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Production of ω-3-Docosahexanoic Acid (DHA) by Using Oleaginous Marine Algae

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Abstract

In the present investigation, algal samples were collected along the coastal areas of Southern part of India (Thondi, Ramanad District) and screened for oleaginous nature. Of 47 isolates, 9 strains were found oleaginous with 2 isolates having the ability to produce docosahexanoic acid (DHA). These two strains were identified as *Schizochytrium* or *Crypthcodium* species. In the laboratory, media manipulation experiments were carried out in the fermentation of these marine microalgae by optimizing the nutrients conditions in shake flasks (SFS). It was found a strain labeled MRICC No-59 (Microcore Research Laboratories in house Culture Collection) were capable of yielding higher DHA content in presence of glucose (carbon source) and glycerol in a ratio of 100:25 in Erlenmeyer flask with working value of 30 ml with adequate dissolved oxygen (DO) at temperature of 27^0 C and 34^0 C along with 9gm/L nitrogen source and 35gm/L Na_2SO_4 as on alternate sodium source instead of Nacl. The results were tabulated and discussed.

Key words: Marine, docosahexanoic acid (DHA), *Schizochytrium, Crypthcodium*

INTRODUCTION

Algae are highly variable and very specialized group of organisms. They play a major role in many eco systems such as marine and freshwater environment, desert sands, hot springs, snow ice and so on. Thousands of eukaryotic algal species are grouped into 9 divisions with five large classes. The microalgae provide a variety of metabolic products like lipids, enzymes, pigments, food supplements, etc.(Lee.2001).Harun *et al.,* (2010) have pointed out that the microalgae can be cultivated on diverse mineral media, organic substrates and synthetic or real waste waters. The main aim of

growing the microalgae commercially is to harvest metabolic products, feed, food supplements, biofuels and so on.(Perez Garcia *et al.,* 2011).

Lipids are dynamic bioactive molecules which regulate a number of complex system in living cells. According to Beopoulos *et al.,* (2009). The microorganisms which accumulate substantial amount of lipids are called oleaginous stains. Ratledge (2004) has opined that this microbes require very short cultivation time, utilize various substrates and produce high level of intra cellular lipids mainly triacyl glycerol (TAG). So that they are the important alternatives to the traditional source of lipids. TAG is a non-polar water-insoluble tri-ester of glycerol with fatty acids. It plays an important role in storage and lipid homeostasis. So that the disruption of equilibrium between lipid synthesis and catabolism would lead to metabolic disorder animals, The oleaginous microorganisms produce TAG containing long-chain poly unsaturated fatty acids (LCPUFAs) like arachidonic acid. Leman (1997) has shown that these LCPUFAs act as precursors to a number of hormones especially prostaglandins, leukotriens and Thromboxanes in man and so they are important both nutritionally and pharmacologically.

Eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) are the two important long-chain poly unsaturated fatty acids produced via 3 path way. These are involved in human metabolism at early stages and world and are utilized for prevention and treatment of heart and inflammatory diseases as well as for the nutritional supplements in human. In man, DHA plays a major role in heart and brain function. At present the plant seed oils are the main commercial sources of poly unsaturated fatty acids (PUFAs). But the higher plants cannot synthesisze These fatty acids above C_{18} owing to a lack of required enzymes. As a result, marine fish oils are utilized as main source of commercial EPA and DHA.

However, the PUFA yields from agricultural and animal products are meager in quantity and vary with season climate and geographical location. Moreover, They have objectionable taste and odour, high cholesterol content and some toxic impurities. In line with the opinion of chi *et al.,* (2007), the fish oil which is the common source of EPA and DHA fails to meet the increasing the demand of these two fatty acids. Therefore alternative sources for large scale production of high quality EPA and DHA with reduced cost of production for commercially important. Wynn (1998) has found that DHA could be produced commercially using the marine algae. Some oleaginous microorganisms have been intensively studied in recent years as alternatives for the lipid production (Certik, *et al.,* 1999). Ruenwai *et al.,* (2010) have showed that the lipid production in oleaginous species would be enhanced by controlling cultivation or nutritional conditions. Since the cultivation of microalgae for the economically useful metabolites is the need of the hour, the present study has been carried out to collect, isolate and optimize condition and nutrients for increased lipid biosynthesis and DHA in oleaginous marine algae.

MATERIALS AND METHODS

The algal samples were collected from different regions in the coastal areas of Southern part of India located at Thondi (Ramnadh District) as depicted in table-1.

The samples were brought to the laboratory in sterile poly ethylene bottles kept in Ice cold thermocoal containers. In the laboratory, each sample was shaken well and filtered through a filter unit. The filtrate was used for axenic culture purification techniques (SF or shake flask mode) employing serial dilution and pour plate methods, (Table-2)

Selective isolation of oleaginous algae was obtained through the antibiotic selection and the oleaginous activity in the algae was detected by the addition of tetrazolium chloride. Out of 417 short listed isolates, 9 isolates were selected to be oleaginous marine algae. Among them, two of the isolates were found to be the major producers of DHA fatty acids. In the next step, process development activities were carried out by optimizing the conditions and nutrients for increased biosynthesis of DHA. Both qualitative and quantitative analyses were done in the isolates by employing thin layer chromatography and silica gel column chromatography techniques and the results were tabulated and discussed.

RESULTS AND DISCUSSION

In the present investigation about 417 marine algal isolates were obtained via the systematic isolation procedures. Among them, 9 were found to be oleaginous including two DHA producers. Based on the morphological characteristic, the DHA producing oleaginous marine algae was *Schizochytrium* species.Production of very long-chain poly unsaturated fatty acids has been evidenced in many marine microalgae (Tonon *et al.,* 2002; Leblont *et al.,* 2005). De swaff *et al.,* (1999) have found that the heterotropic marine alga *crypthecodium cohini* is the source of docosahexaenoic acid (DHA). Certain thraustochytrids are currently shown to be potential sources of very long-chain PUFAs (Lewis *et al.,* 1999) and substantial amount of DHA (Jiang *et al*., 2004). An improved protection of DHA has been demonstrated by Iida *et al.,* (1996) in a culture of *Thraustochytrium aurem* through media optimization process. In the present algal strain studies, the media manipulation by optimizing the nutrients and conditions have resulted in a significant improved yield of DHA. The lipid analytical methods are carried out as mentioned in Morris Kates, Elsevier, Techniques of Lipidology isolation, analysis, and identification of lipids 2nd rev.ed, Elsevier Science Pub. Co. in Amsterdam, New York 1986.

In the present study 100gm/l glucose (carbon source) resulted in a maximum yield of 165.0 mg/l DHA) Table-3. It is noticed that there was a gradual enhancement of lipid production as the glucose concentration is increased. The highest level of DHA in increased glucose might be due to the maximum level of glucose utilization by the algae. Glycerol also serves as precursor for the fatty acid biosynthesis. So that the optimization of glucose in combination with glycerol was also carried out. It was noticed that the ratio of concentration of glucose and glycerol namely 100:25 worked well and resulted in maximum output of DHA (181 mg/l) (Table-4).

MRLICC No 59 species of the present study exhibited a gradual increase in lipid production as the concentration of the nitrogen source (soya flour) was increased. A concentration of 9gm/L soya flour had resulted in a maximum of 205 mg/l DHA(Table-5). There was an excellent increase in DHA production (225 mg/L) at the

phase temperature of 27^0 C and at later temperature of about 34^0 C (Table-6). The present work clearly shows that 10 and 15 ml sample in 250 ml shake flask were suitable working volume to obtain the highest yield. (320.0 and 345.5 mg/l) of DHA. The available dissolved oxygen (DO) is the main parameter in the fermentation process. So that the baffled flasks containing gradient quantity of volume was used in the present study. It was noticed that an increasing the working volume of the sample resulted in poor aeration with decreased lipid yield as in Table-7. A highest yield of DHA in 15 ml working volume of the sample could be due to the presence of required available oxygen in the fermenter. In the presence of investigation, an alternative sodium source (sodium sulphate) instead of Nacl was also tried in optimizing process sulphate is normally used to reduce the cell aggregation in fermentation process But it is also seen that sulphate worked well to increase the lipid yield in the present study. That is a high yield of DHA(440 mg/l) was produced by the algae at 35mg/l $NaSo_4$ administration to the culture (Table-8).

From the present study it is clear that the media manipulation techniques through optimizing the conditions and nutrients during the fermentation caused by the marine algal strain *Schyzochytrium* species. have resulted in improved yield of DHA significantly. Similar reports have also been given by Yaguchi *et al.,* (1997) in *Schyzochytrium* species and by De Swaff *et al.,* (1995., 2003) in *Crypthecodium* species. As Schizochytrids accumulate large amount of DHA they could also be used as model organisms to study the mechanisms of DHA synthesis.

Table-1 Samples collected at various regions of the study area

Nature of sample	Quantity (gm)	pH	Temperature (^{0}C)	Salinity (%)
Water	10	7.3	28	36
Mangroove	10	7.4	26	31
Leaf	10	7.8	24	34
Grass	10	7.5	28	36
Sea weeds	10	7.4	25	38
Soil	10	7.6	24	27
Sewage mix	10	7.8	24	27
Shallow water	10	7.3	25	28
Marine tide pool	10	7.2	26	32
Estuaries	10	7.3	27	29
Distillery ETP mix	10	7.7	25	31
Sea wood	10	7.2	25	38
Marine plant	10	7.1	26	39

Table-2 MRLICC No 59 Carbohydrate sources (gm/lit) in carbon optimization process in shake flasks

S.No	Raw Materials	Shake flask numbers							
		1	2	3	4	5	6	7	8
1	Glucose	100	-	-	-	-	-	-	-
2	Sucrose	-	100	-	-	-	-	-	-
3	lactose	-	-	100	-	-	-	-	-
4	Maltose	-	-	-	100	-	-	-	-
5	Fructose	-	-	-	-	100	-	-	-
6	Maltodextrin	-	-	-	-	-	100	-	-
7	Glycerol	-	-	-	-	-	-	100	--
8	Starch	-	-	-	-	-	-	-	100
9	Peptone(soya)	3	3	3	3	3	3	3	3
10	Yeast extract	2	2	2	2	2	2	2	2
11	KH_2PO_4	0.05	0.05	0.05	0.05	0.05	0.05	0.05	0.05
12	Soyaflour	6	6	6	6	6	6	6	6
13	Crude salt	34	34	34	34	34	34	34	34
14	KPO_4	3	3	3	3	3	3	3	3
15	$(NH_4)2SO_4$	2	2	2	2	2	2	2	2
16	CSL	0.7	0.7	0.7	0.7	0.7	0.7	0.7	0.7
17	PUP	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5
18	Sodium acetate	1	1	1	1	1	1	1	1

Table-3 Yield of lipid and DHA from MRLICC No 59 at the glucose optimization

S.No	Quality of glucose (gm/l)	Cell mass (gm/kgm)	Total lipid (mg)	Neutral lipid (mg)	Phospho lipid (mg)	Total DHA (mg)	Neutral lipid %	Phospho lipid %	DHA (mg/l)
1	40	1.7	75	68	9	1.1	90	12	52.5
2	60	2.1	125	114	13	1.8	91	1.0	87.5
3	80	3.2	175	161	12	2.5	92	7	123.0
4	100	3.5	236	219	10	3.3	93	7	165.0
5	125	3.0	214	193	15	3.0	90	1	150.0

Table-4 Yield of lipid and DHA from MRLICC No 59 at the glycerol optimization

S.No	Glucose: Glycerol (Ratio)		Cell mass (gm/kgm)	Total lipid (mg)	Neutral lipid (mg)	Phospho lipid (mg)	Total DHA (mg)	Neutral lipid %	Phospho lipid %	DHA (mg/l)
1	100	25	4.1	258	235	4	3.6	91	1.55	181
2	75	25	3.2	200	180	9	2.8	90	4.50	140
3	80	20	3.4	210	191	13	2.9	91	6.19	147
4	50	50	3.4	211	191	12	3.0	91	5.69	148
5	25	75	3.2	226	219	16	3.2	97	7.08	158

Table-5 Yield of lipid and DHA from the MRLICC No 59 at the nitrogen optimization

S. No	Glucose :Glycerol (Ratio)		Soya flour	Cell Mass (gm/ kgm)	Total Lipid (mg)	Neutral Lipid (mg)	Phospho Lipid (mg)	Total DHA (mg)	Neutral Lipid %	Phospho Lipid %	DHA (mg/l)
1	100	25	3	4.2	176	160	13	1.8	90	8	90
2	100	25	5	4.3	202	184	12	2.2	89	10	110
3	100	25	6	4.2	201	183	16	3.0	91	9	150
4	100	25	9	4.6	269	245	19	4.1	91	7	205
5	100	25	13	4.1	236	215	19	2.6	93	7	130

Table-6 Yield of lipid and DHA from the MRLICC No 59 at the temperature optimization

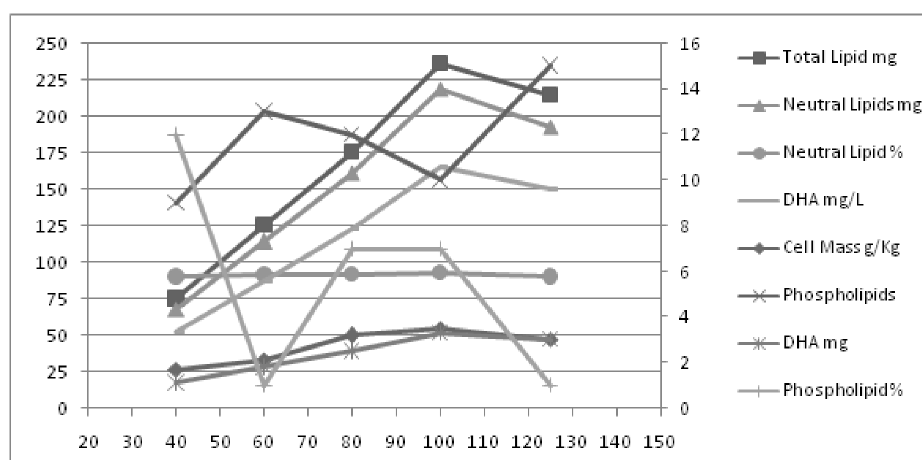
S. No	Glucose: Glycerol (Ratio)		Temperature (^{0}C)	Cell mass (gm/ kgm)	Total lipid (mg)	Neutral lipid (mg)	Phospho Lipid (mg)	Total DHA (mg)	Neutral lipid %	Phospho lipid %	DHA (mg/ml)
1	100	25	24	34	222	181	34	3.1	87	8.89	155
2	100	25	26	4.4	258	68	9	3.2	90	12.0	160
3	100	25	28	4.2	252	114	13	3.6	91	10.4	180
4	100	25	30	4.5	196	161	12	2.3	92	6.86	113
5	100	25	27	6.5	296	219	16	4.5	93	6.78	225

Table-7 Yield of lipid and DHA from the MRLICC No 59 at the volume of the sample and oxygen optimization

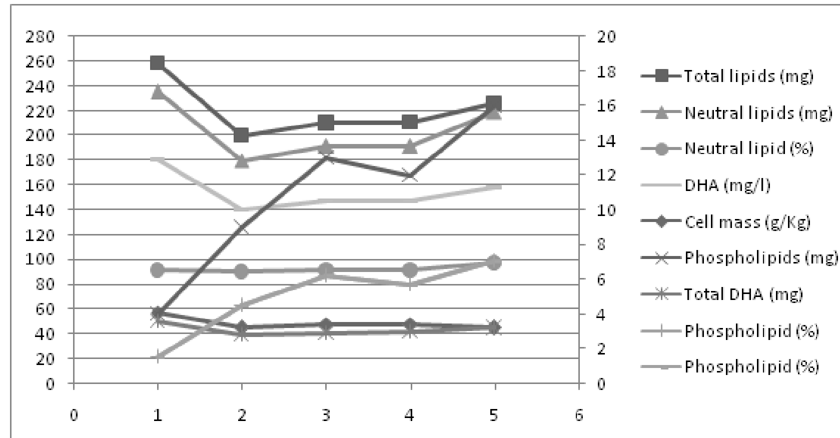
S. No	Glucoe: Glycerol (Ratio)		Volume of sample (ml)	Cell mass (gm/ kgm)	Total lipid (mg)	Neutral lipid (mg)	Phospho lipid (mg)	Total DHA (mg)	Neutral lipid %	Phospho lipid %	DHA (mg/l)
1	100	25	10	3.1	341	310	31	6.4	91	9	320.0
2	100	25	15	3.7	386	351	31	6.9	92	8	345.3
3	100	25	20	4.7	321	292	29	5.1	91	9	255.0
4	100	25	25	4.2	289	263	26	4.5	93	7	255.0
5	100	25	30	3.6	254	231	23	3.9	91	9	195.0

Table-8 Yield of lipid DHA from the MRLICC No 59 at the Na_2So_4 Optimization (replacement of Nacl)

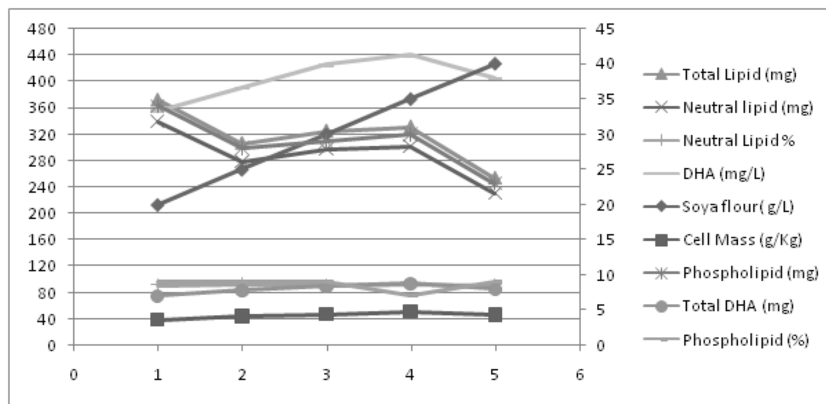
S. No	Glucose :Glycerl (Ratio)		Na_2So_4 (mg)	Cell mass (gm/ kgm)	Total lipid (mg)	Neutral lipid (mg)	Phospho lipid (mg)	Total DHA (mg)	Neutral lipid %	Phopho lipid %	DHA (mg/l)
1	100	25	20	3.6	373	339	34	7.1	91	9	355
2	100	25	25	4.1	307	279	28	7.8	92	9	390
3	100	25	30	4.4	326	297	29	8.5	91	9	425
4	100	25	35	4.8	332	302	30	8.8	93	7	440
5	100	25	40	4.3	254	231	23	8.1	91	9	405



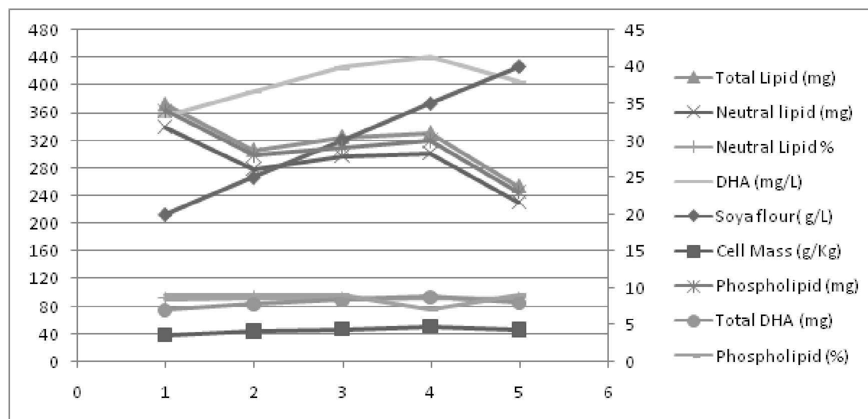
Graph-1 Yield of lipid and DHA from MRLICC No 59 at the glucose optimization



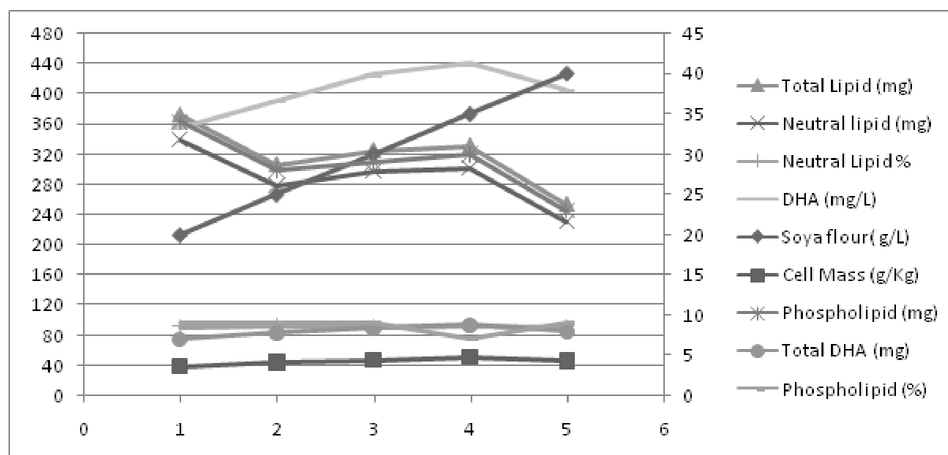
Graph-2 Yield of lipid and DHA from MRLICC No 59 at the glycerol optimization



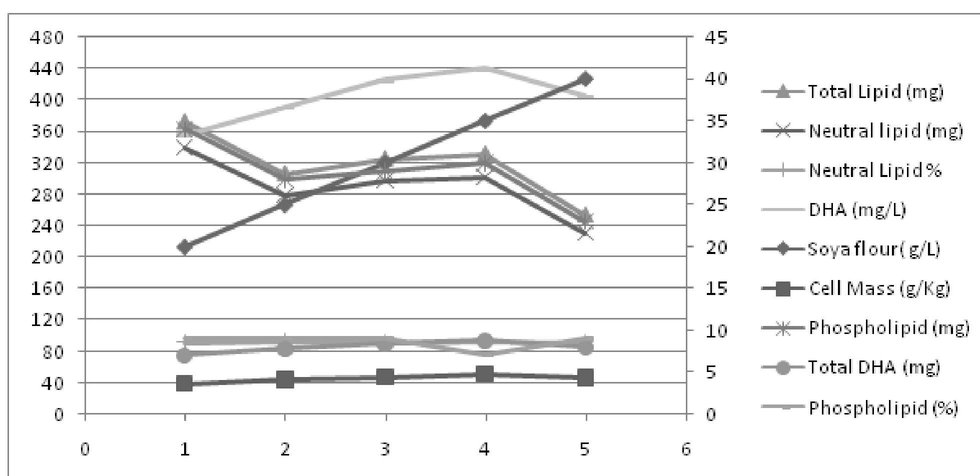
Graph-3 Yield of lipid and DHA from the MRLICC No 59 at the nitrogen optimization



Graph-4 Yield of lipid and DHA from the MRLICC No 59 at the temperature optimization



Graph-5 Yield of lipid and DHA from the MRLICC No 59 at the volume of the sample and oxygen optimization



Graph-6 Yield of lipid DHA from the MRLICC No 59 at the Na_2So_4 Optimization (replacement of Nacl)

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