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Full Length Research Paper

Effect of growth media modifications on cell biomass and polyunsaturated fatty acids (PUFAs) production from *Shewanella frigidimarina*

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Polyunsaturated fatty acids (PUFAs) are chemically present as esters, ethers, glycerides glycolipids, phospholipids, phosphonolipids, glycosphingolipids, sulpholipids and lipoproteins in storage oils and membranes lipids. Marine microorganisms such as *Shewanella frigidimarina* are important sources of polyunsaturated fatty acids with promising biomedical applications, commercial value and the potential ability to be used in the bioremediation of environments contaminated with petroleum hydrocarbons. The growth media dependency of *S. frigidimarina* in terms of its growth behavior in response to modifications made to the media as well as its potential to produce PUFAs was evaluated. *S. frigidimarina* was cultured in conventional shake-flasks and controlled bioreactors with a batch-type procedure using different media compositions. The media used included artificial sea water, modified artificial sea water 1, modified artificial sea water 2, Luria Bertani, modified Luria Bertani, sodium pyruvate-yeast (PYS) and marine broth. The highest cell biomass was obtained from artificial sea water media with an optical density (OD_{600}) value of 0.15 and subsequent studies were carried out using this medium. To evaluate the potential for PUFA production, RNA transcripts of polyketide synthases (PKS) genes were isolated and reverse-transcriptase polymerase chain reaction (RT-PCR) with *S. frigidimarina* specific primers carried out. The results of the successful RNA extraction and subsequent RT-PCR revealed that modifications made to growth media compositions can affect the potential for PUFA production.

Key words: Polyunsaturated fatty acids, *Shewanella frigidimarina*, media modification.

INTRODUCTION

Polyunsaturated fatty acids (PUFA; long-chain fatty acids with 18 or more carbon atoms containing two or more nonconjugated *cis* double bonds) have recently enjoyed scientific research and ever-increasing consumption from the general public. This is due to the fact that lipids (oils and fatty acids) are indispensable for the growth and survival of all living organisms (Gill and Valivety, 1997; Sijtsma and Swaaf, 2004). Physiologically, they are important structural components of membranes (such as the brain and the eye), they are crucial in energy storage in many organisms and most recently, they are used in pharmaceutical, nutraceuticals and food supplements, and as biofuels (Simopoulos, 1991 and Okuyama et al., 2007). Polyunsaturated fatty acids especially dietetic,

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Abbreviations: PUFA, Polyunsaturated fatty acids; **EPA,** eicosapentaenoic acid; **DHA;** docasaesaenoic acid; **MA,** marine agar; **SDS,** sodium dodecyl sulfate; **CTAB,** cetyl trimethylammonium bromide; **PCR,** polymerase chain reaction; **PKS,** polyketide synthases; **DEPC,** diethyl pyrocarbonate; **ASW,** artificial sea water; **MB,** marine broth.

Table 1. Liquid media used for the cultivation of *S. frigidimarina*[a].

Component	Artificial sea water (ASW; g/L)	Luria Bertani (LB; g/L)	Modified ASW 1 (g/L)	Modified ASW 2 (g/L)	Modified LB (g/L)	PYS Media (g/L)	Marine Broth (MB) (g/L)
Peptone	3.50	10.00	3.50	3.50	10.00	10.00	5.00
Yeast extract	3.50	5.00	10.00	3.50	5.00	5.00	1.00
NaCl	23.00	19.45	23.00	11.50	0.58	23.00	19.45
$MgCl_2$	5.08	5.90	5.08	5.08	5.90	-	5.90
$MgSO_4$	6.16	-	6.16	6.16	-	-	-
Fe(III)citrate	-	0.10	-	-	0.10	-	0.10
$Fe_2(SO_4)_3$	0.03	-	0.03	0.03	-	-	-
Na_2SO_4	-	3.24	-	-	3.24	-	3.24
$CaCl_2$	1.47	1.80	1.47	1.47	1.80	-	1.80
KCl	0.75	0.55	0.75	0.75	0.55	-	0.55
Na_2CO_3	-	0.16	-	-	0.16	-	0.16
Na_2HPO_4	0.89	0.008	0.89	0.89	0.008	-	0.008
$(NH_4)NO_3$	-	0.0016	-	-	0.0016	-	0.0016
NH_4Cl	5.00	-	5.00	5.00	-	-	

[a]Adapted from Lang et al. (2005).

ω-3 (omega-3) fatty acids principally extracted from fish oil and which constitute an economic alimentary feed stock for the aquaculture industries, are produced by many psychrophilic bacteria (Fernandes and Ventatraman, 1993). Eicosapentaenoic acid (EPA), and docasaesaenoic acid (DHA) are the most important and essential molecules to cellular and tissue metabolism, with regulatory activities on cholesterol and triglyceride transport, plaque accumulation, inflammatory develop-ments and on nervous cells (Clark and Jump, 1996; Gentile et al., 2003; Amiri-Jami, et al., 2006). In fact, PUFA form a unique class of food constituents with a very wide range of functions in biomedical and nutra-ceuticals (that is, food and nutritional products having specific health-promoting biological activities) especially in relation to certain disease conditions (Gill and Valivety, 1997). There is medical evidence that diseases such as artheriosclerosis and heart disease can be prevented by PUFA consumption. Furthermore, PUFA producing organisms have possible biotechnological applications, especially the potential to be used as ‘environmental cold-adapted cleaners’ capable of bioremediating petroleum hydro-carbons. These oils are comparatively cheap and are generally considered to be healthier than those derived animal fats, due to their relatively high amounts of unsaturated fatty acids. However, it has been established that the higher plants rarely contain PUFA above C_{18} owing to lack of the requisite of enzymes richest in diversity of PUFA as encountered in microorganisms, especially algae, bacteria and fungi (Gill and Valivety, 1997). These are some of the reasons why psychrophilic and psycrotrophic bacteria are receiving growing interest (Gentile et al., 2003). However, only a restricted number of the bacterial genera posses the capability to produce PUFA, most of which have been isolated from polar regions or from deep sea. *Shewanella* sp. has been found to produce significant amounts (up to 24% of total fatty acids) of EPA (Gentile et al., 2003; Dague, et al., 2006).

In this study, the effects of different growth media on the production of polyunsaturated fatty acids by *S. frigidimarina* isolated from the seawater samples collected from Seabait Ltd. carried out in shake-flask scale was investigated and described.

MATERIALS AND METHODS

Shaking liquid culture

Pre-germinated spores of *Shewanella* strains grown on marine agar (MA) plates were inoculated into 7 different modified liquid media in duplicates (Table 1) and incubated in an orbital shaker at 28°C for 3 days. Four different trials were conducted with different concen-trations of inoculants of 0.063, 0.065, 0.072 and 0.085 nm in trials 1, 2, 3 and 4, respectively, at various time intervals in order to determine the cell biomass production at the different growth stages of the *Shewanella* sample. Optical density (OD_{600}) readings were taken at various intervals. The samples were transferred into 50 ml tubes and centrifuged at 4000 rpm for 10 min and the pellets separated.

Fermentation

Applikon® Autoclavable Bio Reactor Systems with a 3 L total volume vessel (ADI 1010 Bio Controller, ADI 1025 Bio Console and ADI 1032 Stirrer Controller) was used for the fermentation of the *S. frigidimarina*. The general procedures provided by operating manual were used for cultivation.

DNA extraction and amplification of PUFA gene

Bacterial genomic DNA was extracted from the Seabait Ltd strain using a procedure modified from the sodium dodecyl sulfate (SDS)-based method (Zhou et al., 1996). Deviations from the original method included a second chloroform isoamyl alchohol extraction (24:1, v/v) step. Due to better performance of cetyl trimethylammonium bromide (CTAB) in reducing humic contamination, it was used in the buffer for SDS-based extractions. 5 g sediment samples were mixed with 13.5 ml DNA extraction buffer (100mM Tris-HCl, pH 8.0; 100 mM sodium EDTA, pH 8.0; 100 mM sodium phosphate, pH 8.0; 1.5 M NaCl; and 1% CTAB) in Oakridge tubes by horizontal shaking at 225 rpm for 30 min at 37℃. 1.5 ml of 20% SDS was added after the shaking treatment and the samples incubated in a water bath at 65℃ for 2 h with gentle end-over-end inversions every 15 to 20 min. Samples were then centrifuged at 6,000 x g for 10 min at room temperature and supernatants transferred into 50 ml centrifuge tubes. The soil pellets were re-extracted two more times by adding 4.5 ml extraction buffer and 0.5 ml 20% SDS, vortexing for 10 s, incubating at 65℃ for 10 min and centrifuging as before. Supernatants from the three cycles of extractions were combined and mixed with an equal volume of chloroform isoamyl alcohol (24:1, v/v). The aqueous phase was recovered by centrifugation and precipitation with 0.6 volume of isopropanol at room temperature for 1 h. The pellet of crude nucleic acids was obtained by centrifugation at 16,000 x g for 20 min at room temperature, washed with cold 70% ethanol and resuspended in sterile deionised water to give a final volume of 500 ml. DNA was precipitated with ethanol.

The presence, concentration and purity of bacterial DNA preparations were checked by agarose gel electrophoresis by running 2 µl aliquots of each product in a 1%, w/v agarose gel. The gels were prepared using 0.5 x tris/Borate/ ethylenediaminetetraacetic acid (TBE) buffer and 0.5 µg/ml ethidium bromide and run for 45 min at 100V in 0.5 x TBE buffer. They were then visualised using a computerised UV transilluminator equipped with an automatic camera and a BioRad Fluor-S™ MultiImager gel image documentation system. Bacterial DNA preparations were kept at -20℃ until required.

Polymerase chain reaction (PCR) were performed with sample dilutions of 1:50 and 1:100 of 100-200 ng DNA, 10 µM of polyketide synthases (PKS) primer, 10 mM dNTP, 0.5 mg/ml non-acetylated bovine serum albumin (BSA), 5 ml 10X buffer (Biolab), 0.5 µl Biolab standard Taq, MilliQ water to a final volume of 20 µl. Thermal cycling protocol was 96℃ for 3 min, 45 cycles of 96℃ for 1 min, annealing for 1 min. The PCR products were assessed by gel electrophoresis.

RNA extraction

TRI Reagent®- RNA/DNA/Protein isolation protocol by Helena BioSciences was employed for the RNA isolation. 5^{-10} x 10^{6} cells were added to 1 ml TRI reagent and mixed several times through a pipette to allow proper homogenization. The homogenate was stored for 5 min at room temperature to permit complete dissociation of nucleoprotein complexes. 0.2 ml chloroform per 1 ml TRI reagent was added and vortexed vigorously for 15 s. The resulting mixture was stored at room temperature for 15 min and centrifuged at 12,000 X g for 15 min at 4℃, the mixture separates into a lower red layer, interphase containing DNA and proteins, and an upper aqueous phase exclusively containing the RNA. The aqueous phase was carefully transferred into a new vial tube and the RNA precipitated by adding 0.5 ml isopropanol per 1 ml of TRI reagent used for the initial homogenization. Samples were then stored at room temperature for 10 min and centrifuged at 12,000 X g for 8 min at 25℃, the supernatant was discarded and 1 ml of TRI reagent used for the initial homogenization 75% v/v ethanol was added to gel-like white pellet RNA sample. The mixture was vortexed and centrifuged at 7,500 X g for 5 min at 25℃. The ethanol wash was discarded and the RNA pellets were briefly air-dried for 5 min by placing them on the table (note: not by centrifugation under vacuum). The RNA pellets were then dissolved by adding RNase-free diethyl pyrocarbonate (DEPC) treated water and passing the solution a few times through a pipette tip and incubated for 15 min at 60℃.

Integrity of RNA

The integrity of the RNA was quantified by running a gel electrophoresis; 0.6 g agarose was melted in 45 ml DEPC water and allowed to cool. 5 ml of 10 X 3-[N-morpholino] propanesulfonic acid (MOPS) buffer and 2 µl ethidium bromide (10 mg/ml) were added, gently swirled and poured in the gel tank. RNA cocktail was prepared in a separate tube by adding 200 µl formamide, 70 µl formaldehyde, 20 µl of 10 X MOPS buffer, 2 µl ethidium bromide (10 mg/ml) and 20 µl of 10 X RNA loading buffer. RNA loading buffer was also prepared in a separate tube by filtering 2.5 mg bromophenol blue, 2.5 mg xylene cyanol FF and DEPC water through a 0.45 µM prior to adding 5 ml sterile glycerol and 20 µl of 0.5M ethylenediaminetetraacetic acid (EDTA) (pH 8.0).

10 µl of RNA sample was mixed by vortexing and spun down with 20 µl RNA cocktail in a centrifuge, incubated at 65℃ for 10 min and then kept on ice for 3 min before running on agarose gel electrophoresis with 1 X MOPS as running buffer at 80V for 1.30 h (Hurt et al., 2001). The integrity of the RNA samples was visualized using a computerized UV transilluminator equipped with an automatic camera and a BioRad Fluor-S™ MultiImager gel image documentation system. RNA samples were kept at -20℃ until required.

Reverse-transcriptase (RT)-PCR

The protocol outlined in the Omniscript® reverse transcription handbook produced by QIAGEN was employed for the reverse transcription. Prior to reverse transcription, the RNA extracts were treated against any DNA by adding 2 µl of DNase inhibitor to 10 µl of RNase-free water containing 2 µg RNA templates. The mixture was incubated at 37℃ for 30 min and then at 75℃ for 5 min. It was immediately placed on ice. RNase inhibitor was diluted to a final concentration of 10units/µl in ice-cold 1 X Buffer RT, thoroughly and carefully mixed by vortexing for no more than 5 s and centrifuged briefly to collect the residual liquid from the sides of the tubes.

A fresh master mix was then prepared on ice, mixed carefully and thoroughly by vortexing for no more than 5 s and centrifuged briefly to collect the residual liquid from the sides of the tube. 12 µl of the DNase-treated RNA template was added to 8 µl of the reverse

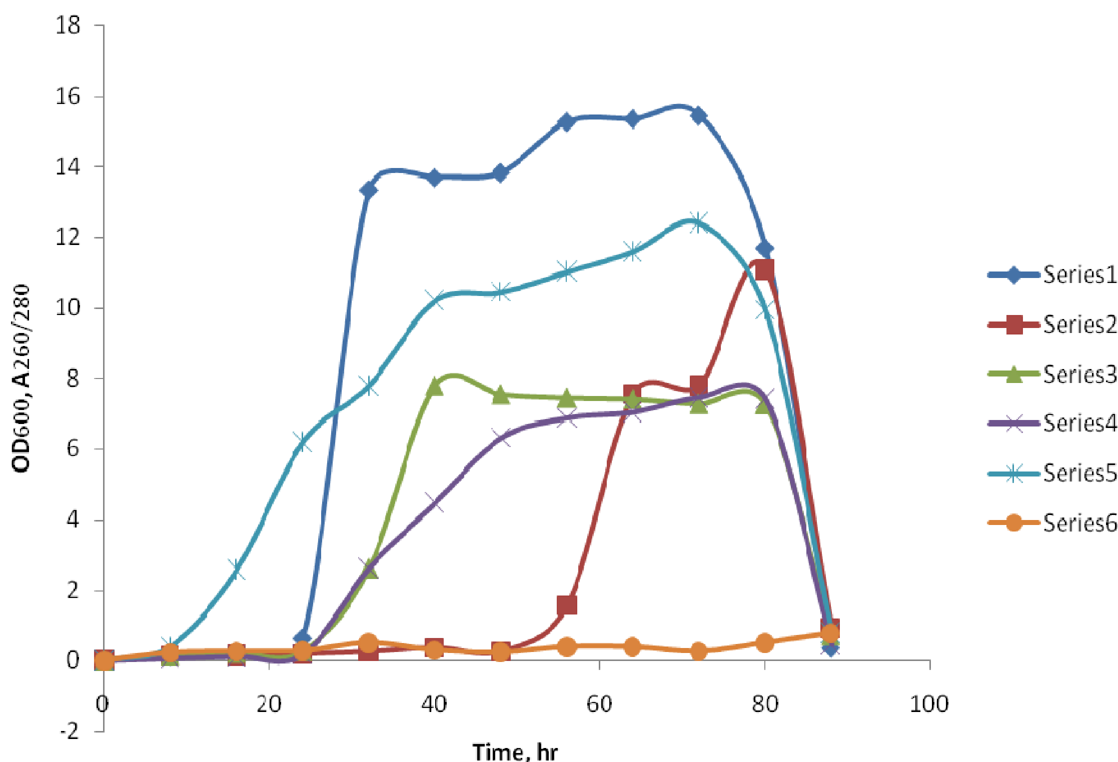

Figure 1. Plot of all the growth curves against time for comparison. Series1: ASW; 2: modified LB; 3: modified ASW with 10g YE; 4: MB; 5: LB and 6: PYS media. The index of OD_{600} readings on the y-axis is to be multiplied to 10^{-2}.

reverse transcription master mix. The mixture was mixed carefully by vortexing for no more than 5 s and centrifuged briefly to collect the residual liquid from the sides of the tubes. The reaction mixture was then incubated at 37ºC for 60 min. and stored at -20ºC.

Two-tube RT-PCR was performed sequentially in separate tubes. This method allows for the storage of the cDNA for later analyses. Aliquots for a 50 µl PCR assay, only ≤5 µl of the finished reverse-transcription reaction were added to PCR mix. The appropriate volume of the master mix was distributed into individual reaction tubes placed on ice and the template cDNA was added to the individual tubes and placed in the PCR machine to run for 45 cycles (Loffert et al., 1997; Guthier et al., 2000).

RESULTS AND DISCUSSION

Shake-flask experiments

Series of shake-flask experiments in duplicates, containing different growth media were used to assess which is best for cell biomass production for the subsequent gene expression of *S. frigidimarina* and effects on PUFA production. From the optical density (OD_{600}) readings, it was found that the highest concentration of cell biomass was obtained in artificial sea water (ASW) and a similar concentration and growth curve from the marine broth. This is due to the fact that ASW has a similar nutritional composition to the environment *S. frigidimarina* was isolated from, containing approximately 3% of sodium chloride and small amounts of other minerals, and the fact that microorganisms having the sea as their natural habitat usually have a specific requirements for sodium ion. This growth requirement is best understood in relation to the microorganisms' sodium motive force (smf)-dependence for active transport and stability (Lang et al., 2005). On the contrary, other growth media neither followed the same growth pattern nor produced good cell biomass concentrations (Figure 1). This leads to the presumptive conclusion that apart from temperature, growth requirements of carbon and nitrogen sources and other inorganic ion components are also important factors for the genes expressing PUFAs. Therefore, ASW was chosen for the subsequent cultivation in the bioreactor.

The variation in growth with response to growth media modifications is best analysed when the curves obtained are plotted on the same graph (Figure 1). ASW media produced the best cell mass with optimal growth achieved within the span of one to three days. Marine broth (MB) had a cell production next to ASW media even though maximum growth was achieved after about two days. Modified ASW media also had a good cell growth but was half that obtained from ASW media.

Table 2. RNA optical absorbance, OD_{600} at 8 h interval.

Sample	Concentration (µg/ml)	Ratio (260/280)	Ratio (260/230)
Shake-flask S1	429.3	1.58	1.49
Shake-flask S2	483.6	1.78	2.16
Shake-flask S3	1091.0	1.81	1.58
Fermenter F1	500.2	1.60	1.55
Fermenter F2	925.0	1.67	2.07
Fermenter F3	1860.1	1.86	2.17

RNA extraction

Unlike DNA extraction, there was great difficulty in RNA extraction, purification and subsequent reverse transcription despite the various RNA isolation methods so far developed for the various kinds of organisms and tissues. This could partly be due to the fact that there are no universal protocols applicable for the wide range of marine microorganisms (Seonock et al., 2005). Another possibility could be due to the fragility of RNA molecule which arises primarily from (i) the ubiquitous and exceptionally stable nature of ribonuclease activity within the living organisms, which easily gets contaminated during RNA preparations and (ii) the fact that thermo-dynamically, RNA is a fragile molecule because of its 2'-OH group on the ribose ring which promotes hydrophilic attack on the 5'-3' phosphodiester bond forming cyclic phosphate which subsequently encourages RNA degradation (http://stanxterm.aecom.yu.edu/wiki/index.php?page=RNA_handling).

The TRI reagent RNA isolation method based on the original acid guanidine thiocyanate-phenol-chloroform (APGC) protocol was employed in this research to isolate and purify the RNA. However, this method was not successful in isolating *S. frigidimarina* RNA from any of the (modified) liquid growth media (shake-flasks and fermenter). These results probably indicate that *S. frigidimarina* have unique contents of specialized proteins, lipids and/or carbohydrates that affect RNA extraction and purification from liquid culture. Furthermore, the media compositions may have inhibited the RNA extractions due to the quantity of peptone and yeast extract present in relation to the TRI reagent used. These results further confirm that a protocol to isolate RNA must be efined depending on the organism (Seonock et al., 2005).

Hence, cells from the shake-flasks and fermenters were inoculated on agar plates and the RNA indirectly isolated, purified and reversed transcribed into cDNA. The indirect method was successful as 28S and 18S bands were seen (not shown here). All the results of $A_{260/280}$ obtained for this study agree with the literature range of 1.6 to 1.9. Moreover, high purity RNA was obtained with $A_{260/280}$ ratio of 1.86. Absorbance readings to reflect the RNA concentration were taken from both shake-flasks and fermenters at 8-h intervals and these showed that the PUFA producing genes are increasingly being expressed (Table 2). Reverse transcription of the isolated RNA from the shake-flask and fermenter was assessed on 1% agarose/formaldehyde gel. RT-PCR of purified RNA into cDNA was successful and the size of the bands corresponded to those of the amplicon produced following PCR with the PKS primers designed for *Shewanella.*

Conclusion

This study further confirmed the fact that gene expression during PUFA production is affected by growth conditions, especially in response to growth media modifications. Cell biomass produced by *S. frigidimarina* grown on varying carbon source and other nutrients at different times along a curve showed a direct dependence of growth requirement to sodium motive force (smf). Growth media such as ASW with nutrient composition similar to that obtainable from the marine environment produced more cell biomass compared to other growth media used. This study was also able to successfully isolate, purify and reverse transcribe high quality RNA into cDNA with the same size as the PCR product. Concentrations of RNA isolated at three different times (immediately after the lag phase, growth phase and the exponential phase) along the cultivation from shake-flask were 429.3, 483.6 and 1091.0 µg/ml, respectively. Also, similar RNA concentrations were observed from fermenter cultivation of *S. frigidimarina* recorded at the post lag phase, growth phase and exponential phase which were observed to be 500.2, 925.0 and 1860.1µg/ml, respectively. The RNA extracted also had high purity OD_{600}, $A_{260/280}$ (nm) which fell within the range of 1.6 to 1.9 as stipulated by the TRI reagent protocol for the final preparation of total RNA free

from DNA and proteins.

REFERENCES

Amiri-Jami M, Wang H, Kakuda Y, Griffiths MW (2006). Enhancement of polyunsaturated fatty acid production by Tn5 transposon in Shewanella baltica. Biotechnol. Lett. 28: 1187-1192.

Clark SD, Jump DB (1996). Polyunsaturated fatty acid regulation of hepatic gene transcription. Lipids, 31: 7-11.

Dague E, Jorand F, Thomas F, Gaboriaud F (2006). Probing surface structures of Shewanella spp. by microelectrophoresis. Biophys. J. 90: 2612-2621.

Fernandes G, Ventatraman JT (1993). Role of omega-3 fatty acids in health and disease. Nutr. Res. 13: 19-45.

Gentile G, Bonasera V, Amico C, Giulian L, Yakimov MM (2003). Shewanella sp. GA-22, a psychrophilic hydrocarbonoclastic Antarctic bacterium producing polyunsaturated fatty acids. J. Appl. Microbiol. 95: 1124-1133.

Gill I, Valivety R (1997). Polyunsaturated fatty acids, part 1: Occurrence, biological activities and applications. TIBTECH. 15: 401-409.

Guthier JN (2000). DNA extraction from coral reef sediment bacteria for the polymerase chain reaction. J. Microbiol. Methods, 43: 73-80.

Hurt RA (2001). Simultaneous recovery of RNA and DNA from soils and sediments. Appl. Environ. Microb. 67(10): 4495-4503.

Lang S, Huners M, Lurtz V (2005). Bioprocess Engineering Data on the Cultivation of Marine Prokaryotes and Fungi. Adv. Biochem. Eng. Biotechnol. 97: 29-62.

Loffert D, Stumps S, Schaffrath N, Berkenkopt M, Kang J (1997). PCR: Effects of templates quality QIAGEN. News, pp. 1-8.

Okuyama H, Orikasa Y, Nishida T (2007). Significance of antioxidative function of eicoasapentaenoic and docosahexaenoic acids in marine organisms 1128/AEM.02256-07. Appl. Environ. Microb. 10: 1-26.

Seonock W, Seungshic Y, Moongeun Y, Sa Heung K, Jongrak L, Jeong Ha K, Taek-Kyun L (2005) Efficient Isolation of Intact RNA from the Soft Coral Scleronephthya gracillimum (Kükenthal) for Gene Expression Analyses. Integrative Biosci. 9: 205-209.

Sijtsma L, de Swaaf ME (2004). Biotechnological production and application of -3 polyunsaturated fatty acid docosahexaenoic acid. Appl. Microb. Biotechnol. 64: 146-153.

Simopoulos AP (1991). Omega-3 fatty acids in health and disease and in growth and development. Am. J. clin. Nutr. 54: 438-463.

Zhou J, Bruns MA, Tiedie JM (1996). DNA Recovery from Soils of Diverse Composition. Appl. Environ. Microb. 62: 316-322.

http://stanxterm.aecom.yu.edu/wiki/index.php?page=RNA_handling [retrieved: 04/04/2008].