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Halomonas sp. nov., an EPA-Producing Mesophilic Marine Isolate from the Indian Ocean

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Abstract

Marine samples from the Indian Ocean were used to isolate and characterize the organisms with respect to their fatty acid profiles. Six mesophilic isolates (MBRI 6, MBRI 8, MBRI 9, MBRI 10, MBRI 12 and MBRI 13) were obtained from three different water samples. They were i) Gram-negative, ii) catalase positive, iii) produced acid from glucose and maltose, iv) tolerated 5 to 15% NaCl v) except MBRI 9, showed pH tolerance in the range of 5.0 to 9.0 with optimum pH 7.0 to 8.0 v) grew well at 30°C and were able to grow in the range of 15 to 45°C. EPA, an essential omega-3 fatty acid, was produced by these isolates in the range of 12 to 60% at 30°C. MBRI 12 was found to be a potential source as it produced 60% EPA. This isolate was further identified by partial 16S rDNA sequencing and phylogenetic analysis revealed that the strain belonged to *Gammaproteobacteria* and was closely related to *Halomonas bolviensis* (96% sequence similarity, 570 bp). Thus a new genus of *Halomonas* may be included in earlier reported EPA- producing prokaryotic genera affiliated to the *Gammaproteobacteria*.

Key words: *Gammaproteobacteria*, *Halomonas bolviensis*, Alpha-linoleic acid, nutraceuticals, polyunsaturated fatty acid

Introduction

Long chain polyunsaturated fatty acids (LC-PUFAs) are essential components of membrane lipids of various organisms (Berge and Barnathan, 2005). They are well documented for their beneficial physiological effects in human health including i) lowering of plasma cholesterol and triglycerols ii) prevention of certain cardiovascular diseases (atherosclerosis and thrombosis) and iii) reducing the risk of breast, colon and pancreatic cancer (Robert *et al*., 2009). Alpha-linoleic acid (ALA), ecosapenthaenoic acid (EPA) and docosahexaenoic acid (DHA) are the major LC-PUFAs of general nutritional importance, suggested by numerous nutritional bodies (Simopoulos *et al*., 1999). Although the recommended ratio of dietary omega-6 and omega-3 fatty acids is 5:1 (Sargent, 1997), it has shifted heavily towards omega-6 acids in the current western diet and by some estimate, is up to 30-fold too high (Simopoulos, 1999). The main reason being an increase in the consumption of vegetable oils rich in omega-6 fatty acids. Since omega-6 and omega-3 fatty acids are not interconvertible in the human body, the ratio of linoleic acid (LA)/ALA in our diet influences the ratio of omega-6/omega-3 fatty acids LC-PUFAs. To correct this imbalance, the needed omega-3 LC-PUFAs, can be obtained in our diet from external sources such as fish oil.

The current commercial sources of EPA and DHA are restricted to fish and algal-derived oils. Problems exist with both of these sources. Commercial fish stocks are likely to decline in the future, since the demand for fish oil in the aquaculture industry alone is estimated to exceed the supply by 2010 due to increase in global population (Meyers and Worm, 2003). Besides the concern for the presence of contaminants, such as mercury and polychlorinated biphenyls, in some fish oils, makes it evident that alternative sources of LC-PUFAS must be found (Jacobs *et al.*, 2004). Algal-derived oils require a relatively high investment in technology compared to bacterial fermentation, although bacteria contain a lower proportion of lipid (Nichols *et al*., 1996). A key advantage of bacterial PUFA production is that only a single PUFA is produced, rather than the complex mixture yielded from fish or algal oils (Russell and Nichols, 1999). Thus bacterial sources of PUFA remove the expense

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of preparative purification in the production of high-purity PUFA oils. In addition to their potential use as "cell factories" bacteria in particular offer the biotechnological opportunity to study the structure and regulation of the genes and enzymes responsible for PUFA production.

Numerous bacterial species of marine origin producing PUFAs are particularly prevalent in high-pressure, low temperature and deep-sea habitats (Yano *et al*., 1997). This is an important adaptation for countering the effects of hydrostatic pressure and low temperature on fluidity or phase of membrane lipid (Allen and Bartlett, 2002). Halophilic organisms are also known to be good sources of different bioactive compounds (Margesin and Schinner, 2001). Recently we have reported halotolerant organisms from different food samples (Jadhav *et al*., 2010a) as well as from wall scrapings of historical building in India (Jadhav *et al.*, 2010b). They were novel owing to their abilities to fix atmospheric nitrogen and produce industrially important enzymes.

Considering the significance of PUFA in human health and limitations in using fish oils, microorganisms prove the best suitable alternative. However use of psychrophilic or piezophilic organisms producing EPA, necessitates the need of low temperature and high pressure facilities (Gentile *et al*., 2003). Hence use of organisms producing these nutraceuticals at room temperature will be efficient and reasonably priced. The present work was aimed at isolation of marine organisms from the Indian Ocean and characterizing them with respect to their fatty acid profiles. Being mesophilic in nature, high PUFA producing isolates could be conveniently used for pilot scale production and for further scale-up. To our knowledge this is the first report of a high EPA- producing marine isolate from the Indian Ocean.

Experimental

Material and Methods

Sampling. Water samples were collected during summer 2008 from different locations in the Indian Ocean (Table I). All chemicals were procured from Himedia and Merck, India. All were of A.R. grade.

Isolation and characterization of microorganisms. 10 ml of each individual sample were inoculated in 100 ml Marine Salt Medium (MSM) (Composition per litre: 81.0 g NaCl, 10.0 g yeast extract, 9.6 g $MgSO_4$, 7.0 g $MgCl_2$, 5.0 g protease peptone no 3, 2.0 g KCl, 1.0 g glucose, 0.36 g $CaCl_2$, 0.06 g $NaHCO_3$ and 0.026 g NaBr with pH adjusted to 7.0±0.2) and incubated at 30°C, 120 rpm for 48 hrs. After 48 hrs, 0.1 ml of each sample was spread on MSM agar plates and incubated for another 48 hrs. The isolated colonies were maintained on MSM agar slants.

The isolates were characterized morphologically, physiologically and biochemically. Acid production was studied by using different sugars (glucose, fructose, sucrose, lactose, mannitol and maltose). They were also studied qualitatively for their ability to secrete extracellular enzymes (amylase, catalase, urease, protease, and gelatinase) (Collee *et al*., 1989).

MSM broth supplemented with various concentrations of NaCl (ranging from 1–25%) was used to examine salt-tolerance of the isolates. The cultures were incubated at 30°C, 120 rpm for 24 hrs and cell growth was determined by measuring the absorbance at 660 nm. Temperature tolerance was examined by incubating the cultures at temperatures 15°C, 30°C and 45°C for 24 hrs. Further these isolates were screened for pH tolerance in MSM broth adjusted to pH 5.0–11.0. The media at different pH were inoculated with overnight grown inoculum (10^7 cells/ml). Cell growth was determined by measuring the absorbance at 660 nm.

Preparation of fatty acid methyl esters (FAMES). 10% inoculum of all the isolates was used to inoculate MSM and incubated at 30°C in an orbital shaker (Remi, India) at 120 rpm. Cells were harvested after 24 hrs by centrifugation (Plastocraft, India) at 10,000 rpm for 12 mins. Following centrifugation, the supernatant was discarded, the cell pellet resuspended in 1.0% NaCl (w/v), and recentrifuged. Each bacterial culture tube was capped and stored at 4°C. Cells were reweighed, to which a fresh solution of the transesterification reaction mix (methanolic HCl (0.6 N) 4 ml) was added in the tubes (Carrapiso and Garcia, 2000). The tubes were capped tightly and the solutions were vortexed for 5 s to 10 s and heated in an 80°C ± 2°C water bath for 2 hrs. The tubes were then cooled quickly in ice.

Table I
Different sampling locations

Location	Latitude	Longitude	Depth (m)	pH	Temperature (°C)	Salinity	Conductivity (ms)	D.O. (mg/l)	Isolates
6NT/LT/BOT	18°48"52"N	72°46'30"E	13	8.02	30.4	71.61	974.7	3.98	MBRI 9, MBRI 10 and MBRI 12
2NT/LT/BOT	15°33.01"6"N	73°56.58"E	9.5	7.62	31.8	27.05	412.4	4.72	MBRI 8
2ST/LT/BOT	15°33.01"6"N	73°56.58"E	11	7.62	31.8	27.05	412.4	4.72	MBRI 6 and MBRI 13

The resultant FAMES were extracted twice by adding 2 volumes of hexane and then 1 volume of hexane by centrifugation at 5000 rpm for 15 mins. The upper phase of hexane layer was separated and stored for gas chromatography analysis.

Gas chromatography analysis of bacterial extracts. Analyses of the FAMES were performed with a Chemito GC 1000 equipped with a 50 m × 0.25 mm internal diameter cross-linked methyl silicone fused-silica CP – SIL 88 capillary column and flame ionization detector. Samples were injected at 100°C in the split mode. After 5 mins the oven was temperature-programmed from 100°C to 198ºC at the rate of 1.5°C min^{-1} and hold for 9 mins. Nitrogen was used as a carrier gas, and the injector and detector were maintained at 225 and 250°C respectively. Peak areas were quantified using chromatography software (IRIS 32, India).

16S rDNA sequencing. The isolate producing high amount of PUFA was used for identification by 16S rDNA sequencing. The genomic DNA was isolated as described by Ausubel *et al.* (1987). The PCR assay was performed using Applied Biosystems, model 9800 with 1.5 µl of DNA extract in a total volume of 25 µl. The PCR master mixture contained 2.5 µl of 10X PCR reaction buffer (with 1.5 M $MgCl_2$), 2.5 µl of 2 mM dNTPs, 1.25 µl of 10 pm/µl of each oligonucleotide primers 27f (5'CCAGAGTTTGATCGT GGCTCAG3'), 1488r(5'CGGTTACCTTGTTACGA CTTCACC 3'), 0.24 µl of Taq DNA polymerase and 15.76 µl of glass distilled PCR water.

Initially denaturation accomplished at 94°C for 3 min. Thirty-five cycles of amplification consisted of denaturation at 94°C for 1 min, annealing at 55°C for 1 min and extension at 72°C for 1 min. A final extension phase at 72°C for 10 min was performed. The PCR product was purified by PEG-NaCl method. The sample was mixed with 0.6 times volume PEG-NaCl [20% PEG (MW 6000), 2.5 M NaCl] and incubated for 40 min at 37°C. The precipitate was collected by centrifugation at 3,800 rpm for 28 min. The pellet was washed with 70% ethanol, air dried and sequenced using 96 well sequencing plate as per manufacturer's instructions.

The thermocycling for the sequencing reactions was performed with a 9800 PCR model (Applied Biosystems). It began with an initial denaturation at 94°C for 2 min, followed by 35 cycles of PCR consisting of denaturation at 94°C for 10 sec, annealing at 50°C for 10 sec and extension at 68°C for 4 min.

The samples were purified using standard protocols described by Applied Biosystems, Foster City, USA. To this, 10 µl of Hi-Di formamide was added and vortexed briefly. The DNA was denatured by incubating at 95°C for 3 min, kept on ice for 5–10 min and was sequenced in a 3730 DNA analyzer (Applied Biosystems) following manufacturer's instructions.

Nucleotide sequence accession number. The partial 16S rDNA sequence generated in this study was deposited in Gen Bank + EMBL + DDBJ + PDB under the accession number GU593323. Sequence was compared with the compilation of 16S rDNA genes available in the Gen Bank + EMBL + DDBJ + PDB library by BLASTN 2.2.17 searching.

Results and Discussion

Isolation and characterization. Total six bacterial isolates were isolated from the three samples of Indian Ocean (Table I). The isolates were grown and maintained on MSM. They were named as MBRI 6, MBRI 8, MBRI 9, MBRI 10, MBRI 12 and MBRI 13. All 6 MBRI isolates were characterized morphologically, biochemically and physiologically. They i) were Gram-negative ii) produced catalase, iii) produced acid from glucose and maltose, iv) tolerated 5 to 15% of salt, v) except MBRI 9, showed pH tolerance in the range of 5.0–9.0 with optimum pH of 7.0–8.0, vi) grew well at 30°C and v) were able to grow in the range of 15 to 45°C. MBRI 6, MBRI 8, MBRI 9 and MBRI 10 produced acid from fructose while MBRI 6, MBRI 8 and MBRI 13 used lactose. Only MBRI 11 was able to utilize sucrose as carbon source. MBRI 10 and MBRI 12 could grow on citrate as carbon source. MBRI 9 tolerated a wide pH range of 6.0–9.0.

Fatty acid profile. All six isolates were analysed for their fatty acids profiles (Table II). The results indicated that total saturated fatty acids, monounsaturated fatty acids and polyunsaturated fatty acids were in the range of 21 to 100%, 11 to 30% and 1 to 62% respectively. Interestingly, MBRI 10 did not show presence of unsaturated fatty acids. Linoleic acid was the only omega-6 fatty acid detected in MBRI 8, MBRI 9 and MBRI 12. It was detected in the range of 1 to 6%, while omega-3 fatty acids were ALA and EPA (0.5 to 16% and 12.0 to 60%, respectively). Arachinoidic acid and docosahexanoic acid were not detected in any of the isolates. Trans-fatty acids were also not detected in these isolates. So far, EPA has mainly been reported to occur in eukaryotes and some peizophilic or psychrophilic microorganisms (Freese *et al*., 2009). Bacteria are known to alter the composition of their phospholipid fatty acids side-chains in order to maintain appropriate membrane organization and function (Suutari and Laakso, 1994). At low temperatures or high pressures, the content of unsaturated fatty acids often increases with a concomitant decline in saturated fatty acids (Allen *et al.*, 1999). Polyunsaturated fatty acids (PUFAs), such as EPA and DHA, are particularly effective in the adjustment of membrane fluidity due to their low melting points (Hazel, 1995). Hence several EPA producing marine organisms isolated

Table II
Fatty acid profile of MBRI isolates

Fatty acids	MBRI 6	MBRI 8	MBRI 9	MBRI 10	MBRI 12	MBRI 13
C 4:0	0.2	7.7	11.6	21.9	6.8	19.3
C 6:0	0.4	2.2	2.7	8.4	2.0	7.3
C 8:0	2.2	4.5	8.3	2.5	N.D.	2.4
C 10:0	0.5	0.7	3.8	5	1.2	N.D.
C 11:0	0.7	0.8	1.2	6.1	1.4	1.1
C 12:0	0.8	1.0	N.D.	N.D.	N.D.	0.8
C 13:0	N.D.	N.D.	1.4	N.D.	N.D.	N.D.
C 14:0	18.1	34.3	8.7	14.1	1.7	4.1
C 15:0	N.D.	N.D.	N.D.	N.D.	N.D.	1.9
C 16:0	25.1	19.7	22.0	42.1	8.5	8.1
C 17:0	N.D.	0.9	0.9	N.D.	N.D.	4.8
C 18:0	7.2	11.7	0.8	N.D.	N.D.	2.5
Total Saturated Fatty acids	55.3	83.5	61.4	100	21.6	52.3
C 16:1	1.6	2.7	25.7	N.D.	12.1	10.7
C 18:1	6.9	0.9	2.7	N.D.	4.7	2.5
C 22:1	0.6	N.D.	N.D.	N.D.	N.D.	N.D.
C 17:1	2.3	11.7	1.1	N.D.	N.D.	N.D.
Total Unsaturated Fatty Acids	11.4	15.3	29.5	N.D.	16.8	13.2
C 18:2 n6t	N.D.	1.1	5.9	N.D.	1.5	N.D.
C 18:2 n6c	4.7	N.D.	0.8	N.D.	N.D.	N.D.
C 18:3 n6	15.9	N.D.	2.5	N.D.	0.7	N.D.
C 20:5 n3	12.8	N.D.	N.D.	N.D.	59.5	34.4
Total Polyunsaturated Fatty Acids	33.8	1.1	9.2	N.D.	61.7	34.4

N.D. – Not detected

were psychophilic and peizophilic from polar regions and deep sea, as mentioned earlier. However the recent isolation of mesophilic EPA-producing *Shewanella* species from a temperate estuary (Skerratt *et al*., 2002) and from shallow seawater samples (Frolova *et al*., 2005; Freese *et al.*, 2008) suggested that EPA may not be restricted to psychrophiles and piezophiles.

Figure 1 shows the comparison of % EPA produced by our isolates with that of microalgae and mesophilic bacterial cultures. Vazhappilly and Chen (1998) have reported upto 34.2% EPA in various microalgae at 25°C. However recovery of EPA from these sources is difficult along with poor yields. Freese *et al.* (2009) have documented up to 1.2% EPA in various organisms at 30°C (Fig. 1). Different sp. of *Shewanella* were reported to produce EPA up to 6.5% at 28°C (Ivanova *et al*., 2003; Ivanova *et al.*, 2004; Hirota *et al*., 2005), probably indicating that temperature sensitive enzymes were involved in biosynthesis. Thus our isolates appear novel owing to their ability to produce high EPA at 30°C. Also these studies contradict the notion that only barophilic or cold-adapted species are able to produce significant levels of PUFAs such as EPA.

Oleaginous fungi are also known to store large amounts of lipid, mainly triglycerols, and *Mortierella* spp. are noteworthy for the n-3 and n-6 PUFA contents of their stored lipid. Typically up to 40% of the fungal dry weight may be triacylglycerol in which the acyl chains are up to 15% EPA or 55% AA (Singh and Ward, 1997). Our results are comparable to these observations besides the advantage of rapid cultivation and easy recovery.

Branched chain fatty acids (BCFAs) have repeatedly been reported to promote cold adaptations (Chattopadhyay and Jagannadham, 2001). Our results showing comparatively low levels (1 to 34%) of BCFAs in all the isolates except MBRI 10 support these observations. However in some other bacteria no clear changes in BCFAs with varying growth temperature were observed (Nichols *et al.*, 2002).

Phylogeny. MBRI 12, the highest EPA producing isolate was identified using partial 16S rDNA sequencing. The sequence was deposited in EMBL+Genbank under accession number GU593323. BLAST analysis of partial 16S rDNA sequence revealed that the strain belonged to *Gammaproteobacteria* and was closely related to *Halomonas bolviensis* (96% sequence similarity, 570 bp). All so far known EPA-producing prokaryotes affiliate with only a few genera within two bacterial phyla: The *Gammaproteobacteria* (*e.g.* genera *Shewanella*, *Moritella*, *Colwellia*, *Alteromonas*,

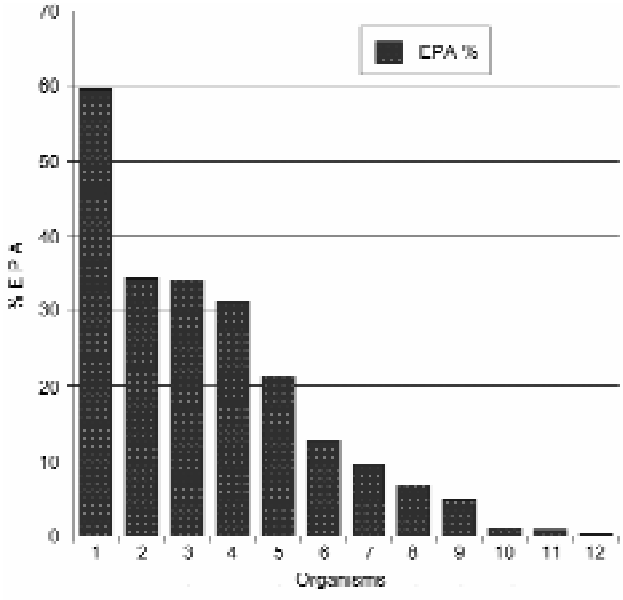

Fig. 1. Comparison of % EPA produced by MBRI isolates (this work) with other mesophilic marine bacteria and microalgae. 1. MBRI 12 ; 2. MBRI 13; 3. *Monodus subterraneus* UTEX 151, 4. *Chlorella minutissima* UTEX 2341, 5. *Phaeodactylum tricornutum* UTEX 642 (Vazhappilly and Chen, 1998); 6. MBRI 6 ; 7. *Shewanella pneumatophori* (Hirota *et al.*, 2005); 8. *Shewanella waksmanii* (Ivanova *et al.*, 2003); 9. *Shewanella pacifica* (Ivanova *et al.*, 2004); 10. *Photobacterium* sp. SAMA2, 11. *Vibrio* sp. NB73, 12. *Shewanella* sp. NB72 (Freese *et al*., 2009).

and *Photobacterium*) and the *Bacteroidetes* (*e.g Flexibacter* and *Psychroserpens*). Therefore, EPA may be a potentially useful marker for these genera in environmental microbial communities (Freese *et al*., 2009). Our results suggest that a new genus of *Halomonas* may be added to earlier reported EPA producing prokaryotic genera belonging to *Gammaproteobacteria*.

Conclusions. 1. The occurrence of high levels of EPA in MBRI isolates (present work) with MBRI 12 being highest producer of EPA which could be commercially exploited. 2. This is probably the first report of *Halomonas* sp. producing EPA at 30°C in the presence of high salt-concentration.

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