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NEW MICROBIAL SOURCES OF PUFA FROM THE ARABIAN SEA AND FRESH WATER HABITATS OF INDIAN TERRITORY

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Keywords:

Sudan black B staining, Lipids, Polyunsaturated Fatty Acids, Screening for PUFA producers, Rivers and Lakes, GC, GC/MS, 16S rRNA.

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ABSTRACT: Polyunsaturated Fatty Acids (PUFA) have been shown to be of major importance in the prevention and treatment of a range of human diseases and disorders. A number of microorganisms have been shown to produce and accumulate PUFA. In the current study we have isolated 86 lipid producing microbes from various sources like a river from northern India, lakes from southern India and marine environments off the western coast of India. The isolates were shortlisted following a preliminary screening scheme by studying a set of 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. Lipid sample was extracted and derivatized for GC analysis using standard procedure. Further, GC/MS analysis confirmed that 5 isolates produce PUFA of omega-6(LA) and omega-3(ALA, ETA) type. Selected cultures have been identified by 16S r-RNA sequencing and are being optimized for PUFA production by the technique of fermentation.

INTRODUCTION: The potential for identifying and extracting novel bio-actives valuable to the medical and food industries has made marine environment attractive to researchers[1]. In the quest to identify newer and economical sources of the highly valuable polyunsaturated fatty acids (PUFA) many researchers have identified numerous psychrophilic taxa of microorganisms possessing this unusual ability [2]. Although fish oils and plants were primarily considered to be the main source of polyunsaturated fatty acids (PUFA) since the last two decades the production of PUFA by bacteria is being developed as an alternative technique [2, 3, 4, 5].

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PUFAs constitute a large group of fatty acids containing long chain carbonic molecules that include ω-3 and ω-6 fatty acids a few of which are listed here: Alpha-Linolenic Acid (ALA) 18:3 ω-3, Eicosatrienoic Acid (ETA) 20:3 ω-3, Eicosapentaenoic Acid (EPA) 20:5 ω-3, Arachidonic Acid (ARA) 20:4 ω-6, Docosahexaenoic acid (DHA) 22:6 ω-3. Some important PUFA along with their structures are depicted in **Figure 1**.

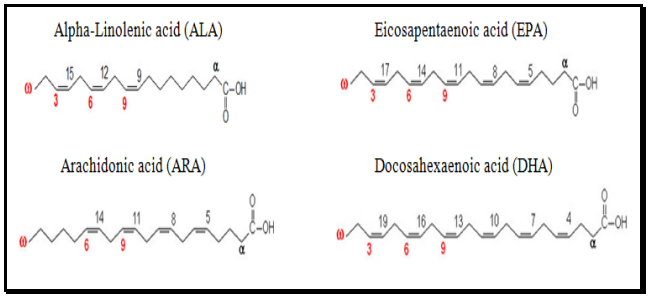


FIGURE: 1 STRUCTURES OF SOME NUTRITIONALLY AND PHYSIOLOGICALLY IMPORTANT PUFA

Omega, 'ω' is the position of the first double bond when counted from the methyl end and the number '3/6' refers to the number of carbon atoms at that position from the methyl end [1, 3].

Animals, including humans are not capable of building long chain omega fatty acids, and must acquire them from their diets. Humans and other animals obtain some DHA and intermediate products such as EPA through bioconversion of α-linolenic acid (18:3, ω-3), and some from direct consumption of DHA itself. PUFA are found in certain vegetable sources like chia, perilla, pursalane, lingoberry, olive, flax seed and hemp seed oil [6].

A variety of fish species such as herring, mackerel, sardine and salmon are even better sources of omega-3 fatty acids. Due to the many shortcomings of fish-derived oil including undesirable taste and odour, diminishing supplies, reluctance to consumption by vegetarians, its non-ecofreindly chemical processing methods like bleaching and deodorizing, and the likely presence of contaminants such as mercury, dioxins and polychlorinated biphenyls, research has been diverted towards the exploitation of other marine species for the development of suitable alternatives. Numerous organisms including bacteria, yeast, filamentous fungi, and microalgae have been reported to produce different types of polyunsaturated fatty acids[1, 2, 5, 7].

Human health benefits of PUFA have been extensively evaluated through a wide range of clinical studies. Omega-3 fatty acids have been shown to help prevent heart attack as well as decrease the overall risk of cardiovascular disease. DHA is 'physiologically essential' for brain and eye development, particularly for infants, which has led to the addition of DHA to most infant formulae and many other infant related food products.

A diet with inadequate DHA during infancy and childhood might lead to inhibited development of the brain. The effect of dietary ω-3-fatty acids on human colon cancer cells is positive, acting to hinder the escalation of the deadly carcinomas[1]. PUFA also play an important role in cell signaling and pregnancy. They help alleviate Type II diabetes and disorders like arthritis and auto immune conditions [6].

Increased recognition of the biotechnological potential of microorganisms has occurred over recent years[8]. Screening microbial flora from diverse aquatic sources has become a major thrust area of research for both industry as well as academia[7]. It is hoped that novel oleaginous organisms (microorganisms which produce more than 25% lipid on dry cell weight basis) can be tailored to produce high amounts of omega-3 fatty acids, particularly docosahexaenoic acid (DHA) and eicosapentaenoic acid (EPA) [1, 9].

Traditional sources of PUFA of plant and animal origin, offer relatively low yields and cost of production is generally high; thus a consistently available source of high quality raw material is critical for production of n-3 polyunsaturated fatty acid concentrates. A promising cost-effective alternative source of PUFA is bacterial production [2]. A number of marine microorganisms have been reported to be capable of producing PUFA. Several strains belonging to the genus *Shewanella* isolated from cold water environments have been reported as being high-level PUFA-producing microorganisms. Besides these, strains belonging to the genera *Colwellia, Photobacterium, Flexibacter, Vibrio* and *Moritella* are also PUFA-producing microorganisms [2, 4, 5, 10].

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 PUFA.

In the current research work we have aimed to isolate microorganisms capable of producing PUFA from both marine and fresh water environments. Water as well as muddy water samples from the Arabian Sea along the coastal regions of Maharashtra particularly the Konkan Strip and Kerala were studied. Owing to the few yet encouraging reports from researchers working on isolation of PUFA producers from fresh water sources; we have also studied water samples from fresh water rivers in India and Lakes in Karnataka

for the possibility of PUFA producing microbial inhabitants [4, 8, 11].

MATERIALS AND METHODS:

Sampling: Mid-sea waters and shore muddy water samples from Arabian Sea were collected from a depth of 5-10 feet below sea level along the western coast of Maharashtra, particularly from Kelve, Mumbai, Alibaug, and Konkan strip and Kerala. Fresh water samples from a cold river Pindhari (Uttarakhand) and the river Ulhas (Thana, Maharashtra) were collected for this study.

Water was sampled similarly, from fresh water lakes, namely Hebbal Lake and Ulsoor Lake, Bengaluru for isolation of lipid producers. All the water samples were collected in sterilized airtight 100cm^3 plastic screw capped bottles and stored at 0^{o}C till analysis. Sample collection was carried out in the period of August to December 2011.

Isolation of microbes: Isolation of microbes was carried out by serial dilutions of water samples in 0.85% saline and pour plate technique using Salt Water Complete Medium containing 0.5g Peptone, 0.3g Yeast Extract, 0.3 cm^3 Glycerol, 3g Agar in 75% Sea Water for marine samples. After incubation at 25^{o}C for 120h colonies were selected on the basis of differences in colony characteristics. Nutrient agar and M9 medium were used as plating media for fresh water samples. The samples from Pindhari river were incubated at 20^{o}C, owing to the year round cooler temperatures of the river. Whilst the rest of the fresh water (Ulhas River, Hebbal and Ulsoor Lakes) sample plates were incubated at 25^{o}C. All microbiological media were obtained from Himedia®, India and the chemicals were procured from Merck® Specialty chemicals limited.

Preliminary Screening for Lipid Accumulation: The isolates were shortlisted following a preliminary screening scheme by studying as set of sequential parameters like intensity of lipid accumulation by Sudan Black B staining, amount of biomass yielded on nitrogen limited semi-synthetic medium, percentage of lipid quantity against biomass. The shortlisted cultures were checked for the reproducibility of above said parameters by running respective experiments three times[12].

Induction of Lipid Accumulation: The obtained isolates were transferred to solid to induce lipid accumulation under nitrogen limitation and incubated at 25^{o}C for 120h. Lipid producers were selected by Sudan Black B staining method. The composition of the Semi synthetic minimal medium used for culturing isolates obtained from both marine and fresh waters is given in **Table.1**.

TABLE.1 COMPOSITION OF SEMI SYNTHETIC MINIMAL MEDIUM

Medium Components	Quantity for culturing Marine water isolates	Quantity for culturing Fresh water isolates
Yeast Extract	1.5g	1.5g
NH_4NO_3	0.286g	0.286g
KH_2PO_4	0.75g	0.75g
$MgSO_4$	0.4g	0.4g
$CaCl_2$	0.4g	0.4g
Glucose	40.0g	40.0g
pH	5.8	5.3
Sea Water	500.0 cm^3	0.0 cm^3
Distilled Water	500.0cm^3	1000.0 cm^3
Agar	1.5g	1.5g

Intensity of Lipid Accumulated: 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 total magnification. Sudan Black B stains the intracellular lipid granules bluish-black and the counter stain Saffranine stains cells pink. The selected isolates were cultured thrice on the above stated agar medium, stained and observed to confirm the reproducibility of lipid accumulation.

Amount of Biomass on Semi-Synthetic Medium: Isolates were cultured in 250 cm^3 Erlynmeyer flasks in the above mentioned semi-synthetic broth medium at 25^{o}C for 120 hr at 180rpm on a temperature controlled orbital shaker having 25mm stroke. 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.

Lipid extraction: Accumulated lipid was extracted by an organic solvent mixture. The lipids were extracted from the biomass in sealed conical flasks having 20 times volume of Methanol: Chloroform (2:1 v/v) by shaking overnight at 60rpm in a shaker at 25^{o}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 weights of cells[12].

Gas Chromatography: After short listing by Sudan Black B staining only strongly positive lipid accumulators capable of giving reproducible accumulators capable of giving high amount of lipid reproducibly were considered for GC analysis. Fatty acid methyl esters (FAME) were prepared from the obtained lipid by transmethylation with methanolic HCl in a water bath at 80^{o}C.

The esters were extracted in hexane and dried under nitrogen. FAME were analyzed by Shimadzu 2014 Gas Chromatograph (Shimadzu Corporation, Kyoto, Japan) equipped with Flame Ionization Detector and Rt®-2330 (Restek Corporation, USA) capillary column (30m × 0.32mm ID × 0.2µm df). Nitrogen was used as carrier gas. Initial column temperature was set at 140^{o}C which was later raised to 230^{o}C at 4^{o}C min^{-1} and final hold of 5min. The injector and detector were kept at 240^{o}C with an injection volume of 0.2 µl. Chromatographic comparison with authentic FAME standards from Supelco was used for identifying the FAME from cultures.

The relative 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[12].

GC/MS: GC/MS analysis was performed as confirmatory technique for selected PUFA producers. FAME were analyzed on Shimadzu GCMS QP 2010 ULTRA equipped a Quadrupole Mass Spectrophometer (Shimadzu Corporation, Kyoto, Japan). Rt®-2330 (Restek Corporation, USA) capillary column (30m × 0.32mm ID × 0.2µm df) was used for analysis. The oven temperature program was same as on GC analysis. Helium was used as the carrier gas at a linear velocity of 44.4 cm/sec. The ion source temperature was maintained at 200^{o}C and analysis performed in the EI (Electron Impact Ionization) Scan mode at 70eV. Scans from 40 to 600 m/z were run with an event time of 0.30 seconds and scan speed of 2000 u/sec. The GC/MS analysis of the FAME samples was performed at Shimadzu Analytical India Pvt. Ltd., Mumbai, India.

Phenotypic Characteristics: Confirmed PUFA producers were studied for their morphologies, Gram characters and motility. 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, 2013.[13]

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) 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 and 907R CCGTCAATTCMTTTRAGTTT. Cycle sequencing clean up was performed subsequently. Samples were loaded on the machine ABI 3730 Xl [12, 14]. The 16S rRNA sequencing of cultures was carried out at the National Centre for Cell Sciences, Pune, India.

The obtained 16S rRNA from isolates ranging from 800-1200 bp allowed direct comparisons with Database sequences. The programme 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 86 lipid producers were isolated from various environments from Indian western coastal region and rivers and lakes. The locations of sample collection and corresponding isolates obtained are listed in **Table 2** and **3**. Salt water samples from the Arabian Sea were collected and studied for the occurrence of lipid producing microbes[15]. These halophilic bacteria were isolated from upper sea layer having temperatures around 25°C round the year, thus the samples were processed and incubated at near ambient temperatures.

Fresh water samples were collected from the Pindhari River, Uttarakhand owing to its year round cool temperature. Water samples from Ulhas River, Maharashtra were checked for lipid producers as well. Lakes being recognized as sources of PUFA producers by a few researchers previously [8, 11], we have studied water samples from two fresh water lakes from Bengaluru, Karnataka as well; namely Hebbal Lake and Ulsoor Lake.

TABLE. 2 SALT WATER AND COASTAL MUDDY WATER SAMPLES COLLECTED FROM ARABIAN SEA.

Sr.No.	Sample source	No. of phenotypic variants	No. of Lipid producers	Media used	Incubation temperature
Salt water: Western coast of India					
1	Kelwa	08	5	Salt Water	25°C
2	Mumbai: Worli, Off Gateway of India	15	7	Complete Medium	
3	Alibaug(Kihim)	12	4		
4	Konkan Strip- Harnai (Mid Sea Water), Murud (Shore Muddy Water), Murud (Sea Water), Palande (Shore Muddy Water), Palande(Sea Water)	37	27		
5	Kerala (Cochin)	4	4		
	Total	**76**	**47**		

TABLE. 3 FRESH WATER SAMPLES COLLECTED FROM INDIAN RIVER AND LAKE SOURCES.

Sr.No.	Sample source	No. of phenotypic variants	No. of Lipid producers	Media used	Incubation temperature
Fresh Water Rivers					
1	Pindhari(Uttarakhand)	26	13	Nutrient Agar, M9 Agar	20°C
2	Ulhas(Thane, Maharashtra)	12	4	Nutrient Agar, M9 agar	25°C
Fresh Water Lakes					
1	Ulsoor (Bengaluru, Karnataka)	22	9	Nutrient Agar, M9 agar	25°C
2	Hebbal (Bengaluru, Karnataka)	23	13	Nutrient Agar, M9 agar	25°C
	Total	**83**	**39**		

Preliminary Screening:

Sudan Black B Staining: Cells were stained pink and the intracellular lipid granules were stained bluish-black contained within the cells. The images of some isolates are shown in **Fig. 2**. The isolates which 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 negative and studied no further.

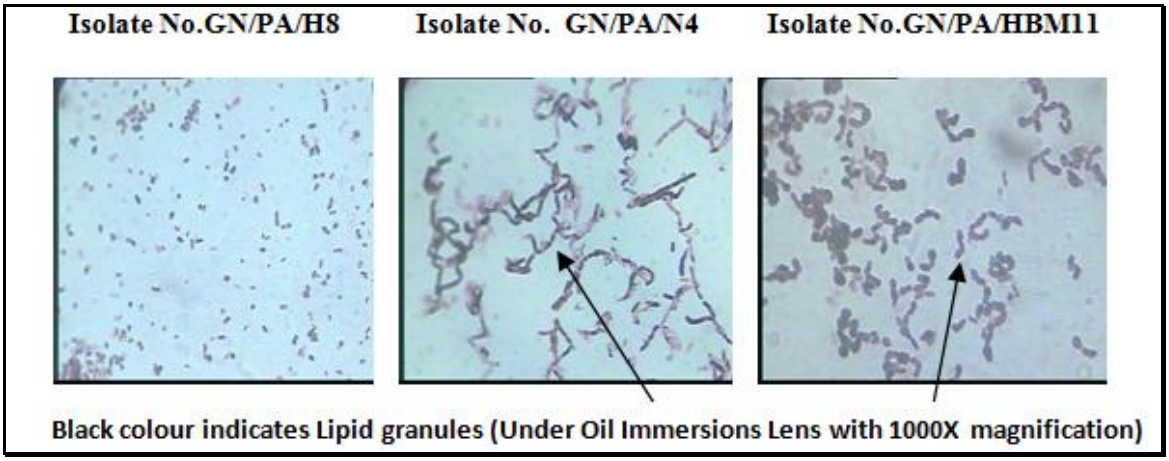


FIGURE 2: SELECTED BACTERIAL CELLS AS SEEN UNDER THE OIL IMMERSION LENS (1000X) AFTER SUDAN BLACK B STAINING PROCEDURE

As shown in the histogram in **Figure 3**, approximately 62% of the cultures isolated from the salt water samples taken from the Arabian Sea were found to be having the capacity of accumulating lipid. Water samples from the Rivers Pindhari, Uttarakhand and Ulhas, Maharashtra gave a considerable of number of lipid accumulating bacteria i.e. 45% of the total cultures obtained from these river water samples. Though, a higher percentage of lipid producers were observed in water samples from the River Pindhari as compared to the River Ulhas. Also, a notable 49% percent of the isolates obtained from the Hebbal and Ulsoor lakes in Bengaluru, Karnataka were positive for lipid accumulation. A total of 27 isolates from both marine and fresh water sources were seen to be strongly positive for lipid accumulation.

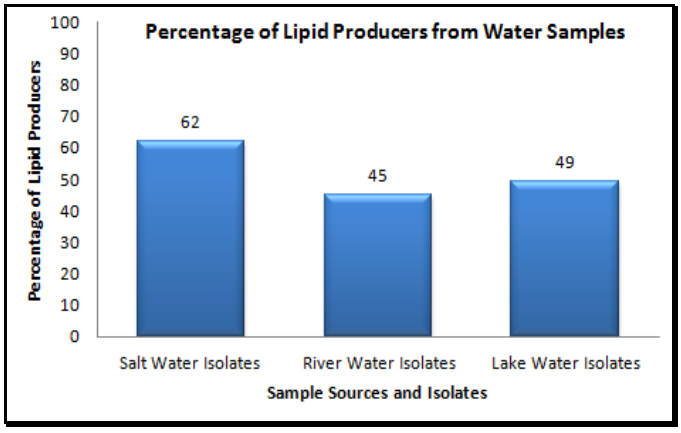


FIGURE.3 THE PERCENTAGE OF LIPID PRODUCERS ISOLATED FROM THREE AQUATIC ENVIRONMENTS

Percentage of Lipid Content w.r.t. Biomass: The 27 shortlisted cultures were studied for the percentage of lipid they could accumulate upon culturing in nitrogen limited liquid medium as described in the material and methods section. 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 5 shortlisted isolates is seen in **Figure 4.** From a total of 27 shortlisted isolates, 20 were isolated from Arabian Sea waters particularly along the Konkan strip, 04 from the fresh water River, Pindhari (Uttarakhand) and 03 from fresh water lakes in Bengaluru namely Hebbal Lake and Ulsoor Lake.

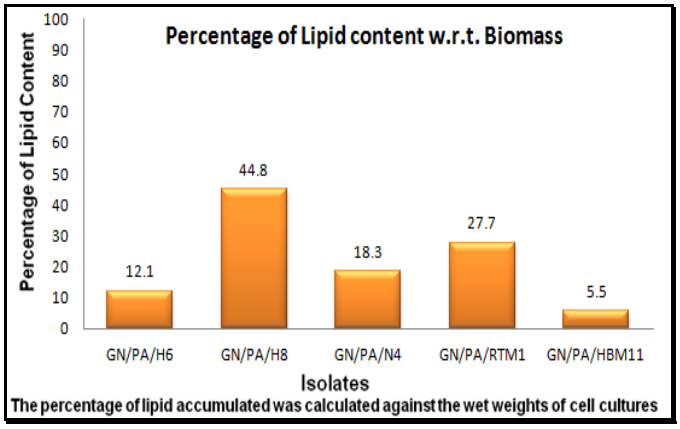


FIG 4: HISTOGRAPHICAL REPRESENTATION OF PERCENTAGE LIPID CONTENT AGAINST THE CELL BIOMASS OF SELECTED 5 ISOLATES

The 5 cultures presented in the histogram showed comparatively the highest lipid percentages as against the other isolates under study. Highest

percentage i.e 45% of lipid was seen in the isolate No. GN/PA/H8 obtained from the Arabian Sea off the Konkan Strip. Around 28% lipid content was noted for the isolate No. GN/PA/RTM1 and about 18% lipid content for isolate No. GN/PA/N4 isolated from the cold River Pindhari in northern India. Furthermore, isolate No. GN/PA/H8 and isolate No. GN/PA/RTM1 can be considered as oleaginous microorganisms due to their capacity to accumulate more than 20% of their biomass as lipid[9].

Gas Chromatography: The 27 shortlisted isolates were screened for PUFA producing ability by lipid extraction and derivatization for Gas Chromatography Analysis. Identification of fatty acid methyl esters (FAME) derived from the hexane extracts of bacteria was based on the matching of GC retention times with those of FAME standards. The lipid profiles obtained by Gas Chromatography were studied and the isolates with maximum LA, ALA were shortlisted. The results are presented in **Table 4**.

The gas chromatograms demonstrating the presence of Linoleic acid methyl ester in the FAME prepared by the lipid obtained from two isolates namely isolate No. GN/PA/H8 and GN/PA/N4 are shown in **Figure 5 and 6**. The peak corresponding to LA-methyl ester was observed in the lipid from all the five bacterial species presented in the **Figure. 4**.

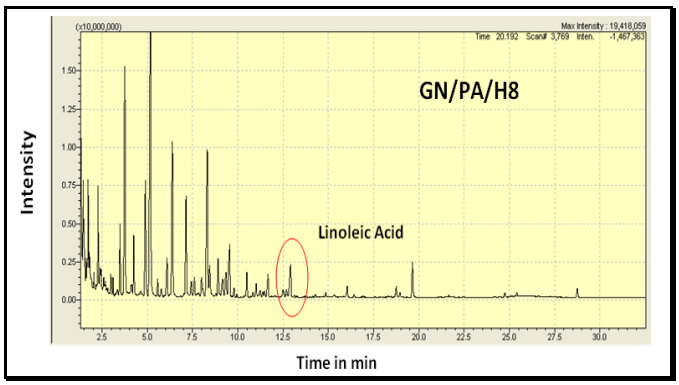


FIG.5 GAS CHROMATOGRAM OF FAME FROM ISOLATE NO. GN/PA/H8

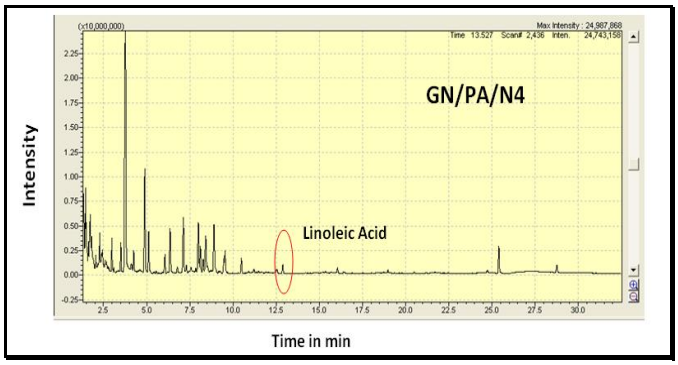


FIG. 6 GAS CHROMATOGRAM OF FAME FROM ISOLATE NO. GN/PA/N4

TABLE 4: FATTY ACID PROFILE OF TOTAL LIPIDS FROM SHORTLISTED ISOLATES BY GAS CHROMATOGRAPHY

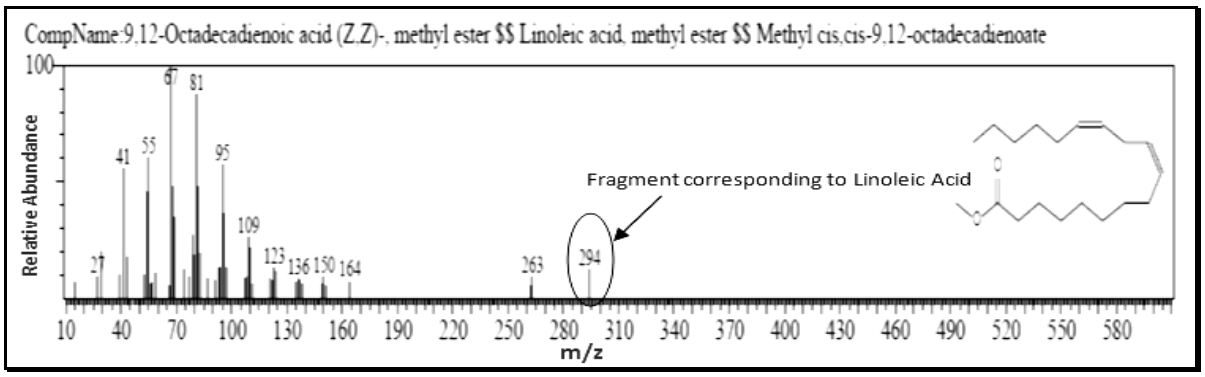
Isolates	GN/PA/ H6	GN/PA/ H8	GN/PA/ N4	GN/PA/ RTM1	GN/PA/ HBM11
Sample Source **Fatty acids**	**Konkan, Harnai**	**Konkan, Harnai**	**Pindhari River**	**Pindhari River**	**Hebbal Lake**
Myristic acid (14:0)	+	+	+	+	+
Myristoleic acid (14:1)	+	+	+	+	+
Pentadecanoic acid (15:0)	+	+	+	+	+
Palmitic acid (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)	+	+	+	-	-
Eicosatrienoic acid (20:3n-3)	-	+	-	-	-
Behenic acid (22:0)	-	+	+	-	-
Lignoceric acid (24:0)	-	+	+	-	-

Also, as can be seen in **Table 4** 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 Myristoleic acid (14:1) 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. Oleic acid (18:1) was produced by all the isolates except isolate No. GN/PA/N4. 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/H6 isolated from sea water collected offshore Harnai, Konkan,

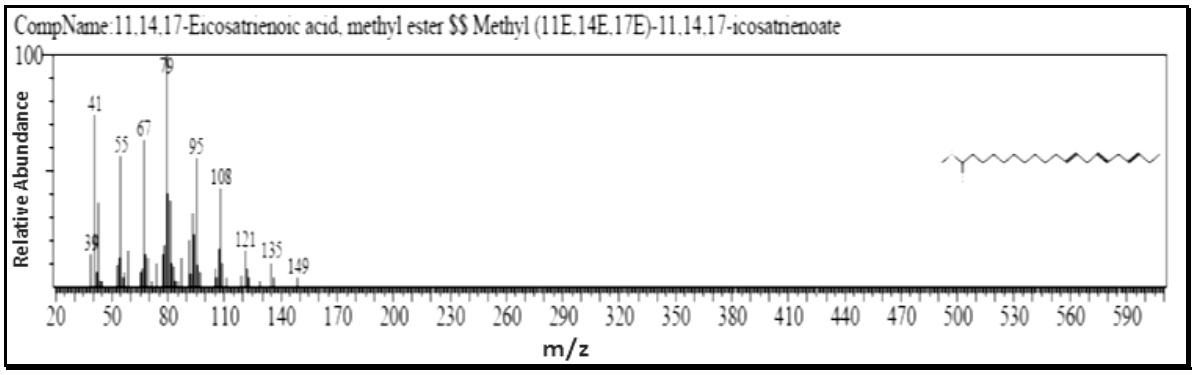
Maharashtra. Also, a rare long chain polyunsaturated fatty acid 11, 14, 17-Eicosatrienoic acid (20:3n-3) (ETA) was detected in the isolate No. GN/PA/H8 isolated from Harnai offshore.

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). The GC/MS spectra obtained for the methyl esters of the detected PUFA are shown in **Figure 7** and **8**.

Fragment 294 corresponding to Linoleic acid methyl ester was observed in all five isolates. Hence the presence of LA in all five isolates was confirmed. The GC/MS spectrum showing the presence of methyl esters of LA and ETA in the derivatized lipid from isolate No. GN/PA/H8 is shown in **Figure 7a**. Culture No. GN/PA/H8 was isolated from sea water collected offshore Harnai, Maharashtra



a) (Z,Z)-,9,12-Octadecadienoic acid methyl ester (Linoleic Acid) MOL. WT 294



b) (Z,Z,Z)-11,14,17-Eicosatrienoic acid, methyl ester

FIGURE 7: GC-MASS SPECTRA OF FAME FROM ISOLATE NO. GN/PA/H8

Figure 7b above depicts the typical fragmentation obtained for Eicosatrienoic acid methyl ester from the extracted and derivatized lipid of isolate No. GN/PA/H8, thus the presence of ETA was confirmed in the said bacterial species.

The GC-MS spectrum showing the presence of methyl ester of ALA in the extracted and derivatized lipid obtained from isolate No. GN/PA/H6 is shown in **Figure 8.**

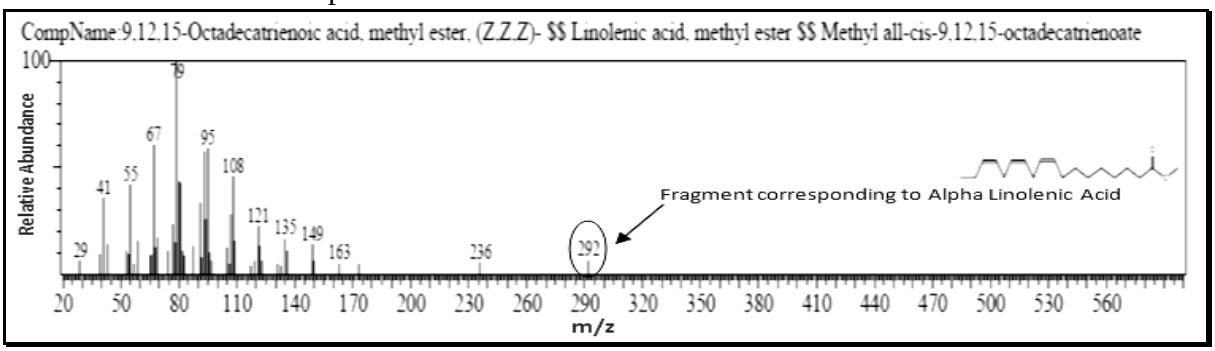


FIG. 8: GC-MASS SPECTRA OF FAME FROM ISOLATE NO. GN/PA/H6

(Z, Z, Z)-9, 12, 15-Octadecatrienoic acid, methyl ester (Alpha LinolenicAcid Mol. Wt 292

Phenotypic Characteristics: (Biochemical and Physiological): A summary of the biochemical characteristics of the isolated cultures are shown in **Table 5**. The isolates were tested for their sugar utilization capabilities. No gas was detected during fermentation. All of the strains produced catalase and dehydrogenase enzymes. Culture No. GN/PA/H6, GN/PA/H8, GN/PA/N4 and GN/PA/RTM1 were oxidase positive. All the cultures were Gram Positive except GN/PA/H8 which is Gram Negative coccobacilli. Motility was tested by microscopically examining cell wet mounts. All cultures except culture No. GN/PA/H6 were found to be motile. H_2S production if any was determined with triple-sugar iron (TSI) agar.

TABLE.5 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 Iron	Urease	Catalase	Oxidase	Dehydrogenase	Ammonia Production	Lipase	Motility	Gram Character
(GN/PA/H6)	-	-	-	-	-	-	-	-	W+	-	++	K/K	+	+	+	W+	+	-	-	+
(GN/PA/H8)	+	+	+	-	-	-	-	-	++	-	+	A/A	+	++	+	+	+	+	+	-
(GN/PA/N4)	+	+	+	-	-	-	+	-	-	-	+	K/K	-	+	+	+	+	+	+	+
(GN/PA/RTM1)	-	-	+	-	+	-	-	-	-	-	+	A/A_G	-	+	W+	+	+	+	+	+
(GN/PA/HBM11)	+	+	+	+	-	-	-	-	-	-	+	A/A	-	+	-	W+	-	++	+	+

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

Identification by 16S rRNA sequencing: The 16S rRNA region sequences obtained from isolates under consideration were aligned using the NCBI BLAST Search. The accession numbers generated in this study and corresponding bacterial Strains are listed in **Table 6**. The phylogenetically (Percent Identity 97-99 %) closest species corresponding to our isolates have been listed.

TABLE 6 RESULTS FOR IDENTIFICATION BY 16S rRNA SEQUENCING AND SUBSEQUENT BLAST SEARCH

ISOLATE	Morphology	16S rRNA Sequence bp Length	NCBI Reference Sequence Accession. No	Percent Identity	Highest Similarity Hit By NCBI Blast Search
(GN/PA/H6)	Bacilli in chains	819	JQ659884.1	99%	*Bacillus* sp. R7-532
(GN/PA/H8)	Coccobacilli	841	JF416844.1	100%	*Halomonas* sp. QY113
(GN/PA/N4)	Bacilli	838	CP003889.1	99%	*Bacillus thuringiensis* Bt407
(GN/PA/ RTM1)	Bacilli	700	JX949777.1	99%	*Bacillus* sp. TMT1-56
(GN/PA/ HBM11)	Thick Bacilli	841	KC153298.1	99%	*Bacillus* sp. CK-6

As seen from the alignment results one of our isolates, culture No. GN/PA/H8 has 100% sequence similarity with *Halomonas* sp. QY113. Also, a close identity (99%) is seen among the rest of the four isolates and the already reported bacterial species, thus these aquatic isolates may be novel variants of variants of the listed bacteria. *Halomonas* sp. QY113 was recently described at the NCBI database by Li S., Han F. and Yu W. in 2011. It is a gram negative halophilic bacterium close to the *Cobetia marina* species. *The Halomaonas* strain isolated in our study is capable of producing Linoleic acid and a rare n-3 PUFA, the 20 carbon tri-unsaturated Eicosatrienoic acid. There is so far, to our knowledge, no report on production of such a PUFA by this *Halomonas*

strain. However, a different strain of *Halomonas* isolated from the Indian Ocean was shown to be capable of producing EPA by Salunkhe D. and Bhadekar R., 2011 [16]. Furthermore, a *Bacillus pocheonensis* strain isolated in this study from Arabian Sea and is capable of producing two nutritionally relavant omega 3 and omega 6 PUFA. We are probably the first to report a *Bacillus pocheonensis* like species isolated from marine environment to be capable of producing PUFA.

Also, it is an interesting finding that a *Bacillus thuringiensis* strain isolated in our study from the cold water River Pindhari, Uttarakhand is found capable of producing PUFA. Again, we are most likely the first ones to show that a *Bacillus thuringiensis* strain isolated from an Indian fresh water river is capable of producing PUFA.

Interesting findings were obtained from our study on marine waters from Arabian Sea and fresh water from Indian rivers and lakes studied for presence of possible PUFA producers. Approximately 62% of the total isolates obtained from sea water were found to be capable of accumulating intracellular lipids at room temperature. Around 45% of the phenotypically distinct isolates obtained from the Pindhari and Ulhas River samples and 49% isolates from lakes were positive for lipid accumulation indicating a substantial prevalence of lipid producers in the fresh water habitats as well.

Isolate No. GN/PA/H6 and isolate No. GN/PA/H8 obtained from mid Arabian Sea waters produced Linoleic Acid. Also isolate no. GN/PA/H6 a *Bacillus pocheonensis* like strain was capable of also producing Alpha Linolenic acid as well. Interestingly isolate No. GN/PA/H8 a *Halomonas* strain was also shown to be producing a rarely reported omega 3 fatty acid namely Eicosatrienoic acid.

Delong and Yayanos,1986; have pointed out that PUFA are not only found in deep sea barophilic bacteria but also in *Vibrio* spp isolated from relatively shallow depths[17]. The presence of PUFA in such barely barophilic bacteria most likely represents an adaptation to low temperature rather than to high pressure. The marine bacteria isolated by us here gives further evidence in support of this hypothesis.

Almost half the total types of isolates from Pindhari River as well as those from Hebbal and Ulsoor fresh water lakes were seen to be positive for lipid accumulation. Isolate No. GN/PA/N4 as well as GN/PA/RTM1 from the cold north Indian river were capable of producing PUFA. The isolate GN/PA/HBM11 from Hebbal lake, Bengaluru was seen to be capable of producing Linoleic acid. This is an interesting finding as only a few researchers have considered or reported fresh water sources as a means for isolation of lipid producing microbes[8, 11].

Work by Shulse and Allen, 2011 has demonstrated that the biosynthetic pathways involved in PUFA production are not confined solely to a narrow group of marine bacteria, as previously believed; but that these pathways are also present in microbes found in sediments, soil and other terrestrial environments[18]. The fact that, in the present study more than 40% of the isolates obtained from fresh waters were lipid accumulators, indicates that fresh water reservoirs can also be a good source of lipid producing microbes and PUFA producers.

All the 3 fresh water isolates enlisted above were seen to be capable of producing an important omega 6 PUFA (Linoleic Acid) upon culturing at around 25^{o}C. This is a revealing finding as much attention has not been paid on fresh water fish as sources of PUFA producers. Having these fresh water mesophilic bacteria capable of producing PUFA is appealing as they can be possibly cultured at ambient temperatures without sea salts thus cutting down on the cost of fermentation.

The discovery by DeLong and Yayanos, 1986; that numerous deep-sea bacterial isolates contain substantial quantities of omega-3 PUFA, led to the speculation that polyenoic fatty acids are specifically involved in the adaptation of piezophilic bacteria to the high-pressure, low-temperature conditions prevalent in the deep-sea environment[17]. Since then, it has been confirmed that PUFA production occurs in numerous bacterial species isolated from Antarctic regions as well as temperate marine environments. Attention of many

researchers has thus been centered on cold seas and deep oceans[2, 5, 8, 19]. However, we have been successful in isolating PUFA producers from the Arabian Sea along the Indian western coast; falling in the tropical belt generally having ambient temperatures around the year. This information goes contrary to the common notion that only psychrophilic and halotolerant microorganisms from the colder regions of the world are able to produce PUFA[2, 5, 19].

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 mentioned the occurrence of PUFA producers in warmer waters as well[20]. A report demonstrating rapid isolation of PUFA producers from water sampled from Arabian Sea has been previously reported by Tilay and Annapure, 2012 [15]. Our report has also affirmed the occurrence of PUFA producing bacteria in the Arabian Sea waters adjoining Indian Territory.

Moreover, we are the first to report the isolation of PUFA producers from Pindhari River in northern India. The isolation of a *Shewanella* species from the Amazon River that is capable of producing PUFA has been discussed in a mini review by Nichols D., 2003 [4]. Our report brings to light the possibility of presence of PUFA producers in cold water rivers in India. To confirm our findings more cold water rivers in India need to be explored.

Projects by David Nichols and fellow workers in 1999, 2003 [4, 8]; have firmly established the presence of PUFA producing *Shewanella*, *Colwellia* and *Psychroflexus* spp. in fresh water rivers and lakes in Antartica and America. Scientific information on isolation of PUFA producing microflora from Indian Lakes has yet not been published by other workers. We have isolated *Bacillus* sp. CK-6 like strain producing Linoleic acid from Hebbal Lake, Bengaluru. Lakes, which exhibit a vast biodiversity in themselves, present a promising possibility of presence of microflora valuable in the field of PUFA research.

CONCLUSIONS: A total of 86 isolates were isolated from various aquatic environments in and around the Indian Territory. Twenty seven isolates were selected as efficient lipid producers. Five isolates were confirmed as potential PUFA producers by GC/MS. These screened isolates were identified by Biochemical and Molecular Characterization Techniques. The biochemical studies of the organisms revealed valuable information about their substrate utilization and metabolic activities. Complete identification of isolates along with their strain numbers was accomplished by 16S rRNA sequencing, a modern confirmatory identification tool.

Microalgae have long been considered as the only de novo source of PUFA. However, DeLong and Yayanos (1986) correctly pointed to the potential role of prokaryotic PUFA production in marine food webs, regardless of this position, the production of PUFA by bacteria is often ignored [8]. It is evident that, while the strains analyzed do not contain high levels of PUFA found in some algae and fish oils, it is quite possible that modification of culture conditions and genetic manipulations will increase these levels.

At present, only certain fish oils and algal species are utilized as industrial sources of PUFA. Bacterial sources of PUFA, however, possess distinct advantages as majority of algal species generally require strictly controlled growth conditions in terms of nutrients, light quantity and quality, oxygenation and carbon dioxide levels; these factors result in considerable production expense. In contrast, most bacteria are not fastidious, and can often be grown on the waste products of agricultural or industrial processes. Also, as bacteria can be cultured and maintained almost indefinitely, they can be considered as a more easily renewable resource for the production of PUFA [2]. In addition, the PUFA production in PUFA-producing mutants generated from parent strains can be further enhanced by changing the composition of growth media and cultural conditions to make them better candidate for use in industry [21, 22].

In the present study newer bacteria capable of producing PUFA were isolated from coastal areas, inland lakes and rivers from India. Bacteria model a cheap and reliable source of PUFA and are therefore of great interest in many industrial and health fields. This research we believe will bring up

an attractive prospect of having bacteria that and health fields. This research we believe will bring up an attractive prospect of having bacteria that may prove to be viable for industrial scale production of PUFA and which may prove to be superior to the existing algal and fungal PUFA producing microbial cultures [23]. Furthermore, a bacterial fermentation based process could provide a reliable economically attractive PUFA source for research and clinical trials intended for dietary supplementation and pharmaceutical applications in the near future. Also, PUFA derived from microbial sources shall help in contributing to the prevention of the current dwindling animal and plant derived PUFA supplies.

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