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Soil microorganisms produce omega-3 fatty acids

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Abstract

Studies on the application of functional lipids such as Polyunsaturated Fatty Acids (PUFAs) have proceeded in various fields in search of novel and rich sources for health and dietary requirements. Natural limitations favour a novel approach for the production of omega-3 fatty acids. A series of PUFAs including Eicosapentanoic acid (EPA) and Docosahexanoic acid (DHA) have widespread nutritional and pharmaceutical values. This study investigated the potential production of these two economically important fatty acids from fungi. The microorganisms used were *Trichoderma sp and Aspergillus niger*, isolated from soil. The use of *Trichoderma* sp. is preferred since it produced considerable amounts of EPA and DHA. This paper presents the results on the ratios of EPA and DHA produced by these microorganisms and recovery aspects.

Keywords: PUFA, Omega-3, *Trichoderma* sp., *Aspergillus* sp.

Introduction

Omega-3 fatty acids are considered as essential nutrients since human body cannot synthesize them, but have to be provided through food. Omega-3 fatty acids can be found in fish, such as, salmon, tuna and halibut, other seafoods including algae and krill, some plants and nut oils. Also known as Polyunsaturated Fatty Acids (PUFAs), these omega-3 fatty acids play a crucial role in brain function as well as normal growth and development.

Omega-3, the highly unsaturated fatty acids are of significant commercial interest in that they have been recently recognized as important dietary compounds for preventing arteriosclerosis and coronary heart disease, for alleviating inflammatory conditions and for retarding the growth of tumor cells. It is important to have a balance of omega-3 and omega-6 (another essential fatty acid) in the diet. Currently, the commercially available dietary source of Omega-3 PUFAs is from certain fish oils which contain up to 20-30% of these fatty acids. Consequently, large quantities of fish oil are processed and encapsulated for sale as dietary supplements. However, there are several significant problems with these fish oil supplements, including bioaccumulation of fat-soluble vitamins and high levels of saturated and omega-6 fatty acids, both of which have deleterious health effects (Bajpai & Bajpai, 1993). A report from Packaged Facts (a division of MarketResearch.com) and the US Food and Beverage Market, predicts that foods with added omega-3 will reach $ 7 b in sales by 2011. This signifies that the demand for these fatty acids is increasing and thus it becomes necessary to study and search for novel and rich sources containing omega-3 fatty acids.

Alternative sources of omega-3 fatty acids are the microflora. The microflora have the advantages of being heterotrophic and capable of producing high levels of omega-3. There still exists a need, however, for improved methods for fermentation of these microflora and identification of improved uses of the microflora products. It has been reported that marine protists and dinoflagellates, such as, species of *Thraustochytrium, Schizochytrium and Crypthecodinium* are the rich sources of docosahexaenoic acid (DHA), whereas microalgae like *Phaeodactylum and Monodus* are good sources of Eicosapentanoic acid (EPA). Species of lower fungi are also able to accumulate a high percentage of EPA in the lipid fraction (Ward & Singh, 2005). Perusal of literature indicates the production of PUFAs from marine fungi. Several filamentous fungi belonging to the genus *Mortierella* were found to produce large amounts of EPA in their mycelia when grown at low temperature (Sakayu *et al*., 1988).

The oleaginous yeast *Yarrowia lipolytica* is known to inhabit various lipid-containing environments. *Y. lipolytica* is used as a model organism to study the mechanisms involved in lipid metabolism associated to fat uptake, storage, deposition, mobilization and regulation (Anthanasios *et al*., 2009). Omega-3 fatty acids, especially DHA, have profound health effects on humans and animals. DHA serves as a health supplement for humans and is also incorporated in aquaculture feeds in the form of fish oil to maintain vitality and enhance the survival rate of aquaculture species. A marine protist, *Schizochytrium mangrovei*, has been isolated which can produce a very high yield of DHA (Chen *et al*., 2007).

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Materials and methods

Fungal cultures isolated from soil were used in the present study. Soil samples were collected from different places in and around our laboratory and serially diluted in sterile saline solution. A 100 µl aliquot of the final dilution was spread over potato dextrose agar (PDA) plates. The plates were incubated at room temperature and at the end of 8 days, the isolates obtained were subcultured and purified on PDA plates and identified by following standard protocol.

Fish oil (omega-3) preparations were obtained from capsules available commercially from local pharmacies. Methylene chloride, hexane and methanol were used for extraction of the samples. HCl, 1.25 M in methanol, sodium hydroxide and sodium chloride (AR grade) were used for sample esterification, Hexane was used for dissolving and diluting the samples before GC analysis.

Fungal mycelium was crushed in a mortar and pestle and 20 g of that was subjected to saponification, using 2 g of NaOH and 10 mL ethanol. After saponification, unsaponified materials were removed using 25 mL of methylene chloride using a separating funnel. Addition of 50 mL acetic acid liberated the free fatty acids from their salt forms. To remove the saturated fatty acids, 50 mL acetone was added. Finally to obtain a concentrated form of only omega-3 fatty acids, a base and alcohol mixture was used (David, 2005; Kang & Wang, 2005).

The fatty acids thus extracted and the soft gelatin capsules were weighed and 1.25 M HCl in Methanol and hexane were added and heated at 80 C for 1 h. It was then cooled to room temperature and water was added. After a short centrifugation, hexane layer containing the derivatized fatty acids was separated (Antolin et al, 2007). Thus obtained methyl esters of the fatty acid fractions were subjected to GC analysis (Mohammed & Klein, 2007).

The chromatographic column used was a fused silica (Rtx1 fused silica) capillary column (30 m x 0.25 mm ID). Nitrogen was used as the carrier gas at a flow rate of 1.84 mL/min. The column temperature was 180° C and the detector temperature was 250° C. The injection was performed in split mode 50:0.

Results and discussion

Fatty acids are abundant in most organisms. Each class or group of organisms is known to have distinctive fatty acid profile which enables us to use them to produce biologically important fatty acids as well as to use them as biomarkers of the typical class of organisms *(Moss et al, 1993).* It has been reported earlier that several filamentous fungi are able to produce large quantities of EPA and DHA fatty acids (Sakayu *et al*., 1988).

In the present study, ten fungal cultures were isolated from soil samples and initially screened for the production PUFA and two were shortlisted for further studies. The growth conditions were kept uniform to minimize the variation in fatty acid composition. Both the fungi was grown in potato dextrose broth initially and later transferred to a medium containing oleic acid. Oleic acid is considered as a possible precursor for the synthesis of highly unsaturated fatty acids. However, in the present study the concentration of EPA and DHA was lower in oleic acid containing medium than that was grown in potato dextrose broth. According to Yongmanitchai and Ward (1991), even though oleic acid acts as a precursor for EPA and DHA, higher concentrations of the same inhibited cell growth and synthesis of EPA. Detailed studies are now undertaken to optimize the dosage levels of oleic acid.

Of the two cultures, *Trichoderma* sp. produced 7.47 mg/g of DHA and 0.298 mg/g of EPA (Fig. 2), when compared to the standards (derived from fish oil capsules) (Fig.1). Interestingly, both oleic acid containing medium and potato dextrose broth inoculated with *Trichoderma* sp revealed higher concentrations of DHA than EPA (Fig.2 & 3). According to Sakayu *et al*. (1988), fungi belonging to genus *Mortierella* were able to produce up to 27 mg/g of EPA and many of these are good producers of fatty acids. The major fatty acid produced by most of the fungi is the 18 C α-linolenic acid. According to Devi *et al*. (2006), *Penicillium* sp was able to produce high concentrations of this linolenic acid.

The low levels of EPA in our study might be due to the inactivation of EPA synthesizing enzymes at room temperatures. Certain fungus like, *Pythium irregular,* was able to produce EPA even at room temperatures (Cheng *et al*., 1999). This study has clearly shown that substantial amounts of DHA as compared to EPA were produced by *Trichoderma* sp. and to a lesser extent *Aspergillus* sp. (Fig. 4). Of the two, *Trichoderma* sp. was a better producer of DHA. Earlier reports on fungal fatty acids showed the presence of very long chain fatty acids of 22, 23, 24, 26 and 28 carbons (Patricia *et al*., 2000).

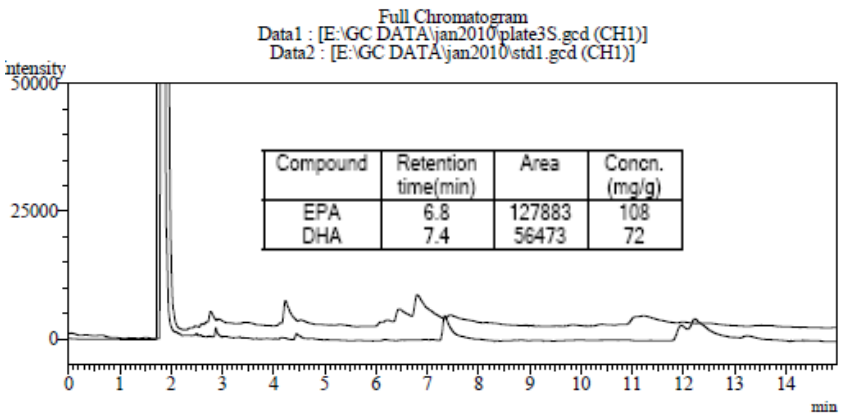

Fig.1.GC analysis of EPA & DHA standards from fish oil capsule

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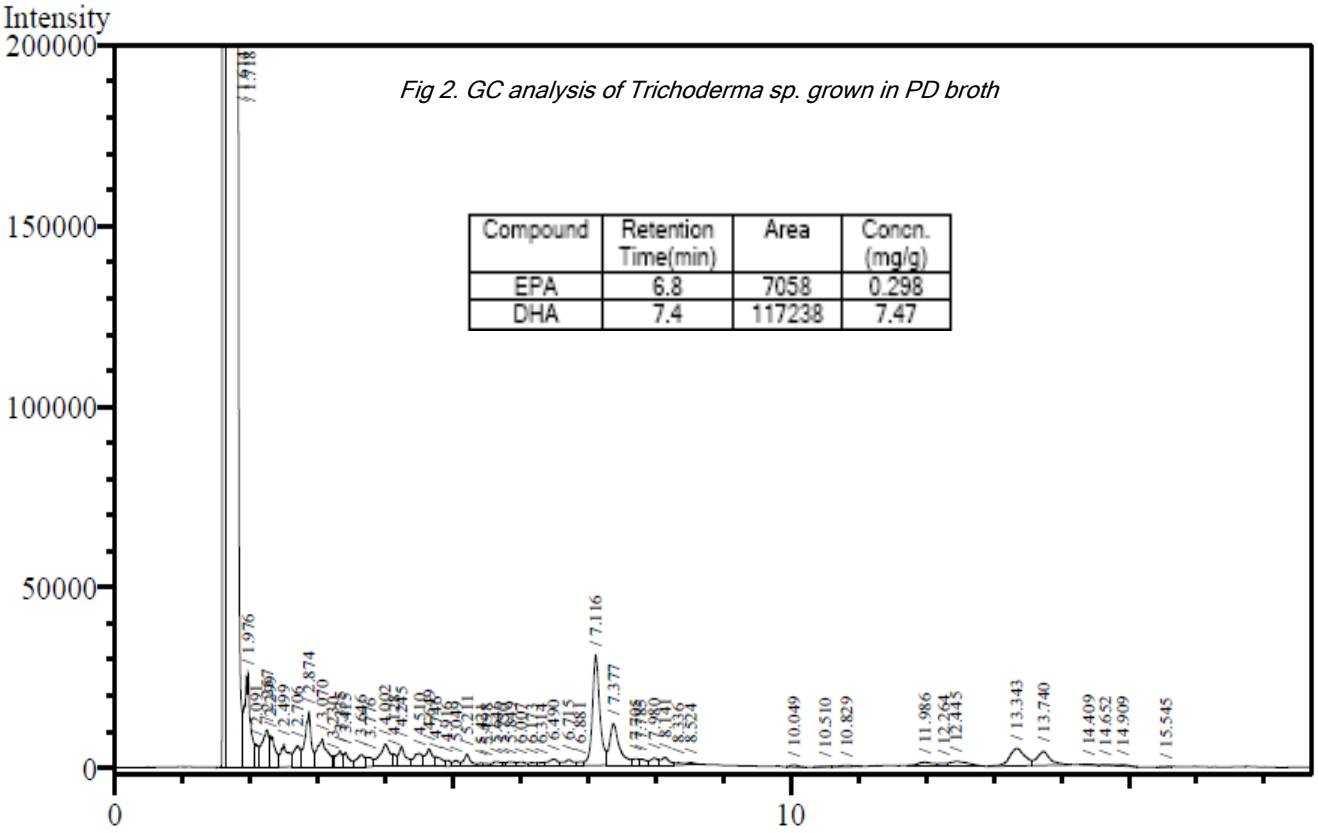

Fig 2. GC analysis of Trichoderma sp. grown in PD broth

Compound	Retention Time(min)	Area	Concn. (mg/g)
EPA	6.8	7058	0.298
DHA	7.4	117238	7.47

Peak#	Ret.Time	Cmpd Name	Area	Height	Area%
1	1.614		8015852	4116336	1.2097
2	1.718		653012284	54976284	98.5447
3	1.976		161322	25871	0.0243
4	2.091		21735	6360	0.0033
5	2.257		71480	10370	0.0108
6	2.299		51310	8750	0.0077
7	2.499		49886	6223	0.0075
8	2.706		41347	5943	0.0062
9	2.874		98360	15276	0.0148
10	3.070		77569	7706	0.0117
11	3.230		7132	2850	0.0011
12	3.335		24922	4356	0.0038
13	3.415		23629	3872	0.0036
14	3.646		28123	3375	0.0042
15	3.776		17533	2531	0.0026
16	4.002		64029	6215	0.0097
17	4.128		13744	3530	0.0021
18	4.245		42299	5680	0.0064
19	4.510		32772	3620	0.0049
20	4.649		33884	4764	0.0051
21	4.746		20258	2643	0.0031
22	4.916		9435	1659	0.0014
23	5.049		9427	1644	0.0014
24	5.211		24178	3257	0.0036
25	5.421		4076	762	0.0006
26	5.498		3766	684	0.0006
27	5.646		10096	1261	0.0015
28	5.750		2387	874	0.0004
29	5.849		10010	1234	0.0015
30	6.007		8890	1110	0.0013
31	6.173		5748	822	0.0009

Peak#	Ret.Time	Cmpd Name	Area	Height	Area%
32	6.314		5458	829	0.0008
33	6.490		18527	1842	0.0028
34	6.715		16360	1659	0.0025
35	6.881		7058	1107	0.0011
36	7.116		237035	30549	0.0358
37	7.377		117238	11510	0.0177
38	7.705		12262	1838	0.0019
39	7.785		11372	1748	0.0017
40	7.980		16265	1976	0.0025
41	8.141		17940	2091	0.0027
42	8.336		4705	572	0.0007
43	8.524		5334	592	0.0008
44	10.049		4075	533	0.0006
45	10.510		1744	177	0.0003
46	10.829		3676	274	0.0006
47	11.986		15743	974	0.0024
48	12.264		6072	722	0.0009
49	12.445		23948	1363	0.0036
50	13.343		74954	4831	0.0113
51	13.740		51644	3802	0.0078
52	14.409		1270	160	0.0002
53	14.652		1023	129	0.0002
54	14.909		3341	351	0.0005
55	15.545		1107	117	0.0002
Total			662655634	59305608	100.0000

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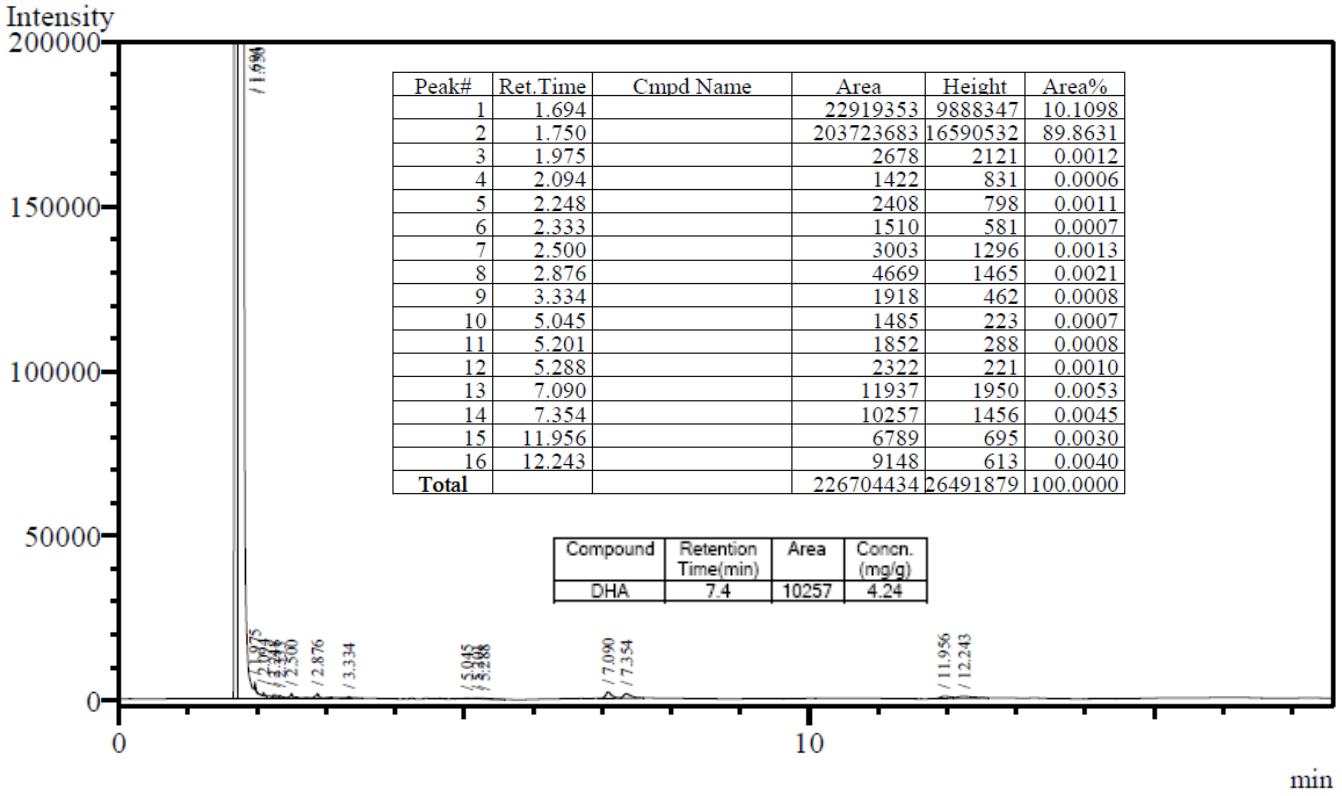
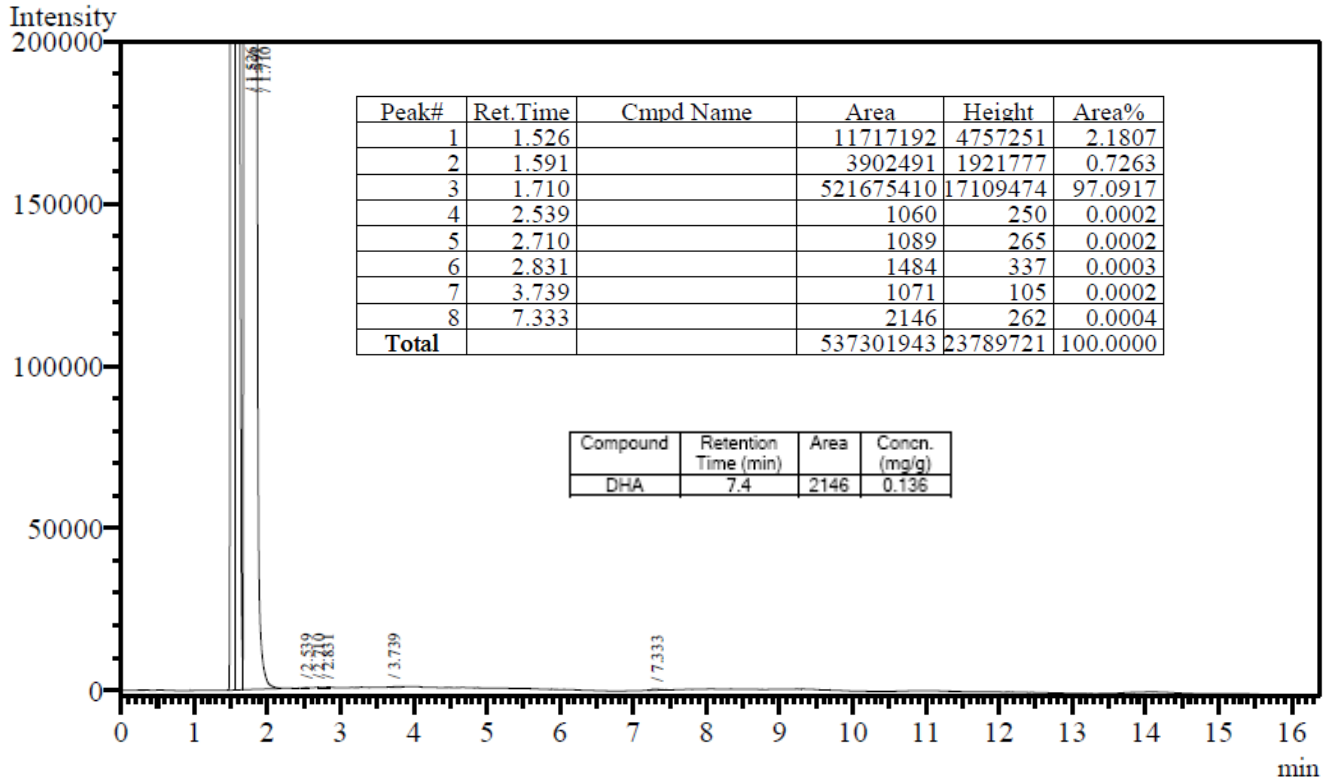
Fig 3. GC analysis of Trichoderma sp. grown in oleic acid medium



Peak#	Ret.Time	Cmpd Name	Area	Height	Area%
1	1.694		22919353	9888347	10.1098
2	1.750		203723683	16590532	89.8631
3	1.975		2678	2121	0.0012
4	2.094		1422	831	0.0006
5	2.248		2408	798	0.0011
6	2.333		1510	581	0.0007
7	2.500		3003	1296	0.0013
8	2.876		4669	1465	0.0021
9	3.334		1918	462	0.0008
10	5.045		1485	223	0.0007
11	5.201		1852	288	0.0008
12	5.288		2322	221	0.0010
13	7.090		11937	1950	0.0053
14	7.354		10257	1456	0.0045
15	11.956		6789	695	0.0030
16	12.243		9148	613	0.0040
Total			226704434	26491879	100.0000

Compound	Retention Time(min)	Area	Concn. (mg/g)
DHA	7.4	10257	4.24

Fig 4. GC analysis of Aspergillus niger grown in oleic acid medium



Peak#	Ret.Time	Cmpd Name	Area	Height	Area%
1	1.526		11717192	4757251	2.1807
2	1.591		3902491	1921777	0.7263
3	1.710		521675410	17109474	97.0917
4	2.539		1060	250	0.0002
5	2.710		1089	265	0.0002
6	2.831		1484	337	0.0003
7	3.739		1071	105	0.0002
8	7.333		2146	262	0.0004
Total			537301943	23789721	100.0000

Compound	Retention Time (min)	Area	Concn. (mg/g)
DHA	7.4	2146	0.136

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