chain polyunsaturated fatty acid (n-3 LC-PUFA), is concentrated in membrane phospholipids at synapses, other neural cellular membranes, and retinal photoreceptors (Bazan N. G, 2006). Results in Table 1, for polar lipids and phospholipids having 25.1% DHA yield indicate that phospholipids, in particular that containing docosahexaenoic acid (DHA) is significantly higher and important.

All four lipid fractions were also noted having trace amounts of linoleic acid (LA, 18:2(n-6)) ranging from 0.5-2.6% (Table 1). Linoleic acid is essential dietary fatty acid found in grains and seeds. It is also a precursor for a number of long-chain PUFAs. These two fatty acids—alpha-linolenic acid (ALA, an omega-3) and linoleic acid (an omega-6)—are the two essential fatty acids that the human body needs and cannot manufacture. When sufficient ALA is supplied in the diet, the body can make enough DHA and EPA for the eicosanoids that form our metabolic "thermostat" system—or the body can use DHA and EPA directly from diet as well.

Table 1. Fatty Acid Composition of the four lipid fractions of *Schizochytrium sp.* POL01 strain analyzed using gas chromatography

Fraction	Fatty Acid Composition (%)											
	C14:0	C15:0	C16:0	C17:0	C18:0	C18:2n 6	C20:4 ω-3	C20:4 ω-6	C20:5 ω-3	C22:4 ω-6	C22:6 ω-3	Others
						LA		AA	EPA	DTA	DHA	
1	8.5171	4.0873	26.9824	-	-	1.2741	-	-	-	-	3.3064	55.8327
2	1.2305	5.2925	14.5519	0.8906	0.7758	0.6853	-	-	-	9.3013	47.6126	19.6595
3	1.7697	7.6576	22.0316	2.8714	0.1251	0.5419	-	-	-	6.0991	35.4379	23.4657
4	5.3116	2.7912	28.7337	0.5186	-	2.5890	-	-	-	3.0179	25.0930	31.9450

Legend: 1 – n-alkanes, n-alkenes, free fatty acids; 2-neutral lipids; 3-glycolipids, monoglycerides, diglycerides; 4-polar lipids, phospholipids; 13:0 – 14:0, myristic acid, tetradecaenoic acid; 15:0, pentadecyclic acid, pentadecaenoic acid; 16:0, palmitic acid, hexadecaenoic acid; 18:0, stearic acid, octadecaenoic acid; 18:1, oleic acid, cis - 9 – octadecenoic acid; 18:2(n-6), linoleic acid, cis - 9,12 - octadecadienoic acid; 18:3(n-3), α- linolenic acid (LA), 9,12,15 - octadecatrienoic acid; 20:4(n-6), arachidonic acid (AA), cis - 5,8,11,14 - eicosatetraenoic acid; 20:5(n-3), EPA cis -5,8,11,14,17 – eicosapentaenoic acid; DTA 22:4, cis -7,10,13,16 - docosatetraenoic acid; 22:6(n-3) DHA cis - 4,7,10,13,16,19 - docosahexaenoic acid.

*Dashes indicate negative values.

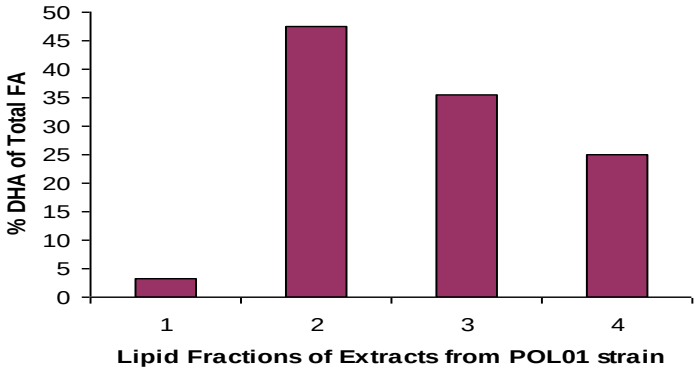

Figure 2. Histogram representation of DHA levels of four lipid fractions from *Schizochytrium* sp POL01 strain

Hence, above results indicate that all fractions of *Schizochytrium* sp. have trace amounts of linoleic acid making thraustochytrid a significant source of the essential fatty acid.

All lipid fractions had no EPA (Table 1) content compared to PUFA-rich fish with EPA values ranging from 9.6 to 14.6% (Seto *et al*., 1984; Li and Ward, 1993; Ackman *et al*., 1988). However, the high levels of EPA in fish oil may act as an inhibitor of the infant's own endogenous ARA biosynthesis. Therefore, fish oil supplementation of infant formula may require an ARA co-supplementation to overcome the detrimental effect of EPA (Kinsella, 1990). Hence, oils from thraustochytrids are still better than PUFA-rich fish oils.

According to Brenna, J T (2007), DHA concentration in breast milk worldwide is lower than and more variable than that of AA. The highest DHA concentrations were primarily in coastal populations and were associated with marine food consumption. Hence, the high DHA concentration of *Schizochytrium* sp. POL01 is far greater than the DHA concentration in milk.

Also, tetradecaenoic (C14:0), pentadecaenoic (C15:0), and hexadecaenoic (C16:0) acids were never absent in the fatty acid of all the fractions and were high ranging from, 1.2-8.5%, 2.8-7.7%, and 14.5-29.7%, respectively, with hexadecaenoic acid as the predominant unsaturated fatty acid.

Heptadecaenoic (C17:0) acid was also found in three out of four lipid fractions of the fatty acid profiles. Fraction two and three also produced significant quantities of docosatetraenoic acid (C22:4n6) having the amount of 9.3% and 6.1%, respectively (Table 1).

In terms of the total lipid fractions extracted by column chromatography, fraction two of neutral lipids revealed the highest average value of 400 mg/L which is 46.5% of the total lipid extracted, followed by fraction three having 233.2 mg/L of glycolipids, monoglycerides and diglycerides which is 27.2% of the total extracted lipid, respectively. Fraction four has 160 mg/L of phospholipids and polar lipids, 19.1% and fraction one of n-alkenes, n-alkanes and free acids having the least value of 66.7 mg/L, 7.2% of the amount of total lipid extracted (Table 2). Each fraction yielded significant amount of DHA content per liter of culture medium, which is expressed in mg/g of dried cells (Figure 3). The DHA yield of neutral lipids gained the highest content having 16.1 mg/g dried cell, followed by 7.5 mg/g dried cell of glycolipids, monoclycerides and diglycerides, respectively. Fraction four and one gained lesser DHA yield of 1.8 mg/g and 1.1 mg/g since n-alkenes, n-alkanes, phospholipids and polar lipids has lesser amount of fatty acids compared to neutral lipids and glycolipids, MAG and DAG indicated by the results summarized in Table 2.

Table 2. Biomass production, extracted lipid, esterified lipid %DHA and DHA yield of *Schizochytrium* sp. POL01 strain after culturing 72 hrs

Biomass (g/L)	Extracted Lipid (mg/L)	Fraction	Lipid Fractions (mg/L)	Lipid Fractions (%)	Esterified Lipid (mg/L)	DHA (%)	DHA Yield (mg/g dried cell)
4.8	900	1	66.7	7.2	16.6	3.3	1.1
		2	400	46.5	161.6	47.6	16.1
		3	233.3	27.2	83.7	35.4	7.5
		4	160	19.1	44.2	24.9	1.8

The isolated strain used in this research, *Schizochytrium* sp.POL01 in Polo, Dapitan City of Western Mindanao , showed 4.8 g/L of oven-dried biomass, 860 mg/L of total lipid extracted, 306.1 mg/L of esterified lipid and 26.5 mg/g of dried cell in the actual DHA Yield (Table 3). DHA yield per liter of

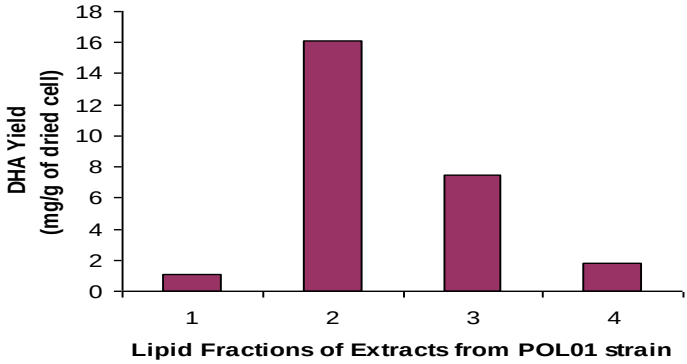

Figure 3. Histogram representation of DHA Yield of four lipid fractions of extracts from *Schizochytrium* sp.POL01

culture medium is a crucial consideration in targeting strains potential for mass production, intended for enrichment studies in aquaculture and nutrition studies in humans.

Table 3. Biomass, average extracted lipid, average esterified lipid and actual DHA yield of oven-dried *Schizochytrium* sp. POL01 strain after culturing 72hrs

Isolate (Strain)	Biomass (g/L)	Ave. Extracted Lipid (mg/L)	Ave. Esterified Lipid (mg/L)	Actual DHA Yield (mg/g dried cells)
Schizochytrium sp. POL01	4.8	860	306.1	26.5

The presence of abundant n-3 fatty acids (Findlay *et al.,* 1986, Fan *et al*., 2000) put forward the value of this protist as food sources from marine organisms, such as crabs, shrimps and fish that live in mangroves (Fan, 2002). Further, the role of thraustochytrids in nutrient recycling in mangroves has been recommended by studies: thraustochytrids can secrete degrading enzymes such as cellulase and amylase (Raghukumar *et al.,* 1994, Bremer and Talbot, 1995) and consume a wide range of carbon and nitrogen sources (Bahnweg, 1979).

In general, Philippine thraustochytrid was found to be better alternative

sources of DHA than of fish when seen in lipid components of n-alkenes, n-alkanes, free fatty acids, neutral lipids, glycolipids, MAG, DAG, phospholipids, and polar lipids. A high proportion of DHA in the total lipids fractions of thraustovhytrids and relatively lower levels or absence of structurally related PUFA's would simplify downstream processing of DHA (Singh and Ward, 1996).

4. Conclusion and Recommendation

Though fish oil have been the traditional source of DHA, large scale production of DHA were recommended due to the low proportion of DHA in fish oil and the difficulties in extraction and purification of omega-3 fatty acids. Thus, this study was conducted to search for microorganisms rich in DHA, such as thraustochytrids, as alternative commercial sources. Fatty acid compositions and distribution of lipid components of *Schizochytrium* sp. POL01 were harvested, was studied, with special emphasis on the distribution of docosahexaenoic acid (C22:6 *n*-3, DHA). The isolate obtained 4.8 g/L of oven-dried biomass in 100-mL medium for 72 hrs of incubation. Lipid components were collected into fractions and fraction two and three were among the highest in percent proportion and content in DHA level according to gas chromatographic analysis. Fraction two, composed of neutral lipids, was the highest in DHA level and accounted for 47.6% of the total fatty acids which is 16.1 mg/g of dried cell. Neutral lipids were the major lipid constituents in which triacylglyerol (TAG) was the predominant component. Fraction three, composed of glycolipids, mono- and diglyceride, also indicate significant value of DHA, 35.4%, 7.5 mg/g dried cell, followed by fraction four of phospholipids and polar lipids, 25.1%, 1.8 mg/g dried cell as DHA yield, respectively. However, the DHA content of fraction one of n-alkanes, n-alkenes and free fatty acids had the least amount with a value of 3.3%, 1.1 mg/g dried cell. DHA was found to be distributed in all lipid fractions and was found to be the major polyunsaturated fatty acid. Moreover, *Schizochytrium* sp. POL01, looking on its lipid components as different fractions can become the new mark for biotechnological studies for industrial use; and research into further applications is continuing.

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