Purification and Characterization of Polyunsaturated Fatty Acids (PUFA) Produced by Psychrotolerant Bacteria Isolated from Different Glaciers of Pakistan

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Islamabad, 2017

Purification and Characterization of Polyunsaturated Fatty Acids (PUFA) Produced by Psychrotolerant Bacteria Isolated from Different Glaciers of Pakistan

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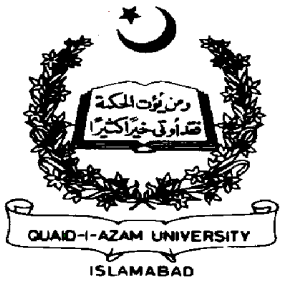

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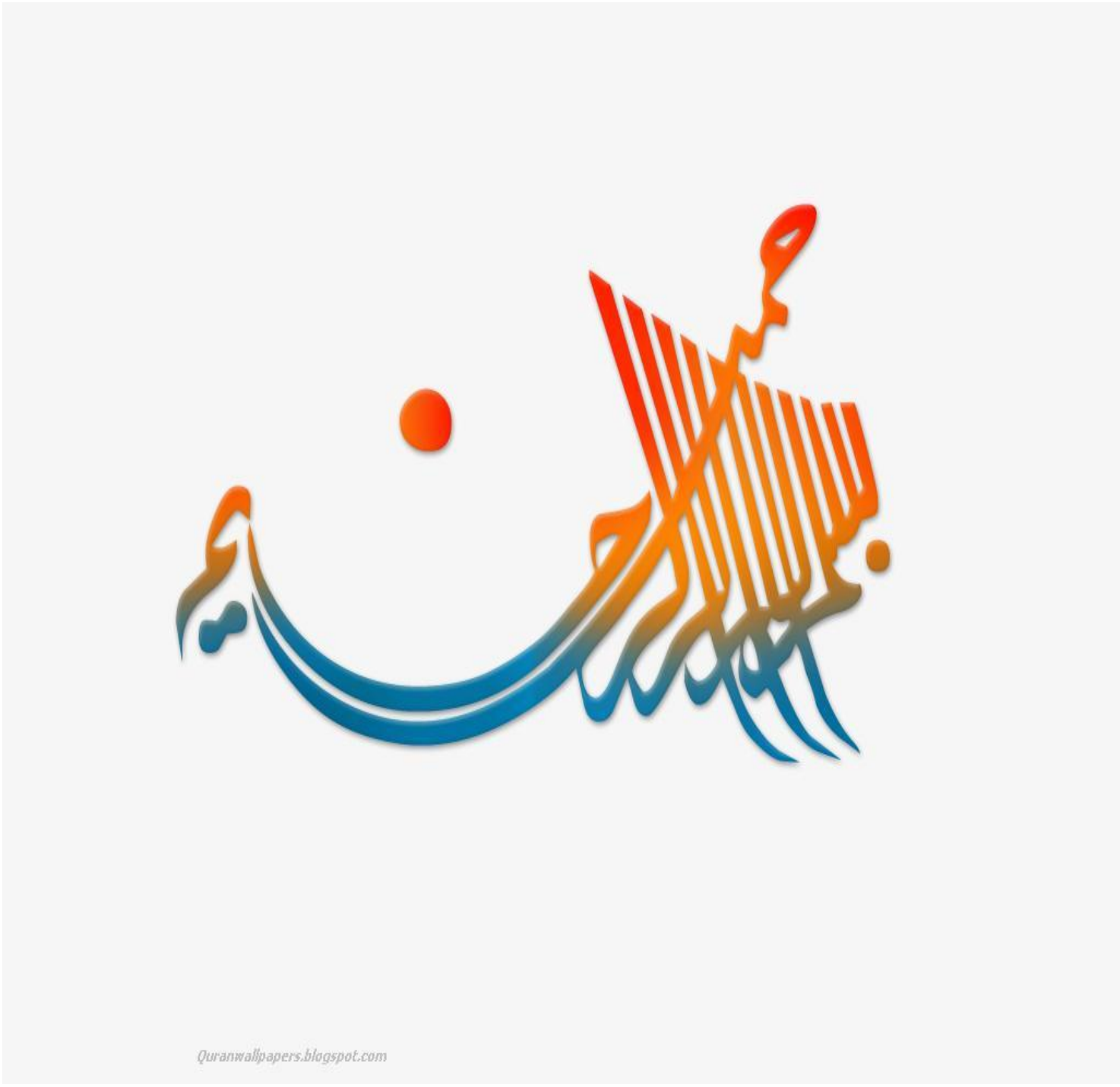

In the name of ALLAH, Most Gracious, Most Merciful.

Dedicated to

My Beloved Family

Declaration

The material and information contained in this thesis is my original work. I have not previously presented any part of this work elsewhere for any other degree.

Atta Ullah

Certificate

This thesis, submitted by **Mr. Atta Ullah** is accepted in its present form by the Department of Microbiology, Faculty of Biological Sciences, Quaid-i-Azam University, Islamabad, as satisfying the thesis requirements for the degree of Master of Philosophy in Microbiology.

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List of Contents

Sr. No.	Titles	Page No.
1.	List of Tables……	i
2.	List of Figures……	ii
3.	List of Acronyms……	iii
4.	Acknowledgements……	iv
5.	Abstract……	v
6.	Introduction……	01
7.	Aims and Objectives……	08
8.	Review of Literature……	06
9.	Material and Methods……	24
10.	Results……	29
11.	Discussion……	56
12.	Conclusions……	61
13.	References……	.62
14.	Appendix……	73

Aims and Objectives

The aim of the current research work was isolation of Psychrotolerant bacteria of HKKH region for the production and characterization of polyunsaturated fatty acids (PUFAs)

Objectives of the study

- Isolation of bacteria from HKKH glaciers and their identification via morphological as well as molecular approaches
- Screening of the isolated Psychrotolerant bacteria for the production of PUFA
- Optimization of pH, temperature and media for the production of PUFA
- Characterization of PUFA using FTIR and various chromatographic techniques

List of Tables

S. No	Titles	Page. No.
1	Types and Sources of PUFA	3
2	PUFA products and manufacturing Companies	4
3	PCR programmed used for amplification of the gene of interest	30
4	Physiochemical characteristics of samples	32
5	No of total and distinct colonies appeared upon spreading	33
6	Gram characteristic and colony morphology of the isolates	33
7	No of selected and PUFA positive isolates	34
8	Media optimization for growth and PUFA production by isolate PLPI 1	37
9	Media optimization for growth and PUFA production by isolate PDPI 2	38
10	Media optimization for growth and PUFA production by isolate PDPI 3	39
11	Media optimization for growth and PUFA production by isolate TSC 2	40
12	Media optimization for growth and PUFA production by isolate PSC3	41
13	pH optimization for the selected isolates	42,43

List of Figures

S. No.	Titles	Page. No.
1	Conventional biosynthesis pathway of PUFA formation	17
2	PUFA production by PKS pathway	19
3	Isolated bacterial colonies obtained from Batura,TirichMir samples (sediment, and water)	32
4	Rapid TTC assay of the isolated bacteria FUFA Production	34
5	Temperature optimization for the isolates PLPI 1, PDPI 2	35
6	Temperature optimization for the isolates PDPI 3, TSC 2 and PSC 3	36
6	TLC analyses of isolate PLPI1, PDPI2, PDPI3, TSC2 and PSC3.	43
7	5 FTIR analysis of Standard PUFA obtained from The Vitamin Company USA.	44
8	FTIR analysis of PUFA extracted from bacterial isolate PLPI-1, PDPI-2	46
9	FTIR analysis of PUFA extracted from bacterial isolate PDPI-3, TSC-2	47
10	FTIR analysis of PUFA extracted from bacterial isolate PSC-3	48
11	DNA bands of isolate PLPI1, PDPI2, PDPI3, TSC2 and PSC3	48
12	Agarose gel electrophoresis with specific band size of 16S rRNA gene	49
13	Phylogenetic tree of the Isolates PLP-1, PDPI-2, PDPI-3, TSC-2 and PSC-3	50

List of Acronym/abbreviations

ALA	Alpha-Linolenic acid
BG11	Blue Green Algae Medium
DHA	Docosahexaenoic acid
EDTA	Ethylene diaminetetraacetic acid
EPA	Eicosapentaenoic acid
EDTA	Ethylene diaminetetraacetic acid
FA	Fatty Acid
FAME	Fatty acid methyl ester
FAS	Fatty Acid Synthase
FTIR	Fourier Transformed Infrared spectroscopy
GC	Gas Chromatography
GC-MS	Gas Chromatography Mass Spectroscpy
GLA	Gamma-Linolenic acid
GPS	Global positioning system
HKKH	Hindu Kush Karakoram Himalaya
LB	Lauria broth
MAFFT	Multiple Alignment using Fast Fourier Transform
MEGA	Molecular Evolutionary Genetics Analysis
MSM	Minimum salt medium
NA	Nutrient Agar
nm	Nanometer
OD	Optical density
ORFs	Open Reading Frames
PCR	Polymerase Chain Reaction
PKS	Polyketide Synthase
PM	Production medium
PUFAs	Polyunsaturated fatty acids
PYM	Peptone Yeast Extract Meat Extract
pH	Potential of Hydrogen
Rpm	Revolutions per minutes
Rf	Retardation factor
TF	Triphenyl formazan
TTC	Triphenyltetrazolium chloride
TLC	Thin layer chromatography
UV	Ultra violet

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Abstract

Polyunsaturated fatty acids (EPA, DHA) are a member of Omega-3 family is essential fatty acid. Omega-3 can be isolated from various source like plants, microalgae, fungi and various fish but they all have certain problems associated with them. Human cannot synthesize omega-3 due to deficiency of enzyme Δ5 desaturase and obtain it through external source. A promising and cost effective alternative source of Omega-3 is bacterial production.. Bacteria can be easily cultured in simple medium and have short growth and production time. A total of 140 bacteria were isolated from previously collected samples of glaciers sediments, water and ice of Batura, Passu and Tirchmir of the Himaliya, Karakoram and Hindukosh regions of Pakistan. The isolates were screened through a calorimetric method 2,3,5-triphenyltetrazolium chloride (TTC) for the production of PUFA. We selected five isolates and labeled as PLPI-1, PDPI-2, PDPI-3, TSC-2 and PSC-3. The selected bacterial isolates were identified by 16S rRNA gene sequence analysis. PLPI-1 was identified as *Alcaleene fecalis*, PDPI-2 identified as *Alcaleene fecalis* PDPI-3 identified a *Alcaleene fecalis*, TSC-2 identified as *Alcaleene fecalis* and PSC-3 was identified as *Alcaleene fecalis* but the strain was different. Production of the PUFA by the selected bacteria was verified by Thin Layer Chromatography (TLC) and Fourier transformed infrared spectroscopy (FTIR) using EPA and DHA standards. An investigation of the effects of variables like media, incubation time, temperature and pH for the growth and optimum production of PUFA was checked, using one factor at a time approach (OFAT). It was observed that all the isolates produce maximum PUFA at lower temperature and at neutral pH. For isolates PLPI-1, pH 7, 15˚C temperature and NB medium was found best for PUFA production. Isolate PSC-3, PDPI-1 and TSC-2 showed optimum PUFA production on pH 7, 15˚C temperature and NB medium. Moreover, PDPI-1 has shown maximum PUFA production on wider pH ranging from 5 to 8. It was observed that bacteria were able to produce PUFA. PUFA producing ability in bacteria make it valuable and are better alterative of fish oil will cope the high global demand of omega-3 fatty acid. It has many advantages that heavy metals are absent in their biomass as well suitable for vegetarian. To our knowledge this is the first report of a bacterial isolate producing Omega-3 glaciers samples.

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Introduction

Psychrotolerant are microorganisms that can live their life above 20°C. These microorganism live in cold environment and have an optimal temperature of about 15°C and maximum 20°C while psychrophiles are cold loving fungi, archaea and bacteria that require an optimal temperature of 15°C or lower and having maximal temperature up to 20°C but having minimal temperature of 0°C (Moyer and Morita *et al*., 2007).

Earth is a cold environment and approximately 80-85 % of its temperature is below 5°C that includes oceans, Arctic, Antarctic and mountainous regions (Himalayans, Alpines and rocky mountains) and some other mesospheric and stratospheric regions (Casanueva *et al*., 2010; Hinsa and Tiedje *et al*., 2010; Margesin and Miteva *et al*., 2011). The largest portion of the low temperature environment comprise of oceans i.e. occupied 71% of the planet having temperature -1 to 4°C, then the second largest portion is of snow that covers almost 35% of the Earth's surface, permafrost that covers 24% of the land, sea ice i.e. 13% and glaciers that comprise 10% of land, temperature of which is below 5°C (Margesin and Miteva *et al.,* 2011). Environments like caves, subsoils, cold deserts and lakes are also considered as cold habitats (Aislabie *et al*., 2008). Low temperature habitats are wide spread extreme environments and inhabited by diverse groups of microorganisms that thrive there, while facing various stress conditions (Rummel *et al.,* 2014). In order to cope with extreme conditions like low nutrients, desiccation, osmotic pressures, radiations, high and low pH, they exhibit certain adaptive strategies (Rodrigues and Tiedje *et al*., 2008; Morgan-Kiss *et al*., 2006). As these environments are less explored and probability of discovering new species is always there, that attracts the scientist's attention (Kim *et al*., 2012).

Microorganisms like bacteria, archaea, fungi, microalgae and yeast are successfully colonized in cold environments. *Vibrio marinus* and *vibrio* are the true psychrophiles discovered and described taxonomically in 1964 and in 1972. Bacterial domain showed rich diversity and comprise of both Gram positive and Gram negative groups and may contain heterotrophic and autotrophic, non-photosynthetic, aerobic, anoxic, spore formers and non-spore forming psychrophiles (Moyer and Morita *et al*., 2007). Adaptive strategies of psychrophiles include solute concentration (Morgan-Kiss *et al*.,

2006), enzymes, membrane stability (Rodrigues and Tiedje *et al*., 2008) and macromolecule interaction in order to cope with extreme cold environment (Tehei *et al*., 2005; Piette *et al*., 2011). Psychrophilic microorganisms also produce extracellular and intracellular metabolites having medical and industrial applications (Margesin and Feller *et al*., 2010). One of the potential applications of psychrophilic microorganisms is the production of polyunsaturated fatty acid (PUFA), commonly known as Omega-3, mostly consist of Eicosapentanoic acid (EPA) and Decosahexanoic acid (DHA), that play role in bacterial membrane stability and also have vast medical applications (Usui *et al*., 2012). Due to high biotechnological applications of PUFA psychrophilic bacteria attract the scientist's interest to explore these regions for best alternatives (Gentile G. *et al*., 2003).

Human beings have the ability to synthesize saturated and monounsaturated fatty acids which are called non-essential fatty acids, while cannot synthesize polyunsaturated fatty acids due to lack of de-saturase enzyme and are required from outside source in the form of food, therefore termed as essential fatty acids (Bajpai *et el*., 1993). Hence PUFA is said to be natural product due to difficulty in production of double bond formation synthetically (Meyer *et al*., 1999). As mammals are unable to synthesize PUFA, therefore, direct uptake is necessary. Currently, primary source of PUFA is marine fish such as Sardines, Mackerel, Salmon and Mullet that mostly consist of DHA and EPA (Gunstone *et al*., 1996; Arab-Tehrany *et al*., 2012).

Although, the consumption of fish oils and their introduction in market for medical and dietary supplementation, pose certain problems like they have unpleasant taste, flavor and odor (Barclay *et al*., 1994). They also face stability as well as sustainability problems along with problems of purification due to low amount of Omega-3 PUFA (Barclay *et al*., 1994; Ratledge 2004; Foran *et al*., 2005). Contamination of marine ecosystems with heavy metals, chemicals, and other compounds that impede the use of fish oil for PUFA production, lead to carcinogenic, mutagenic, and terotogenic effects which decrease fish stock (Domingo *et al*., 2007; Sidhu, 2003, Foran *et al*., 2005; Tricon, 2006; Cole *et al*., 2009; Ward *et al*., 1995). These contaminants may lead to health related problems in children and lactating women (Park and Johnson *et al*., 2006). It has been observed that fish oil consumption reduces the malignant cell's sensitivity to chemotherapeutic agents (Roodhart *et al*., 2011). Due to overfishing and decreased fish stock, fish oil does not meet the growing global demands (Medaas *et*

al., 2014). Therefore, researchers search for an alternative source like plants, microalgae and bacteria for PUFA production, in order to meet the increased global demand (Abbadi *et al*., 2004; Sayanova, 2004; Robert, 2005; Wu *et al*., 2005). Genetically modified plant maybe a good alternative for PUFA production but they also face problems of viability and slower growth (Ruiz-López *et al*., 2012; Napier *et al*., 2007). Similarly, banning of genetic modification and cloning's might face certain challenges in some countries (Certik and Adamechova *et al*., 2009). Plant also need arable land for their growth and might be susceptible to pathogens (Ursin *et al*., 2003). Fungi also require longer growth periods and various organic carbon sources which is not economical for PUFA production (Barclay *et al*., 1994). Microalgae may be used for PUFA production but they require controlled condition for their cultivation, specific light quality and quantity, and certain nutrients which make it not economical (Seto *et al*., 1984; Mirón, 2002; Grima *et al*., 2003). Therefore, the best alternative for PUFA production will be bacteria.

Table 1.1 Types and Sources of PUFA

Habitat	**Source**	**Types**	**Reference**
Psychrophilic	Chlorella	EPA, DHA	K Skjånes *et al*., 2013
Psychrophilic	*Colwellia spp*	EPA, DHA	Amiri-Jami *et al*., 2006
Psychrophilic	*S. affinis*	EPA, DHA	Ivanova *et al*., 2004
Psychrotolerant	*Photobacterium*	EPA, DHA	Zhang *et al*., 2011
Cold adapted	salmon, sardine,	DHA, EPA	McManus *et al*., 2011.
Psychrotolerant	Mucorales fungi	GLA,EPA, DHA, AA	Bajpai *et al.*, 1991
Cold adapted	Primrose seed oil	GLA	Mahajan *et al*., 1988
Psychrotolerant	Fungal sources	GLA	Diramin *et al*., 2009
Cold adapted	Wild fish	EPA , DHA	Miller *et al*., 2008
Cold adapted	Canola and Soybean	EPA, DHA	Sayanova *et al.*, 2011

Table 1.2 PUFA products and manufacturing Companies

Product Name	Company	Reference
Ultimate Omega 3	Nordic Naturals	http://www.brainreference.com/ultimate-omega-2/
Optimum Omega 3	Dr. Tobia	www.**drtobias**.net
Vitafusionomega3	Walgreen	https://www.walgreens.com/store/c/vitafusion-omega-3
Essential Omega 3	Steele Spirit	http://www.steelespirit.com/essential-omega-3
Ellite Omega 3	Carlson	http://www.carlsonlabs.com/p-255-elite-omega-3-gems.aspx
Omegavia	BETTYS	http://www.bettyshealthstore.com/product/omegavia-pharma-grade-omega-3

Bacterial production of PUFA may be a promising alternate approach (Bianchi, 2014; Gorissen et al., 2015; Shin *et al*., 2007). As bacteria have many advantages of easily stored, cultured and renewable source of PUFA (Sijtsma and de Swaaf *et al*., 2004; Nichols *et al*., 1993). The producing strain may be genetically modified for increased production of PUFA (Amiri-Jami *et al*., 2006; Berge *et al*., 2005; Hinzpeter, 2006; Nichols *et al*., 1999). Producing strains are known to be frequently found in low temperature habitats. Production of PUFA in such environments is known to be part of the adaptive strategy to survive in such harsh conditions. These bacteria can modify sterol, protein, hopanoids and fatty acid contents of their cell membrane. Homoviscous is an adaptation process that is frequently found in these bacteria, where degree of fatty acid saturation and unsaturation take place to maintain membrane fluidity (Shulse *et al*., 2011). The adaption may involve change in chain length, cis-trans isomerization as well saturation and unsaturation of fatty acids. Similarly, these microorganisms incorporate specific fatty acid molecules in their membrane for fluidity that lead easy transportation of nutrients (Fernandes *et al*., 1993; Tamegai *et al*., 2011). Some of the PUFA producing bacteria previously reported are *Shewanella, Colwellia, Alteromonas, Mortillia* and *Photobacterium* (Bergé and Barnathan *et al*., 2005; Freese *et al*., 2009; Nichols *et al*., 1993; Nogi *et al*., 2013). Among these *Shewanella* species are more studied for PUFA production (Kawamoto *et al*., 2009; Shulse *et al*., 2011; Lee *et al*., 2009; Morita *et al*., 2005; Gladyshev *et al*., 2007). Most of the studies showed that microorganisms isolated from Antarctica produced

more PUFA due to extreme cold environments (Lewis *et al*., 1999; Russell *et al*., 1999; Fogliano *et al*., 2010).

PUFA is eicosanoids precursor mainly composed of thromboxane and prostaglandin that plays important role in regulation of immune system, neurotransmitter, structure of membrane phospholipids of retina and brain, cholesterol metabolism and blood clotting. It also play role in the inhibition of low density lipoprotein (LDL) and its elimination (Steffens and Wirth *et al*., 2005), that help in reducing blood pressure, platelets reduction and prolong bleeding time. PUFA also help preventing skin disease (Steffens and Wirth *et al*., 2005), cardiovascular disease, asthma, arthritis (Lands *et al*., 1986; Kremer *et al*., 1988), multiple sclerosis (Bates *et al*., 2000), lupus erythromatosis (Kelley *et al*., 1985) and nephritis (Thais and Stahl *et al*., 1987).

Hindu Kush, Karakoram and Himalaya (HKKH) regions are considered the third pole of the world and their glaciers cover total area of 16933 Km^2, and are inhabited by psychrophilic microorganisms. Psychrophiles acquire certain mode of adaptation to low temperature for their survival and they must produce PUFA for adaptation because it play important role in adaptation to low temperature. As these regions have not been explored for the production and isolation of PUFA previously, therefore, in the current research work the particular areas were selected in order to find out promising alternative in terms of bacteria for the production of PUFA to meet the need of the day.

Literature Review

Polyunsaturated fatty acids are long chain fatty acids having two or more double bonds. They belong to the class of biomolecules, known as lipids which are the broad groups of organic compounds occurring naturally and include; phospholipids, waxes, oils, steroids and fats. These molecules show solubility in non-polar organic solvents i.e. acetone, benzene, ether and chloroform while generally insoluble in water (Fahy *et al*., 2005). Lipids play very essential biological functions like structural component of cell membrane, energy storage and signaling molecules (Gurr *et al*., 1991).

Fats may be solid or liquid (oils) that comprise fatty acids of 12 to 22 carbons. Among all the lipid molecules, fats provide highest amount of energy, approximately double of the protein and carbohydrates. The basic building block of fats and oils are two types of molecules i.e. glycerol and fatty acid esters. Fatty acids have many types but they are generally grouped into three main divisions: saturated, monounsaturated and polyunsaturated fats. Saturated fat has highest number of hydrogen atoms and has no double bond, generally solid at room temperature, mainly found in animal products like meat, cheese and butter while palm oil and coconut are the common vegetable source. Saturated fat has an unwanted effect on human body that lead to rise in cholesterol level as compared to any other diets (Brasaemle *et al*., 2007). One or more double bonds unsaturated fatty acids. Those having one double bond are termed as monounsaturated while those which have more than one double bond are termed as polyunsaturated fatty acid. Mostly, monounsaturated fatty acids are found in canola, peanut and olive oils, avocados, nuts and olives, while polyunsaturated fatty acids are found in sunflowers, corn and some animals like fish.

2.2 Polyunsaturated fatty acids (PUFA)

Polyunsaturated fatty acids are long chain fatty acids that contain more than 18 carbon atoms in their chain and have two or more double bonds in their backbone. These fatty acids have raised interest of general public for their consumption. This is because of the fact that lipids are very essential for the healthy growth and survival of living organisms

(Gill and Valivety *et al.*, 1997; Sijtsma and Swaaf *et al.*, 2004). The fact is well understood when beneficial effects of lipids analyzed critically in human and other animals where it play a role as storage molecules, become part of the cell membrane, and most recently evolved that used in nutraceutical and pharmaceutical industries (Okuyama *et al*., 2007a). In fact, PUFA constitutes unique form of food constituents that play variety of functions in nutraceutical and biomedical as food and nutritional products that pose promising beneficial health activity, help preventing certain lethal diseases (Gill and Valivety *et al.*, 1997). Depending of the first double bond position, PUFA are classified into two main groups, Omega-3 which has first double bond at carbon number 3 when counting from the methyl terminal and Omega-6, where the first double bond found at carbon number 6 when counting from the methyl end (Sijtsma and Swaaf *et al.*, 2004).

2.3 Polyunsaturated Fatty Acid (PUFA) sources and associated problems

2.3.1 *Vegetables*

Vegetables have high amount of shorter chain of Omega-3 fatty acid such as primrose, linseed, hempseed and echium but with low amount of EPA and DHA (Benhaïm *et al*., 2013). Plants are rich in fatty acids like Linolenic Acid (LA), Gamma Linolenic Acid (GLA) and Alpha-linolenic acid (ALA) respectively. Seeds of above mentioned plants are the main vegetable source of PUFA. Although, vegetable sources have low level of fatty acids but its use in human supplements and fish feed will increase their production and dependence on fish oils. Various number of transgenic plants such as safflower, canola and soybean are used for the over production of PUFA (Sayanova and Napier *et al.*, 2011). Genetically modified plant maybe a good alternative for PUFA production but they also face problems of viability and slower growth (Graham *et al.*, 2007; Ruiz-López *et al*., 2012). Similarly, banning of genetic modification and cloning's might face certain challenges in some geographical areas (Certik and Adamechova *et al.*, 2009). Plant also need arable land for their growth and might be susceptible to pathogens (Ursin *et al.*, 2003).

2.3.2 *Marine Fish*

Marine fish like salmon, sardine, mackerel and herring are well known source of PUFA. It is considered as the major source of PUFA till date and comprise of DHA and EPA essential fatty acids. Various studies showed that seafood are the major source of PUFA particularly fish oils (McManus *et al*., 2011). Due to increased market demand of PUFA, marine fish are highly consumed for PUFA production and supply to markets (Molendi *et al*., 2011).

Fish consumption attracted interest of human for being the source of PUFA due to their beneficial effects on health. Whereas, fish source pose daunting challenges like decrease in fish stock, global warming and provision of supplements, which may lead to extinction of fish (Dulvy *et al*., 2003). Although, the consumption of fish oils and their introduction in market for medical and dietary supplementation, pose certain problems like they have unpleasant taste, flavor and odor (Barclay *et al*., 1994; Venegas-Caleron *et al*., 2010). They also face stability as well as sustainability problems along with problems of purification due to low amount of Omega-3 PUFA (Simopoulos *et al*., 2000; Ratledge, 2004; Jenkin *et al.,* 2009). Contamination of marine ecosystems with heavy metals, chemicals, and other compounds that impede the use of fish oil for PUFA production, lead to carcinogenic, mutagenic, and terotogenic effects which decrease fish stock (Undeland *et al*., 2009; Sidhu. 2003, Foran, 2005; Hu *et al.,* 2009). These contaminants may lead to health related problems in children and lactating women (Park *et al.*, 2011). It has been observed that fish oil consumption reduces malignant cell's sensitivity to chemotherapeutic agents (Vaughan *et al*., 2013). As these sources poses certain problems there must be a need of alternative for PUFA production that should be microorganisms.

2.3.3 *Microorganisms*

Microorganisms are promising producers of various metabolites; one of the most important is polyunsaturated fatty acid, because they have the array of elongase and desaturase activities required for the de novo synthesis of PUFA. Thus the prokaryotic organisms synthesize various types of PUFA and will be the promising source of PUFA for industrial scale. For example, marine phycomycetes especially of mucorales fungi,

that produces GLA, EPA, DHA and AA (Lordan *et al*., 2011). Ogawa *et al*. (2012) described that filamentous fungi like *Mortierella alpina* 1S-4 oils containing Omega-3 and Omega-6 fatty acid but fungi require longer growth period and various organic carbon sources which is not economical for PUFA production (Barclay *et al*., 1994). Macroalgae also considered as potential source of PUFA for large scale production. Researchers investigated chlorophytae, phaeophyta and rhodophyta for fatty acid production. They investigated that Phaeophytes and Rhodophytes have high concentration of essential PUFA as compared to cholorophyta and should be considered a better source (Pereira *et al*., 2012). Microalgae may be used for PUFA production but they require controlled condition for their cultivation, specific light quality and quantity, and certain nutrients which make it costly (Seto *et al*., 1984; Saanchez Miroon, 2000; Molina *et al*., 2003). Therefore, the best alternative for PUFA production will be bacteria.

Until 1990s, scientist considered that only cyanobacteria can produce PUFA while others bactaria are unable to produce PUFA. It is because of the lack of studies regarding physiology, biochemistry and molecular biology of the psychrophilic microorganisms while scientists focus on study of mesophilic organism like *Escherichia coli,* which is unable to produce PUFA. It is then investigated by (Singh *et al*., 1997; Wirsen *et al*., 1986) that PUFA preferably EPA and DHA are distributed in psychrophilic microorganisms (Yano *et al.*, 1994). It is very interesting that, PUFA produced in these bacteria were mostly EPA and DHA and not linolenic and linoleic acids, which are mostly found in plants, fungi and cyanobacteria. However, it was discovered in 1980s but they got no attention (Russel *et al*., 1999; Singh, 1997; Valentine *et al*., 2004). Sequence will be given

Most of the Earth part is a cold environment and approximately 80-85% of its temperature is below 5°C including oceans, Arctic, Antarctic and mountainous regions (Himalayans, Alpines and rocky mountains) and some other mesospheric and stratospheric regions (Casanueva *et al*., 2010; Rodrigues and Tiedje *et al.,* 2008; Margesin and Miteva, *et al.,* 2011). The largest portion of the low temperature environment comprise of oceans i.e. occupies 71% of the planet having temperature -1 to

4°C, then the second largest portion is of snow, permafrost, sea ice and glaciers, that covers almost 35, 24, 13, and 10%, of the land surface, temperature of which is below 5°C (Margesin and Miteva *et al.,* 2011).

Glaciers are the highest persistent dense body of ice which is constantly moving under its, own weight. It is generally consisted of re-crystallized snow and due to the pull of gravity it showed evidence of down slope (Nelson *et al.,* 2003). Glaciers have various regions like deep ice or subglacial part, supraglacial also called cryoconite holes that have variation in hydrological, temperature, physical and geochemical characteristics due to which it has fluctuating diversity (Hodson *et al*., 2008).

Pakistan has numerous types of large and small glaciers that are found in northern mountainous regions of Hindu Kush, Karakoram and Himalaya (HKKH) regions. These glaciers are considered to have highest glaciated mass in the whole world, out of the North and South Poles. These glaciers cover approximately 17000 square kilometers, which is 15% of the mountainous region located in the Upper Indus River Basin. The total length of glaciers on the lap of Karakoram Range in Pakistan is above 6160 square kilometers. According to estimate, almost 37% of the Karakoram region is under the glaciated ice.

Generally, HKKH region of Pakistan consists of six types of glaciers namely mountain, niche, ice caps, ice aprons, valley and cirque. Batura glacier in Hunza region is the fourth largest glacier in the world and its length is about 59 km. The glaciers have 5 main ice flows and 20 smaller tributary glaciers (Batura Glacier Investigation Group, 1979, 1980). Batura and Passu are located in Muztagh, a sub-range of the Karakoram mountain i.e. located in west of the Hunza Valley, District Gilgit, Pakistan. Tirich Mir (7750 m) in the Chitral area of northwestern Pakistan represents the tallest mountain in Hindu Kush. It consists of Upper Tirich and Lower Tirich. The Upper Tirich Glacier basin is formed by Tirich Mir peaks in different directions such as East, West (the main summit peaks) and groups of peak in north; these peaks look like a horseshoe-like semicircle (Sweatman, *et al*., 1969).

2.4 Role of PUFA in bacterial adaptation

Low temperature is one of the most challenging environmental conditions that influences numerous life processes like metabolic activity, cell growth, energy production and gene transcription (Barria *et al*., 2013). Unsurprisingly, a range of metabolic, anatomical and cellular strategies relieve the negative impact of such harsh temperature (Godin-Roulling *et al*., 2015; Garcia-Descalzo *et al*., 2014). The modulation of membrane fluidity is one of the most important strategies for microbial adaptation to low temperature environment (Mykytczuk *et al*., 2010; Najjar *et al*., 2007). In membrane lipids the composition of fatty acid such as *cis or Tran's* fatty acids, the structure, saturation and length of fatty acid are thought to be nearly related to membrane fluidity (Kim *et al.,* 2002). Among all these factors, one of the crucial factors that reduces the melting point and amplifies the membrane fluidity is the rise in polyunsaturated fatty acids (Delong, *et al.,* 1986; Amiri-Jami, 2006; Chintalapati *et al*., 2004).

Currently, member of *Vibrio, Shewanella, Psychromonas, Colwellia* and *Photobacterium* are known to produce PUFAs (Satomy *et al*., 2003; Fang, 2010; Shulse, *et al*., 2011). Majority of these isolates are piezophilic and psychrophilic that generally are PUFA producers and originate from the deep sea Polar Regions (Delong *et al.,* 1986; Gentile, *et al*., 2003).

Increased polyunsaturated fatty acids play a crucial role in their adaptation to these harsh conditions by increasing the membrane fluidity and reducing the freezing point of these fatty acids (Delong, *et al.,* 1986; Skerratt, *et al*., 2002b). Kawamoto, *et al*., (2011) confirmed this theory by producing 2 different strains of EPA deficient mutants of *Shewanella* and that became cold and pressure -sensitive while the wild type was resistant to cold and increased pressure. The EPA synthesis is yet to be fully elucidated; it has been proposed that the enzyme responsible for synthesis of EPA might show increased activity at colder temperature (Gladyshev *et al*., 2009). The EPA has been also shown to play a significant role in bacterial division at coder habitats.

This significant role could be due to specific interaction of EPA with specific protein in cell membrane responsible for cellular processes (Kawamoto, *et al*., 2009). Besides, its

role in cold adaptation, the PUFAs, particularly the EPA also has a role as an antioxidant. This has been confirmed by enhanced protein carbonylation and decreased in *Shewanella marinintestina* IK-1a mutant that lack EPA, when exposed to different H_2O_2 concentration as well as by increased resistance to exogenous H_2O_2 in genetically engineered *E. coli* (Orkasa, *et al*., 2009). Several evidences support that PUFAs in bacterial cell membrane may also affect the movement of molecules across the membrane, like the facilitation of the entry of hydrophobic compounds and inhibition of the hydrophilic one. For instance the *Shewanella marinintestina* IK-1 strain (Nishida, *et al*., 2010) and increased resistance to water soluble antibiotics as compared to hydrophobic inhibitory compounds. The increased resistance to such harmful compounds has been suggested to be due to enhanced production of several proteins like porins and TolC by EPA involved in efflux of these compounds (Blair and Piddock, *et al.,* 2009; Kawamoto, *et al*., 2009). Initially, it was suggested that DHA support bacterial growth, but later it was observed that such effect was due to uptake of EPA that exist in preparation of DHA. There is probability that EPA might be synthesized after DHA is taken up by the cell (Nikaido, *et al.*, 1994).

2.5 Why Bacteria; best alternative source of PUFA

Due to non-fastidious nature PUFA producing Bacteria grows on waste products which lower the production cost. Bacterial isolates also have a potential of bioremediation along with PUFA production in the same process. For instance several psychrophilic bacteria have been reported as cold adapted cleaners with ability to produce PUFAs and bioremidiate hydrocarbon and oil (Lin *et al*., 2009). Bacteria are also good candidate as an alternative to fish oil due to cultivation in a controlled environment, absence of unpleasant taste, odor, structural simplicity of structure that can be used as model organism to study the metabolic pathway of PUFAs that ultimately lead to increased understanding of such process (El razak, *et al*., 2014) and enhancement of Omega-3 PUFA (Gill, *et al.,* 1997; Certik *et al.,* 1999). Besides, these bacteria produce one particular sort of PUFA rather than a mixture; therefore, no further purification steps are necessary that reduces production cost (Abd Elrazak, *et al.,* 2014). Bacterial PUFAs can

be used directly in food supplement, pharmaceutical, indirectly as a food stock for organisms like rotifers (Russell and Nichols, *et al.,* 1999a) or biomass of such bacteria can be introduced into aquaculture for fish supplement or in poultry feed (Harel, *et al*., 2002). The bacterial Omega-3 have high quantity of desired fatty acids; also recommended as Halal, Kosher and as a source of vegetarian as well as higher sensory and increased stability profile (Raghukumar, *et al.,* 2008; Fedorova-Dahms, *et al*., 2010).

2.6 Diversity of PUFAs producing bacteria

2.6.1 *Shewanella*

The genus *Shewanella* is a Gram negative, motile, straight/ curved rod and facultative anaerobic bacteria (Ivanova *et al.*, 2001) including both psychrophilic and mesophilic strains producing PUFA and widely distributed in marine environment. Different strains of *Shewanella* including *S. waksmanii, S. benthica, S. schlegeliana, S. sairae, S. baltica, S. pneumatohori* and *S. pealeana* from marine animals are capable of producing 15 to 37% of EPA or TPA (Gali *et al*., 1992; Shukla, 2003; Amiri-Jami, *et al*., 2006), while *S. hanedai, S. halifaxensis* and *S. frigidimarina* isolated from Antarctic marine habitats were efficient producer of EPA ranging from 7 to 22% of EPA (Bowman, *et al*., 1997). Other researchers have isolated *S. japonica* and *S. affinis* from sea water comprising up to 8% of EPA (Ivanova, *et al*., 2001 and 2004).

2.6.2 *Colwellia*

Species belonging to *Colwellia* are facultative anaerobic, psychrophilic bacteria classified as *Vibrio* (Deming, et al., 1988). Formerly, all the *vibrio* species capable of producing DHA and *colwellia* were grouped in a new genus (Wilkinson, *et al.,* 1988) and the genus became vibrant after identification of DHA producing four novel species *C. psychrotropica, Colwellia demingiae, C. hornerae* and *C. rossensis* from Antarctica (Raetz et al., 2007), which produces up to 6% DHA of the total fatty acids (yao *et al*., 2006), *C. chukchiensis* (Yu et al., 2011), *C. asteriadis* (Choi, et al., 2010), *C. polaris* (Zhang, et al., 2008) have been reported to synthesized high level of unsaturated fatty acid.

2.6.3 *Photobacterium*

One of the oldest bacterial taxa with one species *Ph. phosphoreum* reported by Thyseen *et al.,* in (2005), are psychrophilic and/ or psychropiezophilic, Gram negative, facultative anaerobic bacilli (kato *et al*., 2011) are prevalent in marine habitats (Shieh, *et al*., 2003). Currently, among 15 isolated species, only two piezophilic species *Ph. profundum, Ph. profundum* (SS9) from cold-seep area (Nogi *et al*., 2013) and *Ph. frigidiphilum* from deep-sea sediment (fang *et al*., 2007) are capable of producing PUFA.

2.6.4 *Moritella*

Moritella is a group of bacteria characterized as piezophilic and/or psychrophilic, majority of which are isolated from marine environment. The 7 known isolates were from marine habitats like fish farms, sea water, sediments and abyssal oceans (Nogi, *et al*., 1998; Benediktsdottir, *et al*., 2000). *Moritella marina* a non- piezophilic from North Pacific ocean (Radjasa *et al*., 2001), while *Moritella japonica* from Japan Trench and *Moritella yayanosii* from Mariana Trench have been isolated and characterized as piezophilic strains and are known to produce PUFA (Nogi *et al*. 2004). *Psychromonas* species, such as *P. agarivorans* and *P. boydii* do not produce either EPA or DHA in its membrane layer (Canion *et al*., 2013).

2.7 Genes responsible for PUFA production in Bacteria

The biosynthesis of PUFA via a bacterial polyketide synthase-type multienzyme complex is controlled by proteins encoded by five *pfa* genes including *pfa*A, *pfa*B, *pfa*C, *pfa*D and *pfa*E (Okuyama, *et al*., 2007b). The successful cloning of genes involved in the biosynthesis of EPA from *Shewanella* sp. strain SCRC-2738 by Tanaka, *et al.,* (1999) can be considered as the first step of research on these genes. Subsequently, a number of cloning experiments were carried out in order to gain deeper understanding of the PUFA genes. From these studies it was found that EPA gene carries at least 18 open reading frames (ORFs), but only five of them are required for the biosynthesis of EPA (Lee, *et al*., 2006; Okuyama *et al*., 2007). Their gene clusters are divided into three types. Type I, where the entire five Pfa genes are present in close vicinity, found in *Shewanella*

pneumatophori SCRC-2738. Type II contains cluster of the four genes pfaABCD, with pfaE separated from the other genes. This type of cluster is found in *Moriella marina* MP-1. The relative direction of *pfa*E has not been determined for this organism. In type III, *pfa*E is integrated into *pfa*C/E, and the cluster is considered to consist of four genes (Gong *et al*., 2014).

2.8 Biosynthesis of Fatty Acid in Bacteria

2.8.1 *Conventional Fatty acid synthase pathway (FAS)*

Synthesis of PUFA depends on presence of specific genes in the organism. Fatty acid biosynthesis pathway in all organisms terminates on the formation of C16 and C18 saturated fatty acids, then via sequence of desaturase and elongase enzymes, these fatty acids are modified into PUFAs (Figure 2.1).

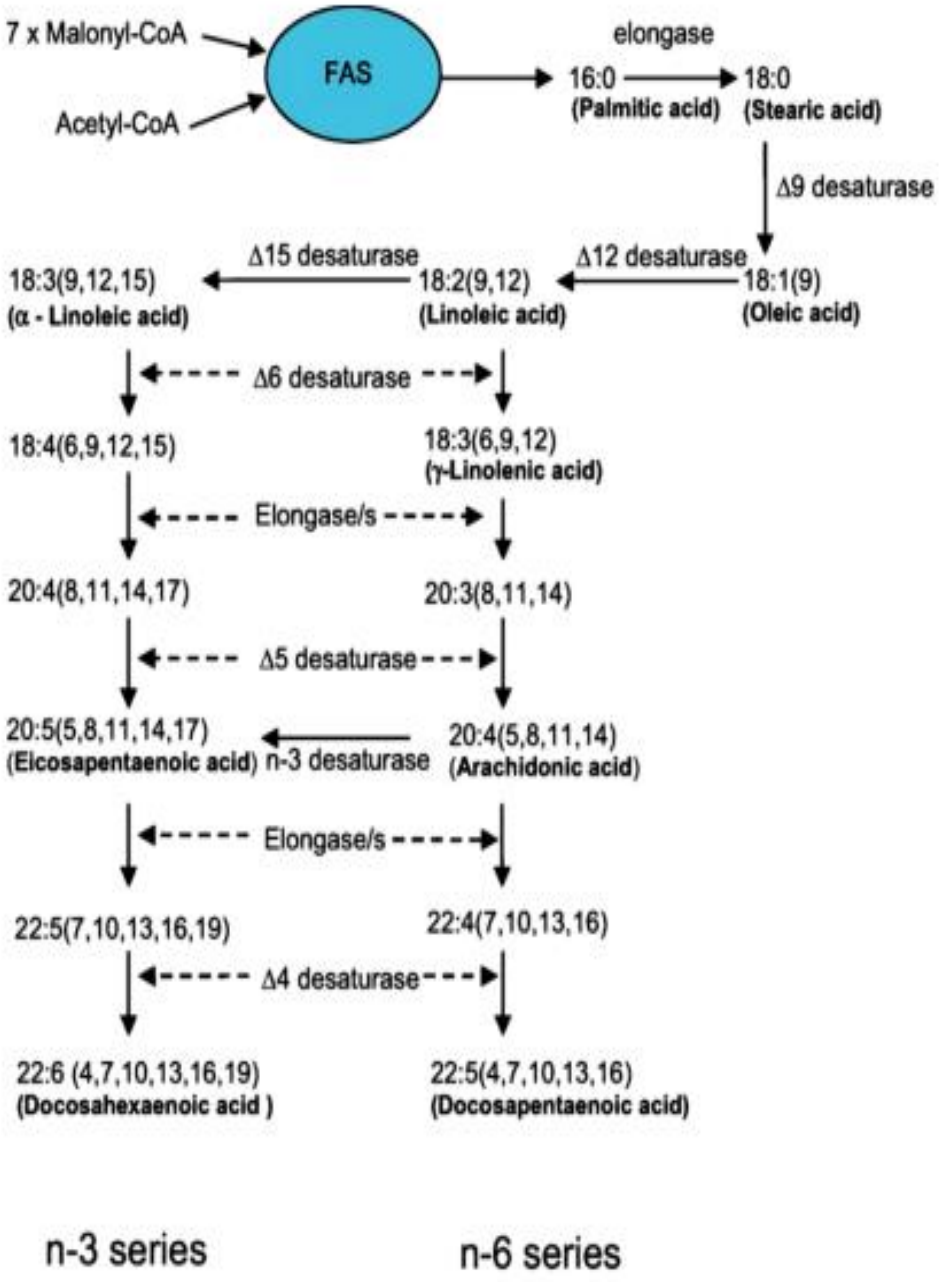


Figure 2.1 Conventional biosynthesis pathway of PUFA formation (Ratledge, *et al.*, 2004)

Figure 2.1.PUFA formation in microorganisms through FAS pathway, where fatty acids are synthesized from malonyl-CoA and acetyl-CoA precursors, using the FAS enzyme complex. Stearic acid, a saturated fatty acid, is then sequentially aerobically desaturated and elongated through a succession of reactions leading to the formation of different PUFA (Ratledge, *et al.*, 2004).

2.8.2 *Novel polyketide synthase biosynthetic pathway*

A novel metabolic pathway for the synthesis of PUFA was suggested by Metz, *et al*., (2001) known as polyketide synthase (PKS) that require multienzyme complex for series of reactions. In this pathway, both malonyl-CoA and acetyl-CoA are still essential but this PKS does not involve in *in situ* reduction of intermediates. The genomic fragment of *Shewanella* spp. as cloned in *E. coli* and five ORF were identified which were necessary for EPA production (Orikas *et al.,* 2006). It was suggested by Yano, et al., (1997) that PUFA synthesis in this strain SCRC2738, include the elongation of 16C or 18C fatty acids produced by FAS pathway and incorporation of double bond through unidentified desaturase pathway. In the five ORF, eleven regions were identified putative enzyme domains. When their sequence was compared in the database it was observed that eight of these resembled with PKS protein than to FAS although three regions were homologous to FAS proteins (Metz, *et al*., 2001; Napier, *et al.,* 2002).

The eight identified PKS domains were malonyl-CoA, ACP acyl transferase, 3-ketoacyl synthase, 3-ketoacyl-ACP, acyltransferase, reductase acyl carrier protein, dehydrase and chain length factor, enoyl reductase. Similar gene clusters were also identified from *Shewanella* spp. isolated from marine source indicated PKS pathway will be common in these organisms (Allen and Bartlett, *et al.,* 2002; Metz, *et al*., 2009). Researchers investigated recombinant *E. coli*, carrying the *Schizochytrium* PUFA synthase clusters, capable of producing PUFA. The genetic examination of *Schizochytrium* showed this organism has PKS system for PUFA production that using malonyl-CoA and acetyl-CoA as precursors for biosynthesis of PUFA (Ratledge, *et al.,* 2004). PKS pathway in *Schizochytrium* is summarized in Figure 2.2.

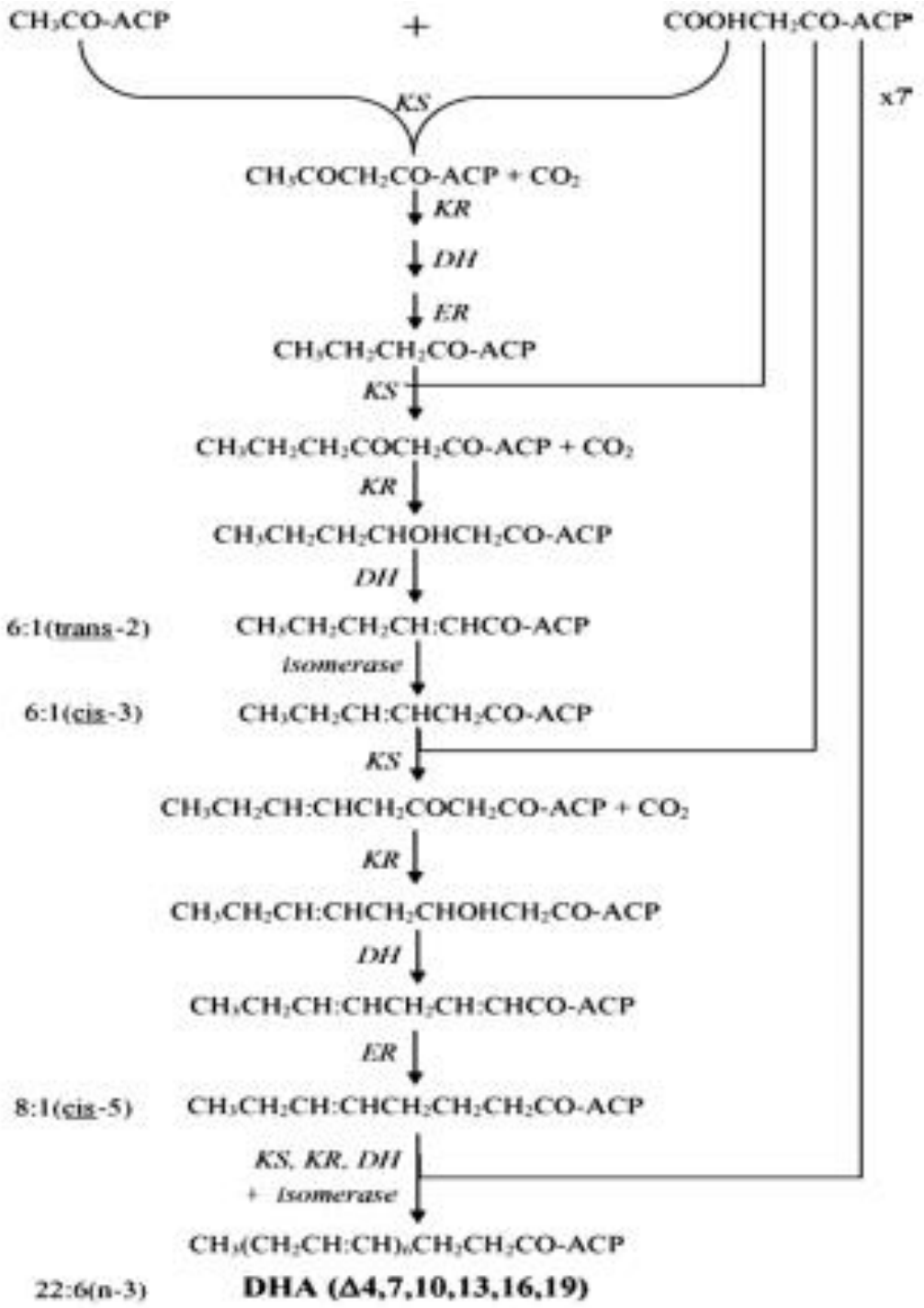


Figure 2.2 PUFA productions by PKS pathway. Ratledge *et al*, 2004.

2.9 Bioactivities of Polyunsaturated Fatty Acids

2.9.1 *Anti-inflammatory Activity*

Polyunsaturated fatty acid is known to affect several inflammatory responses. Inflammation is a normal host response to any kind of infection and injury. Several functions of inflammatory responses could have affected by polyunsaturated fatty acids. In normal host response to injury and infections, inflammation normally occurs. During inflammation chemokines, cytokines, eicosanoids, inflammatory molecules and some types of adhesion molecules are normally produced. Even though inflammation occurs normally, but whenever it takes place in inappropriate or uncontrolled way, disease could be caused due to extreme damage to host tissues. In human, various studies have revealed the inflammation-limiting properties of ω-3 fatty acids. De Caterina, *et al*. (1994) proved that cell surface expression of adhesion molecules is affected by long chain n-3 PUFAs, which are involved in relation between endothelial cells and leukocytes. Synthesis of inflammatory cytokines is also affected by Long chain n-3 PUFAs, a process which is controlled by 4-series LTs and PGE2. The studies of cell cultures showed that the production of interleukin-1 β (IL-1 β) by monocytes and tumor necrosis factor- α (TNF-α) could be affected by both DHA and EPA (Babcock *et al*., 2002; Siriwardhana, *et al*., 2012). Omega-3 PUFAs possessing anti-inflammatory action and its mechanism inside the cell is summarized by Calder, *et al.*, 2006. The work indicated that n-3 PUFAs increase long chain n-3 PUFAs in inflammatory cell membrane phospholipids and decreased arachidonic acid to improve the inflammatory phenotype by changing pattern of eicosanoid and resolvin synthesis, structure of membrane, pathways of signal transduction and expression of inflammatory gene.

After finding the fact that n-3 PUFAs have anti-inflammatory actions, has brought the ideas that complete or comparative less amount of these fatty acids may causally contribute to inflammatory conditions. For patients suffering from inflammatory diseases this idea might bring clinical advantage by providing the n-3 PUFAs. Dietary ω-3 fatty acids have a link with levels of plasma biomarkers, showing lower levels of inflammation and endothelial activation in case of cardiovascular disease and other acute and chronic

diseases, including acute pancreatitis, sepsis and chronic renal disease (Rangel, *et al*., 2012). Evidence are also found regarding treatment of inflammatory bowel disease (IBD) Crohn's disease and rheumatoid arthritis by polyunsaturated fatty acid (Bassaganya-Riera *et al.,* 2010; Zededel *et al.,* 2015).

2.9.2 *Antioxidant Activity*

Though usually information about fatty acids revealed that more the unsaturation, the more will be vulnerability to oxidation, this supposition is not always true due to indication of various lines of evidence. For instance, free radical-scavenging potential of PUFAs and the production of reactive oxygen/nitrogen (ROS/RNS) species by human aortic endothelial cells (HAECs) accompanied by different fatty acids was investigated by Richard *et al.,* 2008). They stated that HAEC accompanied with polyunsaturated fatty acids of the ω3 series yield less formation of ROS, as compared to the cells complemented with monosaturates, saturates or polysaturates of the ω6 series. n-3 PUFAs might act as indirect anti- rather than pro-oxidant in vascular endothelial cells. For the support of antioxidant activity of n-3 PUFAs there are many evidences present (Richard, *et al*., 2008; Tai *et al.,* 2010). There is not much known about the way by which the ω-3 PUFAs attain their protective effect. A proposed mechanism was that the enzyme of oxidative process could be affected by n-3 PUFAs for regulation the balance of oxidative products in vivo. Giordano *et al*., (2014) inspected that whether endothelial dysfunction and oxidative stress reverses with n-3 PUFA in experimental menopause having the background that menopause is linked with oxidative stress and endothelial dysfunction. They demonstrated that due to reversal of alterations in membrane lipid composition induced by ovariectomy and by reduction of vascular oxidative stress n-3 PUFA have a therapeutic advantage. The supplementation of n-3 PUFAs has been used to treat and also to avoid many oxidative injuries such as mild cognitive impairment MID and hence has a role in the protective benefit of health (Ko *et al.,* 2013).

2.9.3 *Cardio- protective Action*

The fatty acid composition of plasma phospholipids influenced by supplementation of DHA and EPA in diet and in a result it may affect *in vivo* cardiac cell functions. The utilization of n-3 PUFA is very useful for primary and secondary prevention of cardiovascular disease and this is confirmed by the studies of n-3 PUFA on cardiovascular health intervention.

Cardio- protective mechanisms by n-3 PUFA were complex because indication from molecular and cellular studies show that it involved pro-resolving lipid mediators, anti-inflammation, reduction of triglycerides, modulation of cardiac ion channels, antithrombotic and antiarrhythmic effects, influence on membrane micro domains and downstream and cell signaling pathways (Adkins and Kelley, *et al.*, 2010; Siddiqui, *et al*., 2008).

In management and prevention of some cardiovascular risk factors, the ω-3 long chain-polyunsaturated fatty acids are used extensively. From various laboratories many evidences revealed that intake of n-3 PUFAs regularly affects many cellular and humoral factors involved in atherogenesis and may prevent arrhythmia, atherosclerosis, cardiac hypertrophy, thrombosis and sudden cardiac death (Siddiqui, *et al*., 2008). The n-3 polyunsaturated fatty acids could prevent vascular and metabolic disorders in fructose-fed rats found by Masson, *et al*., (2008). This pathological status was linked to problems of cardiovascular systems. Recently, for protecting cardiovascular problems, the mechanism by n-3 PUFAs is unconvincing. However, for minimizing the cardiovascular disease's risk, the activity of dietary n-3 PUFAs has been accepted. The regular intake of n-3 PUFAs can minimize cardiovascular disease incidence.

2.9.4 *Anticancer Activity*

Many different types of factors are responsible for the complete process of tumor formation. There are much evidences present that show polyunsaturated fatty acids have a part in cancer risk and development. Dietary supplementation of ω-3 PUFAs or its long-lasting utilization can help us avoid urinary tract tumor risk development proposed

by Eynard, *et al.*, 2013). Many complex biological processes are involved in the effects of the PUFAs on anticancer. To describe their effects, many kinds of molecular mechanisms have been proposed, including metabolic conversion of n-3 PUFAs to novel discovered bioactive derivatives and alterations in arachidonic acid oxidative metabolism, change in cell membrane structure and fluidity, oxidative stress modification and altered metabolism and function of membrane proteins (Calviello, *et al*., 2009). Due to discovery of mechanism of the process, considerable attention was paid to beneficial effect of Omega-3 fatty acids as chemotherapeutic agents and chemo-preventive in cancer treatment. For the possible beneficial effects of Omega-3, much information has been collected nowadays. PUFA should be used in combination with different antineoplastic drugs and radiotherapy against melanoma, leukemia neuroblastoma and colon, breast, prostate and lung cancers.

2.9.5 *Antimicrobial Activity*

Polyunsaturated fatty acids have the potential new topical treatments for Gram-positive infections as these possess anti-inflammatory and antimicrobial properties and hence are attracting attention. Huang, *et al*., 2010) reported that they found novel bioactivity of the three main n-3 PUFAs, DHA, EPA, and ALA, and their ester derivatives and proposed that to make better oral health n-3 PUFA could have a helpful therapeutic outcome due to the antibacterial activities in addition with their anti-inflammatory effects (Huang *et al.*, 2011). The fatty acids due to their potency, absence of traditional resistance mechanisms against the actions of these compounds and broad spectrum of activity have gathered attention now- a- day. Especially, good activity of various long chain polyunsaturated fatty acids was observed against Gram-positive bacteria. Antimicrobial effects of PUFA against *S. aureus* and *P. acnes* were assessed for their potential in treatment of the infections caused by these pathogens. The outcomes revealed that LC-PUFAs could be used in addition with some present day treatment to increase efficiency of therapy to bacterial infections. These kinds of compounds either directly killed the bacteria or inhibit the bacterial growth (Desbois, *et al.*, 2012). Besides, the n-3 PUFA and their derivatives have variation in the pattern of action against bacteria, not restricted to

differences associated with Gram-negative or Gram-positive nature of species. The n-3 PUFA could target the cellular membrane hence destroying the functions of normal cell membrane and death of bacteria could be occurring. Fatty acids possessing antimicrobial activity could be used in agriculture, medicine and industry. Along with these, PUFAs also contribute in prevention and for controlling these infections in at-risk children; hence it can be an innovative approach (Huang and Ebersole, *et al.*, 2010).

Material and methods

3.1 Sample collection

Three different samples (glacier sediments, water and ice) were selected from the collected isolates of Tirich Mir glacier of the Hindu Kush Range, Passu and Batura from Karakoram region. Geographic coordinates and altitude of the sampling sites were determined by using GPS (Garmin).The pH and temperature of all samples were recorded using pH strips and thermometer. All the samples were collected in sterile Nalgene bottles under standard microbiological procedures and were transported to Microbiology Research Laboratory, Department of Microbiology, Quaid-i-Azam University, Islamabad, and stored at -20°C and preceded for isolation of PUFA producing bacteria.

3.2 Isolation of Bacteria

3.2.1 *Direct spreading method*

The previously collected sample of water, sediments and ice of the HKKH were selected for the isolation of bacteria. 100 µl of each sample was spread on MSM and NA medium and incubated at 4 and 20^{o}C for one week. After incubation different colonies were appeared. On the basis of visual observation 140 types of distinct bacterial colonies were subculture on NA and incubated at corresponding temperatures.

3.3 Preservation

For preservation purpose, 30% glycerol was used followed Lee *et al*., (2007) protocol.

Procedure

The selected isolates were grown in NA medium for 2 days at 15°C while used a sterile loop, and aseptic technique, transferring several colonies into the vial of broth with 30% Glycerol. The vial were shake well and allow the tubes containing culture to place at 20°C for approximately 30 minutes prior the long term storage. The culture were then stored at -20 degree.

3.4 Colorimetric screening for PUFAs producers

For EPA determination, 140 isolates were first grown in NA and incubate for 3 days. After 3 days of incubation, 0.1% w/v of the dye 2, 3, 5-triphenyltetrazolium chloride (TTC) was added to the tubes containing 5ml culture broth of the inoculated strain. Tubes were incubated at (15°C) for one hour. The formation of red color was considered to be a positive result (Ryan, *et al*., 2010).

3.5 Optimization of condition for the growth of isolates

3.5.1 *Temperature Optimization*

For optimization of PUFA production, the isolates were incubated at three different temperatures i.e. 5, 15, and 25°C. The selected isolates, PLPI 1, PDPI 2, PDPI 3, TSC 2 and PSC 3 were first grown in NB medium and incubate for 24 hours. After incubation, 1mL of culture was transferred into a sterile conical flasks containing 100 ml NB medium. Each experiment was run in duplicate. The isolates were incubated in shaker incubator with 120 rpm and temperature 5, 15, and 25°C. The Optical Density was observed from initial time till decline phase of organism.

3.5.2 *Media Optimization*

Five different media i.e. Nutrient Broth (NB), Luria bertani (LB), Peptone Yeast Extract Meat Extract (PYM), Minimal Salt Medium (MSM) and Blue Green Algae (BG11) were selected in order to determine their growth pattern and optimum PUFA production. Inoculums were prepared and equal amounts were transferred in to each flask containing 100ml of the respective medium. The flasks were incubated in rotary shaker 120 rpm for five days at 15°C. The O.D was observed after every 24Hrs of incubation.

3.5.3 *pH Optimization*

In order to know the optimum pH for PUFA production, the isolates were grown in duplicate on pH 3, 5, 7, 9 and 11. Nutrient broth was prepared and the pH range was adjusted by using H_2SO_4 and HCl as a buffering agents. After adjustment of particular pH, the media were autoclaved and inoculated with 2ml of the 24 hours old culture. The O.D was recorded after every 24 hour of incubation for five days.

3.6 Confirmation and structural characterization of products

For confirmation of produced PUFA Fatty Acid Methyl Easter was prepared.

3.6.1 *Fatty Acid Methyl Easter (FAME) preparation*

Isolates were grown in NB for 3 days of incubation at 15°C. After incubation periods the media was centrifuge in order to make the cells pellet. The biomass was harvested and treated with suitable solvent for lipid extraction. The cells were allowed to for freeze dryingand were suspended in 2ml of 5% methanolic HCl, heated at 70°C in a water bath for 2 hours. The tubes were allowed to cool at room temperature for 30 minutes, then 1 ml distilled water was added and the tubes were vortexed. To extract the FAME 1 ml hexane was added and vigorously vortexed. The tubes were kept till two layers were formed. The upper layer was transferred into a clean tube and dried under nitrogen. A known volume of hexane was added for knowing final concentration and FAME was analyzed using Thin Layer Chromatography (TLC) and Fourier Transformed Infrared Spectroscopy (FTIR).

3.6.2 *Thin Layer Chromatography*

Thin layer chromatography is a chromatographic technique used for the separation of compounds by disfusing the sample along with mobile and stationary phase. This technique is use for the separation, identification and even partial purification of compound from crude sample as well run in comparison with standard for the identification. Following the Shimajiri *et al*., 2013 procedure. For the TLC procedure lipids were separated by TLC on 20 X 20 cm glass plates coated with silica gel-60 (Merck, Darmstadt, Germany). Chromatography was carried out in light-protected jars under an Ar atmosphere. Lipids were eluted with chloroform/acetone/methanol/acetic acid/water (10:4:2:2:1) as the developing solvent. The plates were labeled by led pencil and the samples were spotted accordingly along with the standard obtained from the Vitamin Company USA. After 10 minute of incubation, developed plate was allowed to dry as the solvent evaporated and visualized under UV lamp in both short and long wavelengths. Separated spots were

marked with the help of lead pencil carefully and Rf values were calculated by dividing their distance travelled to that of the solvent.

3.6.3 *Fourier Transformed Infrared Spectroscopy (FTIR)*

The purified lipid extracted from the PUFA positive isolate was analyzed through FTIR along with the standard obtained through (The Vitamin Company USA). FTIR works on the principle of absorbance and transmittance of infra-red radiations through the sample. The infrared spectrum produced as a result, corresponds to a fingerprint of respective sample where absorption is represented in the form of peaks. These peaks symbolize the frequencies of atomic vibrations occurring in the bonds of functional groups present in the sample. As atomic combination varies with different substances, infrared spectra for two materials can never be alike. Samples can be qualitatively or quantitatively evaluated by the presence of peaks and the size of particular peak respectively. Height directly relates to the quantity of a substance in the sample.

Active frictions of all strains were evaluated by FTIR spectrophotometer (Tensor 27, Bruker, equipped with ZnSe ATR) in the range of 4000-600cm-1. About 10 mg of extracted sample before and after fractionation was positioned on the sample tray and infrared spectrum was obtained. It was then compared to the library for presence of known or unknown components using Software Opus 65.

3.7 Characterization of bacterial isolates

3.7.1 Morphological characterization

3.7.1.1 *Colony morphology*

The colony morphology was observed with respect to colony texture, elevation, shapes, size, margins and pigmentation.

3.7.1.2 *Gram staining*

For gram staining crystal violet, Gram's iodine, alcohol (95%) and safranin were prepared (Table A9).For staining purpose, a colony from fresh culture was mixed in dH_2O and a thin smear was made on grease free slide. The smear was dried and heat fixed. After fixation the smear were flooded with Crystal Violet and remain for the slide for 1minute. The stain was then washed with distilled water and then flooded the slide with iodine solution for 1 minute. The iodine was washed with tap water and then the secondary stain Safranin was applied on the slide and remains the stain for 45 Seconds. Then, the slide was washed with distilled water and air dried and examined under microscope at 100X using cedar wood oil. Gram positive and Gram negative bacteria were differentiate according to their color appearance.

3.7.2 Molecular characterization

3.7.2.1 Extraction of DNA

For molecular characterization and phylogenetic analysis, DNA of the selected isolates, PLPI 1, PDPI 2, PDPI 3, TSC 2 and PSC 3, were extracted by using MO BIO Ultraclean DNA extraction kit. For isolation of DNA the selected isolates were grown on Nutrient Agar to obtain fresh cultures. Bacterial colonies were then transferred to 2 ml beads solution and vertexes gently for complete mixing. Solution S1 (see Appendix) was added and incubated in water bath for some time at 60°C in order to dissolve the present precipitate. After this, 60 µl of solution S1 was added and vertex gently. After incubation, 200 µl of solution, Inhibitor Removal Solution (IRS) was added which is required only when DNA is to be used for PCR. Again overtaxing was performed at maximum speed for 10 minutes in order to lyse the whole bacterial cells. The lyse solution was then transferred to autoclaved 2 ml eppendorf tubes and secured the beads tube. Eppendorf were centrifuge for 30 sec at 4°C, at speed of 10000 rpm. After centrifugation particulates including cell biomass and other debris will form pellet at this point. Supernatant was collected which having our desire DNA and were transferred into autoclaved micro centrifuge tubes. Then 250 µl of solution S2 was added and then vertex for 5 seconds and then incubates at 4°C for 5 minutes. This is step is done basically for the removal of contaminated proteins which bring impurities in DNA, as this solution contain protein precipitation reagents. The tube was centrifuge for 1 minute at 10000 rpm at 4°C. The pellet became removed off and the supernatant become shifted into micro centrifuge tubes. Solution S3 (1.3 ml) was then added as it contains DNA binding salt solution and vortexed it for 5 seconds. Then 700 µl was added upon spin filter and centrifuge at 10000 rpm for 1 minute. Then again the supernatant was passed through the spin filter and centrifuge it for 1 minute. After that 300 µl of solution S4 was poured and centrifuge it for 30 seconds upon 10000 rpm at 4°C. The filtrates were then discarded and the DNA tubes were kept at frozen temperature of about - 20°C

3.7.3 Agarose gel electrophoresis

The presence of extracted DNA was checked on Agarose gel electrophoresis. For this purpose 1% agarose gel were prepared, i.e. 1 g agarose was mixed in 100 ml of TAE

buffer. The mixture was heated in microwave oven until the complete dissolution of gel. The gel was then allowed to cool at room temperature up to 40-50°C, and then added 2 µl of ethidium bromide. The agarose solution then poured into the gel tray having comb, allowed the gel to set completely at room temperature. After gel solidification, 5 µl DNA sample was mixed with 2 µl of loading dye and load properly to the well using micropipette. The electrophoretic tank was filled with 1X TAE buffer; lid of the gel tank was closed and attached electrical leads, so that DNA was migrated towards the positive anode. Gel was run at 115 volts for 30 minutes. For positive results, gel was observed under UV illuminator and photographed.

3.7.4 Polymerase chain reaction (PCR)

3.7.4.1 *16S rRNA amplification*

The extracted genomic DNA was used as a template for the amplification of the 16S rRNA gene. PCR master mixture, containing 5.0 µl 1 X buffer (160 mM $(NH4)_2SO_4$, 670 mM TrisHCl, 0.1% Tween-20), 0.8 µl a mixture of NTPs, 20 µl each of primers 27F (5′AGAGTTTGATCMTGGCTCAG-3′) and 1492R, TACGGYTACCTTGTTACGACTT (Schluenzen, *et al*., 2000), 3.0 µl of 50 mM $MgCl_2$, 0.5 µl of Bio Taq DNA polymerase (Bioline) 1 µl of genomic DNA made up with distilled water to 50 µl was prepared. Controls lacking template DNA were included in each PCR run. The PCR amplification was performed in an Applied Biosystems, Thermocycler according to the following programed

Table.2.1 PCR programmed used for amplification of 16S RNA

Steps	Temperature in °C	Time
First Denaturation	94	5 minutes
Step 1 Denaturation	94	30 seconds
Step 2 Annealing	52	45 seconds
Step 3 Extension	72	1 minute
Final Extension	72	10 minute

The cycles of steps from 1-3 were repeated 35 times. The PCR products were checked by agarose gel electrophoresis (1% w/v; 30 minutes at 100 V, 0.5 X TAE buffer). The amplified fragments were compared with 500bp molecular size marker (Bioron, Germany). The PCR product was stored at -20C.

3.7.5 Sequencing and phylogenetic analysis

3.7.5.1 *Sequencing of the Isolates*

The amplified product was run on 1% gel for confirmation and then sent for sequencing. Sequencing was commercially performed through Macrogen Standard Customer DNA Sequencing Services (Macrogen Inc. Seoul, Korea). For all sequencing reactions, universal primers 27F and 518R were used to sequence the strands of 16S rRNA gene.

3.7.5.2 *Compilation of Sequencing Data*

For the compilation of sequence data, DNA Baser v 4.16.0 software was used. The sequences of both the complementary strands were aligned to each other. The sequence from one isolate was determined by this method. ClustalW software was used for obtaining and aligning multiple sequences belong to each component for each isolates (Thompson, *et al*., 1994). Using the similar procedure all the data were compiled.

3.7.5.3 *Phylogenetic Analysis*

After compilation of the sequences data, similar sequences were obtained from NCBI Gen Bank by BLAST search. Similar sequences were downloaded and aligned by using MAFFT software. The downloaded sequences were used for the construction of phylogenetic tree. Tree was constructed using Neighbor-Joining (NJ) analysis, Maximum Composite-likelihood model with uniform rates among the sites, the 1000 bootstraps replicates were used to evaluate the significance of generated tree using the Molecular Evolutionary Genetics Analysis Program (MEGA) version 6.

4.1 Physiochemical Characteristics of Samples

Water, ice and sediment samples were collected from different sites of Batura, Passu and Tirich Mir glaciers located in Northern Areas of Pakistan. Physical parameters recorded during sampling are given in Table 4.1.

Table 4.1 Physiochemical characteristics of samples

Glaciers	GPS coordinates	Sample	Characteristics of sample	
			pH	Temperature (°C)
Batura	N 36°30.302 E 074°51.138	sediment	7	1
		water	7	1
		ice	7	-2
Passu	N 36°27.424 E 074°52.010	sediment	7	1
		water	7	1
		ice	7	-2
Tirch Mir	N 36°22.616 E 072°08.983	sediment	7	1
		water	7	4
		ice	7	0

4.2 Isolation of bacteria

After incubation different colonies were appeared. On the basis of visual observation 140 types of colonies were selected for further processing showing in figure 4.1

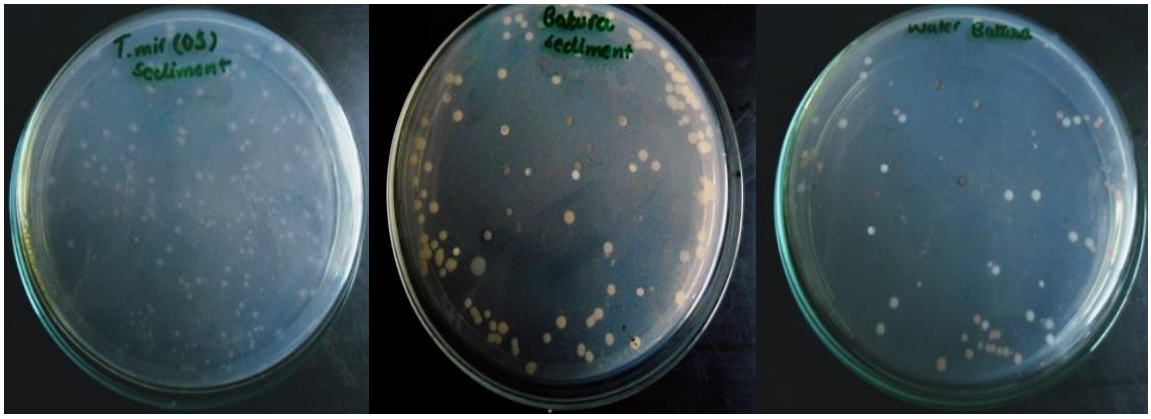


Figure 4.1 Isolated bacterial colonies from Batura, Tirich Mir samples (sediment, and water)

Table 4.2 No of total and distinct colonies appeared upon spreading

Site of sampling	samples	Total No. of colonies		No. of different colonies	
		4°C	20°C	4°C	20°C
Batura	Sediment	>100	>200	11	16
	Water	17	45	7	9
	Ice	14	17	6	11
Passu	Sediment	>200	>200	9	10
	Water	5	32	5	9
	Ice	7	52	5	4
Tirich Mir	Sediment	>300	>300	7	4
	Water	29	73	5	7
	Ice	25	89	8	6

4.3 Gram Staining and morphological characteristics

Gram staining and colonies morphology analysis was carried out for all the isolates. Due to gram characteristic and colonies morphology similar isolates were removed and the remaining 100 isolate were again sub cultured for obtaining pure culture. Then the isolates were subjected for Screening of PUFA. The selected isolates are shown in table 4.3.

Table 4.3 Gram characteristic and colony morphology of the isolates

Isolates	Gram reaction	Morphology						
		Size	Color	Shape	Texture	Elevation	Margin	Pigmentation
PLPI-1	- Rods	Small	Yellow	Round	S mucoid	Raised	Entire	Yellow
PDPI-2		Medim	Cream	Irregular	Mucoid	Convex	Undulate	Creamy
PDPI-3		V small	Yellow	Rounded	Mucoid	Pulvinate	Entire	Yellow
TSC-2								
PSC-3		Medium	Off white	Irregular	S mucoid	Convex	Undulate	Np

4.4 Screening of isolates for PUFA production

A total of 100 isolates were screened which showed that 52 were able to produce PUFA. Among the selected isolates 36 were isolated from Batura, among which 19 showed activity of PUFA production.17 isolates out 30 of the Passau regions showed positive PUFA production while 16 isolate of Tirch Mir out of 34, showed ability to produce PUFA.

Table 4.4 No of selected and PUFA positive isolates

Site of sampling	Samples	Total No. of selected isolates	Total No. of PUFA producers
Batura	Sediment	19	6
	Ice	9	8
	Water	8	5
Passu	Sediment	17	5
	Ice	7	7
	Water	6	5
Tirich Mir	Sediment	14	7
	Ice	11	6
	Water	9	3

4.5 Selection of best producer

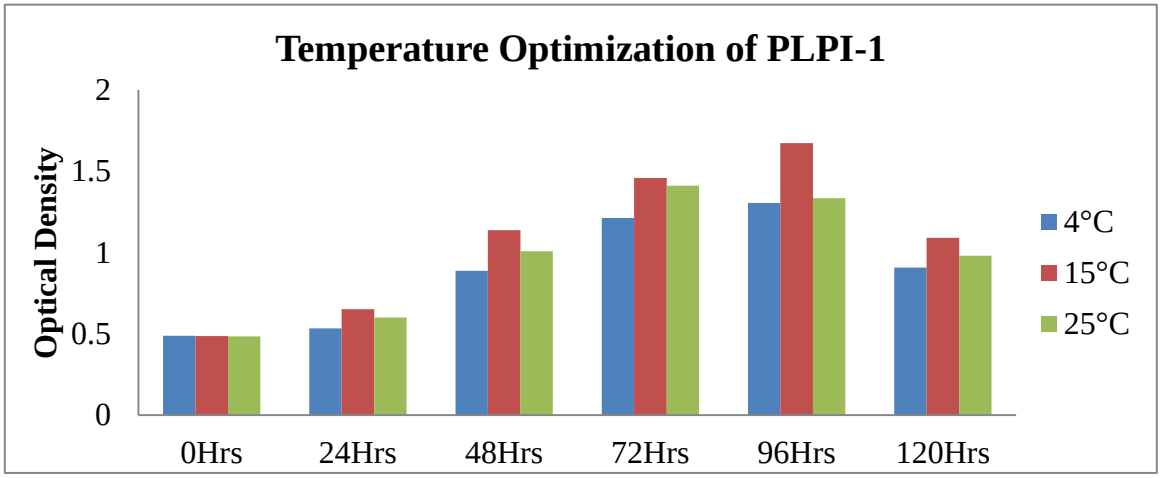
All the isolates that showed PUFA producing ability were again screened in broth as well in solid ager. Five isolates PLPI-1, PDPI-2, PDPI-3, TSC-2 and PSC-3 that showed maximum PUFA production were selected for further study.

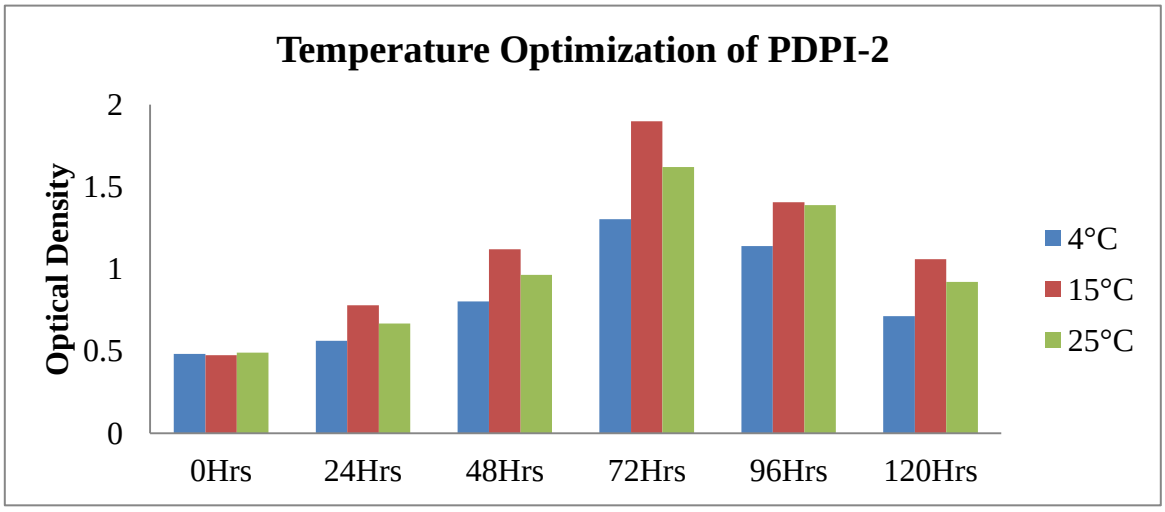
Figure 4.2 Rapid TTC assays of the isolated bacteria for FUFA Production

4.6 Growth Optimization and PUFA production

4.6.1 Temperature optimization

All the selected isolates were checked for optimum growth temperature. Among selected isolates, PLPI-1 and PDPI-2 showed maximum growth at 15°C while the isolate PDPI-3 showed maximum growth at 4°C. Similarly the isolate TSC-2 and PSC-3 showed maximum growth at 15°C followed by 25°C. The rapid TTC test were carried out for the isoaltes which showed optimum production of PUFA at 15°C Maximum growth of PLPI1,TSC-2 and PDPI-3 was observed after 96 hrs while isolates PDPI-2 and PSC-3 showed maximum growth after 72 hrs of incubation. It was observed that all the isolates showed maximum PUFA production at 15^{o}C however their growth pattern was variable.





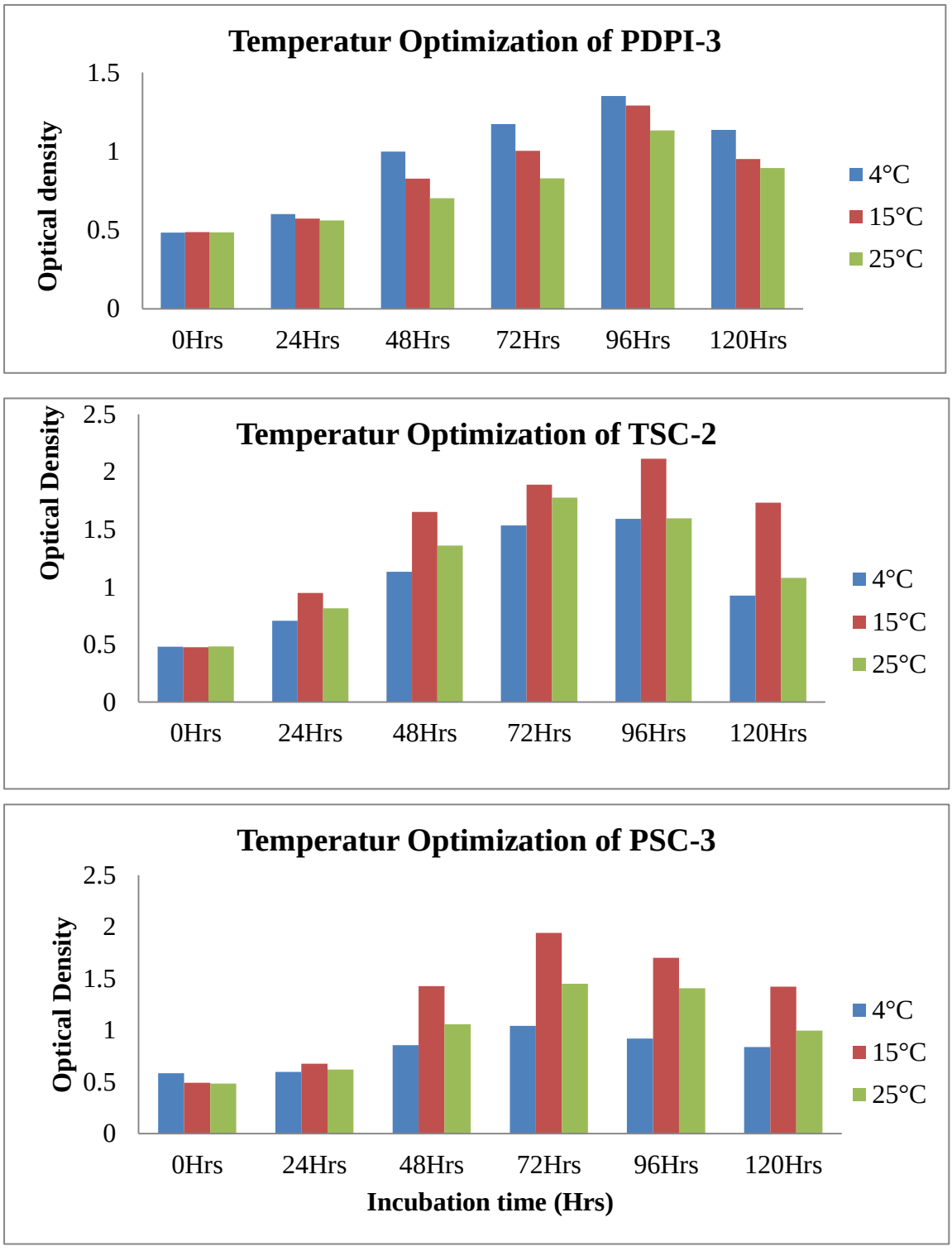


Figure 4.3 Temperature Optimization for the isolates PLPI-1, PDPI-2, PDPI-3, TSC-2 and PSC-3 respectively

4.6.2 Media optimization

Growth of selected isolates was checked on different media and consequently checked for fatty acid production. It was observed that PLPI1 showed maximum growth at NB followed by LB, MSM, PYM and BG11and shoed maximum production of fatty acid in after 72 hrs of incubation, while isolates PDPI-2 and PDPI-3 showed maximum growth on PYM followed by LB, NB, MSM and BG11. Similarly the isolates TSC-2 and PSC-3 showed maximum growth on PYM followed by NB, LB, MSM and BG11. Initially the growth of the isolates was low up to 48 hrs but increased after 72 hrs and reached maximum at 96 hrs. The details are shown in tables (1, 2, 3, 4 and 5) for the isolates respectively. It was noted that production of PUFA was maximum after 72 hrs of incubation.

Table 4.5 Media optimization for growth and PUFA production by isolate PLPI-1

Isolate PLPI-1			**Time of incubation in hrs.**					
Media	**Parameters**		**0**	**24**	**48**	**72**	**96**	**120**
NB	OD		0.486	0.652	1.137	1.458	1.673	1.091
	pH		6.90	6.85	7.33	7.31	7.01	7.02
	TTC Concentration	0.1	-	++	+++	++++	+++	+++
		0.05	-	+	+++	+++	++	++
		0.01	-	+	+++	++++	+++	++
LB	OD		0.542	0.136	1.157	1.158	1.273	1.391
	pH		6.93	6.85	7.20	7.17	6.91	6.95
	TTC Concentration	0.1	-	++	+++	++++	+++	++
		0.05	-	+	+++	++++	++	++
		0.01	-	++	+++	++++	+++	++
PYM	OD		.402	4.001-2	.291	0.146	0.146	0.148
	pH		5.65	5.48	5.51	5.35	5.51	5.45
	TTC Concentration	0.1	-	-	W+	+	+	-
		0.05	-	-	W+	W+	W+	-
		0.01	-	-	W+	+	+	-
MSM	OD		0.152	6.782-2	0.532	0.4978	0.334	0.315
	pH		7.06	7.07	7.39	7.07	7.34	7.54
	TTC Concentration	0.1	-	+	++	++	+	+
		0.05	-	W+	++	++	W+	-
		0.01	-	W+	++	++	W+	-
BG11	OD		0.149	0.2544	0.2336	0.2826	1.417	8.157-3
	pH		7.34	6.89	6.82	7.02	7.03	7.04
	TTC Concentration	0.1	-	-	-	-	-	-
		0.05	-	-	-	-	-	-
		0.01	-	-	-	-	-	-

Table 4.6 Media optimization for growth and PUFA production by isolate PDPI-2

Isolate PDPI-2			**Time of incubation in hrs.**					
Media	**Parameters**		**0**	**24**	**48**	**72**	**96**	**120**
NB	OD		0.476	0.779	1.120	1.899	1.406	1.060
	pH		6.90	6.62	7.32	7.17	7.10	7.22
	TTC Concentration	0.1	-	++	++++	++++	+++	++
		0.05	-	++	++++	+++	+++	++
		0.01	-	++	+++	++++	++	++
LB	OD		0.542	0.339	1.264	1.2891	1.321	0.321
	pH		6.93	6.66	7.00	6.92	7.20	7.01
	TTC Concentration	0.1	-	++	++++	++++	+++	++
		0.05	-	++	+++	+++	++	+
		0.01	-	++	+++	++++	++	+
PYM	OD		0.402	0.179	1.420	2.0899	2.606	2.460
	pH		5.65	5.60	5.33	4.90	4.51	4.72
	TTC Concentration	0.1	-	W+	+	++	W+	-
		0.05	-	W+	+	+	-	-
		0.01	-	W+	+	+	-	-
MSM	OD		0.152	0.458	0.723	0.325	0.302	0.292
	pH		7.06	7.19	7.37	7.56	7.24	7.58
	TTC Concentration	0.1	-	+	++	++	W+	-
		0.05	-	+	++	+	W+	-
		0.01	-	+	++	+	W+	-
BG11	OD		0.149	1.224-2	0.407	0.478	1.764-2	1.695-2
	pH		7.34	7.08	6.83	6.87	6.96	6.23
	TTC Concentration	0.1	-	-	-	-	-	-
		0.05	-	-	-	-	-	-
		0.01	-	-	-	-	-	-

Table 4.7 Media optimization for growth and PUFA production by isolate PDPI-3

Isolate PDPI-3			Time of incubation in hrs.					
Media	**Parameters**		**0**	**24**	**48**	**72**	**96**	**120**
NB	OD		0.487	0.573	0.827	1.004	1.290	0.951
	pH		6.90	6.82	7.40	6.98	7.02	7.09
	TTC Concentration	0.1	-	++	+++	+++	++	+
		0.05	-	++	++	++	++	+
		0.01	-	++	+++	+++	++	+
LB	OD		0.542	0.303	0.827	1.304	1.490	0.351
	pH		6.93	6.59	7.00	6.97	6.92	7.18
	TTC Concentration	0.1	-	++	++++	++++	+++	++
		0.05	-	++	+++	+++	+++	+
		0.01	-	++	+++	++++	+++	++
PYM	OD		0.402	0.202	1.499	2.173	2.351	2.336
	pH		5.65	5.63	4.98	4.41	4.60	5.09
	TTC Concentration	0.1	-	+	+	W+	W+	-
		0.05	-	W+	W+	W+	W+	-
		0.01	-	W+	W+	W+	W+	-
MSM	OD		0.152	0.1051	0.392	0.4224	0.323	0.30
	pH		7.06	7.08	7.30	8.16	8.06	8.06
	TTC Concentration	0.1	-	+	++	+	W+	-
		0.05	-	+	+	+	W+	-
		0.01	-	+	+	+	W+	-
BG11	OD		0.149	8.412-2	0.255	0.590	3.134-2	2.321-2
	pH		7.34	6.73	7.27	6.78	7.02	6.96
	TTC Concentration	0.1	-	-	-	-	-	-
		0.05	-	-	-	-	-	-
		0.01	-	-	-	-	-	-

Table 4.8 Media optimization for growth and PUFA production by isolate TSC-2

Isolate TSC-2			**Time of incubation in hrs.**					
Media	**Parameters**		**0**	**24**	**48**	**72**	**96**	**120**
NB	OD		0.485	0.814	1.360	1.776	1.597	1.079
	pH		6.90	7.67	8.10	8.17	8.37	8.38
	TTC Concentration	0.1	-	++	++++	++++	++++	++
		0.05	-	++	+++	+++	++	++
		0.01	-	++	++++	+++	+++	++
LB	OD		0.542	1.167	1.633	1.6982	1.592	0.325
	pH		9.93	6.46	7.0	6.79	6.81	6.94
	TTC Concentration	0.1	-	++	+++	++++	++++	+++
		0.05	-	++	+++	+++	+++	++
		0.01	-	++	+++	++++	++++	++
PYM	OD		0.402	3. 149	3.125	3.5601	3.352	3.133
	pH		5.65	4.44	4.17	4.08	4.16	4.10
	TTC Concentration	0.1	-	-	-	W+	-	-
		0.05	-	-	-	W+	-	-
		0.01	-	-	-	W+	-	-
MSM	OD		0.152	0.1091	0.518	0.3201	0.299	0.299
	pH		7.06	6.91	7.35	7.27	7.46	7.46
	TTC Concentration	0.1	-	+	++	+	-	-
		0.05	-	W+	+	+	-	-
		0.01	-	+	++	W+	-	-
BG11	OD		0.149	6.0481-3	0.191	2.224-2	2.295-2	2.128-2
	pH		7.34	6.52	6.89	6.88	7.05	6.86
	TTC Concentration	0.1	-	-	-	-	-	-
		0.05	-	-	-	-	-	-
		0.01	-	-	-	-	-	-

Table 4.9 Media optimization for growth and PUFA production by isolate PSC3

Isolate PSC-3			**Time of incubation in hrs.**					
Media	**Parameters**		**0**	**24**	**48**	**72**	**96**	**120**
NB	OD		0.485	0.621	1.058	1.851	1.607	0.997
	pH		6.90	6.80	7.84	7.95	8.18	8.30
	TTC Concentration	0.1	-	++	++++	++++	+++	++
		0.05	-	++	+++	+++	++	++
		0.01	-	++	++++	+++	+++	++
LB	OD		0.542	0.256	1.857	1.843	2.021	0.438
	pH		6.93	6.85	6.88	6.70	6.82	7.00
	TTC Concentration	0.1	-	++	++++	++++	+++	++
		0.05	-	++	+++	+++	+++	++
		0.01	-	++	+++	++++	+++	++
PYM	OD		0.402	2.002-3	2.429	3.543	3.590	3.122
	pH		5.65	5.40	5.10	4.22	4.09	4.08
	TTC Concentration	0.1	-	-	W+	W+	-	-
		0.05	-	-	W+	W+	-	-
		0.01	-	-	W+	W+	-	-
MSM	OD		0.152	0.1957	0.674	0.409	0.368	0.335
	pH		7.06	7.10	7.60	7.27	7.36	7.58
	TTC Concentrat-ion	0.1	-	+	++	++	-	-
		0.05	-	+	+	++	-	-
		0.01	-	+	+	++	-	-
BG11	OD		0.149	1.556-2	0.148	0.1061	2.034-2	1.589-2
	pH		7.34	6.75	6.92	7.05	7.08	6.84
	TTC Concentration	0.1	-	-	-	-	-	-
		0.05	-	-	-	-	-	-
		0.01	-	-	-	-	-	-

4.6.3 pH optimization

After 72 hrs of incubation it was observed that isolate PLPI-1 showed maximum production at 7pH. Very little growth were observed at pH 11 and 3 and showed no activity for PUFA production. The isolate PDPI-2 showed maximum production of PUFA at wide range of pH i.e. at 5, 7, and 9 but maximum production was observed at 7pH. The isolate was able to tolerate a wide range of pH and show active growth at all pH but was highest at 7pH. The isolate PDPI-3 was able to produce maximum PUFA at 7pH after 72 hrs of incubation and similarly showed maximum growth at this pH. The isolate TSC-2 showed maximum production at 7pH and showed highest growth at this temperature while the isolates PSC-3 showed maximum production of PUFA at 5pH, as the isolate was showed highest growth at this pH and similarly showed activity at pH 7 as there growth was also observed at this pH. Throughout the study it was observed that at pH7 the isolate showed maximum production of PUFA as the isolates were able to show highest growth activity at this pH.

Table 4.10 pH optimization for the selected isolates

Isolates	Time of incubation in hrs.											
	0			24			48			72		
	pH	OD	TTC	pH	OD	TTC	pH	OD	TTC	pH	OD	TTC
PLPI-1	3	0.227	-	3.20	0.204	-	3.23	0.228	-	3.66	0.219	-
	5	0.158	-	3.15	0.223	-	3.35	0.210	-	3.66	0.082	-
	7	0.095	-	7.32	0.174	+	7.15	0.429	++	7.18	0.620	+++
	9	0.062	-	3.20	0.228	-	3.24	0.218	-	3.29	0.160	-
	11	0.025	-	3.15	0.183	-	3.16	0.159	-	3.17	0.096	-
PDPI-2	3	0.227	-	6.45	0.174	-	7.19	0.605	-	7.46	1.447	-
	5	0.158	-	6.54	0.156	+	7.27	0.769	+++	7.66	1.645	++++
	7	0.095	-	7.27	0.274	++	7.19	0.440	++	7.26	0.533	+++
	9	0.062	-	6.61	0.208	+	7.23	0.805	++	7.46	1.459	++++
	11	0.025	-	6.48	0.173	-	7.20	0.500	-	7.34	1.318	-
PDPI-3	3	0.227	-	9.19	0.080	-	9.27	0.189	-	7.86	0.640	-
	5	0.158	-	9.23	0.075	W+	8.82	0.197	+	7.98	0.653	++
	7	0.095	-	7.51	0.233	+	7.30	0.398	++	7.35	0.447	++
	9	0.062	-	9.10	0.121	W+	8.55	0.309	W+	7.90	0.649	+
	11	0.025	-	8.92	0.113	-	8.04	0.506	-	8.26	0.426	-

TSC-2	3	0.227	-	10.25	0.045	-	10.21	0.098	-	10.12	0.040	-
	5	0.158	-	10.06	0.050	-	10.07	0.048	-	10	0.061	-
	7	0.095	-	7.68	0.143	+	7.37	0.299	++	7.43	0.508	++
	9	0.062	-	10.04	0.072	-	10	0.062	-	9.85	0.081	-
	11	0.025	-	10.16	0.047	-	10.16	0.052	-	10.10	0.036	-
PSC-3	3	0.227	-	3.22	0.210	-	3.28	0.174	-	3.36	0.198	-
	5	0.158	-	5.99	0.144	-	6.74	0.262	-	7.44	1.228	-
	7	0.095	-	7.36	0.134	+	7.04	0.265	+++	7.19	0.443	+++
	9	0.062	-	9.21	0.073	W+	9.14	0.088	++	8.96	0.096	W+
	11	0.025	-	9.99	0.093	-	9.79	0.078	-	9.07	0.114	-

4.7 Characterization of product

The extracted PUFA was identified and characterized by TLC and FTIR analysis

4.7.1 Thin Layer Chromatography

The TLC analysis of the FAME extract of the selected isolates PLPI-1, PDP-2, PDPI-3, TSC-2 and PSC-3 revealed wide dark spot at Rf 0.87, 0.86, 0.86, 0.87 and 0.86 respectively which was very closely related to the standard EPA, DHA spot appeared at Rf 0.88, (RF the retardation factor, i.e. the ratio of the distance travelled by the center of the spot to the overall distance travelled by the mobile phase). The band was observed under UV using mobile phase chloroform/acetone/methanol/acetic acid/water (10:4:2:2:1).

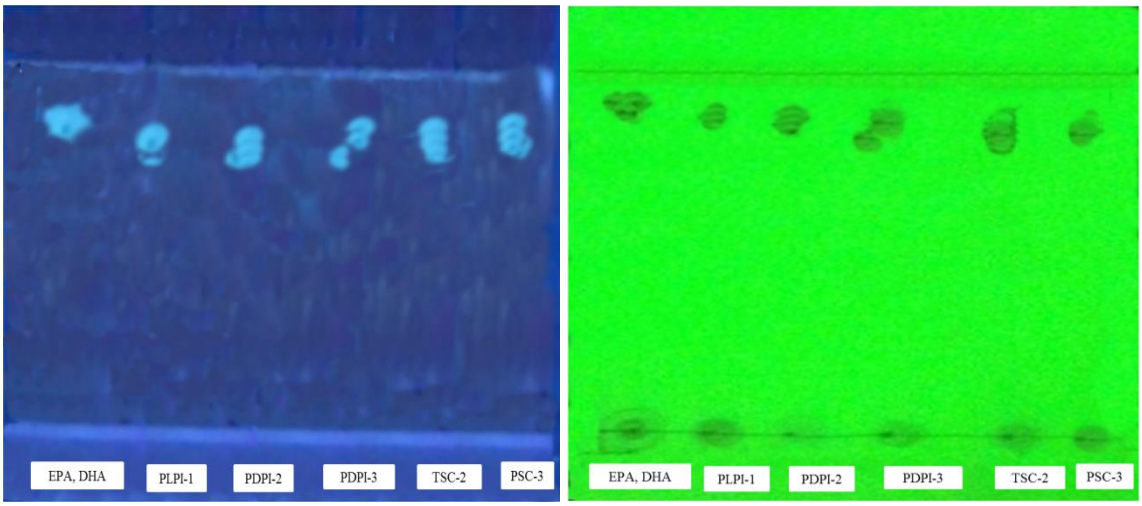


Figure 4.4 TLC analyses of isolate PLPI-1, PDPI-2, PDPI-3, TSC-2 and PSC-3.

4.7.2 FTIR analysis

The FTIR analysis of the isolate PLPI-1, PDPI-2, PDPI-3, TSC-2 and PSC-3, FAME extracts showed different functional groups given in the figures 7.1, 7.2, 7.3, 7.4 and 7.5. Spectrum of all the isolates showed strong evidence of the PUFA presence especially EPA and DHA with the standard as well online FTIR peaks representation table. Peak value 2812–3012cm−1 region which is associated with the =C–H stretching vibration band, the C=O carbonyl band at 1710– 1766 cm−1, multiple C=C double bond at 1400-1600 cm−1 while the fingerprint region below1500 cm−1 represent huge variation for the PUFA are present. The Complete list of functional groups are given in appendix table 7.6

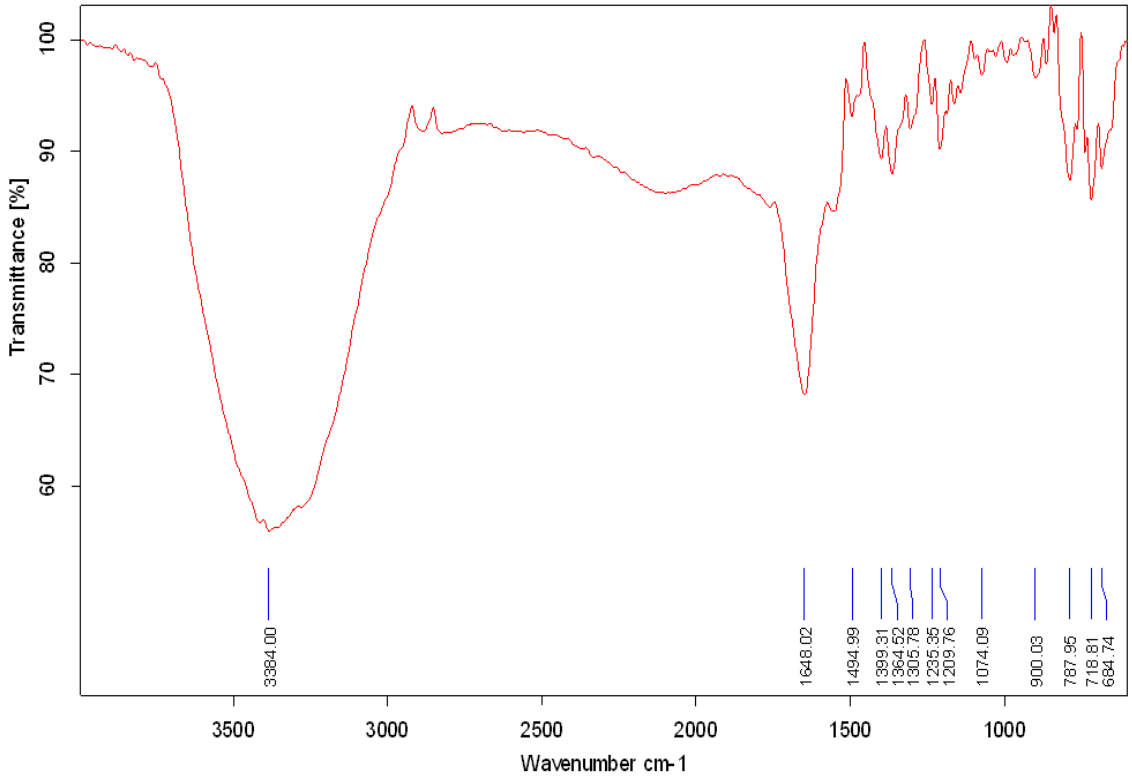


Figure 4.5 FTIR analysis of Standard PUFA obtained from The Vitamin Company USA.

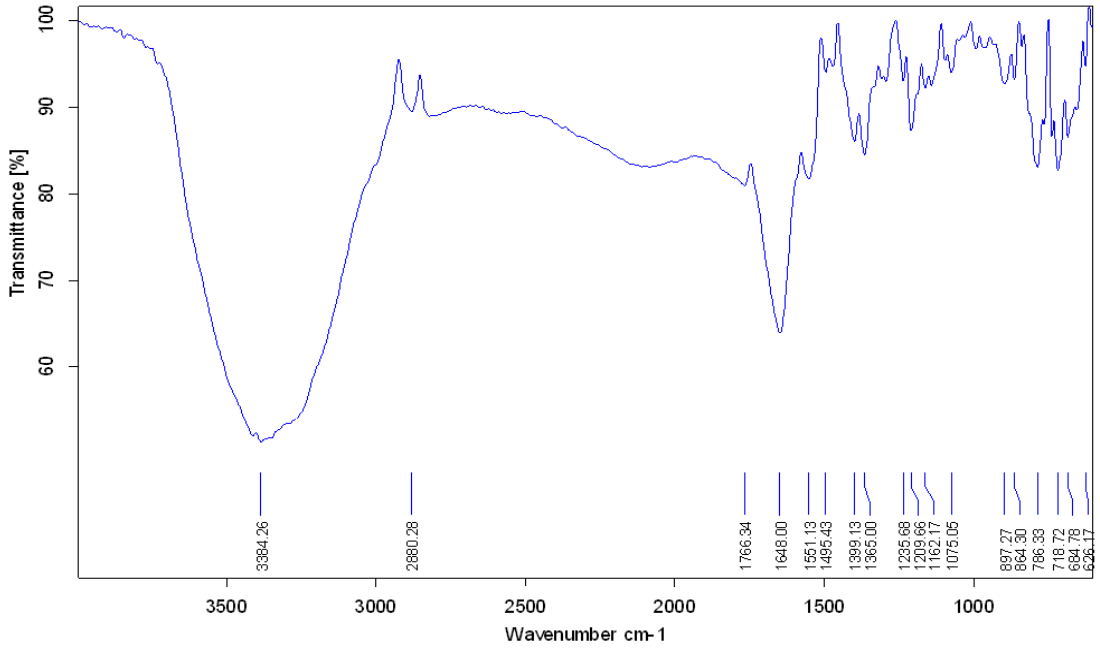


Figure 4.6.FTIR analysis of PUFA extracted from bacterial isolate PLPI-1

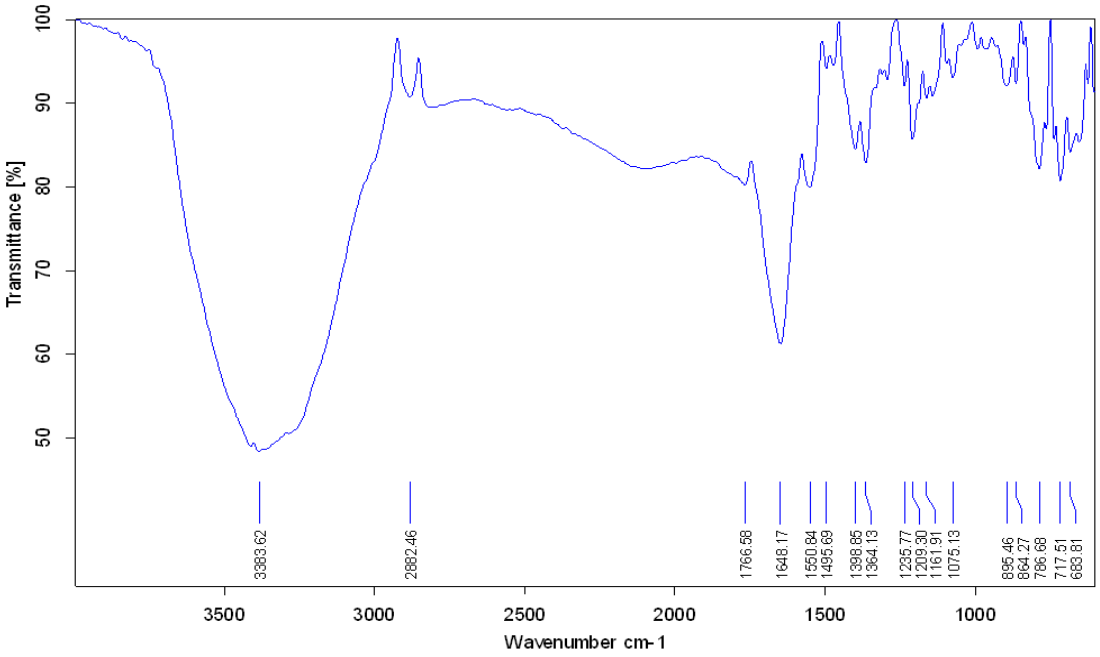


Figure 4.7 FTIR analysis of PUFA extracted from bacterial isolate PDPI-2

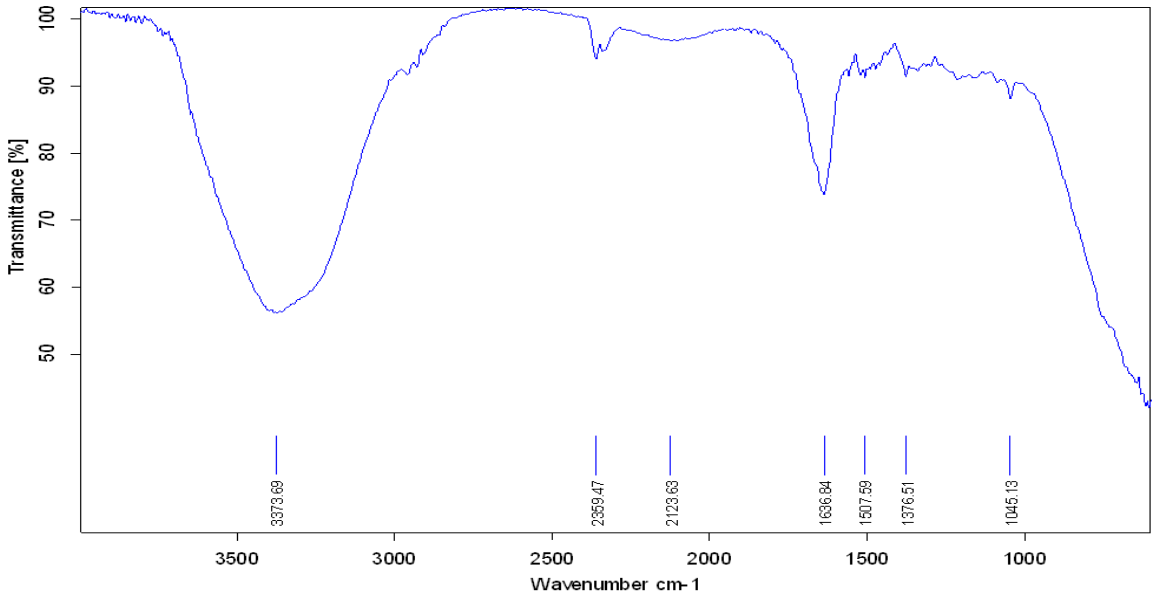


Figure 4.8 FTIR analysis of PUFA extracted from bacterial isolate PDPI-3

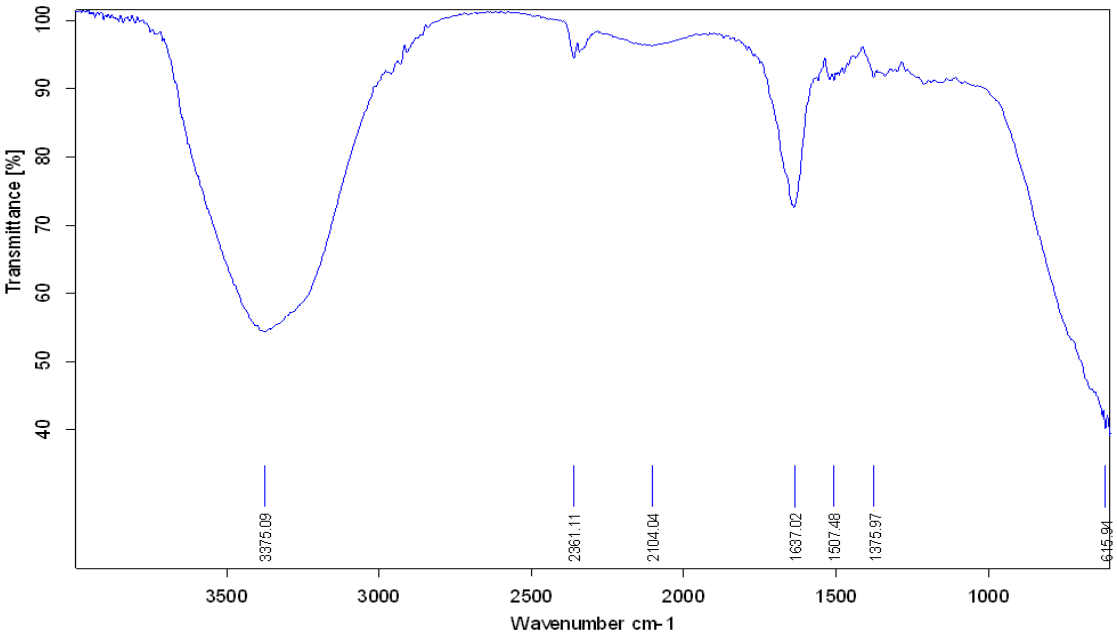


Figure 4.9 FTIR analysis of PUFA extracted from bacterial isolate TSC-2

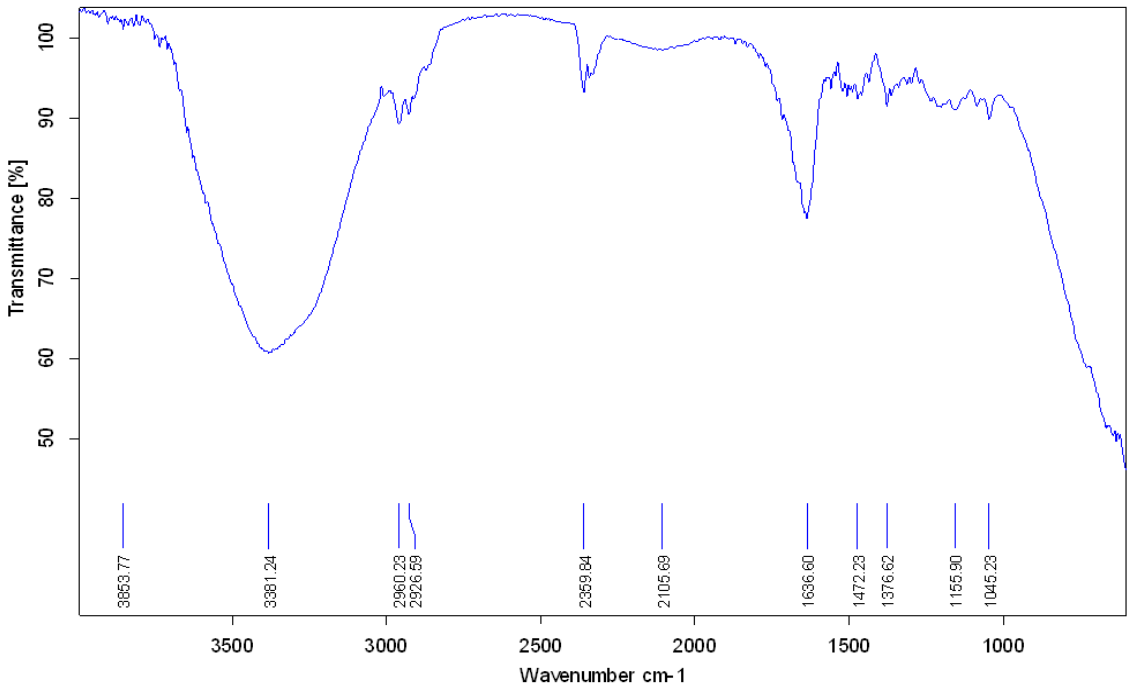


Figure 4.10 FTIR analysis of PUFA extracted from bacterial isolate PSC-3

4.8.1 Molecular characterization

DNA of the selected isolates was extracted and prior to PCR, run on gel for confirmation which is shown in figure 4.11.

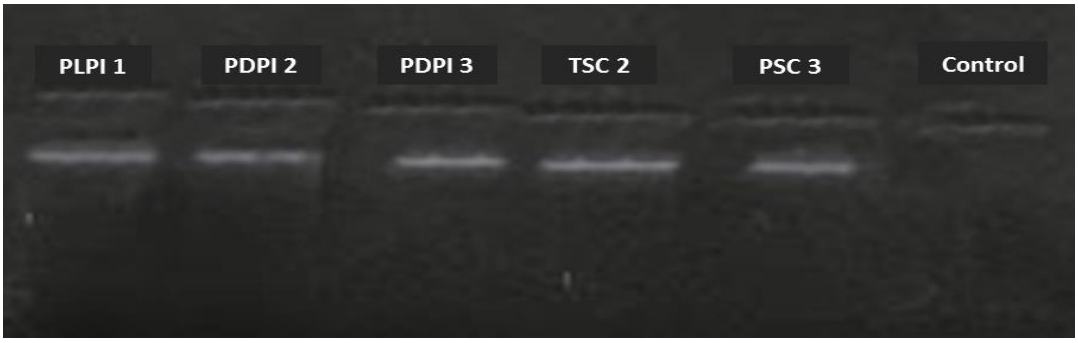


Figure 4.11 Show DNA bands of isolate PLPI-1, PDPI-2, PDPI-3, TSC-2 and PSC-3.

4.8.2 PCR amplification

The extracted DNA was amplified by using 16S rRNA universal primers 27F and 1492R. The gene size approximately 1460bp was confirmed by 1% agarose gel electrophoresis

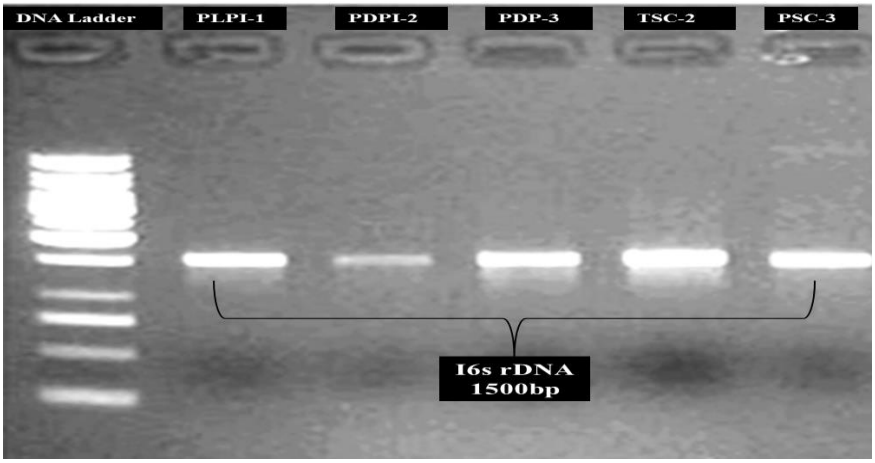


Figure 4.12 Agarose gel electrophoresis with specific band size of 16S rRNA gene

4.8.3 Sequencing and Phylogenetic analysis

After 16S rRNA sequencing, the obtained sequences BLAST in NCBI, found that Isolates TSC-2 showed 99% similarity with *Alcaligene fecalis* and PSC-3 showed 100% similarity with *Alacaligene fecalis*. Maximum Likelihood method according Tamura-Nei procedure was used for the determination of evolutionary history of the isoates. Highest log likelihood (-988.3410) in the tree is mentioned. The %age of trees in which the associated taxa clustered together is shown next to the branches. For obtaining the heuristic initial tree, search were done automatically by applying BioNJ algorithms and Neighbor-Join and to a matrix of pairwise distances estimated using the Maximum Composite Likelihood (MCL) approach, and then selecting the topology with superior log likelihood value. The tree is drawn to scale, with branch lengths measured in the number of substitutions per site (next to the branches). The analysis involved 13 nucleotide sequences. All positions containing gaps and missing data were eliminated. There were a total of 699 positions in the final dataset. Evolutionary analyses were conducted in MEGA6.

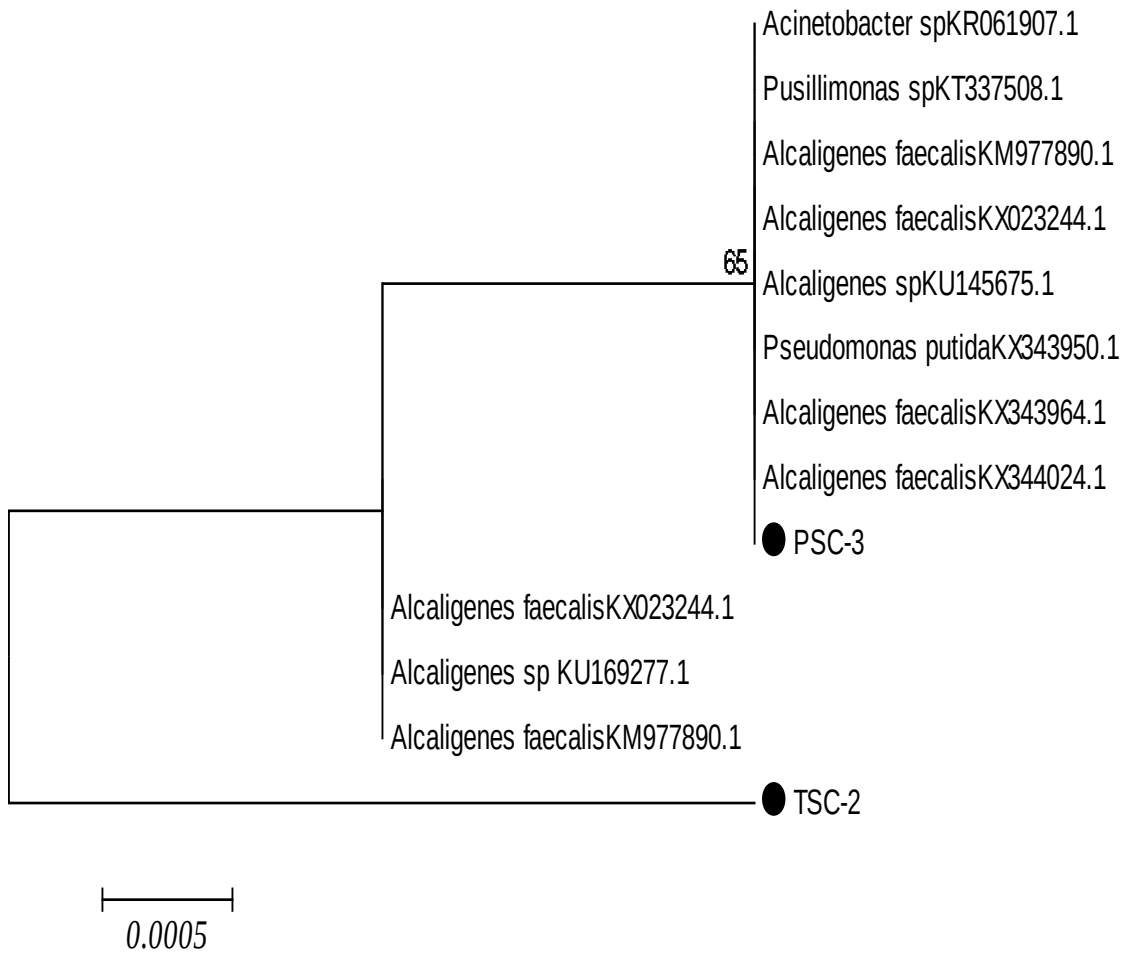


Figure 4.12 Phylogenetic tree of the isolates TSC-2 and PSC-3

Discussion

The aim of the current research work was to isolate psychrophilic bacteria from HKKH regions for the production of polyunsaturated fatty acids. These regions have not been previously explored for the screening and isolation of PUFA producing bacteria which will have a great impact regarding health benefits globally as the global demand are increasing day by day for polyunsaturated fatty acid. In the present study total 140 bacteria were isolated. Out of 140, 5 bacterial isolates were selected for complete identification. Poly phasic approach was followed (considering both morphological and molecular identification methods) and used for the identification. PLPI-1 was identified as *Alcaligene fecalis*, PDPI-2 as *Alcaligene fecalis*, PDPI-3, *Alcaligene fecalis,* TSC-2 as *Alcaligene fecalis* strain M59 showed similarity index 98% and PSC-3 Was identified as *Alcaligene fecalis* strain S26 showed similarity index 99%.

Alcaligene fecalis (TSC-2 and PSC-3) is most commonly occurring bacterium in all habitats ranging from low to high temperature, dry to aquatic habitats. *A. faecalis* strains have been isolated from a various habitats including environmental and clinical samples (Coeny *et al*., 1999; Nakano *et al*., 2013). According to many published reports, fatty acids were the predominant compound observed from various Antarctic bacteria including *Pseudoalteromonas sp*., *MLY15* (Li, *et al*., 2009), *Micrococcus cryophilus*, *Flavobacterium*, (Skerratt *et al*., 1991), *Halomonas species* (Jadhav, *et al*., 2013), *Flectobacillus species* (Dobson, *et al*., 1993) and many other bacterial species (Russell and Nichols, *et al*., 1999).

All the isolated bacteria were screened for the production of PUFA. The screening strategy was divided into two main steps. The first step was applying the TTC calorimetric method (as described in Methodology section) and the second was of TLC and FTIR analysis of prepared FAMEs. The ability to reduce the TTC (colorless) to a red-colored triphenyl formazan (TF) was found to be related to the ability of bacteria to produce EPA (Dinamarca, *et al*., 2017; Ryan, *et al*., 2010). Ryan and his collegue hypothesized that the ability of bacteria to reduce TTC to TF is due to the effect of Δ5-desaturase, an enzyme required for PUFAs biosynthesis. The TTC

method is an easy and fast screening method for PUFA producer's identification (Ryan, *et al*., 2010).

Five isolates PLPI1, PDPI2, PDPI3, TSC2 and PSC3 were selected on the basis of their maximum producing ability by rapid TTC test for further proceedings. The selected isolates were further processed for optimization on different media, pH, and temperature as well as structural characterization of the product. The effect of medium, temperature and pH on the production of PUFA was analyzed in term of incubation time, after adding TTC to the bacterial culture after every 24hrs of incubation at particular medium, temperature and pH.

The isolates were grown in different media like LB, NB, PYM, MSM, and BG 11 for of optimum PUFA production and 15°C was used as incubation temperature as it is reported that PUFA production may vary with growth temperature (Freese, *et al*., 2009; Freese *et al*., 2008). The isolate were screened for PUFA respectively after every 24 hrs of incubation by direct TTC methods similarly done by El razak, *et al*., 2014). Growth OD was taken continuously of each of the isolates after every 24hrs at 600nm. The results showed that the PLPI 1 showed maximum growth on NB after 96 hrs of incubation followed by LB, MSM, PYM and BG 11 respectively. It was noted that maximum PUFA production was observed after 72 hrs of incubation in NB as well as in LB agar. Similar results were also observed by (Ryan, *et al*., 2010; Gad *et al*., 2016) and its co-worker but they isolated bacteria from marine habitat. Similarly PDPI2 showed maximum growth after 96hrs on LB agar followed by NB, PYM, MSM and BG 11 while isolates PDPI 3 showed maximum growth after 96 hrs on PYM followed by LB, NB, MSM and BG11. Similarly the isolate TSC 2 showed maximum growth after 72 hrs on PYM followed by NB, LB, MSM and BG11 while the isolate PSC 3 showed maximum growth on PYM after 72hrs followed by NB, LB, MSM and BG11. Throughout the study it was observed that all the selected isolates showed maximum growth on PYM, NB, and LB broth but least growth was observed in BG11follwed by MSM. It may be due to the highly selectivity of the BG11 media for the growth of *Cyanobacteria* and it is reported that, there is no evidence of PUFA production from these microorganism (Okoyuma, *et al*., 2007). The maximum production was observed up to 96 hrs of incubation as up to these time the cell was in log phase and after that continues decrease was observed in growth as well in PUFA

production because within oleaginous microorganisms storage materials are produced in plenty of times when carbon is sufficient and utilized in times of starvation but when carbon become deficient may further reduce lipid yields (Wu, *et al.,* 2005). Similar results were observed in our study, up to 72 and 96hrs of incubation cell were grown efficiently but as the depletion of carbon source occur growth and PUFA become gradually decrease

The selected isolates were grown on various temperatures including 5, 15 and 25°C using NB as growth medium. Variation in the incubation temperature is the sole tested variable and significantly affects the PUFA production in bacteria. As the high production of PUFA is achieved between 5-25°C while there is no production reported at temperatures higher than 25°C (Nichols and McMeekin, *et al*., 2002; Petri, *et al.,* 2015). Optimum temperatures support growth of microorganism while PUFA are low at higher temperatures. Microorganisms are reported to produce PUFA at low temperature for maintaining their membrane fluidity however; the biomass production may be low at this temperature (Masurkar *et al.,* 2015; Bouzidi *et al.,* 2005). Komazawa *et al.* 2004 suggested that the growth temperature controlled in such a way that produce product of interest in high amount. For this purpose the isolates were kept at different temperature 15 and at 25°C and incubate for 72 hrs as was previously optimized on growth media. After 72 hrs of incubation it was observed that all the isolates showed maximum growth on 15°C and at this temperature the concentration PUFA production was also high. Similar results were also noted by (Ryan, *et al*, 2010) for the marine isolate. The results of (Nichols *et al*, 2002) indicate that the lower the temperature, the higher the EPA production. This is probably due to the ability of EPA to keep the fluidity of the membrane in cold environment. It is due to the adaptation of bacteria to low temperature as PUFA became the part of membrane and help in fluidity, thus helps in adaptation of the bacteria to cope with low temperature. Skerratt, *et al.*, 2002, observed that decreasing the temperature from 24 to 4°C gradually become double the percentage of PUFA production. Similar result were observed by(Michinaka, *et al.* (2003) that the activity of Δ6-destaurase enzyme, obtained from the fungus *Mucor circinelloides* was doubled when the cultivation temperature was decreased from 28°C to 15°C, leading to a significant increase in the percentage of GLA.

The effects of pH on the production of PUFA of selected isolates were checked on different pH 3, 5, 7, 9 and 11. After 72 hrs of incubation it was observed that isolate PLPI 1 showed maximum production at pH7 and their growth was also high at this particular temperature. It may be because of the maximum growth of the isolate at this pH and the temperature and media was also optimized for the PUFA production. Similarly (Yazawa, *et al.* 1988) screened 5,000 strains of marine microorganisms for PUFA production found that 88 of them showed a positive result. The main factor affecting the productivity was pH where he noted that at pH 7 and 20-25°C production was high which correspond to our finding. Very little growth were observed at pH 11 and 3 and showed no activity for PUFA production. The isolate PDPI 2 showed production of PUFA at wide range of pH i.e. at 5, 7, and 9 but maximum production was observed at 7pH where there was no production at pH 3and 11. It may be because the isolate was able to tolerate a wide range of pH and shown active growth at all pH but was highest at 7pH. Lee, *et al.* (2008) reported a bacterium *Shewanella* sp. Strain KMG427, isolated from marine environment are able to produce PUFA at pH 8 and 9 which reflect our finding but our isolate is from glaciers origin. The isolates PDPI 3 and TSC 2 was able to produce maximum production of PUFA at 7pH after 72 hrs of incubation and similarly showed maximum growth at this pH while the isolates PSC 3 showed maximum production of PUFA at 5pH, as the isolate was showed highest growth at this pH. Throughout the study it was observed that pH7 was best for growth and optimum production of PUFA.

Thin layer chromatography of the extracted FAME of the selected isolates was carried out in chloroform/acetone/methanol/acetic acid/water (10:4:2:2:1) as the developing solvent showed a single spot when compared with standard EPA, DHA (Rf value=0.88) having Rf values 0.87, 0.86, 0.86, 0.87 and 0.86 for the isolates PLPI1, PDPI2, PDPI3, TSC2 and PSC3 respectively shown in figure 4.1, 4.2, 4.3, 4.4, 4 and 4.5 respectively. Similar results were noted by Deshpande, *et al*, 2013 while investigating isolation of PUFA from various fish, by using developing solvent Benzene:chloroform (9.5:0.5 ratio) where the Rf value for EPA standard was noted 0.67 and their results were also in the range of 0.65, 0.67, 0.66 for the Omega oil. Nikhade, *et al*, 2014, perform TLC for finding DHA using Benzene as mobile phase and standard DHA, where he obtained Rf value 0.56 for the standard and Extracted

sample, Thus variation in Rf value in the studies is due to the use of different mobile phase while performing TLC analysis.

FTIR analysis of the extracted FAME of the selected isolate shows a strong evidence of presence of EPA, DHA (PUFA) production by the isolates. Spectral comparison of FAME was analyzed. FTIR spectra shown in fig. 3 Important variables were observed and related with positive coefficients correspond with peaks at 3383, stretching vibration band, the C=O carbonyl band 1710 to 1766 and around 696 cm_1 (CH_2)n and cis =C–H symmetric rocking), most of the variable related to high levels of unsaturated fatty acids (Guillen, *et al*., 2008; Guillen, *et al*., 1997). The spectra also show the range from 3030 to 2882 cm_1 and 1810 to 1600 cm_1 and the fingerprint region below 1500 cm_1, 1050–950 cm−1 region, which has been described to =C–H out-of-plane bending, appear to contribute most to the prediction of total n-3 Fas (Plans *et al*., 2015; bekhit, *et al*., 2014). FTIR spectrum of our selected isolates show high resemblance to the previous reported spectrum for fish oil analysis describe above thus research work indicating that bacteria from the HKKH glaciers has the potential of PUFA production and may be good alternative for commercial application.

Conclusions

- Psychrotolerant bacteria were good producers of PUFA
- Optimum PUFA production was observed using Nutrient Agar as growth medium, at 15˚C and pH 7.
- The produced PUFA was confirmed through TLC and FTIR analysis which revealed that bacteria from the glaciers has the ability to produce PUFA
- The selected bacterial isolates were identified as members of genus *Alcaligenes* by 16S rRNA gene analysis.

Future prospects

- Molecular analysis of PUFA producing genes
- Purification of individual PUFA from crude product
- Optimization of various media components for PUFA production via Placket Berman design
- Effect of different metals and salts concentration on production of PUFA
- Stability analysis of PUFA on different pH, metals and temperatures
- Bioactivities of PUFA

References

1. **Abbadi A, Domergue F, Bauer J, Napier JA, Welti R, Zähringer U, Cirpus P, Heinz E.** 2004. Biosynthesis of very-long-chain polyunsaturated fatty acids in transgenic oilseeds: constraints on their accumulation. The Plant Cell **16:**2734-2748.

2. **Abd Elrazak A, Ward AC, Glassey J.** 2016. Effect of inorganic salts on bacterial omega-3 PUFA production. Preparative Biochemistry and Biotechnology**:**1-8.

3. **Adkins Y, Kelley DS.** 2010. Mechanisms underlying the cardioprotective effects of omega-3 polyunsaturated fatty acids. The Journal of nutritional biochemistry **21:**781-792.

4. **Aislabie J, Jordan S, Barker G.** 2008. Relation between soil classification and bacterial diversity in soils of the Ross Sea region, Antarctica. Geoderma **144:**9-20.

5. **Allen EE, Bartlett DH.** 2002. Structure and regulation of the omega-3 polyunsaturated fatty acid synthase genes from the deep-sea bacterium Photobacterium profundum strain SS9. Microbiology **148:**1903-1913.

6. **Amiri-Jami M, Wang H, Kakuda Y, Griffiths MW.** 2006. Enhancement of polyunsaturated fatty acid production by Tn5 transposon in Shewanella baltica. Biotechnology letters **28:**1187-1192.

7. **Arab-Tehrany E, Jacquot M, Gaiani C, Imran M, Desobry S, Linder M.** 2012. Beneficial effects and oxidative stability of omega-3 long-chain polyunsaturated fatty acids. Trends in Food Science & Technology **25:**24-33.

8. **Babcock TA, Helton WS, Hong D, Espat NJ.** 2002. Omega-3 fatty acid lipid emulsion reduces LPS-stimulated macrophage TNF-α production. Surgical infections **3:**145-149.

9. **Bajpai P, Bajpai P, Ward O.** 1991. Optimization of production of docosahexaenoic acid (DHA) byThraustochytrium aureum ATCC 34304. Journal of the American Oil Chemists' Society **68:**509-514.

10. **Bajpai P.** 1997. Microbial xylanolytic enzyme system: properties and applications. Advances in applied microbiology **43:**141-194.

11. **Barclay W, Meager K, Abril J.** 1994. Heterotrophic production of long chain omega-3 fatty acids utilizing algae and algae-like microorganisms. Journal of Applied Phycology **6:**123-129.

12. **Barria C, Malecki M, Arraiano C.** 2013. Bacterial adaptation to cold. Microbiology **159:**2437-2443.

13. **Bassaganya-Riera J, Hontecillas R, Horne WT, Sandridge M, Herfarth HH, Bloomfeld R, Isaacs KL.** 2012. Conjugated linoleic acid modulates immune responses in patients with mild to moderately active Crohn's disease. Clinical nutrition **31:**721-727.

14. **Bekhit MY, Grung B, Mjøs SA.** 2014. Determination of Omega-3 Fatty Acids in Fish Oil Supplements Using Vibrational Spectroscopy and Chemometric Methods. Applied spectroscopy **68:**1190-1200.

15. **Benediktsdóttir E, Verdonck L, Spröer C, Helgason S, Swings J.** 2000. Characterization of Vibrio viscosus and Vibrio wodanis isolated at different geographical locations: a proposal for reclassification of Vibrio viscosus as Moritella viscosa comb. nov. International Journal of Systematic and Evolutionary Microbiology **50:**479-488.

16. **Benhaïm D, Bégout M-L, Péan S, Manca M, Prunet P, Chatain B.** 2013. Impact of a plant-based diet on behavioural and physiological traits in sea bass (Dicentrarchus labrax). Aquatic Living Resources **26:**121-131.

17. **Bergé J, Barnathan G.** 2005. Recent advances in fatty acids from lipids of marine organisms: molecular biodiversity, roles as biomarkers, biologically-active compounds and economical aspects. Marine Biotechnology Adv Biochem Eng Biotechnol, Springer **96:**49-125.

18. **Bergé J-P, Barnathan G.** 2005. Fatty acids from lipids of marine organisms: molecular biodiversity, roles as biomarkers, biologically active compounds, and economical aspects, p 49-125, Marine biotechnology I. Springer.

19. **Bianchi AC, Olazábal L, Torre A, Loperena L.** 2014. Antarctic microorganisms as source of the omega-3 polyunsaturated fatty acids. World Journal of Microbiology and Biotechnology **30:**1869-1878.

20. **Blair JM, Piddock LJ.** 2009. Structure, function and inhibition of RND efflux pumps in Gram-negative bacteria: an update. Current opinion in microbiology **12:**512-519.

21. **Bouzidi L, Dyal SD, Narine SS.** 2005. Maximizing the production of¿-linolenic acid in Mortierella ramanniana var. ramanniana as a function of pH, temperature and carbon source, nitrogen source, metal ions and oil supplementation. Food research international **38:**815-829.

22. **Bowman JP.** Shewanella. Bergey's Manual of Systematics of Archaea and Bacteria.

23. **Brasaemle DL.** 2007. Thematic review series: adipocyte biology. The perilipin family of structural lipid droplet proteins: stabilization of lipid droplets and control of lipolysis. Journal of lipid research **48:**2547-2559.

24. **Calder PC.** 2006. n− 3 polyunsaturated fatty acids, inflammation, and inflammatory diseases. The American journal of clinical nutrition **83:**S1505-1519S.

25. **Calviello G, Serini S, Piccioni E, Pessina G.** 2009. Antineoplastic effects of n-3 polyunsaturated fatty acids in combination with drugs and radiotherapy: preventive and therapeutic strategies. Nutrition and cancer **61:**287-301.

26. **Canion A, Prakash O, Green SJ, Jahnke L, Kuypers MM, Kostka JE.** 2013. Isolation and physiological characterization of psychrophilic denitrifying bacteria from permanently cold Arctic fjord sediments (Svalbard, Norway). Environmental microbiology **15:**1606-1618.

27. **Carton I, Goicoechea En, Uriarte PS.** 2008. Characterization of cod liver oil by spectroscopic techniques. New approaches for the determination of compositional parameters, acyl groups, and cholesterol from 1H nuclear magnetic resonance and Fourier transform infrared spectral data. Journal of agricultural and food chemistry **56:**9072-9079.

28. **Casanueva A, Tuffin M, Cary C, Cowan DA.** 2010. Molecular adaptations to psychrophily: the impact of 'omic'technologies. Trends in microbiology **18:**374-381.

29. **Certik M, Adamechova Z.** 2009. Cereal-based bioproducts containing polyunsaturated fatty acids. Lipid Technology **21:**250-253.

30. **Certik M, Shimizu S.** 1999. Biosynthesis and regulation of microbial polyunsaturated fatty acid production. Journal of bioscience and bioengineering **87:**1-14.

31. **Chintalapati S, Kiran M, Shivaji S.** 2004. Role of membrane lipid fatty acids in cold adaptation. Cellular and molecular biology (Noisy-le-Grand, France) **50:**631-642.

32. **Choi EJ, Kwon HC, Koh HY, Kim YS, Yang HO.** 2010. Colwellia asteriadis sp. nov., a marine bacterium isolated from the starfish Asterias amurensis. International journal of systematic and evolutionary microbiology **60:**1952-1957.

33. **Coenye T, Falsen E, Vancanneyt M, Hoste B, Govan JR, Kersters K, Vandamme P.** 1999. Classification of Alcaligenes faecalis-like isolates from the environment and human clinical samples as Ralstonia gilardii sp. nov. International Journal of Systematic and Evolutionary Microbiology **49:**405-413.

34. **Cohen Z, Reungjitchachawali M, Siangdung W, Tanticharoen M.** 1993. Production and partial purification of γ-linolenic acid and some pigments fromSpirulina platensis. Journal of Applied Phycology **5:**109-115.

35. **Cole DW, Cole R, Gaydos SJ, Gray J, Hyland G, Jacques ML, Powell-Dunford N, Sawhney C, Au WW.** 2009. Aquaculture: Environmental, toxicological, and health issues. International journal of hygiene and environmental health **212:**369-377.

36. **De Caterina R.** 2011. n–3 fatty acids in cardiovascular disease. New England Journal of Medicine **364:**2439-2450.

37. **DeLong EF, Yayanos AA.** 1986. Biochemical function and ecological significance of novel bacterial lipids in deep-sea procaryotes. Applied and Environmental Microbiology **51:**730-737.

38. **Deming JW, Somers LK, Straube WL, Swartz DG, Macdonell MT.** 1988. Isolation of an obligately barophilic bacterium and description of a new genus, Colwellia gen. nov. Systematic and applied microbiology **10:**152-160.

39. **Desbois AP, Lawlor KC.** 2013. Antibacterial activity of long-chain polyunsaturated fatty acids against Propionibacterium acnes and Staphylococcus aureus. Marine drugs **11:**4544-4557.

40. **Dinamarca J, Levitan O, Kumaraswamy G, Lun DS, Falkowski P.** 2017. Overexpression of a diacylglycerol acyltransferase gene in Phaeodactylum tricornutum directs carbon towards lipid biosynthesis. Journal of Phycology.

41. **Diraman H, Koru E, Dibeklioglu H.** 2009. Fatty acid profile of Spirulina platensis used as a food supplement.

42. **Dobson S, Colwell R, McMeekin T, Franzmann P.** 1993. Direct sequencing of the polymerase chain reaction-amplified 16S rRNA gene of Flavobacterium gondwanense sp. nov. and Flavobacterium salegens sp. nov., two new species from a hypersaline Antarctic lake. International Journal of Systematic and Evolutionary Microbiology **43:**77-83.

43. **Domingo JL.** 2007. Omega-3 fatty acids and the benefits of fish consumption: is all that glitters gold? Environment International **33:**993-998.

44. **Dulvy NK, Sadovy Y, Reynolds JD.** 2003. Extinction vulnerability in marine populations. Fish and fisheries **4:**25-64.

45. **El Razak AA, Ward AC, Glassey J.** 2014. Screening of marine bacterial producers of polyunsaturated fatty acids and optimisation of production. Microbial ecology **67:**454-464.

46. **Eynard AR, Navarro A.** 2013. Crosstalk among dietary polyunsaturated fatty acids, urolithiasis, chronic inflammation, and urinary tract tumor risk. Nutrition **29:**930-938.

47. **Fahy E, Subramaniam S, Brown HA, Glass CK, Merrill AH, Murphy RC, Raetz CR, Russell DW, Seyama Y, Shaw W.** 2005. A comprehensive classification system for lipids. Journal of lipid research **46:**839-862.

48. **Fang J, Kato C.** 2007. FAS or PKS, lipid biosynthesis and stable carbon isotope fractionation in deep-sea piezophilic bacteria. Commun Curr Res Educ Top Trends Appl Microbiol **1:**190-200.

49. **Fang J, Zhang L, Bazylinski DA.** 2010. Deep-sea piezosphere and piezophiles: geomicrobiology and biogeochemistry. Trends in microbiology **18:**413-422.

50. **Fedorova-Dahms I, Marone P, Bailey-Hall E, Ryan A.** 2011. Safety evaluation of Algal Oil from Schizochytrium sp. Food and chemical toxicology **49:**70-77.

51. **Fernandes G, Venkatraman JT.** 1993. Role of omega-3 fatty acids in health and disease. Nutrition Research **13:**S19-S45.

52. **Fogliano V, Andreoli C, Martello A, Caiazzo M, Lobosco O, Formisano F, Carlino PA, Meca G, Graziani G, Rigano VDM.** 2010. Functional ingredients produced by culture of Koliella antarctica. Aquaculture **299:**115-120.

53. **Foran JA, Carpenter DO, Hamilton MC, Knuth BA, Schwager SJ.** 2005. Risk-based consumption advice for farmed Atlantic and wild Pacific salmon contaminated with dioxins and dioxin-like compounds. Environmental health perspectives**:**552-556.

54. **Freese E, Rütters H, Köster J, Rullkötter J, Sass H.** 2009. Gammaproteobacteria as a possible source of eicosapentaenoic acid in anoxic intertidal sediments. Microbial ecology **57:**444-454.

55. **Freese E, Sass H, Rütters H, Schledjewski R, Rullkötter J.** 2008. Variable temperature-related changes in fatty acid composition of bacterial isolates from

German Wadden sea sediments representing different bacterial phyla. Organic Geochemistry **39:**1427-1438.

56. **Gad AM, Beltagy EA, Abdul-Raouf UM, El-Shenawy MA, Abouelkheir SS.** 2016. Screening of Marine Fatty Acids Producing Bacteria with Antimicrobial Capabilities. Chemistry of Advanced Materials **1**.

57. **Galli C, Marangoni F, Galella G, Salami M, Petroni A.** Manipulation of Long-Chain n-6 and n-3 Fatty Acids, and Arachidonic Acid Metabolism in Cultured Monocytes and Glial Cells, p 49. *In* (ed), The American Oil Chemists Society,

58. **García-Descalzo L, García-López E, Alcázar A, Baquero F, Cid C.** 2014. Proteomic analysis of the adaptation to warming in the Antarctic bacteria Shewanella frigidimarina. Biochimica et Biophysica Acta (BBA)-Proteins and Proteomics **1844:**2229-2240.

59. **Gentile G, Bonasera V, Amico C, Giuliano L, Yakimov M.** 2003. Shewanella sp. GA-22, a psychrophilic hydrocarbonoclastic antarctic bacterium producing polyunsaturated fatty acids. Journal of applied microbiology **95:**1124-1133.

60. **Gill I, Valivety R.** 1997. Polyunsaturated fatty acids, part 1: occurrence, biological activities and applications. Trends in biotechnology **15:**401-409.

61. **Giordano E, Visioli F.** 2014. Long-chain omega 3 fatty acids: molecular bases of potential antioxidant actions. Prostaglandins, Leukotrienes and Essential Fatty Acids (PLEFA) **90:**1-4.

62. **Gladyshev MI, Sushchik NN, Kolmakova AA, Kalachova GS, Kravchuk ES, Ivanova EA, Makhutova ON.** 2007. Seasonal correlations of elemental and ω3 PUFA composition of seston and dominant phytoplankton species in a eutrophic Siberian Reservoir. Aquatic Ecology **41:**9-23.

63. **Gladyshev MI, Sushchik NN, Makhutova ON.** 2013. Production of EPA and DHA in aquatic ecosystems and their transfer to the land. Prostaglandins & other lipid mediators **107:**117-126.

64. **Godin-Roulling A, Schmidpeter PA, Schmid FX, Feller G.** 2015. Functional adaptations of the bacterial chaperone trigger factor to extreme environmental temperatures. Environmental microbiology **17:**2407-2420.

65. **Gong Y, Wan X, Jiang M, Hu C, Hu H, Huang F.** 2014. Metabolic engineering of microorganisms to produce omega-3 very long-chain polyunsaturated fatty acids. Progress in lipid research **56:**19-35.

66. **Gorissen L, Leroy F, De Vuyst L, De Smet S, Raes K.** 2015. Bacterial production of conjugated linoleic and linolenic acid in foods: a technological challenge. Critical reviews in food science and nutrition **55:**1561-1574.

67. **Graham IA, Larson T, Napier JA.** 2007. Rational metabolic engineering of transgenic plants for biosynthesis of omega-3 polyunsaturates. Current Opinion in Biotechnology **18:**142-147.

68. **Grima EM, Belarbi E-H, Fernández FA, Medina AR, Chisti Y.** 2003. Recovery of microalgal biomass and metabolites: process options and economics. Biotechnology advances **20:**491-515.

69. **Guillen M, Cabo N.** 1997. Characterization of edible oils and lard by Fourier transform infrared spectroscopy. Relationships between composition and frequency of concrete bands in the fingerprint region. Journal of the American Oil Chemists' Society **74:**1281-1286.

70. **Gunstone F.** 1996. Fatty acids—nomenclature, structure, isolation and structure determination, biosynthesis and chemical synthesis, p 1-34, Fatty Acid and Lipid Chemistry. Springer.

71. **Gurr M, Harwood J.** 1991. Fatty acid structure and metabolism, p 23-118, Lipid Biochemistry. Springer.

72. **Harel M, Clayton D, Bullis R.** 2004. Feed formulation for terrestrial and aquatic animals. Google Patents.

73. **Hayes CE.** 2000. Vitamin D: a natural inhibitor of multiple sclerosis. Proceedings of the Nutrition Society **59:**531-535.

74. **Hinsa-Leasure SM, Bhavaraju L, Rodrigues JL, Bakermans C, Gilichinsky DA, Tiedje JM.** 2010. Characterization of a bacterial community from a Northeast Siberian seacoast permafrost sample. FEMS microbiology ecology **74:**103-113.

75. **Hinzpeter I, Shene C, Masson L.** 2006. Biotechnological alternatives for omega-3 polyunsaturated fatty acids production. Grasas y aceites **57:**336-342.

76. **Hodson A, Anesio AM, Tranter M, Fountain A, Osborn M, Priscu J, Laybourn-Parry J, Sattler B.** 2008. Glacial ecosystems. Ecological monographs **78:**41-67.

77. **Hu B, Li J, Zhao J, Yang J, Bai F, Dou Y.** 2013. Heavy metal in surface sediments of the Liaodong Bay, Bohai Sea: distribution, contamination, and sources. Environmental monitoring and assessment **185:**5071-5083.

78. **Hu W, Wang Y, Zhu Z.** 2007. Progress in the evaluation of transgenic fish for possible ecological risk and its containment strategies. Science in China Series C: Life Sciences **50:**573-579.

79. **Huang CB, Alimova Y, Myers TM, Ebersole JL.** 2011. Short-and medium-chain fatty acids exhibit antimicrobial activity for oral microorganisms. Archives of oral biology **56:**650-654.

80. **Huang CB, George B, Ebersole JL.** 2010. Antimicrobial activity of n-6, n-7 and n-9 fatty acids and their esters for oral microorganisms. Archives of oral biology **55:**555-560.

81. **Ivanova EP, Gorshkova NM, Bowman JP, Lysenko AM, Zhukova NV, Sergeev AF, Mikhailov VV, Nicolau DV.** 2004. Shewanella pacifica sp. nov., a polyunsaturated fatty acid-producing bacterium isolated from sea water. International journal of systematic and evolutionary microbiology **54:**1083-1087.

82. **Ivanova EP, Sawabe T, Gorshkova NM, Svetashev VI, Mikhailov VV, Nicolau DV, Christen R.** 2001. Shewanella japonica sp. nov. International journal of systematic and evolutionary microbiology **51:**1027-1033.

83. **Jadhav VV, Yadav A, Shouche YS, Bhadekar RK.** 2013. Isolation and cellular fatty acid composition of psychrotrophic Halomonas strains from Antarctic sea water. Songklanakarin Journal of Science and Technology **35:**287-292.

84. **Jenkins DJ, Sievenpiper JL, Pauly D, Sumaila UR, Kendall CW, Mowat FM.** 2009. Are dietary recommendations for the use of fish oils sustainable? Canadian Medical Association Journal **180:**633-637.

85. **Kato C.** 2011. Distribution of piezophiles, p 643-655, Extremophiles Handbook. Springer.

86. **Kawamoto J, Kurihara T, Yamamoto K, Nagayasu M, Tani Y, Mihara H, Hosokawa M, Baba T, Sato SB, Esaki N.** 2009. Eicosapentaenoic acid plays a beneficial role in membrane organization and cell division of a cold-adapted bacterium, Shewanella livingstonensis Ac10. Journal of bacteriology **191:**632-640.

87. **Kawamoto J, Sato T, Nakasone K, Kato C, Mihara H, Esaki N, Kurihara T.** 2011. Favourable effects of eicosapentaenoic acid on the late step of the cell division in a piezophilic bacterium, Shewanella violacea DSS12, at high-hydrostatic pressures. Environmental microbiology **13:**2293-2298.

88. **Kim O-S, Cho Y-J, Lee K, Yoon S-H, Kim M, Na H, Park S-C, Jeon YS, Lee J-H, Yi H.** 2012. Introducing EzTaxon-e: a prokaryotic 16S rRNA gene sequence database with phylotypes that represent uncultured species. International journal of systematic and evolutionary microbiology **62:**716-721.

89. **Kim Y, Liu R, Rychlik J, Russell J.** 2002. The enrichment of a ruminal bacterium (Megasphaera elsdenii YJ-4) that produces the trans-10, cis-12 isomer of conjugated linoleic acid. Journal of Applied Microbiology **92:**976-982.

90. **Ko J-Y, Lee J-H, Samarakoon K, Kim J-S, Jeon Y-J.** 2013. Purification and determination of two novel antioxidant peptides from flounder fish (Paralichthys olivaceus) using digestive proteases. Food and Chemical Toxicology **52:**113-120.

91. **Komazawa H, Kojima M, Aki T, Ono K, Kawakami M.** 2007. Microorganism having an ability of producing docosahexaenoic acid and use thereof. Google Patents.

92. **Kremer JM, Lawrence DA, Jubiz W, Digiacomo R, Rynes R, Bartholomew LE, Sherman M.** 1990. Dietary fish oil and olive oil supplementation in patients with Rheumatoid Arthritis clinical and immunologic effects. Arthritis & Rheumatology **33:**810-820.

93. **Kremer JM.** 2000. n− 3 Fatty acid supplements in rheumatoid arthritis. The American journal of clinical nutrition **71:**349s-351s.

94. **Lands WE.** 1986. Renewed questions about polyunsaturated fatty acids. Nutrition reviews **44:**189-195.

95. **Lee S, Kim HJ, Chang KC, Baek JC, Park JK, Shin JK, Choi WJ, Lee JH, Paik WY.** 2009. DHA and EPA down-regulate COX-2 expression through suppression of NF-κB activity in LPS-treated human umbilical vein endothelial cells. The Korean Journal of Physiology & Pharmacology **13:**301-307.

96. **Lee S-J, Jeong Y-S, Kim D-U, Seo J-W, Hur B-K.** 2006. Eicosapentaenoic acid (EPA) biosynthetic gene cluster ofShewanella oneidensis MR-1: Cloning, heterologous expression, and effects of temperature and glucose on the production of EPA inEscherichia coli. Biotechnology and bioprocess engineering **11:**510-515.

97. **Lee W-H, Cho K-W, Park S-Y, Shin K-S, Lee D-S, Hwang S-K, Seo S-J, Kim J-M, Ghim S-Y, Song B-H.** 2008. Identification of psychrophile Shewanella sp. KMG427 as an eicosapentaenoic acid producer. J Microbiol Biotechnol **18:**1869-1873.

98. **Lee YK, Hong SG, Cho HH, Cho KH, Lee HK.** 2007. Dasania marina gen. nov., sp. nov., of the order Pseudomonadales, isolated from Arctic marine sediment. JOURNAL OF MICROBIOLOGY-SEOUL- **45:**505.

99. **Lewis TE, Nichols PD, McMeekin TA.** 1999. The biotechnological potential of thraustochytrids. Marine Biotechnology **1:**580-587.

100. **Li Y, Xu X, Dietrich M, Urlacher VB, Schmid RD, Ouyang P, He B.** 2009. Identification and functional expression of a Δ9 fatty acid desaturase from the marine bacterium Pseudoalteromonas sp. MLY15. Journal of Molecular Catalysis B: Enzymatic **56:**96-101.

101. **Lin X, Yang B, Shen J, Du N.** 2009. Biodegradation of crude oil by an arctic psychrotrophic bacterium Pseudoalteromomas sp. P29. Current microbiology **59:**341-345.

102. **Lordan S, Ross RP, Stanton C.** 2011. Marine bioactives as functional food ingredients: potential to reduce the incidence of chronic diseases. Marine drugs **9:**1056-1100.

103. **Margesin R, Feller G.** 2010. Biotechnological applications of psychrophiles. Environmental technology **31:**835-844.

104. **Margesin R, Miteva V.** 2011. Diversity and ecology of psychrophilic microorganisms. Research in microbiology **162:**346-361.

105. **Masson VR, Lucas A, Gueugneau A-M, Macaire J-P, Paul J-L, Grynberg A, Rousseau D.** 2008. Long-chain (n-3) polyunsaturated fatty acids prevent metabolic and vascular disorders in fructose-fed rats. The Journal of nutrition **138:**1915-1922.

106. **Masurkar AA, Vakil BV.** 2015. Optimization of Process Variables for Bacterial PUFA Production by OFAT Method. Int J Curr Microbiol App Sci **4:**160-181.

107. **McManus A, Fielder L, Newton W, White J.** 2011. Health benefits of seafood for men. Journal of Men's Health **8:**252-257.

108. **McManus A, Merga M, Newton W.** 2011. Omega-3 fatty acids. What consumers need to know. Appetite **57:**80-83.

109. **Medaas CP.** 2014. Performing conservation, performing natures: How are multiple natures enacted in marine conservation practices on Maui, Hawai'i?

110. **Metz JG, Kuner J, Rosenzweig B, Lippmeier JC, Roessler P, Zirkle R.** 2009. Biochemical characterization of polyunsaturated fatty acid synthesis in Schizochytrium: release of the products as free fatty acids. Plant Physiology and Biochemistry **47:**472-478.

111. **Metz JG, Roessler P, Facciotti D, Levering C, Dittrich F, Lassner M, Valentine R, Lardizabal K, Domergue F, Yamada A.** 2001. Production of polyunsaturated fatty acids by polyketide synthases in both prokaryotes and eukaryotes. Science **293:**290-293.

112. **Michinaka Y, Shimauchi T, Aki T, Nakajima T, Kawamoto S, Shigeta S, Suzuki O, Ono K.** 2003. Extracellular secretion of free fatty acids by disruption of a fatty acyl-CoA synthetase gene in Saccharomyces cerevisiae. Journal of bioscience and bioengineering **95:**435-440.

113. **Miller MR, Nichols PD, Carter CG.** 2008. n-3 Oil sources for use in aquaculture–alternatives to the unsustainable harvest of wild fish. Nutrition Research Reviews **21:**85-96.

114. **Mirón AS, Garcıa M-CC, Camacho FG, Grima EM, Chisti Y.** 2002. Growth and biochemical characterization of microalgal biomass produced in bubble column and airlift photobioreactors: studies in fed-batch culture. Enzyme and Microbial Technology **31:**1015-1023.

115. **Molendi-Coste O, Legry V, Leclercq IA.** 2010. Why and how meet n-3 PUFA dietary recommendations? Gastroenterology research and practice **2011**.

116. **Morgan-Kiss RM, Priscu JC, Pocock T, Gudynaite-Savitch L, Huner NP.** 2006. Adaptation and acclimation of photosynthetic microorganisms to permanently cold environments. Microbiology and Molecular Biology Reviews **70:**222-252.

117. **Morita N, Nishida T, Tanaka M, Yano Y, Okuyama H.** 2005. Enhancement of polyunsaturated fatty acid production by cerulenin treatment in polyunsaturated fatty acid-producing bacteria. Biotechnology letters **27:**389-393.

118. **Mykytczuk N, Trevors J, Ferroni G, Leduc L.** 2010. Cytoplasmic membrane fluidity and fatty acid composition of Acidithiobacillus ferrooxidans in response to pH stress. Extremophiles **14:**427-441.

119. **Najjar MB, Chikindas M, Montville TJ.** 2007. Changes in Listeria monocytogenes membrane fluidity in response to temperature stress. Applied and environmental microbiology **73:**6429-6435.

120. **Nakano M, Niwa M, Nishimura N.** 2013. Specific and sensitive detection of Alcaligenes species from an agricultural environment. Microbiology and immunology **57:**240-245.

121. **Napier JA.** 2002. Plumbing the depths of PUFA biosynthesis: a novel polyketide synthase-like pathway from marine organisms. Trends in plant science **7:**51-54.

122. **Napier JA.** 2007. The production of unusual fatty acids in transgenic plants. Annu Rev Plant Biol **58:**295-319.

123. **Nelson J, Baker M.** 2003. Charging of ice-vapor interfaces: applications to thunderstorms. Atmospheric Chemistry and Physics **3:**1237-1252.

124. **Nichols D, Bowman J, Sanderson K, Nichols CM, Lewis T, McMeekin T, Nichols PD.** 1999. Developments with Antarctic microorganisms: culture collections, bioactivity screening, taxonomy, PUFA production and cold-adapted enzymes. Current Opinion in Biotechnology **10:**240-246.

125. **Nichols DS, McMeekin TA.** 2002. Biomarker techniques to screen for bacteria that produce polyunsaturated fatty acids. Journal of Microbiological Methods **48:**161-170.

126. **Nichols DS, Nichols PD, McMeekin TA.** 1993. Polyunsaturated fatty acids in Antarctic bacteria. Antarctic Science **5:**149-160.

127. **Nikaido H.** 1994. Porins and specific diffusion channels in bacterial outer membranes. Journal of Biological Chemistry **269:**3905-3908.

128. **Nikhade R, Deshpande S.** 2014. Formulation evaluation and validation of ophthalmic emulsion of docosahexaenoic acid. International Journal of Pharmacological Research **4:**10-17.

129. **Nishida T, Hori R, Morita N, Okuyama H.** 2010. Membrane eicosapentaenoic acid is involved in the hydrophobicity of bacterial cells and affects the entry of hydrophilic and hydrophobic compounds. FEMS microbiology letters **306:**91-96.

130. **Nogi Y, Hosoya S, Kato C, Horikoshi K.** 2004. Colwellia piezophila sp. nov., a novel piezophilic species from deep-sea sediments of the Japan Trench. International journal of systematic and evolutionary microbiology **54:**1627-1631.

131. **Nogi Y, Kato C, Horikoshi K.** 1998. Taxonomic studies of deep-sea barophilic Shewanella strains and description of Shewanella violacea sp. nov. Archives of microbiology **170:**331-338.

132. **Nogi Y.** 2013. Psychrophilic Microorganisms In Marine Environments. Cold-adapted Microorganisms**:**33.

133. **Ogawa J, Sakuradani E, Kishino S, Ando A, Yokozeki K, Shimizu S.** 2012. Polyunsaturated fatty Acids production and transformation by Mortierella alpina and anaerobic bacteria. European Journal of Lipid Science and Technology **114:**1107-1113.

134. **Okuyama H, Orikasa Y, Nishida T, Watanabe K, Morita N.** 2007. Bacterial genes responsible for the biosynthesis of eicosapentaenoic and docosahexaenoic acids and their heterologous expression. Applied and environmental microbiology **73:**665-670.

135. **Orikasa Y, Nishida T, Hase A, Watanabe K, Morita N, Okuyama H.** 2006. A phosphopantetheinyl transferase gene essential for biosynthesis of n− 3 polyunsaturated fatty acids from Moritella marina strain MP-1. FEBS letters **580:**4423-4429.

136. **Orikasa Y, Tanaka M, Sugihara S, Hori R, Nishida T, Ueno A, Morita N, Yano Y, Yamamoto K, Shibahara A.** 2009. pfaB products determine the molecular species produced in bacterial polyunsaturated fatty acid biosynthesis. FEMS microbiology letters **295:**170-176.

137. **Park JK, Kim Z-H, Lee CG, Synytsya A, Jo HS, Kim SO, Park JW, Park YI.** 2011. Characterization and immunostimulating activity of a water-soluble polysaccharide isolated from Haematococcus lacustris. Biotechnology and Bioprocess Engineering **16:**1090-1098.

138. **Park S, Johnson MA.** 2006. Awareness of fish advisories and mercury exposure in women of childbearing age. Nutrition reviews **64:**250-256.

139. **Pereira H, Barreira L, Figueiredo F, Custódio L, Vizetto-Duarte C, Polo C, Rešek E, Engelen A, Varela J.** 2012. Polyunsaturated fatty acids of marine macroalgae: potential for nutritional and pharmaceutical applications. Marine drugs **10:**1920-1935.

140. **Petrie JR, Singh SP, De Feyter RC.** 2015. Process for producing polyunsaturated fatty acids in an esterified form. Google Patents.

141. **Piette F, D'Amico S, Mazzucchelli G, Danchin A, Leprince P, Feller G.** 2011. Life in the cold: a proteomic study of cold-repressed proteins in the Antarctic bacterium Pseudoalteromonas haloplanktis TAC125. Applied and environmental microbiology **77:**3881-3883.

142. **Plans M, Wenstrup MJ, Rodriguez-Saona LE.** 2015. Application of Infrared Spectroscopy for Characterization of Dietary Omega-3 Oil Supplements. Journal of the American Oil Chemists' Society **92:**957-966.

143. **Radjasa OK, Urakawa H, Kita-Tsukamoto K, Ohwada K.** 2001. Characterization of psychrotrophic bacteria in the surface and deep-sea waters from the northwestern Pacific Ocean based on 16S ribosomal DNA analysis. Marine Biotechnology **3:**454-462.

144. **Raetz CR, Reynolds CM, Trent MS, Bishop RE.** 2007. Lipid A modification systems in gram-negative bacteria. Annu Rev Biochem **76:**295-329.

145. **Raghukumar S.** 2008. Thraustochytrid marine protists: production of PUFAs and other emerging technologies. Marine biotechnology **10:**631-640.

146. **Rangel-Huerta OD, Aguilera CM, Mesa MD, Gil A.** 2012. Omega-3 long-chain polyunsaturated fatty acids supplementation on inflammatory biomakers: a systematic review of randomised clinical trials. British Journal of Nutrition **107:**S159-S170.

147. **Ratledge C.** 2004. Fatty acid biosynthesis in microorganisms being used for single cell oil production. Biochimie **86:**807-815.

148. **Richard D, Kefi K, Barbe U, Bausero P, Visioli F.** 2008. Polyunsaturated fatty acids as antioxidants. Pharmacological Research **57:**451-455.

149. **Robert SS, Singh SP, Zhou X-R, Petrie JR, Blackburn SI, Mansour PM, Nichols PD, Liu Q, Green AG.** 2005. Metabolic engineering of Arabidopsis to produce nutritionally important DHA in seed oil. Functional Plant Biology **32:**473-479.

150. **Rodrigues DF, Tiedje JM.** 2008. Coping with our cold planet. Applied and environmental microbiology **74:**1677-1686.

151. **Roodhart JM, Daenen LG, Stigter EC, Prins H-J, Gerrits J, Houthuijzen JM, Gerritsen MG, Schipper HS, Backer MJ, van Amersfoort M.** 2011. Mesenchymal stem cells induce resistance to chemotherapy through the release of platinum-induced fatty acids. Cancer cell **20:**370-383.

152. **Ruiz-López N, Sayanova O, Napier JA, Haslam RP.** 2012. Metabolic engineering of the omega-3 long chain polyunsaturated fatty acid biosynthetic pathway into transgenic plants. Journal of experimental botany **63:**2397-2410.

153. **Rummel JD, Beaty DW, Jones MA, Bakermans C, Barlow NG, Boston PJ, Chevrier VF, Clark BC, de Vera J-PP, Gough RV.** 2014. A new analysis of Mars "special regions": findings of the second MEPAG Special Regions Science Analysis Group (SR-SAG2). Mary Ann Liebert, Inc. 140 Huguenot Street, 3rd Floor New Rochelle, NY 10801 USA.

154. **Russell NJ, Nichols DS.** 1999. Polyunsaturated fatty acids in marine bacteria—a dogma rewritten. Microbiology **145:**767-779.

155. **Ryan J, Farr H, Visnovsky S, Vyssotski M, Visnovsky G.** 2010. A rapid method for the isolation of eicosapentaenoic acid-producing marine bacteria. Journal of microbiological methods **82:**49-53.

156. **Sánchez Mirón A, Garcia Camacho F, Contreras Gomez A, Grima EM, Chisti Y.** 2000. Bubble-column and airlift photobioreactors for algal culture. AIChE Journal **46:**1872-1887.

157. **Satomi M, Oikawa H, Yano Y.** 2003. Shewanella marinintestina sp. nov., Shewanella schlegeliana sp. nov. and Shewanella sairae sp. nov., novel eicosapentaenoic-acid-producing marine bacteria isolated from sea-animal intestines. International journal of systematic and evolutionary microbiology **53:**491-499.

158. **Sayanova O, Napier JA.** 2011. Transgenic oilseed crops as an alternative to fish oils. Prostaglandins, Leukotrienes and Essential Fatty Acids (PLEFA) **85:**253-260.

159. **Sayanova OV, Napier JA.** 2004. Eicosapentaenoic acid: biosynthetic routes and the potential for synthesis in transgenic plants. Phytochemistry **65:**147-158.

160. **Schluenzen F, Tocilj A, Zarivach R, Harms J, Gluehmann M, Janell D, Bashan A, Bartels H, Agmon I, Franceschi F.** 2000. Structure of functionally activated small ribosomal subunit at 3.3 Å resolution. Cell **102:**615-623.

161. **Seeralan A, Murugesan S, Sivaswamy N.** 2016. Profiling of Omega 3 fatty acids from marine green algae Ulva reticulata and Caulerpa racemosa. International Journal of Phytopharmacy **6:**46-50.

162. **Seto A, Wang H, Hesseltine C.** 1984. Culture conditions affect eicosapentaenoic acid content ofChlorella minutissima. Journal of the American oil chemists' society **61:**892-894.

163. **Shieh WY, Chen Y-W, Chaw S-M, Chiu H-H.** 2003. Vibrio ruber sp. nov., a red, facultatively anaerobic, marine bacterium isolated from sea water. International journal of systematic and evolutionary microbiology **53:**479-484.

164. **Shimajiri J, Shiota M, Hosokawa M, Miyashita K.** 2013. Synergistic antioxidant activity of milk sphingomyeline and its sphingoid base with α-tocopherol on fish oil triacylglycerol. Journal of agricultural and food chemistry **61:**7969-7975.

165. **Shin SY, Bajpai VK, Kim HR, Kang SC.** 2007. Antibacterial activity of bioconverted eicosapentaenoic (EPA) and docosahexaenoic acid (DHA) against foodborne pathogenic bacteria. International journal of food microbiology **113:**233-236.

166. **Shukla Y, Pal SK.** 2004. Dietary cancer chemoprevention: An overview. International Journal of Human Genetics **4:**265.

167. **Shulse CN, Allen EE.** 2011. Diversity and distribution of microbial long-chain fatty acid biosynthetic genes in the marine environment. Environmental microbiology **13:**684-695.

168. **Siddiqui RA, Harvey KA, Zaloga GP.** 2008. Modulation of enzymatic activities by n-3 polyunsaturated fatty acids to support cardiovascular health. The Journal of nutritional biochemistry **19:**417-437.

169. **Sidhu KS.** 2003. Health benefits and potential risks related to consumption of fish or fish oil. Regulatory Toxicology and Pharmacology **38:**336-344.

170. **Sijtsma L, De Swaaf M.** 2004. Biotechnological production and applications of the ω-3 polyunsaturated fatty acid docosahexaenoic acid. Applied microbiology and biotechnology **64:**146-153.

171. **Simopoulos A.** 2000. Human requirement for N-3 polyunsaturated fatty acids. Poultry science **79:**961-970.

172. **Singh A, Ward OP.** 1997. Microbial production of docosahexaenoic acid. Adv Appl Microbiol **45:**271-312.

173. **Siriwardhana N, Kalupahana NS, Moustaid-Moussa N.** 2012. 13 Health Benefits of n-3 Polyunsaturated Fatty Acids: Eicosapentaenoic Acid and Docosahexaenoic Acid. Advances in food and nutrition research **65:**211.

174. **Skerratt J, Nichols P, Mancuso C, James S, Dobson S, McMeekin T, Burton H.** 1991. The phospholipid ester-linked fatty acid composition of members of the family Halomonadaceae and genus Flavobacterium: a chemotaxonomic guide. Systematic and applied microbiology **14:**8-13.

175. **Skerratt JH, Bowman JP, Nichols PD.** 2002. Shewanella olleyana sp. nov., a marine species isolated from a temperate estuary which produces high levels of polyunsaturated fatty acids. International journal of systematic and evolutionary microbiology **52:**2101-2106.

176. **Skjånes K, Rebours C, Lindblad P.** 2013. Potential for green microalgae to produce hydrogen, pharmaceuticals and other high value products in a combined process. Critical reviews in biotechnology **33:**172-215.

177. **Steffens W, Wirth M.** 2005. Freshwater fish-an important source of n-3 polyunsaturated fatty acids: a review. ARCHIWUM RYBACTWA POLSKIEGO **13:**5.

178. **Sweatman T, Long J.** 1969. Quantitative electron-probe microanalysis of rock-forming minerals. Journal of Petrology **10:**332-379.

179. **Tai CC, Ding ST.** 2010. N-3 polyunsaturated fatty acids regulate lipid metabolism through several inflammation mediators: mechanisms and implications for obesity prevention. The Journal of nutritional biochemistry **21:**357-363.

180. **Tamegai H, Ota Y, Haga M, Fujimori H, Kato C, Nogi Y, Kawamoto J, Kurihara T, Sambongi Y.** 2011. Piezotolerance of the respiratory terminal oxidase activity of the piezophilic Shewanella violacea DSS12 as compared with non-piezophilic Shewanella species. Bioscience, biotechnology, and biochemistry **75:**919-924.

181. **Tanaka M, Ueno A, Kawasaki K, Yumoto I, Ohgiya S, Hoshino T, Ishizaki K, Okuyama H, Morita N.** 1999. Isolation of clustered genes that are notably homologous to the eicosapentaenoic acid biosynthesis gene cluster from the docosahexaenoic acid-producing bacterium Vibrio marinus strain MP-1. Biotechnology letters **21:**939-945.

182. **Tehei M, Madern D, Franzetti B, Zaccai G.** 2005. Neutron scattering reveals the dynamic basis of protein adaptation to extreme temperature. Journal of Biological Chemistry **280:**40974-40979.

183. **Thais F, Stahl R.** 1987. Effect of dietary fish oil on renal function in immune mediated glomerular injury. Proceedings of AOAC Short Course on Polyunsaturated Fatty Acids and Eicosanoids**:**123-126.

184. **Thyssen A, Ollevier F.** 2005. Photobacterium. Bergey's Manual of Systematics of Archaea and Bacteria.

185. **Tricon S, Burdge GC, Jones EL, Russell JJ, El-Khazen S, Moretti E, Hall WL, Gerry AB, Leake DS, Grimble RF.** 2006. Effects of dairy products naturally enriched with cis-9, trans-11 conjugated linoleic acid on the blood lipid profile in healthy middle-aged men. The American journal of clinical nutrition **83:**744-753.

186. **Undeland I, Lindqvist H, Chen-Yun Y, Falch E, Luten J, Luten J.** 2009. Seafood and health: what is the full story. Marine functional food **1:**17-87.

187. **Ursin VM.** 2003. Modification of plant lipids for human health: development of functional land-based omega-3 fatty acids. The Journal of nutrition **133:**4271-4274.

188. **Usui K, Hiraki T, Kawamoto J, Kurihara T, Nogi Y, Kato C, Abe F.** 2012. Eicosapentaenoic acid plays a role in stabilizing dynamic membrane structure in the deep-sea piezophile Shewanella violacea: a study employing high-pressure time-resolved fluorescence anisotropy measurement. Biochimica et Biophysica Acta (BBA)-Biomembranes **1818:**574-583.

189. **Valentine RC, Valentine DL.** 2004. Omega-3 fatty acids in cellular membranes: a unified concept. Progress in lipid research **43:**383-402

190. **Vaughan V, Hassing M, Lewandowski P.** 2013. Marine polyunsaturated fatty acids and cancer therapy. British journal of cancer **108:**486-492.

191. **Venegas-Calerón M, Sayanova O, Napier JA.** 2010. An alternative to fish oils: metabolic engineering of oil-seed crops to produce omega-3 long chain polyunsaturated fatty acids. Progress in lipid research **49:**108-119.

192. **Ward RD, Zemlak TS, Innes BH, Last PR, Hebert PD.** 2005. DNA barcoding Australia's fish species. Philosophical Transactions of the Royal Society of London B: Biological Sciences **360:**1847-1857.

193. **Wirsen CO, Jannasch HW, Wakeham SG, Canuel EA.** 1986. Membrane lipids of a psychrophilic and barophilic deep-sea bacterium. Current Microbiology **14:**319-322.

194. **Wu S-T, Yu S-T, Lin L-P.** 2005. Effect of culture conditions on docosahexaenoic acid production by Schizochytrium sp. S31. Process Biochemistry **40:**3103-3108.

195. **Yano Y, Nakayama A, Saito H, Ishihara K.** 1994. Production of docosahexaenoic acid by marine bacteria isolated from deep sea fish. Lipids **29:**527-528.

196. **Yano Y, Nakayama A, Yoshida K.** 1997. Distribution of polyunsaturated Fatty acids in bacteria present in intestines of deep-sea fish and shallow-sea poikilothermic animals. Applied and environmental microbiology **63:**2572-2577.

197. **Yao H-T, Chang Y-W, Lan S-J, Chen C-T, Hsu JT, Yeh T-K.** 2006. The inhibitory effect of polyunsaturated fatty acids on human CYP enzymes. Life sciences **79:**2432-2440.

198. **Yazawa K.** 1996. Production of eicosapentaenoic acid from marine bacteria. Lipids **31:**S297-S300.

199. **Yu Y, Li H-R, Zeng Y-X.** 2011. Colwellia chukchiensis sp. nov., a psychrotolerant bacterium isolated from the Arctic Ocean. International journal of systematic and evolutionary microbiology **61:**850-853.

200. **Zendedel A, Habib P, Dang J, Lammerding L, Hoffmann S, Beyer C, Slowik A.** 2015. Omega-3 polyunsaturated fatty acids ameliorate neuroinflammation

and mitigate ischemic stroke damage through interactions with astrocytes and microglia. Journal of neuroimmunology **278:**200-211.

201. **Zhang D-C, Yu Y, Xin Y-H, Liu H-C, Zhou P-J, Zhou Y-G.** 2008. Colwellia polaris sp. nov., a psychrotolerant bacterium isolated from Arctic sea ice. International journal of systematic and evolutionary microbiology **58:**1931-1934.

202. **202 Zhang J.** 2011. The diversity and biotechnological application of marine microbes producing Omega-3 fatty acids.