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SCREENING OF POLY UNSATURATED FATTY ACIDS (PUFA) PRODUCING BACTERIA ISOLATED FROM GUT OF FISH

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Abstract: Polyunsaturated fatty acids especially omega-3 fatty acids that plays an important role in the regulation of biological functions for prevention and treatment of a number of human diseases such as heart and inflammatory diseases. As fish oil fails to meet the increasing demand for purified fatty acids, alternative sources are being sought. *Shewanella putrifaciens* and *Pseudomonas aeruginosa* were isolated and analysed using gas chromatography-mass spectrometry of the respective fatty acid methyl ester. The fatty acids extracted showed free radical scavenging activity (0.67 µg/ml) for *Shewanella sp.,* and 0.53 µg/ml for *Pseudomonas sp.,* by using the method Total antioxidant assay.

Keywords: PUFA, omega- 3 fatty acids, Gas chromatography, Antioxidant assay.

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INTRODUCTION

Fatty acids are organic compounds formed by a hydro carbonated chain and a carboxylic group that is normally bounded with glycerol forming acylglycerides (mono-, di- or triglycerides). Depending on the nature of the hydro carbonated chain, fatty acids can be saturated or unsaturated, which in turn can be monounsaturated or polyunsaturated fatty acids (PUFA). Polyunsaturated fatty acids (PUFAs), such as eicosapentaenoic acid (20:5ω3, EPA), are important for human health. They are the precursors of many essential regulatory molecules in the body as well as being important lipid components of brain and retina cell membranes. They also help to prevent cardiovascular disease (Das, 2008; Shin et al., 2007). Many of the fatty acids can be synthesized by humans, but there is a group of PUFA, the essential fatty acids, that the human body cannot produce: omega-3 (n−3) and omega-6 (n−6) fatty acids. The parent omega-6 fatty acid is linoleic acid (C18:2n−6, LA) and the parent omega-3 fatty acid is α-linolenic acid (C18:3n−3, ALA). Omega-6 fatty acids as arachidonic acid (C20:4n−6; AA) can be synthesized by humans from LA, and omega-3 fatty acids, as eicosapentaenoic acid (C20:5n−3; EPA), docosapentaenoic acid (C22:5n−3, DPA) and docosahexaenoic acid (C22:6n−3, DHA), from ALA; however, the conversion of ALA in EPA, DPA and DHA is low and these omega-3 fattyacids are considered essential fatty acids too. Therefore, both n−3 and n−6 PUFA are entirely derived from the diet and necessary for human health. An n−6:n−3 fatty acid ratio of 5:1 or less is desired, as suggested by nutrition experts (WHO/FAO, 1994). The major source of these essential fatty acids is the consumption of oily fish such as sardines, salmon, tuna, and herring, as well as that of their processed oils. These oils, accumulated by fish, are ultimately derived from microorganisms concentrated up the food chain. One area receiving considerable interest at present is the cultivation of polyunsaturated fatty acid (PUFA)-producing microheterotrophs. Microorganisms have often been considered for the production of oils and fats as an alternative to agricultural and animal sources.

Bacterial production of long chain polyunsaturated fatty acids (LC-PUFAs) is a promising biotechnological approach for the mass production of these valuable compounds, but extensive screening is currently needed to select a strain that meets industrial requirements.

The primary source of these supplements has been fish oil. However, fish oil sources have many disadvantages: they can be contaminated by heavy metals, retain a “fishy” odour in the final product, and are processed from the world's declining fish stocks (Hinzpeter et al., 2006). Sources for the isolation of EPA-producing bacteria use either environmental samples, such as free sea water, sea ice and sea sediments or marine organisms (Bowman et al 1997; Cho and Mo, 1999; Gentile et al., 2003; Ivanova et al., 2003b; Yazawa, 1996) and the screening of specific fish such as blue-backed fish or deep sea fish, has been suggested as the most

promising approach to obtain high yields of these particular bacteria strains (Kawamoto et al., 2009; Valentine and Valentine, 2004; Yazawa, 1996). The most important natural sources of omega-3 PUFA are marine organisms (fish, seafood, algaes…), that are fed, directly or indirectly, from marine phytoplankton, the primary producer of omega-3 in the trophic chain.

Polyunsaturated fatty acids (PUFAs) may be required for renal calcium metabolism and or the regulation of the normal balance between bone and ectopic calcification (Kruger et al., 1995).Some researchers suggest that lipids serve as energy source for displacement and for the production of ectoplasmatic nets. Although DHA is preferentially used as energy source under starving conditions, the PUFAs could also protect cells from oxidative stress when nutrients are depleted, because of their antioxidant properties. It is known that many microorganisms can provide inorganic materials either intra- or extracellularly (Pancholi et al., 2013).

In this study, we isolated the fatty acids producing microbes from guts of fish and their fatty acids were extracted and analysed for their properties.

MATERIALS AND METHODS

ISOLATION AND SCREENING OF FATTY ACIDS PRODUCING MICROBES

The intestines of several fresh marine fishes were collected and their guts were excised and grinded with morter and Pestle. Then they were diluted by serial dilution technique and spreaded onto GYP medium (Glucose-1g, Yeast extract-1g, Poly Peptone-1g, aged sea water-70%, pH 7.0). Strains were incubated at 25˚C for 48hrs and then different types of colonies were isolated and streaked on new plate to obtain homogenous culture. Bacterial colonies were isolated from these plates and re-cultured on to marine and nutrient agar plates to check the purity of the strains and for fatty acids analysis. All colonies tested were submitted to Gas Chromatographic analysis to confirm the presence of fatty acids.

FATTYACIDS EXTRACTION:

Total lipids were extracted with modified Bligh and Dyer extraction method (White et al., 1979; Fang and Findlay, 1996). Approximately 5ml of liquid bacterial culture were added to a test tube filled with 20ml of methanol, dichloromethane (DCM) and phosphate buffer (2:1:0.8) extraction solution. The extraction mixture was allowed to stand overnight in darkness at 4˚C. The lipids were partitioned by adding DCM and water such that the final ratio of DCM-methanol-water was 1:1:0.9. The upper aqueous phase was discarded and lower organic phase was decanted through a filter into a test tube. The solid residue retained on the filter was washed with 3×1ml DCM. The total lipid extract was dried under a gentle stream of nitrogen and then dissolved in methanol.

ANTIOXIDANT ASSAY

Total antioxidant activity of the substances were measured by using the method followed by (Prieto et al., 1999) 7.45ml of sulphuric acid (0.6M solution), 0.992g of sodium sulphate (0.2mM solution) and 1.235g of ammonium molybdate (4mM solution) were mixed together in 250ml distilled water and labelled as Total antioxidant capacity (TAC) reagent. Distilled water was used as blank. The tubes were capped and incubated in a boiling water bath for 95˚C for 90min. Ascorbic acid was used as the reference antioxidant compound. Absorbance was measured at 695nm in a spectrophotometer.

ANALYSIS OF FATTY ACIDS METHYL ESTERS BY GAS CHROMATOGRAPHY

Total lipids extracted by using Bligh and Dyer method were subjected to GC-MS analysis. The esterified samples were applied to a Gas–liquid chromatograph (GC-17A; Shimadzu, Kyoto, Japan) equipped with a flame ionization detector and a split injector, using a TC-70 capillary column (GL Science, Tokyo, Japan) with temperature programming (190 to 220°C at 1°C/min). The fatty acid ester peaks were identified and calibrated with standard fatty acids. Data given are the averages of at least three determinations.

RESULTS

ISOLATION AND IDENTIFICATION OF PUFA PRODUCING MICROORGANISMS

The fish gut microbes were isolated and biochemically tested according to Bergey's manual and identified as *Shewanella putrefaciens, Pseudomonas aeruginosa*.

Mass scale culture of the selected strains was done at optimum temperature 30°C for 3days. From the bacterial culture fatty acids were extracted using methanol: dichloromethane: phosphate buffer solution (2:1:0.8) extraction solution. The extracted fatty acids were then subjected to Gas chromatography to estimate the composition of fatty acids (Fig1 and Fig.2). The total fatty acid composition of *Shewanella putrefaciens* was shown in Table1. and it was estimated as 30.65% , for *Pseudomonas aeruginosa* it was estimated as 11.75% Table 2. Stearic acid, Palmitic acid, Oleic acid, Linolenic acid and Alpha linolenic acid were predominant in the fatty acid extracted from *Shewanella putrifaciens* Stearic acid, Linolenic and Alpha linolenic acids were predominant in the extraction from *Pseudomonas aeruginosa.* The other fatty acids were found in least amount in both the strains.

ANTIOXIDANT ASSAY

Antioxidant of the extracted fatty acids at different concentrations were performed and compared with the ascorbic acid standard and presented in (Fig.3). The extracts were taken at

different concentrations from 100µl to 500µl and absorbances were also in increasing concentration. Free radical scavenging properties of fatty acids increased with increase in concentration of sample. When compared with stranded ascorbic acid (1.64µg/ml), the fatty acids from *Shewanella putrifaciens* showed 0.67µg/ml and *Pseudomonas aeruginosa* showed 0.53µg/ml both strains having lesser activity than stranded.

DISCUSSION

Polyunsaturated fatty acids, particularly 16- to 22-carbon PUFAs, have become high profile in the biomedical and nutraceutical areas because of their specific therapeutic roles in certain clinical conditions. Besides pharmaceutical applications, public awareness on eating healthy has also brought these PUFAs to the attention of the consumer.

Fatty acids were abundant in most of the microorganisms. It has been reported earlier that many bacteria and fungi can able to produce polyunsaturated fatty acids (Sivvaswamy et al., 2010). In the present study, ten bacteria were isolated from fish guts and two were screened and shortlisted for further studies. The growth conditions were kept uniform to minimize the variation in composition of fatty acids. Gram-negative marine bacteria, particularly those found in the gut flora of fish in deep, low temperature waters, produce fatty acids using similar de novo biosynthetic pathways, such as fatty acid synthase pathways for the synthesis of monoenoic fatty acids (Valentine and Valentine, 2004; Watanabe et al., 1997) and polyketide synthase pathways for the synthesis of PUFAs (Kawamoto et al., 2009; Ratledge, 2001; Russell and Nichols, 1999; Valentine and Valentine, 2004).

For the two strains examined greatest recovery of fatty acids were obtained by using modified Bligh and Dyer method. The GC-MS analysis of the fatty acid extract revealed the presence of 9 fatty acids accounting for 30% for *Shewanella putrifaciens* and 11% for *Pseudomonas aeruginosa* of the total extract composition. However, mechanisms of fatty acids biosynthesis vary between different bacterial species.

The consumption of foodstuffs rich in antioxidants provides protection against cancer, cardio and cerebrovascular diseases (Mehta, 2013). This protection can be explained by the capacity of these active compounds to scavenge free radicals, which are responsible for the oxidative damage of lipids, proteins and nucleic acids (Aruoma et al., 1988 and Ramadan et al., 2003). In the present study, we have attempted to rank the antioxidant assay by using Total Antioxidant Assay by (Prieto et al.,1999) the fatty acids extracted were subjected to TAA assay showed free radical scavenging activity lesser than the stranded ascorbic acid. Thus, the fatty acids extracted from gut microbes exhibit anticarcinogenic activity implicates its potential application.

CONCLUSION

Microorganisms from fish microflora can able to synthesis many of the essential fatty acids that the human body cannot synthesis. At present, most of the studies focus on the better production of poly unsaturated fatty acids from microorganisms. After analysing the fatty acids composition it includes several major fatty acids and may be concluded that it has moderate antioxidant activity, which needed the bioactivity guided purification to separate the active compound. Application of genetically modified organisms may be another efficient approach to improve the production of PUFAs.

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TABLES AND FIGURES

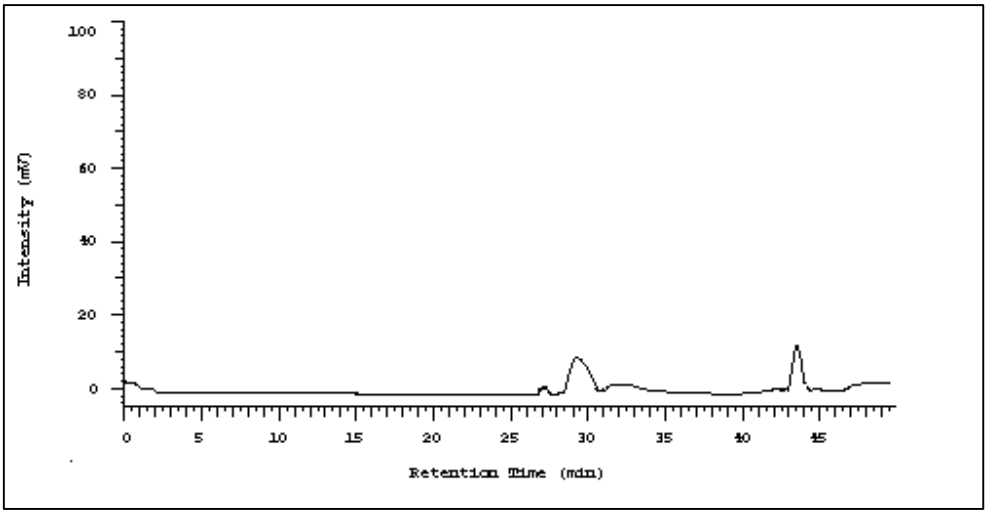
Table. 1 Fatty acids profile for the strain *Shewanella putrifaciens*

Name of the Fatty acids	Percentage
Palmitic acid (16:0)	5.7151
Margaric acid (17:0)	0.2334
Stearic acid (18:0)	7.5296
Oleic acid (18:1)	6.7112
Linolenic acid (18:2)	5.6154
Alpha linolenic acid (18:3)	4.6621
Monoctic acid (18:4)	1.4672
Eicosapentanoic acid (20:5)	0.0098
Docosahexaconic acid (22: 6)	0.0121

Table 2: Fatty acids profile for the strain *Pseudomonas aeruginosa*

Name of the fatty acids	Percentage
Palmitic acid (16:0)	1.5125
Margaric acid (17:0)	0.4272
Stearic acid (18:0)	3.1974
Oleic acid (18:1)	1.2396
Linolenic acid (18:2)	3.4105
Alpha linolenic acid (18:3)	2.0672
Monoctic acid (18:4)	0.5154
Eicosapentanoic acid (20:5)	In traces
Docosahexaconic acid (22: 6)	In traces

Fig.1.GC-MS analysis of fatty acids for the strain *Shewanella putrifaciens*



COMPONENT NAME	R.T	AREA	AREA%
EPA	30.85	1124	92.68
DHA	43.6	89.3	7.32
		1213.3	100

Fig.2 :GC-MS analysis for the strain *Pseudomonas aeruginosa*

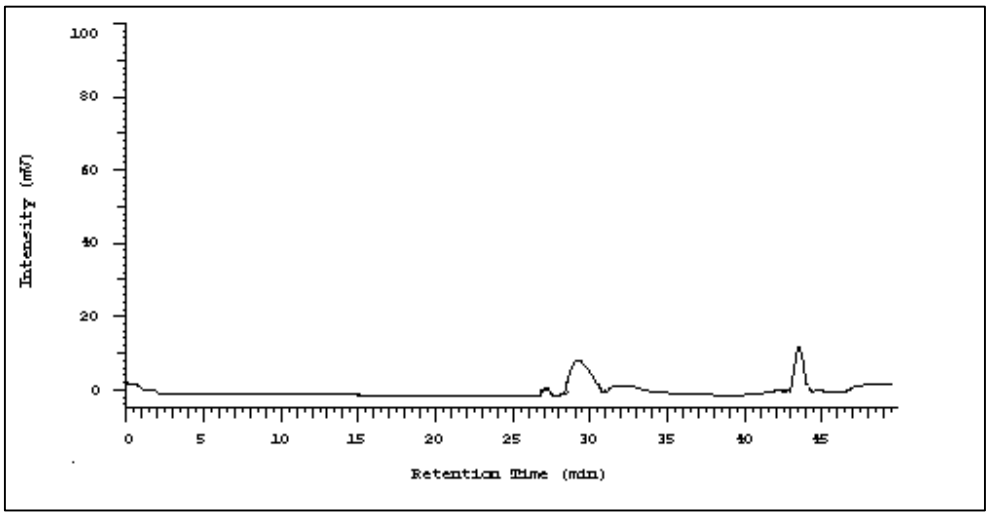


Fig.2. GC-MS analysis of fattyacids for the strain *Pseudomonas aeruginos*

Component Name	R.T	Area	Area%
EPA	30.88	1109	92.61
DHA	43.7	89.5	7.39
		1198.5	100

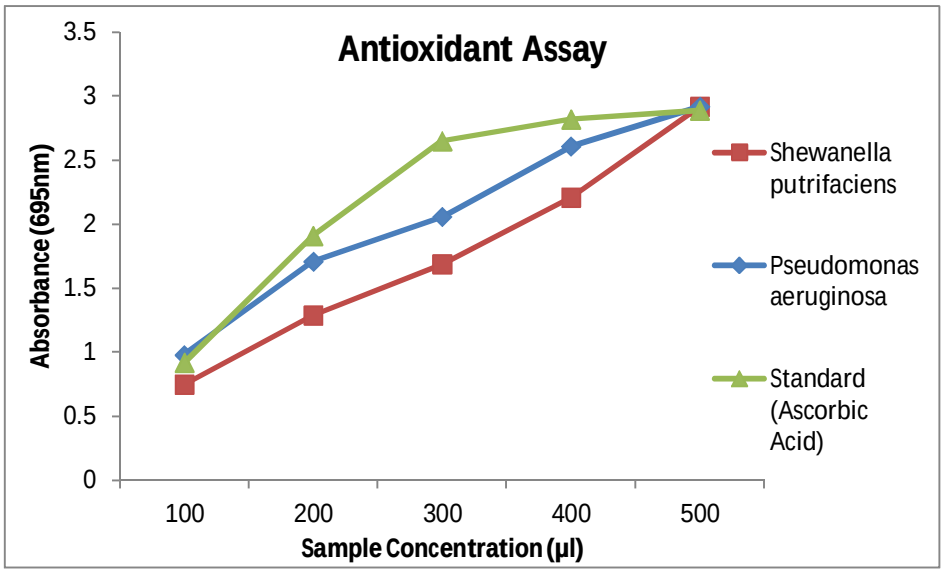


Fig.3: Antioxidant assay for the fatty acids producing strains using TAA assay

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