



Production of Single Cell Oils from Bacterial Sources

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Abstract

Single Cell Oils (SCO) is of profound interest for a variety of purposes ranging from biofuels to nutritional adjuvants, pharmaceutical applications and biotransformation for valuable products. A number of microorganisms have been shown to produce and accumulate SCO. In the present study a methodical attempt was made to isolate potential SCO producers from Indian water sources. Saltwater samples from the Arabian Sea and freshwater samples from an Indian cold-water river (Pindhari River, Uttarakhand) were collected and studied for the occurrence of lipid producing microbes. Of the several isolates shortlisted as lipid producers three isolates from this study were identified as potential SCO producers based on their lipid producing abilities. The types of fatty acids comprising the SCO from selected isolates were studied by Gas Chromatography (GC) and confirmed by Gas Chromatography/Mass Spectrometry (GC/MS). Lipid profiles from the GC analysis showed that the isolates in this study produced economically and nutritionally valuable Monounsaturated fatty acids (MUFA) like Palmitoleic acid and Oleic acid. Also, two isolates from the Arabian Sea were seen to produce a valuable omega-3 Polyunsaturated Fatty Acid (PUFA) like Eicosapentanoic acid. While a freshwater isolate produced Linoleic acid an omega-6 PUFA. Selected isolates were characterized for their biochemical characteristics and identified molecularly by 16S rRNA sequencing. *Ornithinibacillus* sp. Marseille-P3601 strain isolated in our study from the cold-water River Pindhari, Uttarakhand is found capable of producing PUFA.

Keywords: SCO; GC; GC/MS; MUFA; PUFA; 16S rRNA Sequencing

Abbreviations: SCO: Single Cell Oils; GC: Chromatography; MUFA: Monounsaturated Fatty Acids; PUFA: Polyunsaturated Fatty Acid; TAGs: Triacylglycerols; EPA: Eicosapentanoic Acid; AA: Arachidonic Acid; DHA: Docosahexanoic Acid; FAMES: Fatty Acid Methyl Esters.

Introduction

All living organisms produce lipids and oil for various structural and functional roles. Some classes of

microorganisms are known for producing microbial oil or single cell oil (SCO), which are storage lipids accumulated, composed majorly of triacylglycerols (TAGs) [1,2]. SCO formation has been known for over 100 years, it is only within the last 20 years or so that attempts have been made to understand the process of lipid accumulation in the oleaginous organisms [2]. SCOs, in last 2 decades have gained commercial importance for various biotechnological products ranging from food supplements, biodiesel to oleo chemicals and high value products like PUFAs [3].

Microorganisms having high lipid accumulating capability i.e., between 20%-80% of their biomass can be considered as oleaginous microorganisms [1,4]. Lipid accumulation occurs in the stationary phase, and is associated with excess of carbon coupled with limitation of nitrogen, phosphorous etc [3]. Some of the common oleaginous organisms include genus *Mortierella*, *Cryptococcus* etc [5,6].

Polyunsaturated Fatty Acids (PUFA), also known as Omega-3 fatty acids, are a family of unsaturated fatty acids that have in common a final carbon-carbon double bond in the n-3 position like α -Linolenic Acid (ALA) 18:2n-3, Eicosapentanoic Acid (EPA) 20:5n-3, Arachidonic Acid (AA) 20:2n-6, Docosahexanoic acid (DHA) 22:6n3 etc. They are known for significant they play in general well-being of an individual [7]. Being essential fatty acids PUFA are preventive and co-therapeutic for several major diseases such as coronary heart disease, atherosclerosis, cancer and Type II diabetes and disorders like arthritis, mental decline and auto immune conditions. They also reduce the clotting ability of platelets, thus potentially decreasing the incidence of heart attacks and strokes [8-10]. Oily fishes (like mackerel, salmon, striped bass, rainbow trout, halibut, tuna and sardines), vegetable sources and seed oils (like olive oil, rapeseed, chia, perilla, pursalane, lingoberry flax seed oil, linseed and hemp seed oils), are some of the rich sources of PUFAs [8,11,12].

Since, humans cannot synthesize the essential fatty acids, they need to be taken as supplements [13]. Though rich source of fats, fish oils are less preferred because of unpleasant odour, seasonal variations, contamination with heavy metal, toxins and pollutants. Moreover, worldwide fish stocks are under threat, therefore, newer microbial sources are being sought for production of SCO [6,14]. Recently, certain algal, fungal, bacterial sources including marine sources, also have been proven as rich sources of PUFA [3,15]. From a total of 27 isolates from both marine and fresh water sources that were seen to be strongly positive for lipid accumulation, we have presented results for 3 PUFA producers in this paper.

Materials and Methods

All microbiological media were obtained from Himedia®, India and the chemicals were procured from Merck® Specialty Chemicals Limited, Germany.

Sampling

Water samples were collected from 2 sources namely- Arabian Sea water from a coastal village named Pallande situated in western Maharashtra, and fresh water from a cold river Pindhari situated in Uttarakhand, India. The samples were collected in sterilized airtight 100ml plastic screw

capped bottles and stored at 0°C till further analysis.

Isolation of Microbes

Similar to the protocol followed by Patnayak and Sree, 2005 [16] for marine samples, sea water sample collected was serially diluted in saline followed by pour plating in Salt Water Complete Medium (Composition w/v: 0.5g Peptone, 0.3g Yeast Extract, 0.3 ml Glycerol, 3g Agar in 75% Sea Water) The plates were incubated at 25oC for 120h. Similarly, M9 medium and Alkaline M9 (pH adjusted to 9) was used as plating media for freshwater samples [17]. Based on morphological colony characteristics, colonies were shortlisted and transferred to master plate. The samples from Pindhari river were incubated at 20oC, owing to the year-round cooler temperatures of the river.

Preliminary Screening

For Lipid Accumulation: The isolates obtained from water samples needed to be screened for the lipid accumulation ability. Three parameters were selected for the preliminary screening-i) intensity of Sudan Black B staining, ii) biomass yield on nitrogen limited semi-synthetic medium and iii) percentage of lipid quantity against biomass [18]. *All experiments were carried out in triplicates). Sudan Black B staining method was adopted to visually see the lipid accumulation in bacterial cells [19].

Induction of Lipid Accumulation : The isolates obtained upon 24hrs of incubation on complete media [16,18] were transferred to solid Semi synthetic minimal medium; to induce lipid accumulation under nitrogen limitation and incubated at 25oC for 120h.

Intensity of Lipid Accumulated: Three isolates with most intense Sudan Black B stain under 1000X magnification, were shortlisted. The selected cultures were cultured thrice on Semi synthetic minimal agar medium, stained and observed to confirm the reproducibility of lipid accumulation [20]. Amount of Biomass on Semi-Synthetic Medium [21]: The 3 selected isolates were cultured in 250ml Erlenmeyer flasks containing 20ml liquid Semi-synthetic minimal medium at a temperature of 20oC for 120hr at 180rpm on a temperature controlled orbital shaker having 25mm stroke. Biomass was harvested by centrifugation at 8,000 g for 10 min in a cooling centrifuge. Wet weights of the cultures under study were recovered after decanting the broth.

Lipid Extraction

Accumulated lipid was extracted from the cultures under study by the modified Bligh & Dyer Method, 1959. Lipids were extracted from each culture using 20 times

weight of Methanol: Chloroform (2:1 v/v) against weight of collected biomass, and by shaking in sealed conical flasks overnight at 60rpm in a shaker at 25°C [22]. Broth was then 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 [23].

Gas Chromatography

Only 3 strong lipid accumulators capable of giving reproducible amount of SCO 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 PK, et al. [24]. 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°C which was later raised to 230°C at 4°C min⁻¹ and final hold of 5min. The injector and detector were kept at 240°C with an injection volume of 0.2 µl. Chromatographic comparison with authentic FAMES standards (Supelco™, Sigma-Aldrich®) were used for identifying the FAME from cultures. The quantities of individual fatty acids were estimated from the peak areas on the chromatogram.

GC/MS

GC-MS analysis was performed as confirmatory technique for the three selected SCO producers. FAME were analyzed on Shimadzu GCMS QP 2010 ULTRA equipped a Quadrupole Mass Spectrophotometer (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 (mentioned in section 2.5). Helium was used as the carrier gas at a linear velocity of 44.4 cm/sec. The ion source temperature was maintained at 200°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 [18].

Microbial Identification

Morphological and Biochemical Characterization: The two selected isolated were studied for their morphologies, Gram characters, motility as described in the manual

Microbiological Methods by Collins [25]. About 46 biochemical characteristics like sugar fermentation, MR-VP test, nitrate reduction, indole production, citrate utilization, TSI, ammonia production, amino acid metabolism and enzyme tests were studied using the VITEK®2 Microbial Identification System from bioMérieux, France [26].

Molecular Identification: The molecular technique of 16S r-RNA sequencing employing universal PCR primers, was used for molecular identification of the isolate. An isolated colony was used for DNA extraction using Phenol-Chloroform method for PCR Template preparation. Unincorporated PCR primers and dNTPs were removed from PCR products by using Montage PCR Clean up kit (Merck Millipore, USA). Further, the purified PCR product was sequenced by Cycle sequencing using Universal primers 27F AGAGTTTGATCMTGGCTCAG and 1492RTACGGYTACCTTGTTACGACTT. Sequencing reactions were performed using the ABI PRISM® BigDye™ Terminator Cycle Sequencing Kit with AmpliTaq® DNA polymerase (FS enzyme) from Applied Biosystems Inc., USA (Applied Biosystems Inc. © 1998) [27]. To identify the closest similarity hit to our isolates the sequenced 16S rRNA regions from the isolates ranging around 800bp were analysed using the NCBI nBLAST (nr/nt) Database [28].

Results and Discussion

A total of 27 lipid producers were isolated from the fresh water (Pindhari river, Uttarakhand) and sea water (warm Arabian Sea). Screening for PUFA producers from the marine waters from Arabian Sea and fresh water from Indian rivers and lakes were encouraging. Bacterial PUFA producers are of great interest as they are cheap and reliable sources and find applications in industrial and health segments. Thus, the current study was undertaken to isolate that may prove to be superior to the existing algal and fungal PUFA producing microbial cultures. Furthermore, a bacterial fermentation-based process could provide a reliable economically attractive PUFA source.

Preliminary Screening

Sudan Black B Staining: Sudan Black B is a slightly basic dye that interacts with the acidic groups in the lipid compounds, thereby staining the lipoproteins, phospholipids, triglycerides etc., bluish black. The counter stain saffranine stains the rest of the cell pink. Completely pink cells were considered negative and discarded. Amongst the lipid accumulators, 3 isolates (namely PM2, PM5 and AlkM3 were selected for further studies, for they demonstrated intense Sudan black B-stained cells, indicative of maximum lipid build-up (Figure 1).

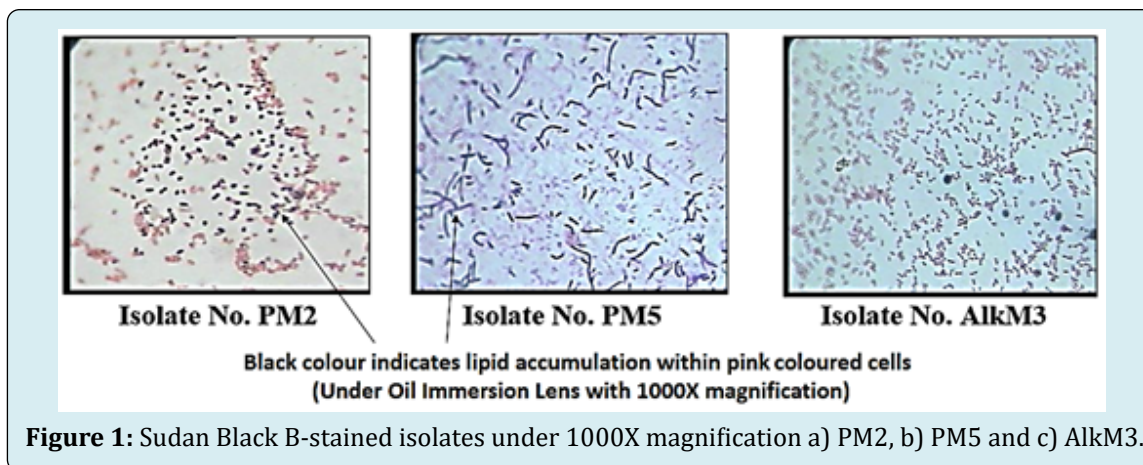


Figure 1: Sudan Black B-stained isolates under 1000X magnification a) PM2, b) PM5 and c) AlkM3.

Percentage of Lipid Content w.r.t. Biomass: In a preliminary attempt to quantitate the lipid production the cultures were cultured in nitrogen limited liquid medium as described in the section 2.3. The percentage of lipid they could accumulate was measured against their wet biomass (Table 1). When the nitrogen gets exhausted, biomass production reduces and lipid accumulation starts [29].

Method of lipid extraction is crucial and depends on the application of the lipids, especially in foods sector. The solvents used in lipid extraction should be removable /

recoverable and non-toxic. They also need to be protected from degradation by oxidation or enzymatic activities. Storage lipids (neutral fatty acids and triglycerols) are extracted with non-polar solvents like hexane and chloroform, as polarity is the key to efficient extractions. On the contrary polar solvents are used for extraction of more polar lipids like the phospholipids and glycolipids [30]. Owing to wide variations in the organisms, pre-treatment and extraction method have been adapted individually, thus several methods have been developed.

Criteria	PM2	PM5	AlkM3
Wet Biomass (mg)	771	896	490
Total Lipid (mg)	212	329	110
Percentage Lipid vs Wet Biomass	27.5	36.7	22.5
Physical Nature of Lipid	White Solid	White Solid	Brown Solid

Table 1: Biomass and Lipid, Percentage Lipid produced by cultures under study.

Cultures namely AlkM3, PM2 and PM5 were selected as they showed a comparably higher biomass and lipid percentage as against the other cultures isolated in this study. Among these three, highest percentage i.e. 27.5% of lipid was produced by the Isolate No. PM2 obtained from the Arabian Sea. Isolate no. PM2 can be considered as oleaginous microorganisms as it can accumulate more than 20% of its biomass as lipid [4]. Around 14.4% lipid content was noted for the Isolate No. PM5 and about 3.06% lipid content for isolate No. AlkM3 isolated from the cold River Pindhari in northern India. Our results are in agreement with study by Delong and Yanos, 1986, which states that PUFA are not only found in deep sea barophilic bacteria but also in *Vibrio* spp isolated from relatively shallow depths. We believe PUFA production by these strains most likely represents an adaptation of low temperature rather than the high pressure. Only a few studies reported so far, have lipid producers isolated from fresh water sources. Thus, our study supports the hypothesis

that fresh water samples also should be equally explored for isolation of lipid procedures. These fresh water mesophiles can be attractive PUFA producers as they can be cultures at ambient temperature without sea salts thus making the fermentation process more economical.

Gas Chromatography

SCO from these 3 shortlisted isolates was analyzed for identifying their fatty acids by lipid extraction and derivatization followed by Gas Chromatography Analysis. Identification of fatty acid methyl esters (FAMES) derived from the hexane extracts of bacteria was based on matching GC retention times of analyses with those of FAMES standards. Gas chromatograms of the FAMES obtained from the 3 cultures under study can be seen in Figure 2. Also, the noteworthy fatty acids identified by GC are presented in Table 2.

Isolate	PM2		PM5		AlkM3	
	F.A.	%	F.A.	%	F.A.	%
Saturated Fatty Acids	Myristic acid (14C)	54	Myristic acid (14C)	12	Myristic acid (14C)	30
	Palmitic acid (16C)	13			Palmitic acid (16C)	23.8
	Stearic acid (18C)	1			Stearic acid (18C)	2.6
Monounsaturated Fatty Acids (MUFA)	Myristoleic acid (14C:1)	10.7	Palmitoleic acid (16C:1)	15	Behenic acid (20C)	1.6
	Palmitoleic acid (16C:1)	16.2			Palmitoleic acid (16C:1)	3.6
Poly-unsaturated Fatty acids (PUFA)	Oleic acid (18C:1)	2	Eicosapentaenoic acid (20C:5)	0.2	Oleic acid (18C:1)	7.8
	Eicosapentaenoic acid (20C:5)	0.3			Linoleic acid (18C:2)	1.7

Table 2: Noteworthy fatty acids identified by Gas Chromatography (GC).

Key: F.A. = Fatty Acid, % = relative peak area percentage in GC chromatogram (%)

As can be seen from Table 2 the bacteria under consideration produced 14-20 carbon saturated fatty acids (SFA). Monounsaturated fatty acids (MUFA) were also produced of which Palmitoleic acid (16:1) was seen in all of the three isolates. Another commercially and nutritionally important MUFA Oleic acid (18:1) was seen in considerable amounts in both Isolate No. PM2 (7.0%) and Isolate No.

AlkM3 (7.8%). Linoleic acid (18:2 n-6), an omega-6 PUFA, was produced by Isolate No. AlkM3. A valuable long chain polyunsaturated fatty acid 5,8,11,14,17-Eicosapentaenoic acid (20:5n-3) (EPA) was detected the two isolates (Isolate No. PM2 and PM5) from the Arabian Sea sampled from Pallande offshore, Maharashtra India.

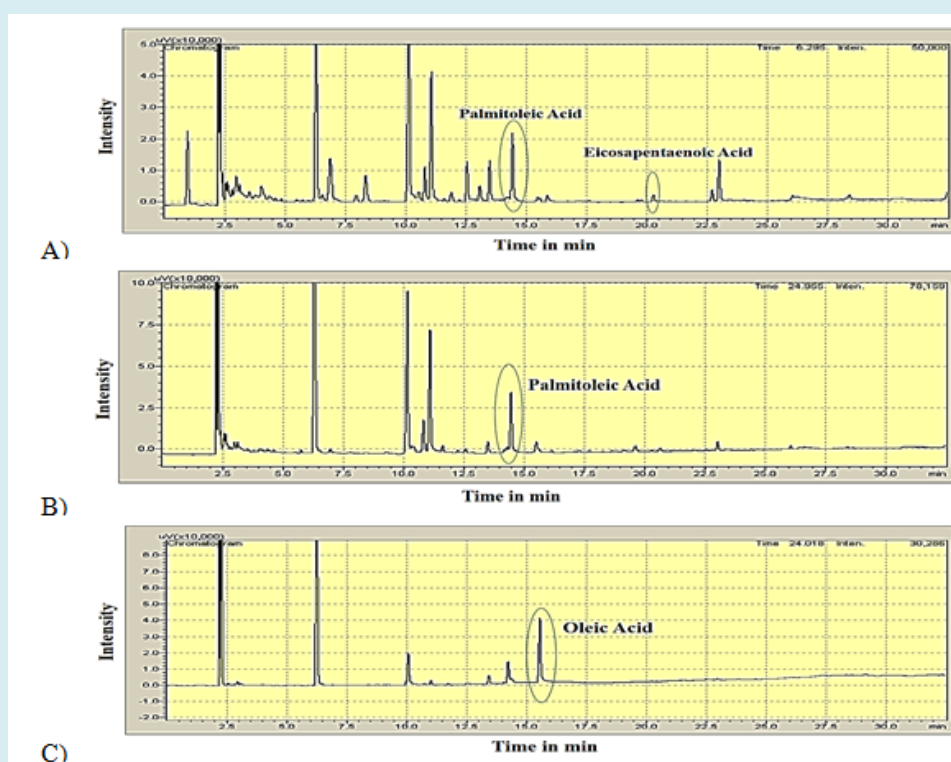
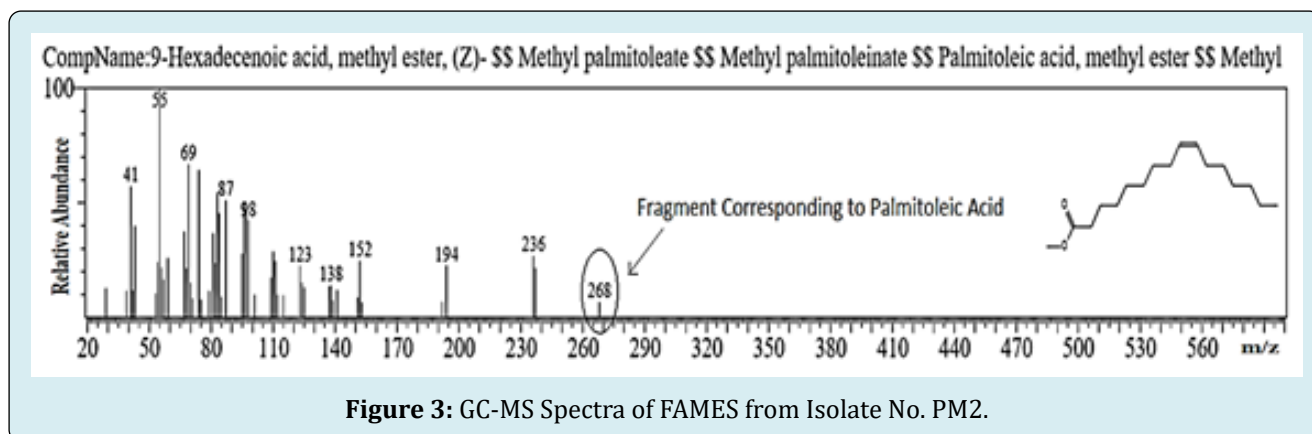


Figure 2: Gas Chromatogram of FAMES from A) Isolate No. PM2 (Salt water isolate), B) Isolate No. PM5 (Salt water isolate) and C) Isolate No. AlkM3 (Fresh water isolate).

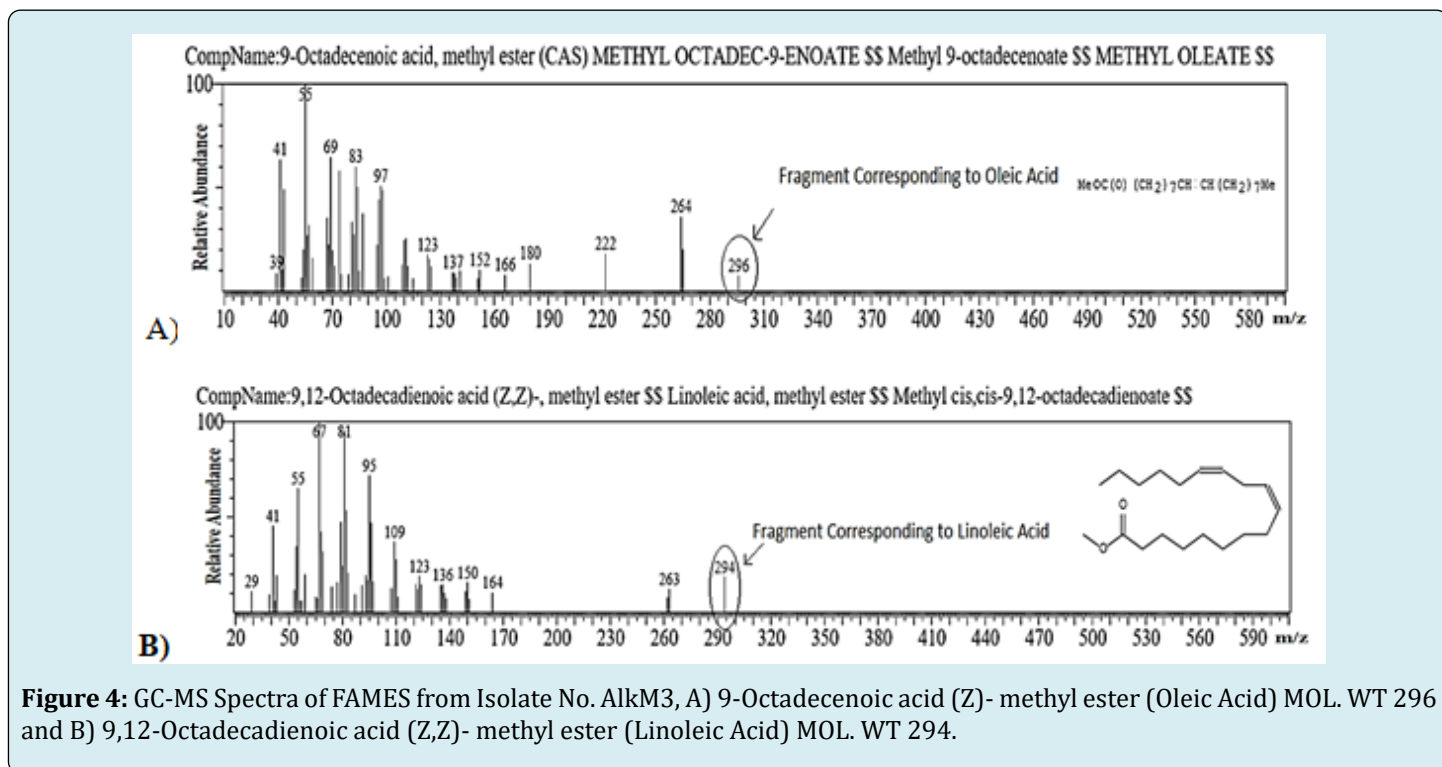
GC/MS: The results obtained from GC analysis were confirmed by GC/MS analysis of the total lipids from the isolates. Mass spectra acquired were matched with the National Institute of Standards and Technology Mass Spectral Reference Library

(NIST08.LIB). The GC-MS spectrum showing the presence of Palmitoleic acid in the lipid extracted from isolate No. PM2 is seen in Figure 3.



The GC/MS spectrum depicts the typical fragmentation obtained for Oleic Acid and Linoleic Acid from the extracted lipid extracted from Isolate No. AlkM3 is seen below in

Figure 4. Fragments 296 and 294 corresponding to oleic acid and linoleic acid, respectively were observed in Isolate No. AlkM3.



Phenotypic Characteristics: (Biochemical and Physiological): A summary of the biochemical and molecular

identification results of the cultures has been tabulated below: (Table 3).

Isolate	Morphology	Biochemical Identification	Molecular Identification by Sequencing & NCBI BLAST Search	Percent Identity by NCBI BLAST Search	16S rRNA Seq bp Length	NCBI Reference Sequence Accession. No.
Isolate No. PM2	Gram Negative thick coccobacilli	<i>Brevibacillus laterosporus</i>	<i>Achromobacter xylosoxidans</i> strain DN002	98.81%	838	CP045222.1
Isolate No. PM5	Gram Positive filamentous bacilli	<i>Unidentified Organism</i>	<i>Cobetia pacifica</i> strain GPM2 chromosome	99.88%	859	CP047970.1
Isolate No. AlkM3	Gram Positive short curved rods	<i>Sphingomonas paucimobilis</i>	<i>Ornithinibacillus</i> sp. Strain XIXJ148 16S rRNA gene partial sequence	100%	801	MH801076.1

Table 3: Biochemical and Molecular Identification results for 3 cultures under study.

One of the theories put forward by DeLong and Yayanos, 1986, suggests that the deep sea bacterial isolates produce polyenoic fatty acids that help them adapt to the high pressure, low temperature conditions existing in the oceans. Bacterial isolates from Antarctic regions and similar low-temperature marine ecosystems demonstrated higher tendencies of lipid accumulation [31-34].

Conclusion

Nowadays the main focus of single cell oil technology development is on production of long chain Polyunsaturated Fatty Acids (PUFA) with applications in human health, as nutraceuticals, pharmaceuticals and pharmaceutical precursors [3]. Bacterial PUFA producers offer a lucrative option for economical production. Also, PUFA are highly priced when obtained from natural sources and hence using bacterial systems for their commercial production may prove to be of great economic advantage. In the present study newer bacteria capable of producing PUFA were isolated from salt and fresh water sources from India. Three isolates were confirmed as potential SCO producers by GC/MS. These screened isolates were identified by Biochemical and Molecular Characterization Techniques. It is an exciting finding that a *Ornithinibacillus* sp. Marseille-P3601 strain isolated in our study from the cold-water River Pindhari, Uttarakhand is found capable of producing PUFA. We are most likely the first one to show that *Ornithinibacillus* sp. isolated from an Indian freshwater river is capable of producing PUFA. 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. It is evident that, while the strains analyzed do not contain high levels of SCO found in some algae and fish oil, they do show valuable PUFA

like EPA in their lipid, hence hold promise for future process development.

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