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Production, characterization and benefits of polyunsaturated fatty acids (PUFAs) derived from halophilic bacteria and their comparison with fish and plant derived PUFA

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Abstract
With the increase in health awareness, the demand for Polyunsaturated Fatty acids (PUFAs) production from fish oil and plants become a necessity. PUFAs play a beneficial role in brain and heart functions, their consumption in the diet is very good for human health. As PUFAs are essential fatty acids, humans can't synthesize them on their own and hence they obtain them from their diet. PUFAs are long-chain hydrocarbons with more than one double bond with a carboxyl group at one end and a methyl group at the other. With the demand for low cost, feasible and no risk production of PUFAs along with the dilemma of fish oil contamination and prevention of plant and marine life exploitation, the focus is concentrated towards halophiles. Halophilic microorganisms live in high saline conditions. They can tolerate salt concentration from 1.7% (0.3M) to 30% (5.1M). Halophiles are efficient in producing large scale PUFAs. The shift from fish-derived PUFAs to microbial PUFAs can become a revolutionary and completely sustainable idea along with solving the issue of fish-derived PUFAs which is faced by most vegans. In this article, we will discuss the production, characterization and benefits of PUFAs like Eicosapentaenoic acids (EPA) and Docosahexaenoic acid (DHA) which are isolated from halophilic bacteria and their comparison with fish and plant-derived PUFAs.

Keywords: PUFA, halophiles, EPA, DHA, omega-3

Introduction

Polyunsaturated fatty acids (PUFAs) are long-chain hydrocarbons with one or more double bonds. PUFAs generally come under essential fatty acids which means they are needed by humans as a dietary source. Two main Omega-3 fatty acids which are widely consumed across the world are EPA and DHA. The abbreviated forms will be discussed from now onwards, their extendable names are already discussed in the abstract. EPA and DHA are much beneficial for human health as they can prevent cardiovascular diseases, diabetes, breast cancer, brain hemorrhage, Alzheimer's disease, bowel inflammation and allergic diseases. The major consumption of EPA and DHA across the world is totally dependent on Fish oil and plants derivatives. But the problem here arises with vegans. Vegans often find consumption problems with fish-derived omega-3 varieties. Similarly using plants parts for PUFAs production are nowadays a serious environmental concern. Thus to overcome this problem, nature has endowed us with a unique life form of marine bodies, namely halophiles. Halophiles as the name suggest, are microorganisms that are residents of high saline conditions. Halophilic microbes can tolerate salt concentration from 1.7% to 30% and hence classified accordingly to the degree of salt requirement by them, blessed with the name halotolerant. These are classified as slight, moderate and extreme halophiles. PUFAs production from halophiles is now becoming a major highlighted research area by many researchers and the industrial world.

EPA and DHA have major health benefits and applications. They are widely used in fish culturing and feeding in domestic purposes, cattle and chick feeding, effective ingredients in drug designing in pharma industries, cosmetic industries, vegetable oil, biofuel etc. DHA is important as nutraceuticals in retina and nervous system development whereas studies found that pure EPA consumption can effectively decrease the triglycerides level in patients suffering from hypertriglyceridemia. These two beneficial PUFAs are precursors to many lipid hormones like eicosanoids and prostaglandins. This article will discuss the production, characterization and applications of PUFAs like Eicosapentaenoic acids (EPA) and Docosahexaenoic acid (DHA) which are isolated from halophiles and their comparison with fish and plant-derived PUFAs.

EPA and DHA Production from Halophilic Bacteria

The major contribution to PUFA production is provided by the class Gammaproteobacteria. This halophilic class includes several members of the family *Shewanella*, *Photobacterium*, *Moritella* etc. in addition to *Flexibacter* and *Psychroserpenes* species. (Moi *et al*., 2018) [11].

PUFAs biosynthesis by halophiles was studied deeply and curiosity arises by some researchers that why PUFAs is a prerequisite for marine habitat? It is explained that halophilic bacteria which are living deep inside the ocean about 1000-2000 km in-depth with very low temperature and high pressure often needs PUFA in their cell membrane, mostly by Psychrophilic (Psychrotropic + Piezophilic) group (Yoshida *et al*., 2016) [24]. These bulk density fatty acids in their cell

membrane acts as insulators from cold shocks and high pressure. Several articles mentioned that EPA is a prerequisite for survival under cold and high-pressure conditions but some have denied it. For eg; the strain *Shewanella livingstonensis* Ac10 at 4°C produces a large amount of EPA, at this temperature it has been observed that EPA becomes 5% of the total fatty acids in their cell membrane. Similarly, the necessity of EPA was proven by taking a mutant strain of Ac10 and its wild type strain and grow it under 4°C. It was shown that the mutant strain was unable to survive at this temperature but the wild type Ac10 was able to survive (Kawamoto *et al*., 2009) [10]. Similarly, it was proved by *Shewanella piezotolerans* WP3which was also a psychotolerant and piezotolerant bacterium isolated from the Pacific Ocean, it was seen that EPA deficient strains are unable to grow between 0.1 MPa- 20MPa and 4°-20°C (Wang *et al*., 2004) [22] (Usui *et al*., 2012) [21]. But as discussed previously, some articles denied that EPA is not required as a prerequisite component for marine microbes. Mutant *Shewanella marinintesina* IK-1 deficient in EPA can grow in deep oceanic vents (Yoshida *et al*., 2016) [24]. Similarly, *Photobacterium profundum* SS9 mutant strain can grow at lower temperatures with the alternative of MUFA in them.

Table 1: List of highest EPA producing halophiles shown.

Bacteria	Type of PUFA	PUFA %	Origin	Reference
Shewanella pneumatohori SCRC- 2738	EPA	36.6	Pacific mackerel	(Yazawa, 1996) [23]
Shewanella hanedai ATCC 33224	EPA	22.2	Arctic Ocean	(J P Bowman *et al*., 1997) [3]
Shewanella benthica ATCC 43992	EPA	16.0	Intestine, holothurians	(J P Bowman *et al*., 1997) [3]
Shewanella gelidimarina ACAM 456	EPA	16.0	Antarctica	(J P Bowman *et al*., 1997) [3]
Vibrio sp. strain 29-1	EPA	19.7	Deep Sea Sediment	(Hamamoto *et al*., 1995) [8]
Vibrio sp. strain 814-4	EPA	17.9	Deep Sea Sediment	(Hamamoto *et al*., 1995) [8]
Photobacterium profundum SAMA2	EPA	15.0	Tidal Flat sediment at Wadden	(Freese *et al*., 2009) [6]
Photobacterium Profundum DSJ4	EPA	13.0	Deep sea sediment	(Y Nogi *et al*., 1998) [12]
Vibrio sp. strain 5710	DHA	22.7	Deep sea sediment	(Hamamoto *et al*., 1995) [8]
Vibrio sp. strain 5705	DHA	21.5	Deep sea sediment	(Hamamoto *et al*., 1995) [8]
Vibrio sp. strain 5703	DHA	18.6	Deep sea sediment	(Hamamoto *et al*., 1995) [8]
Moritella marina MP-1	DHA	12	Deep Sea	(Yuichi Nogi *et al*., 1998) [13]
Colwellia *psychrerythraea* ACAM 550	DHA	8.0	Antarctica	(John P Bowman *et al*., 1998) [4]

PUFA Biosynthesis in Halophiles and Genes Important for PUFA Production

In halophiles, the biosynthesis of PUFA is dependent on a key enzyme known as Polyketide synthase (PKS). The PKS biosynthetic pathway has four major repeating steps for PUFA production. It begins with condensation followed by reduction, dehydration, activation and again reduction (Gong *et al*., 2014) [7] (Ratledge, 2004) [15]. These repeated bioprocessing steps form EPA and DHA. The PKS biosynthetic pathway for EPA and DHA production is illustrated in Fig.1.The PKS pathway is different from the desaturase/elongase pathway. The desaturase/elongase pathway use δ-4 and δ-5 desaturase and elongase in which desaturation by desaturase enzymes of oleic acid occurs followed by elongation by elongase enzyme of stearidonic acid occur which is followed by multiple desaturation and elongation to form EPA and DHA. (Sakuradani *et al*., 2013) [16] The PUFA producing genes are identified in many halophilic bacteria and it was found that there are a total of five PUFA coding genes; PfaA to PfaE. These genes are compared in four bacteria namely; *Photobacterium profundum* SS9, *Shewanella pneumatophori* SSRC-2738, *Moritella marina* MP-1, *Pseudoalteromonas sp.* DS-12. These five genes shared mostly common protein expressions but differ in domains number. These genes code acyl carrier protein (ACP), ketoacyl reductase (KR), ketoacyl synthase (KR), acetyl transferase (AT), dehydratase (DH), Enoyl reductase (ER), phosphopantetheinyl (PT). The different number of domains for PUFA producing gene clusters in these four halophilic bacteria is shown in the following table 2 (Orikasa *et al*., 2006) [14] (Yoshida *et al*., 2016) [24] (Shulse & Allen, 2011) [18] (Yoshida *et al*., 2016) [24] (Allen & Bartlett, 2002) [1] (Gong *et al*., 2014) [7] (Cao & Cao, 2012) [5] (Tanaka *et al*., 1999) [19] (Yazawa, 1996) [23]

Table 2: Different number of domains for PUFA producing gene clusters in these four halophilic bacteria is shown.

Genes Bacteria	PfaA	PfaB	PfaC	PfaD	PfaE
Phobacterium Profundum SS9	5ACP+KR+ KS+AT	AT	2KS+3DH	ER	Absent
Shewanella Pneumatophori SCRC-2738	5ACP+KR+ KS+AT	AT	2KS+3DH	ER	PT
Moritella marina MP-1	5ACP+KR+ KS+AT	KS+AT+DH	2KS+DH	ER	PT
Pseudoalteromonas sp. DS-12	5ACP+KR+ KS+AT	2KS	3DH+ PT	ER	PT

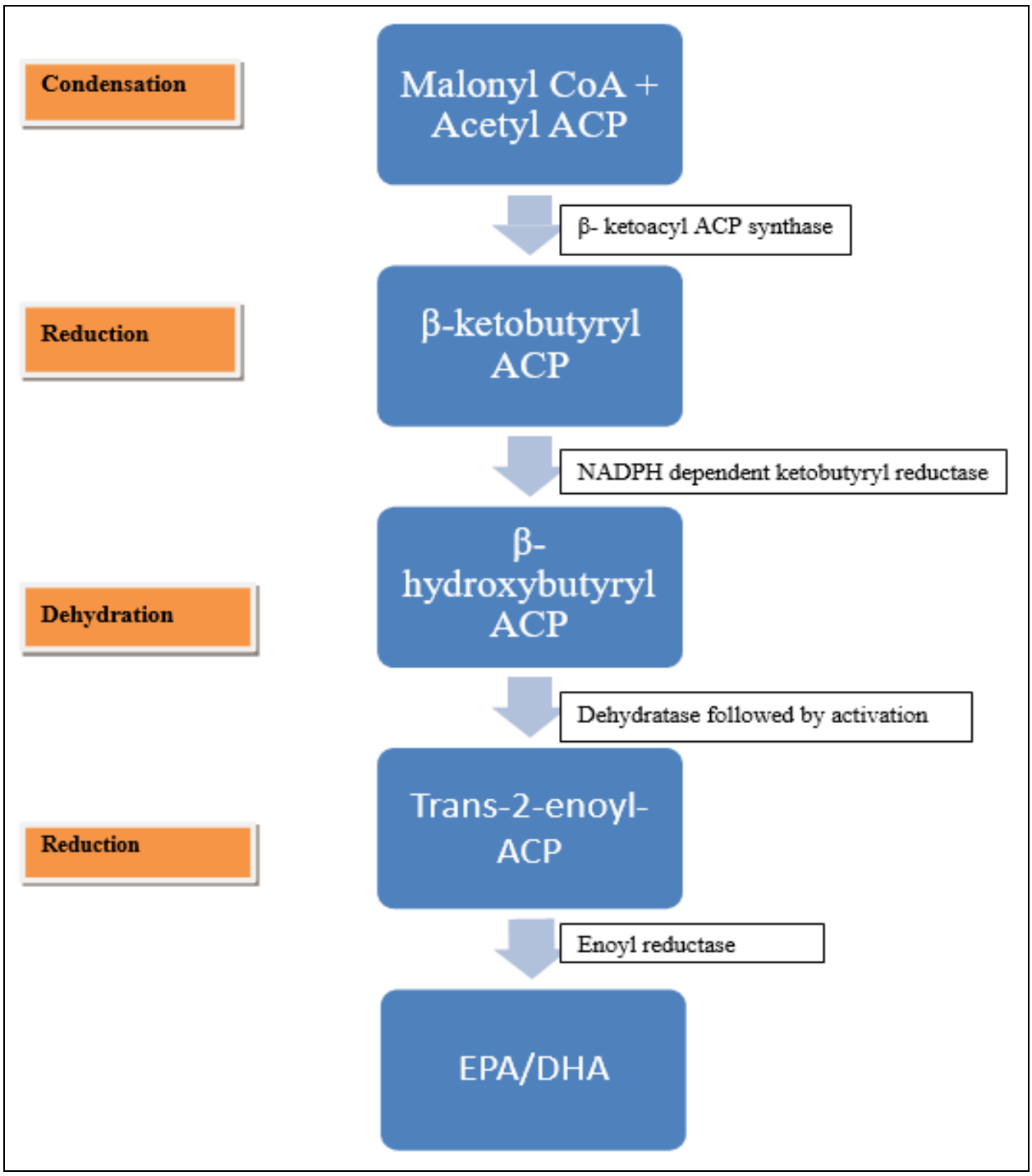

Fig 1: Illustration of PKS pathway for EPA and DHA biosynthesis.

Pufa Characterization

A simpler technique for screening and isolation of PUFA-producing bacteria is direct visualization via the H2O2-plate experiment H_2O_2 assay use for the determination of H_2O_2 in the cell or other biological. Hydrogen peroxide (H2O2) is a reactive oxygen metabolic by-product produce through both enzymatic and non- enzymatic pathways that serves as a key regulator for several oxidative stress- related states. The marine bacteria are cultured in LB (Luria-Bertani) media (1% Tryptone, 0.5% Yeast extract, 1% NaCl composition per litre) at 26-30◦C for 24 hours at 180 rpm with addition to this 0.5% of NaCl is added for proper bacterial growth. By direct inspection, the oxidative balance of PUFAs in growing bacteria in response to additional H2O2 is a significant differentiating characteristic between PUFAs expressing bacteria (without zone of inhibition) and non-PUFAs producing bacteria (zone of inhibition). When it comes to oxygen and reactive oxygen species, PUFAs are the most sensitive to (ROS). (Tilay & Annapure, 2012) [20].

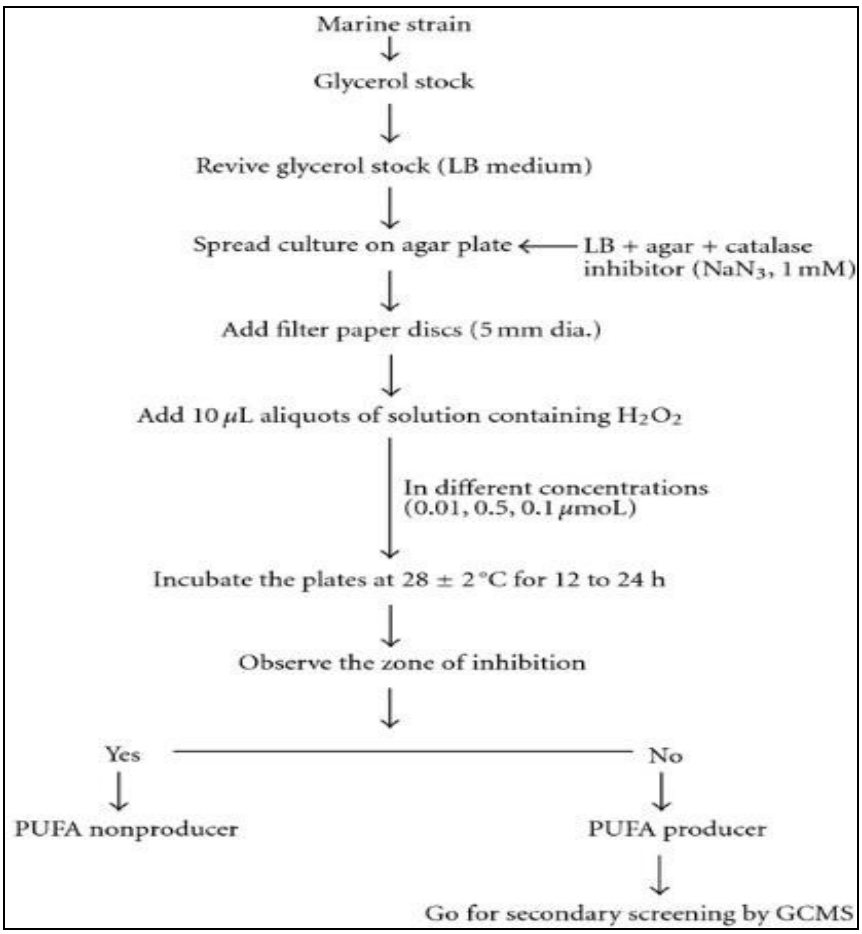

Fig 2: Protocol for primary screening for marine isolates (Tilay & Annapure, 2012) [20]

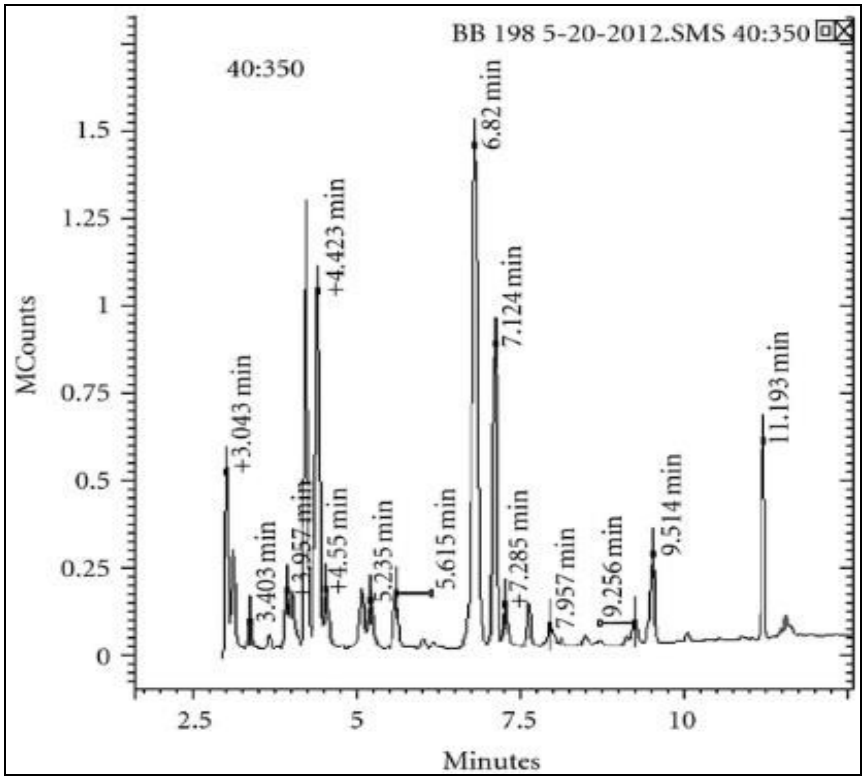

Fig 3: After initial screening, a gas chromatogram displaying the fatty acid profile of a chosen halophilic isolate was created. (Tilay & Annapure, 2012) [20]

Preparation of Fatty acid methylesters (FAMES)

The sample are cultured in Marine Salt Medium (MSM) (81.0 g NaCl, 10.0 g yeast extract, 9.6 g MgSO4, 7.0 g MgCl2, 5.0 g proteose peptone no.3, 2.0 g KCl, 1.0 g glucose, 0.36 g CaCl2, 0.06 g NaHCO3 and 0.026 g NaBr composition per litre and with pH 5-9) and incubated at 15°C, 120 rpm for 48 hours. After 48 hours' cells are harvested by centrifugation at 10,000 rpm for 12 min, the supernatant obtained are discarded, the cell pellets are suspended in 1.0% NaCl (w/v) and again centrifuged. Each bacterial culture tube is stored at 4°C. then the bacterial cells were reweighed, to which a fresh solution of the transesterification reaction mix (methanolic HCl (0.6 N) 4 ml) is added in the tubes and then capped tightly and the solutions were vortexed for 5–10 s and heated in 80°C ± 2°C water bath for 2 h. The tubes were then cooled quickly in ice.

The resultant FAMES were extracted twice by adding 2 volumes of hexane and then 1 volume of hexane by centrifugation at 5,000 rpm for 15 min. The upper phase of hexane layer use for analysis by gas chromatography. (Jadhav *et al*., 2010)

Analysis of FAME by GCMS

The analyte FAME was analyzed by coupling Varian 220-MS with Varian 450 GC based on their retention times and the known standards were taken as referenced for the comparison. The coupled set of equipment was equipped with a capillary column of internal diameter 25 m x 0.25 mm and OD of 0.39 mm, made up of CP- SIL 88 silica fused with methyl silicone (Jadhav *et al*., 2010). The injector was held at 250°C, and the column oven was programmed to go from 160 to 220°C in seven minutes, and then stay at 220°C for ten minutes. Helium was used as the carrier gas, and the flow rate was kept at 1 mL/min. The GCMS had a mass range of 40–350 atomic mass units, with a 70 eV ionization voltage and a 220°C trap temperature (Tilay & Annapure, 2012) [20].

A broad range of bacteria may be reliably investigated utilizing molecular biology techniques such as PCR (Polymerize Chain Reaction), RAPD (Random Amplified of Polymorphic DNA), and sequencing of 16S ribosomal polymer genes. Random amplification of polymorphic DNA (RAPD) analysis, that involves nine arbitrary primers to assess genetic linkage, is the easiest, shortest, and requires lesser amount of DNA.

Fatty acid methyl Esterification (FAME) transesterification process involves the following steps:

1. Harvest cells properly and save the cell pellet.
2. Finely grind the dried cell pellet and transfer 10–20 mg to a 1.8 mL salt glass sample ampule with a PTFE-lined lid.
3. Add 0.5 mL of 5% H2SO4 in wood spirit to the dried biomass in the ampule, more or less.
4. Close the ampule firmly and incubate it at 90°C for 90 minutes before allowing it to cool.
5. Invert the mixture and add 0.4 of dissolvent.
6. Dissolve 0.8 mL of 100 percent NaCl in water and well mix.
7. Allow the solution to split into two layers.
8. Transfer the upper dissolvent layer to a fresh ampule, then repeat the dissolvent extraction on the remaining liquid layer and pool the dissolvent phases.
9. Perform GC–MS or GC–FID analysis as needed. (Allen & Bartlett, 2002) [1]

Comparing halophiles derived PUFA with Fish and Plants derived PUFA

Table 3: Showing % of EPA and DHA production from fish as source

Fish Resources	% of EPA production	% of DHA production	Reference
Sardine	6.6	19.0	(Alonso & Maroto, 2000) [2]
Herring	8.5	8.3	(Alonso & Maroto, 2000) [2]
Shad	0.12	0.43	(Alonso & Maroto, 2000) [2]
Atlantic Salmon	6.2	5.8	(Alonso & Maroto, 2000) [2]
Pink Salmon	1.7	3.3	(Alonso & Maroto, 2000) [2]
Brown trout	0.9	3.5	(Alonso & Maroto, 2000) [2]
Surf smelt	3.6	5.7	(Alonso & Maroto, 2000) [2]
Alaska pollock	1.0	2.4	(Alonso & Maroto, 2000) [2]
Gibel carp	0.6	1.0	(Alonso & Maroto, 2000) [2]
Sudan catfish	0.1	0.7	(Alonso & Maroto, 2000) [2]
Garfish	0.01	0.15	(Alonso & Maroto, 2000) [2]

Table 4: Showing % of EPA and DHA producing plants as source

Plant source	% of EPA production	% of DHA production
Chia seeds	0	0
Flax seeds	0	0
Walnuts	0	0
Brusells sprouts	0	0
Hemp seeds	0	0
Perilla oil	0	0

Plants produce PUFA as α linoleic acid (ALA) and linoleic acid (LA) which are also a form of omega 3 fatty acids but they can't able to produce EPA and DHA which are essential edible polyunsaturated fatty acids and mainly consume by

humans. Hence, with respect to EPA and DHA production we can name them as negligible producer. However, they can be effective source for other applications which involves the usage of ALA and LA. Humans that are why have to depend on microalgae and macroalgae and other halophilic groups for producing PUFA. Hence it can be proved from the above tables that plants can't be highlight as effective producer of EPA and DHA and they should give less importance in comparison to halophilic bacteria which are potent PUFA producer especially for EPA and DHA. Also, cutting down of trees and increase in global warming level are causing deficiency of plants across the world and it is our responsibility to lower their usage as much as we can and hence a great alternative to replace them are halophiles which are enormous in number across the world and with great capacity to produce PUFA.

Table 5: Showing % of EPA and DHA producing halophiles as source

Halophiles source	% of EPA production	% of DHA production	Reference
Shewanella pneumatohori SCRC-2738	36.6	12	(Yazawa, 1996) [23]
Shewanella hanedai ATCC 33224	22.2	8.0	(J P Bowman *et al*., 1997) [3]
Shewanella benthica ATCC 43992	16.0	23.7	(J P Bowman *et al*., 1997) [3]
Shewanella gelidimarina ACAM 456	16.0	20.0	(J P Bowman *et al*., 1997) [3]
Vibrio sp. strain 29-1	19.7	14.8	(Hamamoto *et al*., 1995) [8]
Vibrio sp. strain 814-4	17.9	19.7	(Hamamoto *et al*., 1995) [8]
Photobacterium profundum SAMA2	15.0	23.3	(Freese *et al*., 2009) [6]
Photobacterium Profundum DSJ4	13.0	17.4	(Y Nogi *et al*., 1998) [12]
Vibrio sp. strain 5710	13.0	22.7	(Hamamoto *et al*., 1995) [8]
Vibrio sp. strain 5705	13.8	21.5	(Hamamoto *et al*., 1995) [8]
Vibrio sp. strain 5703	12.4	18.6	(Hamamoto *et al*., 1995) [8]

Benefits of EPA and DHA

As discussed previously, essential polyunsaturated fatty acids like omega-3 fatty acids including Eicosapentaenoic acid and Docosahexaenoic acid are not synthesized by humans on their own. EPA and DHA have various health benefits like in brain development, preventing cardiovascular disease, anti-inflammatory properties, antioxidant properties, feed for cattle and a more cost-effective process as compared to biofuel production etc. Docosahexaenoic acid plays a vital role in fetal brain development as compared to EPA. It has been proven that DHA deficiency in pregnant rats causes impaired brain function and lower brain networking capacity in response to external stimuli. DHA intake by pregnant women causes an increase in the problem-solving skills of their children, reduces allergic reactions, more immune to disease, improvised retinal development, excellent coordination of eyes and hands and even eliminates the risk of asthma (Sharma *et al*., 2020) [17].

EPA and DHA are much beneficial than Arachidonic acid and are useful in preventing Myocardial infarction, sudden cardiac death, atherosclerosis, coronary artery disease and also increases the high-density lipoprotein to low-density lipoprotein ratio, lower the level of triglycerides and hence reduces cholesterol level. From the past 6 years, the consumption of PUFA has been increased by 3 folds, ranging between 120-140 thousand metric tonnes consumption has been recorded between years 2013 to 2015. Modern society has started consuming EPA and DHA ranging between 0.2-4.0 g/day (Sharma *et al*., 2020) [17].

Eicosapentaenoic acid plays a major role in preventing atherosclerotic plaques, reducing macrophages aggregation and hence preventing autoimmune disease, increase HDL to LDL ratio, increase collagen content and smooth muscle cells, decreases internal inflammation by inhibiting adhesion molecules expression, monocyte chemoattractant protein-1 and matrix metalloproteinase which are produced by macrophages. It also inhibits the expression of tumor necrosis factors, dendritic cells etc. which are expressed during atherosclerosis conditions. The secondary metabolites in the form of radicals produced during atherosclerosis conditions are also scavenged by EPA, and hence, it has antioxidant property (Sharma *et al*., 2020) [17].

Hence, PUFA plays a beneficial role in human health. EPA and DHA like omega 3 fatty acids are prerequisites for the nervous system and vascular system development and should be intake regularly. Fish derived dietary PUFA can best be replaced by halophiles derived PUFA and can be consumed by vegans across parts of the world. Halophiles derived PUFA has no side effects, can be produced in higher amounts, economically feasible, no harm to marine life and also no bad impact on the environment.

Discussions

This study provides knowledge regarding the production of PUFA (mainly EPA and DHA) from halophiles and their characterization and benefits. To increase human health concerns, we have shifted the idea of EPA and PUFA production from fish and plant- derived PUFA to halophiles derived PUFA. In this article, the importance of halophiles is highlighted. Halophiles are large factories that can produce EPA and DHA even in a higher amount than fish. They can replace plants as plants can't produce EPA and DHA and instead produce ALA and LA. Halophiles can also be a good choice over microalgae, although microalgae are the leading producer of EPA and DHA the extraction of EPA and DHA and their downstream processing is very difficult. As we have discussed, the major contribution to PUFA production is provided by the class *Gammaproteobacteria*. Halophilic bacteria *Shewanella pneumatophori* SCRC-2738 is the highest EPA producing strain followed by *Shewanella hanedai* ATCC 3324 and *Vibrio species* strain 29-1. Similarly for DHA, *Vibrio species* strain 5710 leads with 22.7% followed by

Vibrio sp. strain 5705 and 5703 producing 21.5% and 18.6% Docosahexaenoic acid. As discussed in Table 1.

We have also mentioned the Polyketide synthase (PKS) biosynthesis pathway which is only found in halophiles. The pathway has the following steps: condensation; reduction; dehydration; activation and reduction. Repetition of these steps forms DHA and EPA. Four Polyunsaturated fatty acids coding genes are found in halophiles namely PfaA to PfaE which codes for ACP, KR, KS, AT, DH, ER, PT proteins. These genes differ in their domains and hence based on the difference in the domain we have compared them in four bacteria namely *Photobacterium profundum* SS9, *Shewanella pneumatophori* SSRC- 2738, *Moritella marina* MP-1, *Pseudoalteromonas sp.* DS-12. (Discussed in Table 2)

For PUFA screening and isolation, a direct visualization method uses H2O2 assay which differentiates PUFA producing organisms and non-PUFA producing organisms on their ability to react with reactive oxygen species in the presence of catalase inhibitor. FAME preparation and analysis by Gas chromatography is discussed in which the halophiles are cultured in a marine salt medium followed by their harvesting through centrifugation and their transesterification reaction in the presence of methanolic HCL 0.6N. The extracts were finally recovered with double treatment of 2 volumes and 1 volume of hexane for analysis of gas chromatography.

Finally, we have compared the halophiles derived PUFA with fish and plants derived PUFA and it was found that Shewanella pneumatohori SCRC-2738 produces the highest amount of EPA and DHA which is 36.6% and 12% respectively which is much higher than fish species like Sardine (6.6% EPA and 19% of DHA), Herring (8.5% EPA and 8.3% DHA), Atlantic Salmon (6.2% EPA and 5.8% DHA) etc. which proves that Halophiles derived PUFA can be produced in a larger amount as compare to Fish derived PUFA. Also as shown in Table 1, Shewanella hanedai ATCC 33224 produces 22.2% EPA and 8.0% DHA, Shewanella benthica ATCC 43992 produces 16.0% EPA and 23.7% DHA etc.

These data suggest that halophiles can be an effective and potent source for dietary PUFA and should be promoted to commercial production due to their cost-effective downstream processing and growth.

Conclusion

With the growing demand for healthy beneficial lifestyles and naturally derived nutraceuticals, EPA and DHA play a major important role in brain development, preventing cardiovascular disease, Alzheimer's disease, acts as anti-inflammatory, antioxidant, anti-allergic, and anti-cancerous products etc. Thus to produce omega 3 fatty acid is a large scale is demanding as the population increases. Hence, halophiles derived PUFA can be a better option and a good replaceable to fish- derived PUFA in terms of environmental concern and vegan ease consumption. Halophiles derived PUFA are non-toxic, can be produced at a large scale, can eliminate the vegan consumption problem, cost-effective downstream processing as compared to microalgae derived PUFA, large factories to EPA and DHA etc. Hence, we can conclude that halophiles derived PUFA has great economic and research scope and can replace the need and dependency of fish and plant- derived PUFA are non-toxic, can be produced at a large scale, can eliminate the vegan consumption problem, cost-effective downstream processing as compared to microalgae derived PUFA, large factories to EPA and DHA etc. Hence, we can conclude that halophiles derived PUFA has great economic and research scope and can replace the need and dependency of fish and plant- derived PUFA. If systematic screening, proper media optimization and optimum growth condition are evaluated and maintained then it will motivate us to isolate more unique genera of halophiles that can produce an even higher amount of PUFA in terms of EPA and DHA.

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