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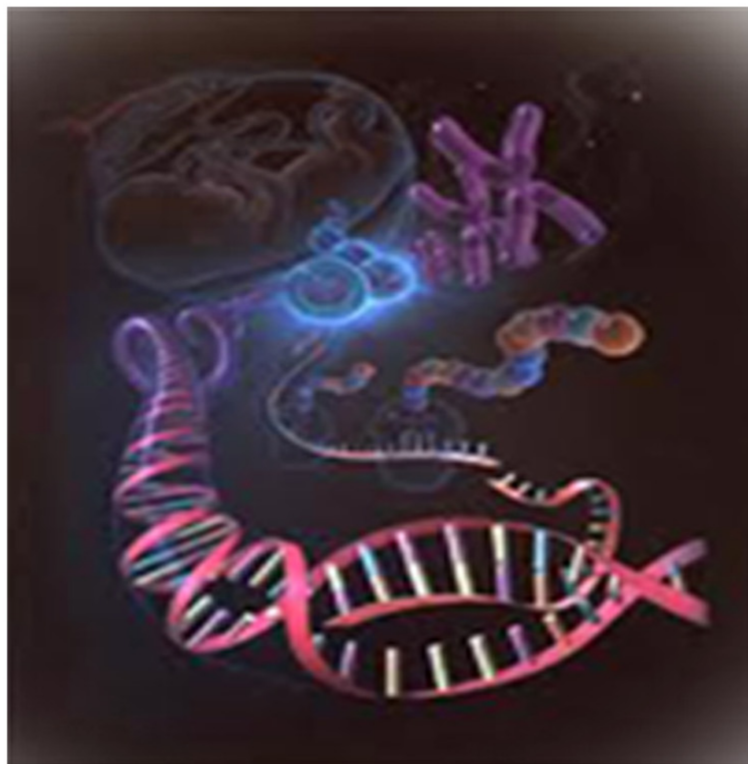

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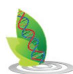

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Research Paper

SCREENING AND IDENTIFICATION OF PUFA PRODUCERS FROM VARIOUS FISH OF INDIAN COASTAL AND FRESH WATER HABITATS

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Polyunsaturated Fatty Acids (PUFA) derived from fish exhibit beneficial physiological effects and have well-known nutritional and pharmaceutical significance and are commercially important as additives in food supplements. Microorganisms isolated from fish gut have been reported to be capable of producing PUFA. In our study 49 lipid producing microbes were isolated from intestines of various fresh water and salt water oily fish. The isolates were shortlisted following a preliminary screening scheme by studying a set of sequential parameters like intensity of lipid accumulation as judged by Sudan Black B staining, amount of biomass on nitrogen limited semi-synthetic medium, lipid quantity per gram biomass, relative quantities of saturated and unsaturated fatty acids and reproducibility of results. Ten shortlisted isolates were screened for PUFA production by Gas Chromatography after samples for GC analysis were prepared by extracting the lipids in organic solvent mixture followed by transmethylation with methanolic HCl to form Fatty Acid Methyl Esters (FAMEs). Further, GC/MS analysis confirmed the GC results as five isolates were shown to be capable of producing omega-6(LA) and omega-3(ALA) PUFA. Selected cultures have been identified by 16S r-RNA sequencing. These cultures are being optimized for PUFA production by the aerobic fermentation route.

Keywords: Polyunsaturated Fatty Acids (PUFA), Fresh Water and Salt Water Fish, Screening for Microbial PUFA Producers, GC, GC/MS

INTRODUCTION

There is an increasing interest in fish consumption recently due to the wide range of health benefits being discovered in long chain marine fatty acids, specifically eicosapentaenoic acid (EPA) 20:5n-3 and docosahexaenoic acid (DHA) 22:6n-3 (Okada and Morrissey, 2007). EPA and DHA are Poly Unsaturated Fatty Acids (PUFA), a family of long chain unsaturated fatty acids that have at least two carbon-carbon double bonds. These are found mainly in fish like mackerel, salmon, striped bass, rainbow trout, halibut, tuna and sardines and also in some plant oils like olive and linseed oil. Few genera of algi,

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fungi and bacteria have also been reported to produce PUFA.

Some of the more commonly occurring PUFA are Linoleic Acid (LA) 18:2n-6, Alpha Linolenic Acid (ALA) 18:3n-3, Arachidonic Acid (AA) 20:4n-6, etc. Humans cannot synthesize these fatty acids and these have to be necessarily supplemented through diet (Dyal *et al.*, 2005). Dietary PUFA have beneficial effects on diverse physiological processes impacting normal health and controlling several diseases and disorders. There is general consensus about the beneficial effects of consumption of PUFA rich fish meat and the reduction in instances of Coronary Heart Disease (CHD). The consensus is based on the studies from over two decades ago when epidemiologists observed the low rate of coronary heart disease among Alaskan and Greenland Eskimos who consume large amounts of fish. In both the US and Europe, several studies have also confirmed the positive effects of fish consumption in reducing CHD among diverse populations (Okada and Morrissey, 2007).

PUFA also play an important role in vision, pregnancy, infant development and cell signaling. Being essential fatty acids they are also preventive and co-therapeutic for several major diseases such as atherosclerosis, cancer and Type II diabetes and disorders like arthritis, mental decline and auto immune conditions (Nichols, 2003). PUFA provide fluidity to cell membranes and improve communication between brain cells. They also reduce the clotting ability of platelets, thus potentially decreasing the incidence of heart attacks and strokes (Doughman *et al.*, 2007; Sahelian Ray, 2014).

With growing public awareness of the clinical benefits of PUFA, various types of products and supplements have been developed. PUFA supplements from fish oils are widely used personally on a daily basis, generally taken in the form of capsules. The oils are also added to infant milk formula with claims of enhancing mental ability. Sales of fish oil supplements have grown almost 10-fold over the last decade, reaching sales of $310 mn in 2004. Most of these represent fish oil capsules made from various seafood sources including fish byproducts (Okada and Morrissey, 2007). No doubt other nutraceutical and pharmaceutical uses will be developed, and, as fish stocks continue to dwindle worldwide, there will be even more pressure to seek alternative, microbiological solutions to the supply of PUFA (Russell and Nichols, 1999).

Thus a consistently available source of high quality raw material is critical for production of n-3 polyunsaturated fatty acid concentrates. Though a sustainable supply of the necessary raw material could be provided, the majority of it is sold for non-human food uses like animal feeds (Okada and Morrissey, 2007). Fish oil which is the best source of PUFA is often contaminated with heavy metals and toxic substances due to environmental pollution and hence, may pose a serious health risk to the consumers (Yaguchi *et al.,* 1997). Often unpleasant smell associated with fish and animal origins of PUFA are barriers for consumption of fish oil by vegetarian people. Furthermore, large scale exploitation of fish as sources of PUFA may lead to shortage of fish as food source.

Traditional sources of PUFA are of plant and animal origin, offer relatively low yields and cost of production is generally high (Dyal *et al.*, 2005). A number of marine microorganisms have been reported to be capable of producing PUFA (Nagao

Toshihiro, 2009). Therefore, it is of interest to isolate, culture and identify microorganisms with PUFA producing ability and to develop an efficient production process for obtaining large quantities of these PUFA. Several strains belonging to the genus *Shewanella* isolated from intestines of blue backed fish rich in PUFA and cold water environments have been reported as being high-level PUFA-producing micro-organisms. Besides these, strains belonging to the genera *Colwellia, Photobacterium, Flexibacter, Vibrio and Moritella* are also PUFA-producing micro-organisms (Kikue Hirota *et al.*, 2005). Thus, screening from diverse sources as well as marine environments and fishes is likely to be good source of microbes capable of producing PUFA.

Of late finding alternate economical sources and novel dietary options to supplement Essential Fatty Acids (EFAs) has become a major thrust area of research for both industry as well as academia (Dyal *et al.*, 2005). Microbial production of PUFA is of immense interest because of the ease with which microbes can be cultivated using modern biotechnology equipment like bioreactors equipped with temperature, pH and Dissolved Oxygen probes along with computer based monitoring and control of fermentation process (Yano Yutaka *et al.*, 1994).

In the current research work we have attempted to isolate microorganisms capable of producing PUFA from intestines of oily fish inhabiting both the marine and fresh water environments. Oily fish from the Indian coastal regions particularly the Arabian Sea were studied. Moreover considering the oily nature of two river fish from the carp family, they were examined for PUFA producing intestinal microflora.

MATERIALS AND METHODS

Sampling: Lipid producers were isolated from intestines of oily fish of salt water and fresh water habitats (Yaguchi *et al*. 1997, Nichols, 2003). The fish (enlisted in table No. 1 and 2) were collected from local fish markets adjoining the Maharashtra Konkan strip. All fish samples were collected in zip locked sterilized plastic bags and stored in ice till analysis. The fish were brought to the laboratory within 24 h for processing. The Baby Shark, Indian Mackerel and Sardines were caught directly from a fishing vessel offshore Harnai, Konkan, Maharashtra.

Isolation of Microbes: For isolation of microbes from fish, the fish surface was washed with sterile water and slit aseptically along the ventral side downwards from the pectoral fin. The gut was removed, washed, cut and homogenized in 0.85% saline using a mortar and pestle. Serial dilutions were prepared from this homogenate and plated on Zobell's Marine Agar and Tryptone Soy Agar for salt water and fresh water fish samples respectively (Yano Yutaka *et al.*, 1994; Gentile, 2003; Jostensen Jens-Petter, Bjarne Landfald, 1997). All above procedures were performed under aseptic environment. After incubation at 25°C for 120 h colonies were selected on the basis of differences in colony characteristics (Patnayak and Sree, 2005). All microbiological media were obtained from Himedia®, India and the chemicals were procured from Merck® Specialty chemicals limited.

Preliminary Screening for Lipid production and Other Desirable Attributes: The isolates were shortlisted following a preliminary screening scheme by studying certain parameters like intensity of lipid accumulation by Sudan Black B staining, amount of biomass on nitrogen limited

semi-synthetic medium and percentage of lipid quantity produced against biomass. The shortlisted cultures were checked for the reproducibility of results for the above said parameters by running the experiments three times.

Induction of Lipid Accumulation: The obtained isolates were transferred to solid Semi synthetic minimal medium as described in Patnayak and Sree (2005), to induce lipid accumulation under nitrogen limitation and incubated at 25°C for 120 h. Lipid producers were selected by Sudan Black B staining method (Burdon, 1946).

Extent of Lipid Accumulation: Isolates were grouped as strongly positive, positive and weakly positive depending on the intensity of lipid accumulation as observed microscopically post Sudan Black B Staining under 1000X magnification. Sudan Black B stains the intracellular lipid granules bluish black and the counter stain Saffranine stains cells pink. The selected cultures were cultured thrice on the above stated agar medium, stained and observed to confirm the reproducibility of the extent of lipid accumulation.

Amount of Biomass On Semi-Synthetic Medium: Isolates were inoculated in 250 mL Erlenmeyer flasks containing 50 mL of above mentioned semi-synthetic broth medium and incubated at 25°C for 120 h at 180 rpm on a temperature controlled orbital shaker having a stroke of 25 mm. The biomass was collected by centrifugation at 8,000 g for 10 min in a cooling centrifuge. The wet weights of the respective cultures were recovered after decanting the broth (Bowles *et al*., 1999).

Lipid extraction: Accumulated lipid was extracted by the modified Bligh and Dyer Method (1959). The lipids were extracted from the biomass in sealed conical flasks having 20 times volume Methanol: Chloroform (2:1 v/v) by shaking overnight at 60 rpm in a shaker at 25°C, filtered through Whatmann No. 1. The total extracted lipid was weighed after separating and evaporating the chloroform layer containing the lipid. The percentage of lipid accumulated was calculated against the biomass, i.e., mg total extracted lipid/ mg wet weight of cells (Nichols and McMeekin , 2002).

Gas Chromatography: After short listing by Sudan Black B staining only Strongly positive lipid accumulators capable of giving reproducible amount of high lipid accumulation were considered for GC analysis. Fatty Acid Methyl Esters (FAMEs) were prepared from the obtained lipid by transmethylation with methanolic HCl in a water bath at 80°C as described by Ranjekar (2003). The esters were extracted in hexane and dried under nitrogen. FAMEs were analyzed by Shimadzu (2014) Gas Chromatograph (Shimadzu Corporation, Kyoto, Japan) equipped with Flame Ionization Detector and Rt®-2330 (Restek Corporation, USA) capillary column (30 m × 0.32 mm ID × 0.2 µm df). Nitrogen was used as carrier gas. Initial column temperature was set at 140°C which was later raised to 230°C at 4°C min^{-1} and final hold of 5 min. The injector and detector were kept at 240°C with an injection volume of 0.2 µL (Ranjekar *et al*., 2003). Chromatographic comparison with authentic FAMEs standards from Supelco was used for identifying the FAMEs from cultures. The quantities of individual fatty acids were estimated from the peak areas on the chromatogram. Each of the shortlisted cultures was cultured in the said

liquid medium three times on separate occasions and their lipids were tested by Gas Chromatography to confirm the reproducibility of the results.

GC-MS: GC-MS analysis was performed as confirmatory technique for identifying the type of PUFA produced by the short listed cultures. FAMEs were analyzed on Shimadzu GC-MS QP 2010 ULTRA equipped with Quadrupole Mass Spectrophotometer (Shimadzu Corporation, Kyoto, Japan). Rt®-2330 (Restek Corporation, USA) capillary column (30 m × 0.32 mm ID × 0.2 µm df) was used for analysis. The oven temperature program was same as for GC analysis. Helium was used as the carrier gas at a linear velocity of 44.4 cm/s. The ion source temperature was maintained at 200°C and analysis performed in the EI (Electron Impact Ionization) Scan mode at 70 eV. Scans from 40 to 600 m/z were run with an event time of 0.30 s and scan speed of 2000 u/s.

Phenotypic Characteristics: Confirmed PUFA producers were studied for their morphology, Gram character, motility as described in the manual "Microbiological Methods by Collins and Lyne (1999)". Biochemical characteristics like sugar fermentation, MR-VP test, nitrate reduction, Indole production, citrate utilization, TSI, Ammonia production and enzyme tests were performed as described in the Microbiology Laboratory Manual by Cappuccino and Sherman (2006).

Identification of isolates by 16s rRNA sequencing: The selected cultures were identified by 16S r-RNA sequencing using multiple PCR primers in Applied Biosystems 3730XL sequencing machine (Applied Biosystems Inc., USA). Pure isolate(s) in the form of single colony cultured on nutrient agar was used for processing. The genetic material was extracted by Phenol-Chloroform method for PCR Template preparation. Amplification was achieved by using 16S rRNA region primers. The amplification was checked by agarose gel electrophoresis. The amplified PCR products were purified by PEG-NaCl method. Cycle sequencing was performed using Universal primers 8F AGAGTTTGATCCTGGCTCAG (Turner *et al.*, 1999) and 907R CCGTCAATTCMTTTRAGTTT (Lane, 1991). Cycle sequencing clean up was performed subsequently. Samples were loaded on the sequencing machine ABI 3730XL.

The obtained 16s rRNA from isolates ranging from 800-1200 bp allowed direct comparisons with Database sequences. The program used for analysis was nBLAST available at NCBI. The alignment of query sequences was performed with the Nucleotide Collection (nr/nt) Database to obtain the Highest similarity hits for each isolate.

RESULTS AND DISCUSSION

Isolation and Screening: A total of 49 lipid producers were isolated from various oily fishes from Indian western coastal region, river environments. The locations of sample collection, the names of fishes used for isolating lipid producers and corresponding isolates obtained are listed in Table 1 and 2. The salt and fresh water fish collected inhabited the Arabian Sea along the western coast of Maharashtra and rivers near Panvel, Maharashtra in India, respectively.

These fish were selected on the basis of their well known oily nature and they being reported to be rich in PUFA. The shark, mackerel, sardines and salmon are predominantly savored and acknowledged oily marine fish along the Indian

coastal regions (Spiller, 1996; Gupta *et al.* 2012). Hence, these fish were probed for the likely presence of lipid producing microorganisms. The shrimp was studied considering the high PUFA content in its tissues (Oksuz, 2009). Fresh water carps are recognized for having high oil and high PUFA content in their flesh and thus seemed interesting for this study (Bieniarz, 2001; Spiller, 1996). Indian river carps Rohu and Catla and were selected for this study and bought from fisherwomen in Mumbai.

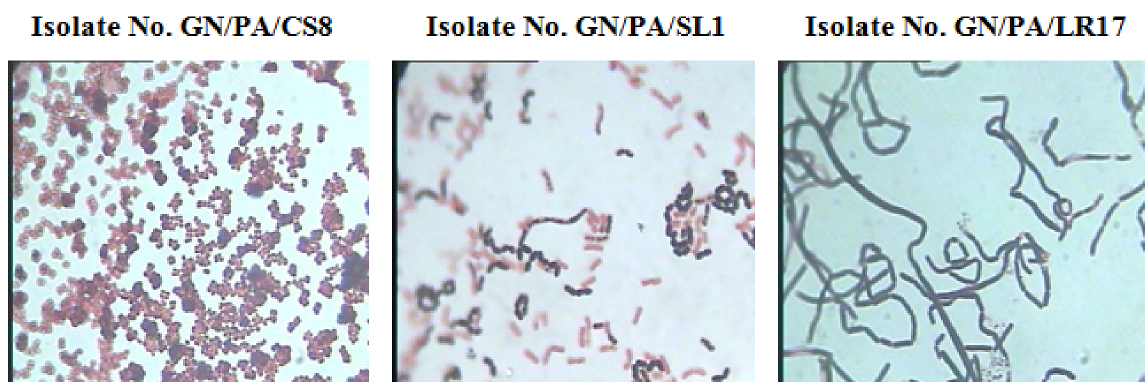
Preliminary Screening

Sudan Black B Staining: Cells were stained pink and the intracellular lipid granules were stained bluish-black contained within the cells. Figure 1 shows images of some isolates. The isolates which on the average visually showed more than half the cells containing lipid granules under the 1000 times magnification were grouped as Strongly Positive cultures. Those with almost half the cells filled with lipid were grouped as positive. Those cells with scarce lipid were weakly positive for lipid production. Completely pink cells were considered as negative.

As seen in Tables 1 and 2, following the staining procedure, a higher number of bacteria isolated from the intestines of marine fish were identified as lipid producing, constituting up to 30% of the total marine fish isolates. In comparison, the intestinal microflora of fresh water fish amounted up to 28% as lipid producers among the total fresh water fish isolates. A total number of 10 isolates from both marine and fresh water fish were seen to be strongly positive for lipid accumulation.

Percentage of Lipid Content w.r.t. Biomass: The ten shortlisted cultures were studied for the percentage of lipid they could accumulate upon culturing in nitrogen limited liquid medium. Varying quantities of lipid were obtained from the cultures under study. Five isolates showing reproducibly higher percentage of lipid against their wet biomass were selected for further GC study. Percentage of lipid produced by cell cultures of isolates is shown in Figure 3. Highest percentage, i.e., 67% of lipid was seen in the isolate from the Indian Salmon, *Polynemus heptaductylus.* Around 48% was obtained from Baby Shark, *Carcharius melanopterus.* Both these cultures may be

Figure 1: Selected Bacterial Cells as Seen Under The Oil Immersion Lens After Sudan Black B Staining Procedure



Black Colour within the Cells Indicates Lipid Deposit (Under Oil Immersion Lens with 1000X total Magnification)

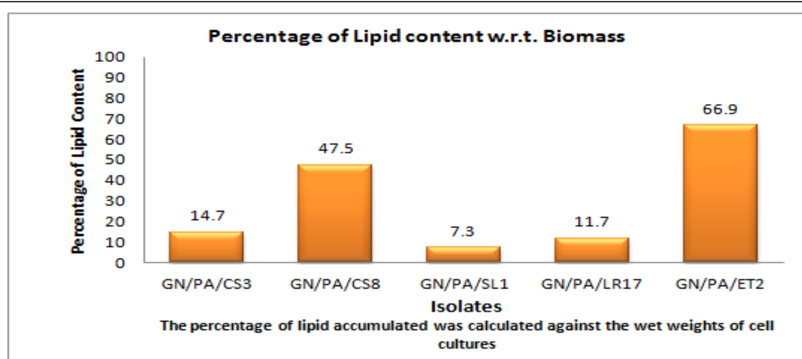
Table 1: Lipid Producing Intestinal Microflora Of Salt Water Fishes Studied From The Arabian Sea

S. No.	Sample Source		No. of phenotypic variants	No. of Lipid producers	Media used	Incubation temperature
	Scientific Name	Common Name				
1.	*Carcharius melanopterus*	Baby Shark(Konkan) Baby Shark(Mumbai) Indian Mackerel(Konkan) Indian Mackerel(Mumbai)	19	09	Zobell's Marine 2216e Agar	25°C
2.	*Rastrelliger kanagurta*	Shrimp	24	03		
3.	*Fenneropenaeus indicus*	Indo-Pacific King Mackerel	12	06		
4.	*Cybium guttatum*	Sardine(Mumbai)	09	01		
5.	*Sardinia longiceps*	Sardine(Konkan)	14	04		
6.	*Polynemus heptaductylus*	Indian Salmon	24	08		
	Total		102	31		

Table 2: Lipid Producing Intestinal Microflora Of Fresh Water Fish Obtained From Indian Rivers:

S. No.	Sample Source		No. of phenotypic variants	No. of Lipid producers	Media used	Incubation temperature
	Scientific Name	Common Name				
1.	*Labeo rohita*	Rohu(Mumbai) Rohu (West Bengal, Ganges)	34	08	Tryptone Soy Agar	25°C
2.	*Catla catla*	Catla(Mumbai) Catla(West Bengal, Ganges) Catla(West Bengal, Katwa)	30	10		
	Total		64	18		

Figure 2: Histographical representation of Percentage Lipid Content Against the Cell Biomass of Selected 5 Isolates



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promising candidates for future commercial production PUFA owing to their high lipid accumulating capability i.e. more than 20% of their biomass and can thus be considered as oleaginous microorganisms (Wynn and Ratledge, 2005).

GC Analysis: Ten shortlisted isolates were screened for PUFA producing ability by lipid extraction and derivatization for Gas Chromatography Analysis. Identification of fatty acid methyl esters derived from the hexane extracts of bacteria was based on the identity of GC retention times vis-à-vis those of standards. The lipid profiles obtained by Gas Chromatography were studied and the isolates with maximum LA, ALA were shortlisted.

Fatty acid profile in extracted total lipid from each of the five shortlisted PUFA producing cultures are shown in Table 3.

All the 5 bacteria under consideration produced 14, 15, 16 and 18 carbon Saturated Fatty Acids (SFA). Most of the corresponding Monounsaturated Fatty Acids (MUFA) were also produced of which oleic acid (18:1) was seen in all of the five isolates. Linoleic acid (18:2 n-6), an omega-6 PUFA, was produced by all the above cultures. The GC chromatogram showing the presence of LA in the lipid extracted from Isolate No.GN/PA/SL1 is shown in Figure 3. The peak matching the retention time with the methyl ester standard of Alpha Linolenic acid (18:3 n-3) an omega-3 PUFA was observed in isolate No. GN/PA/CS3 from Baby shark caught from the Arabian Sea and isolate No. GN/PA/LR17 from the fresh water carp *Labeo rohita.*

GC-MS analysis of the total lipids from the mentioned five isolates confirmed their ability to produce omega-6 (LA) and omega-3 (ALA) PUFA as indicated by GC results. The Mass spectra were matched with the National Institute of Standards And Technology Mass Spectral Reference Library (NIST08.LIB). Fragment 294 corresponding to Linoleic Acid was observed in all five isolates. The GC/MS spectrum showing the presence of LA in the lipid from isolate No. GN/PA/SL1 is seen below, culture No. GN/PA/SL1 is shown in Figure 4, was isolated from the intestine of the marine fish *Sardinia longiceps*.

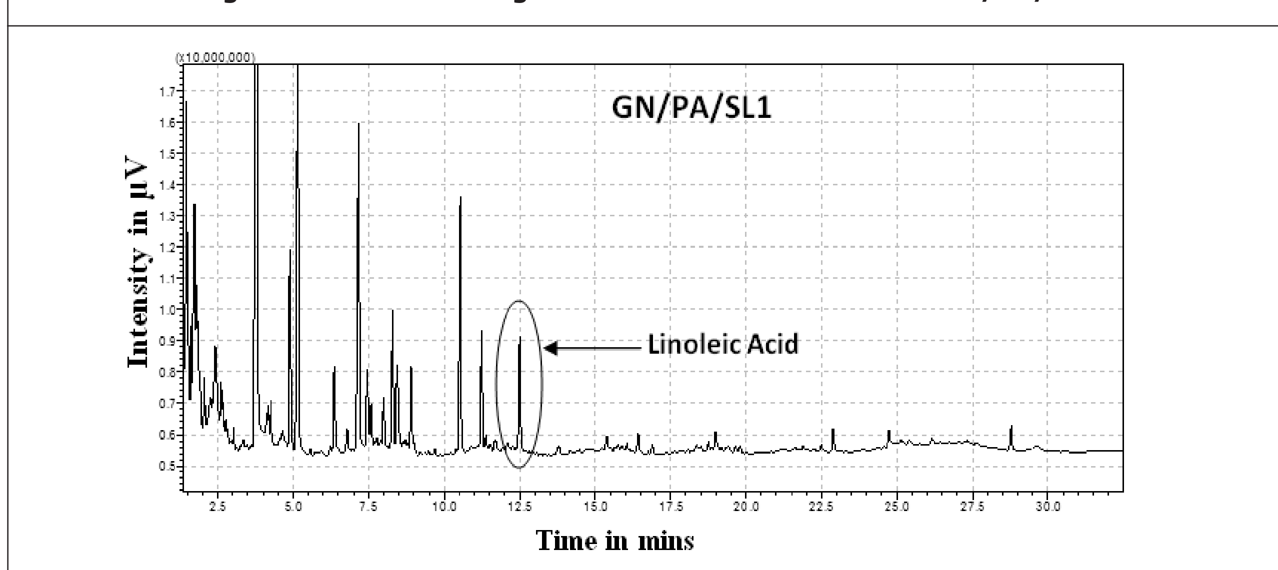
Also, the presence of Fragment 292 corresponding to Alpha Linolenic Acid in the GC/MS spectrum of lipid from isolate No. GN/PA/CS3 isolated from Baby shark and isolate No. GN/PA/LR17 isolated from the fresh water carp *Labeo rohita* established that nutritionally important PUFA like LA and ALA are produced by the isolates obtained from the fish intestinal bacterial flora. This in agreement with the reports published by Yano Yutaka (1997) and Nichols (2003); stating that these microflora are probably living in a symbiotic relationship with the fish, producing and adding to the accumulation of PUFA in the fish muscles in addition to their biosynthesis and intake from diet.

Phenotypic Characteristics (Biochemical and Physiological): A summary of the biochemical characteristics of the isolated cultures are shown in Table 4. The isolates were tested for their sugar utilization capabilities. No gas was detected during fermentation. All of the strains produced catalase and dehydrogenase enzymes. Cultures GN/PA/CS8, GN/PA/SL1 and GN/PA/LR17 were oxidase positive. All the cultures were Gram Positive and motile. Motility was tested by microscopically examining cell wet mounts. H_2S production if any was determined with Triple-Sugar Iron (TSI) agar.

Table 3: Fatty Acid Profile Of Total Lipids From Shortlisted Isolates By Gas Chromatography

Isolate	GN/PA/CS3	GN/PA/CS8	GN/PA/SL1	GN/PA/LR17	GN/PA/ET2
Sample source Fatty acids	Baby Shark	Baby Shark	Sardine	Rohu	Indian Salmon
Myristic acid (14:0)	+	+	+	+	+
Myristoleic acid (14:1)	+	-	-	+	+
Pentadecanoic acid (15:0)	+	+	+	+	+
Palmitic acid (16:0)	+	+	+	+	+
Palmitoleic acid (16:1)	-	+	+	+	-
Stearic acid (18:0)	+	+	+	+	+
Oleic acid (18:1)	+	+	+	+	+
Linoleic acid (18:2 n-6)	+	+	+	+	+
Alpha linolenic acid (18:3 n-3)	+	-	-	+	-
Arachidic acid (20:0)	-	-	+	+	+
Behenic acid (22:0)	+	-	+	-	-
Lignoceric acid (24:0)	-	-	+	-	-

Figure 3: Gas Chromatogram of FAMEs from Isolate No. GN/PA/SL1



Identification by 16S rRNA sequencing: The 16S rRNA region sequences obtained from isolates under consideration were aligned using the NCBI BLAST Search and the accession numbers generated in this study and corresponding bacterial strains are listed in Table 5. The phylogenetically (Percent Identity 97-99 %) closest species corresponding to our isolates have been listed.

Figure 4: GC-Mass Spectra of FAMEs from isolate No. GN/PA/SL1

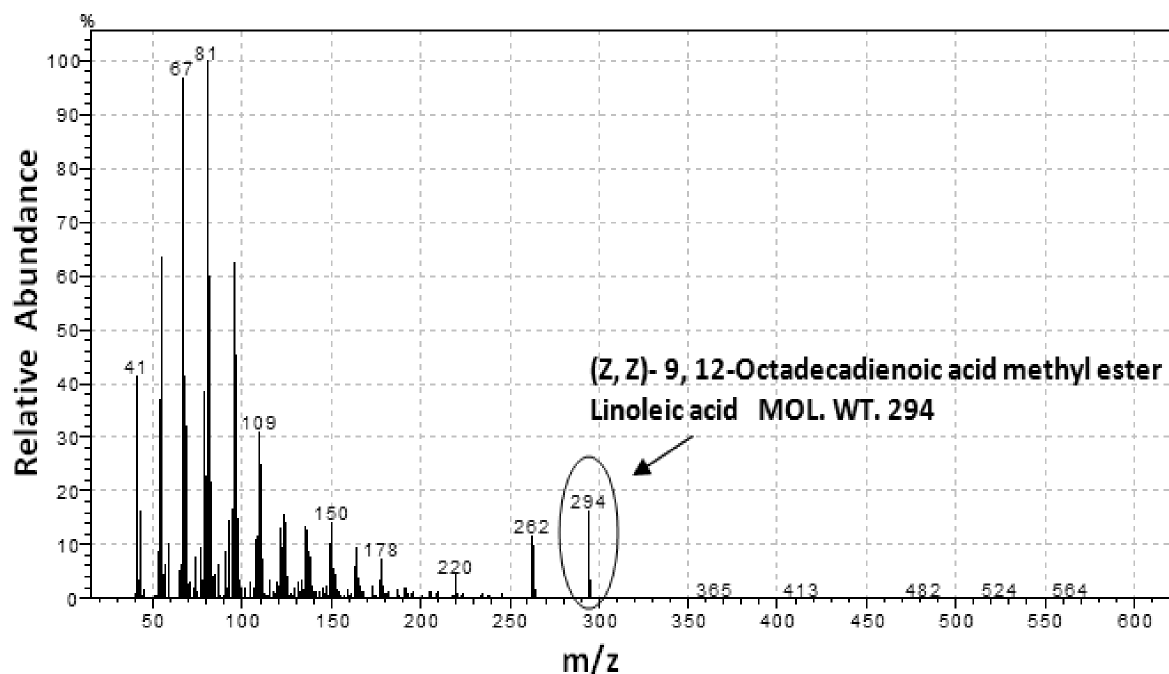


Table 4: Results for Biochemical Testing, Motility and Gram Character

Isolate	Glucose	Sucrose	Maltose	Lactose	Mannitol	Xylose	Methyl Red	Voges-Proskauer	Nitrate Reduction	Indole Production	Citrate Utilization	Triple Sugar Ion	Urease	Catalase	Oxidase	Dehydrogenase	AmmoniaProduction	Lipase	Motility	Gram Character
(GN/PA/ CS3)	-	+	-	-	-	-	-	-	W+	-	-	K/K_{H2S}	-	++	-	W+	+	++	+	+
(GN/PA/ CS8)	-	-	-	-	-	-	-	-	-	-	W+	K/K	-	+	+	W+	-	-	+	+
(GN/PA/ SL1)	+	+	-	+	+	-	-	-	-	-	+	A_G/A	+	+	W+	+	+	+	+	+
(GN/PA/ LR17)	+	-	+	-	+	-	-	-	W+	-	+	K/A	+	+	+	+	+	+	+	+
(GN/PA/ ET2)	+	+	+	-	-	-	+	+	++	-	+	$K/A_{H2S\,G}$	+	++	-	W+	+	+	+	+

Legend: + : Positive; - : Negative; ++ : Strongly Positive; W+ : Weakly Positive; A/A: Acid Slant/ Acid Butt; K/K: Alkaline Slant/ Alkaline Butt; H_2S: Hydrogen Sulphide Production; K/A: Alkaline Slant/ Acid Butt; G : Gas Production

As seen from the alignment results in Table 5, a close identity (97-99%) is seen among our isolates and the already reported bacterial species, thus these fish isolates may be close relatives of these reported bacteria. Isolate No. GN/PA/CS3 a *Microbacterium* sp. isolated from Baby Shark is noticeably distinct showing only 97% identity with the previously recognized *Microbacterium* sp. LC409 on the nBLAST database. Whereas the other four show 99% similarity with *Micrococcus* sp. and *Bacillus* sp. as enlisted in Table 5. Also, it is an interesting finding that *Bacillus endophyticus* strain SCSGAB0048 isolated in our study from a fresh water fish *Labeo rohita* is found capable of producing omega 3 and 6 PUFA. We are probably the first ones to report a *Bacillus endophyticus* species isolated from a river fish intestine to be capable of producing PUFA.

In most cases, the main source of commercially available PUFA is fish oil. However, several problems exist in terms of PUFA production from fish (Kikue Hirota *et al.*, 2005). Marine resources are unstable due to limited fishing seasons and geographic locations. In addition, cholesterol and some objectionable tastes and odors are still difficult to remove from fish oil concentrates and other animal sources. Blue backed fish have gained considerable attention in the recent past for the possibility of harboring PUFA producers in their gut. Quite a few researches in this field have reported intestinal microflora from marine fish and sponge associated microbes to be capable of producing PUFA (Okada and Morrissey, 2007; Kikue Hirota *et al.*, 2005, Patnayak and Sree, 2005). Therefore, in the current study PUFA producers were isolated from the intestines of fish. PUFA obtained from these microbes would be more desirable than fish oil for use as a food additive, supplement, or feedstock (Cheng *et al.*, 1999).

Interesting findings were obtained from our study in which oily fish were studied for possible PUFA producers. Approximately 30% of the total isolates obtained from marine fish were found to be capable of accumulating intracellular storage lipids at room temperature. Around one third of the phenotypically distinct isolates from sardine and salmon could accumulate lipid within their cells. Isolate No. GN/PA/SL1 from sardine (*Sardinia longiceps)* and isolate No. GN/PA/ET2 from the Indian Salmon (*Polynemus*

Table 5: Results for Identification by 16S rRNA Sequencing and Subsequent BLAST Search

Isolate	Morphology	16S rRNA Sequence bp Length	Reference Sequence NCBI Accession. No.	Percent Identity	Highest Similarity Hit By NCBI Blast Search
(GN/PA/CS3)	Coccobacilli	636	JN863509.1	97%	*Microbacterium* sp. LC409
(GN/PA/CS8)	Large Diplococci	828	EU308453.1	99%	*Micrococcus* sp. TA014
(GN/PA/SL1)	Thick Bacilli	860	JX867957.1	99%	*Bacillus* sp. FPZSP13
(GN/PA/LR17)	Long curved rods	830	JX315299.1	99%	*Bacillus endophyticus* strain SCSGAB0048
(GN/PA/ET2)	Large cocci in tetrads	800	GQ157872.1	99%	Uncultured bacterium clone 16slp117-1d11.w2k

heptaductylus) produced Linoleic Acid. Almost half the total types of isolates from shark as well as those from shrimp were detected to be positive for lipid accumulation. Isolate No. GN/PA/CS3 as well as GN/PA/CS8 from the baby shark, i.e., *Carcharius melanopterus* were capable of producing PUFA. This is an interesting finding as these fish have not been considered or reported earlier as a source of lipid producing microbes although sharks have been widely exploited for their PUFA rich oils for nutritional supplements (Gupta *et al.*, 2012).

Also, the fact that one third of the isolates (28%) obtained from carp intestines were lipid accumulators indicates that fresh water fish can also be a good source of lipid producing microbes. Isolate No. GN/PA/LR17 from Rohu, i.e., *Labeo rohita* a fresh water carp was seen to be capable of producing two important omega 3 and 6 PUFA upon culturing at 25°C. This is a revealing finding as much attention has not been paid on fresh water fish as sources of PUFA producers. Having fresh water mesophilic bacteria capable of producing PUFA is appealing as they can be cultured at ambient temperatures without sea salts thus cutting down on the cost of fermentation.

Work aiming to the isolation of PUFA producers from salt water and fresh water fish available in aquatic habitats along western India has not yet been reported. While most contemporary workers have focused on Poikilotherms from cold seas and oceans like polar and temperate regions (Yano Yutaka *et al.*, 1994; Nichols , 2003; Gentile *et al.*, 2003; Kikue Hirota *et al.*, 2005) we have been successful in isolating PUFA producers from both marine and fresh water fish inhabiting the Indian coastal regions falling in the tropical belt generally having higher temperatures around the year. This information counteracts the common notion that only psychrophilic and halotolerant microorganisms from the colder regions of the world are able to produce PUFA (Russell and Nichols, 1999). Our study puts forth a notable finding that even mesophilic bacteria are capable of producing PUFA, which supports the report by Yano Yutaka *et. al*. (1997) that ascertains the occurrence of PUFA producers in warm sea fish as well.

Through the encouraging findings from our study and by harnessing the available knowledge of genetics and biochemistry related to synthesis and regulation of biochemical pathways we hope to develop a fermentation process to bring down the cost of production; thus making the PUFA available to even lower strata of the society.

CONCLUSION

A total of 49 cultures were isolated from various aquatic environments and fishes. Ten isolates were selected as efficient lipid producers. Of these, five isolates were confirmed as potential PUFA producers by GCMS. Intestinal bacterial flora from Indian fish species was studied for potential PUFA producers which are probably symbiotic with the fish (Yutaka Yano, 1997; Russell and Nichols, 1999; Nichols, 2003). Bacterial strains earlier not reported for PUFA production have been found that can be used for faster, easier and economic commercial production of PUFA in future. Bacterial PUFA producers from Indian western coastal areas were sought and studied.

Genus and species level identification for the five isolates under study was carried out by traditional biochemical testing and 16S rRNA

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Sequencing. The biochemical studies of the organisms revealed valuable information about their substrate utilization and metabolic activities. Most of the findings from phenotypic characterization matched with the 16S rRNA sequencing results. Complete identification of isolates along with their strain numbers was accomplished by 16S rRNA sequencing, a modern confirmatory identification tool.

In conclusion, the research work has thrown up interesting possibility of having bacteria with PUFA producing ability that have immense commercial value which may prove to be superior to the contemporary algal and fungal microbial cultures (Russell and Nichols, 1999; Doughman *et al.*, 2007). Algae and fungi being tedious and time consuming to culture, the isolated bacteria producing PUFA will certainly be of choice due to the ease with which they can be cultured, controlled and processed. Moreover, PUFA can be produced in a shorter production time to a guaranteed quality on a year-round basis as productions do not depend on the vagaries of the climate. The results presented in this work would definitely be helpful in bringing forth novel, reliable and efficient microbial sources of PUFA that will necessarily be important in conserving the current rapidly depleting animal and plant sources.

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