Production of Polyunsaturated Fatty Acids from Marine Microorganisms

A Thesis Submitted to Newcastle University for the

Degree of Doctor of Philosophy

By

Ahmed Abdo Ahmed Abd Elrazak

Under the Supervision of

Dr. Jarka Glassey **Prof. Alan Ward**

October 2012

School of Chemical Engineering and Advanced Materials

Newcastle University

Author's Declaration

This thesis is submitted in fulfilment of the requirements for the degree of Doctor Philosophy at Newcastle University, United Kingdom. All the studies described within are solely my work unless expressly stated otherwise, and were undertaken at the School of Chemical Engineering and Advanced Materials under the guidance and supervision of Dr. Jarka Glassey and Prof. Alan Ward between November 2008 and January 2012.

I certify that none of the material offered in this thesis has been previously submitted for a degree or any other qualifications at the above or any other university or institute.

Neither the author nor the University of Newcastle accepts any liability for the contents of this document.

Dedication

I would like to dedicate this work to my lovely wife for her support and sacrifice throughout this long and difficult journey and to my lovely boys too.

I also dedicate this work to my dear parents who are the source of enlightenment and through them I have known the true importance of seeking knowledge.

Finally, I would like to dedicate this work to the souls of 25th January, 2011 Egyptian Revolution Martyrs who sacrificed their precious lives for the sake of real democracy, better future for our beloved country Egypt, social justice, freedom and better quality of life for all Egyptians.

Acknowledgment

I would like to thank **Allah** for giving me the knowledge to complete this work and for giving me the patience and strength to continue throughout my research journey.

Then first and foremost, I would like to convey my most sincere gratitude to Dr. Jarka Glassey for her continuous advices, invaluable support, guidance, enabling me to grow with the freedom of thoughts and expressions, her assistance in my writing and for being more than just a supervisor throughout my tenure.

I would also like to express my gratitude to my supervisor, Prof. Alan Ward, whose expertise, understanding, and patience, added considerably to my graduate experience.

My sincere thanks go to Dr Ronan O'Kennedy for his kind support in my laboratory work.

Much respect goes to my officemates, at room C500, for being good colleagues and more than friends. Also, deep thanks for bio-pharmaceutical lab members for their kind and continuous help during my work.

I extend my sincere thanks to my wife's uncle Mr. Gamal Elmohamadi for his kind assistance in collecting the sea water samples in Egypt.

I am immensely thankful to the officers and crew of RRS James Cook W. D. K. Reid, Dove marine lab members and Dr B. Wigham for the provision of sediment samples made available for this study.

Last but not least, I would also like to acknowledge the continuous care and valuable financial support from my sponsor; Egyptian Cultural Bureau in London on behalf of the Ministry of Higher Education and Mansoura University in my beloved country Egypt.

Attended conferences

ESBES + ISPPP + ISB Symposia, September 5-8, 2010 in Bologna, Italy, Poster presentation.

15th European congress of Biotechnology. October 23-26 2012 in Istanbul, Turkey, Oral presentation.

Prepared Papers

1- 'Polyunsaturated Fatty Acid Production by Marine Microorganisms'
Ahmed Abd Elrazak, Alan C. Ward, Jarka Glassey
Submitted to Bioprocess and Biosystem engineering journal.

2- 'Response Surface Methodology for Optimising the Culture Conditions for Eicosapentaenoic Acid Production by Marine Bacteria'
Ahmed Abd Elrazak, Alan C. Ward, Jarka Glassey
Submitted to Industrial Microbiology and Biotechnology Journal.

3- 'Process Development of Eicosapentaenoic acid production'
Ahmed Abd Elrazak, Alan C. Ward, Jarka Glassey
Submitted to Biochemical Engineering Journal.

Acronyms

ALA (alpha-Linolenic acid)

ASW (Artificial sea water)

BLAST (Basic Local Alignment Search Tool)

CCD (Central composite design)

DHA (Docosahexaenoic acid)

DOE (Design of experiment)

EDX (Energy dispersive X-ray)

EPA (Eicosapentaenoic acid)

FA (Fatty Acid)

FAME (Fatty acid methyl ester)

FAS (Fatty Acid Synthase)

FFD (Full Factorial Design)

GC (Gas chromatography)

GLA (gamma-Linolenic acid)

MA (Mid atlantic ridge)

NIR (Near infra red)

OD (Optical density)

ORFs (Open Reading Frames)

PB (Plackett-Burman)

PCR (Polymerase chain reaction)

PKS (Polyketide Synthase)

PM (Production medium)

PUFAs (Polyunsaturated fatty acids)

RS (Red sea)

RSM (Response surface methodology)

SCOs (Single Cell Oils)

SDS (Sodium dodecyl sulphate)

TF (Triphenyl formazan)

TTC (Triphenyltetrazolium chloride)

Table of Contents

List of Tables

List of Figures

Abstract

Polyunsaturated Fatty Acids (PUFAs), especially eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) are increasingly attracting scientific attention due to their significant health promoting role in the human body. However, the human body lacks the ability to produce them *in vivo*. The limitations associated with the current sources of ω-3 fatty acids and ω-6 fatty acids from animal and plant sources have led to increased interest in microbial production. Marine bacteria provide a suitable alternative, although the isolation of production strains and the identification of operating conditions must be addressed before manufacturing processes become economically viable. Sea sediment samples were collected from three different environments including Mid Atlantic Ridge, Red Sea and Mediterranean Sea. The isolates were screened for PUFA production using a fast colourimetric method and verified by gas chromatography/mass spectroscopy. The isolated PUFA producers were characterised and identified on the basis of 16S rRNA gene sequencing and analysis. Three different isolates were chosen for this study. These were labelled as 717, 66 and Hus-27. The chosen isolates were subjected to an optimisation study to maximise their productivity. This optimisation strategy included identifying a suitable production medium by applying a statistical design of experiment methodology (Plackett-Burman and Central Composite Design). A chemically defined media was identified for isolates 717 and 66 in order to determine the limiting media components and to study the effect of carbon/nitrogen ratio on the productivity of isolates. As an important step in the process development of the microbial PUFA production, the culture conditions at the bioreactor scale were optimised for isolate 717 using a Response Surface Methodology (RSM) revealing the significant effect of temperature, dissolved oxygen and the interaction between them on the EPA production. Two sets of continuous stirred-tank reactor (CSTR) experiments were also performed to test the effect of growth rates on EPA production and the effect of temperature at constant growth rate as this was identified as the most significant factor affecting EPA production. This optimisation strategy led to a significant increase in the amount of EPA produced by isolates under investigation, where the amount of EPA increased from 9 mg/g biomass, 33 mg/l representing 7.6% of the total fatty acids to 45 mg/g, 350 mg/l representing 25% of the total fatty acids using isolate 717. A significant

increase was also achieved using isolate 66 with the amount of EPA increased from 5.5 mg/g, 14 mg/l representing 3.5% of the total fatty acids to 32 mg/g, 285 mg/l representing 15% of the total fatty acids. For isolate Hus-27 the amount of EPA increased from 0.6 mg/g, 3 mg/l representing 2.2% of the total fatty acids to 8 mg/g, 36 mg/l representing 8% of the total fatty acids. The stability of the produced oil and the complete absence of heavy metals in bacterial biomass are considered as an additional benefit of bacterial EPA compared to other sources of PUFA. To our knowledge this is the first report of a bacterial isolate producing EPA with such high yields making large scale manufacture much more economically viable.

Aims and Objectives

The main strategy to enhance the participation of the microorganisms in the industrial production of PUFA is screening for high PUFA producers, the manipulation of culture conditions to optimise PUFA production and the improvement of downstream processes (Lewis *et al.*, 1999). Process optimisation may include exploring many biochemical and physical parameters including media composition and cultivation conditions (Chodok *et al.*, 2010).

This project aimed to optimise the production of PUFAs from marine micro-organisms using bioprocessing approaches. Any optimisation of culturing conditions has to be underpinned by thorough understanding of the metabolic processes leading to PUFA production. Hence a part of this project will be dedicated to identifying and measuring the impact of the optimisation of culture conditions upon PUFA production. One of the objectives of this project is to isolate novel PUFA producing organisms from deep sea core samples. For this purpose the Newcastle University team have developed degenerate primers for key PUFA genes in the PUFA gene cluster to extract DNA from environmental samples and to identify potential PUFA producers.

On the bioprocessing side of the project, statistical design of experiments was used to optimise aspects of the process, such as media composition, temperature, pH, oxygen requirement, and other conditions in order to enable increased accumulation of PUFA in these marine isolates. Whilst initial optimisation was carried out in shake flasks, a suite of state-of-the-art bioreactors with full control was also available to carry out investigations at larger (up to 5 l) scale.

An important aspect of the optimisation part of the project is the quantification of the growth and PUFA production under a range of cultivation conditions. The methylated fatty acids were analysed via means of GC and GC/MS.

The hypothesis in this research was that the marine bacteria isolated from deep sea core samples would be capable of producing lipids, particularly long chain PUFAs at increased levels by optimising the conditions required to carry out economically viable manufacture in a bioreactor.

The main objectives of this research were:

- Culturing of marine bacterial samples from deep sea core samples using different culture media using basic culturing techniques.
- Screening of bacterial isolates for PUFA production from different environments and identifying high and low producing organisms also searching for new isolates if present.
- Creating a suitable production media for maximum production of PUFAs.
- Optimising cultivation conditions at bioreactor scale such as temperature, pH, dissolved oxygen, cultivation method (batch, continuous or fed batch).
- Lipid extraction by suitable and efficient extraction method and lipid analysis using GC and GC-MS.

Applying relevant research methodologies to achieve these objectives expected to lead to a suitable and robust bioprocess for industrial purposes.

Thesis Plan

This thesis is presented as a series of chapters including one literature review, one material and methods chapter and one references chapter for the whole thesis, while results and discussion were divided into five chapters.

Chapter 3 investigates the screening and characterisation of PUFA producers from different environments. Hundreds of bacterial strains isolated from different water samples, collected from the Red Sea, Mediterranean Sea and Mid Atlantic ridge, were screened for the ability to produce PUFAs specially eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA). The screening was performed using a colorimetric method by reduction of the colourless 2, 3, 5-triphenyltetrazolium chloride (TTC) into red-coloured triphenyl formazan (TF) and by gas chromatography (GC), gas chromatography-mass spectroscopy (GC/MS). The PUFA producers were characterised and the best producers were identified on the basis of 16S rRNA sequencing and analysis which led to construction of phylogenetic trees using bioinformatics tools.

In Chapter 4 different media (forty two) components including different carbon sources, nitrogen sources and metal ions were screened for their main effect on the PUFA production and the growth of isolate 66. Plackett-Burman was applied as a statistical method to screen the main effect of these tested components and by using

the statistical analysis the effect of each component was calculated and compared in order to choose the components with the most potential to be used in a production medium for maximum PUFA production. Once the media components were chosen other statistical design of experiments were applied to determine the optimum combination for maximum PUFA production and to reveal the interactions. This design called Central Composite Design (CCD). A chemically defined media for isolate was created to allow a tight control of the cell environment during the fermentation process. Applying a chemically defined medium facilitate examining the effect of Carbon-Nitrogen (C/N) ratio on the growth and productivity of isolate 66.

In Chapter 5 the media components were screened and optimised for the Egyptian isolate Hus-27 by the same strategy applied to isolate 66. Plackett-Burman design of experiments were applied for screening and CCD to optimise the production medium.

Chapter 6 deals with the optimisation and development of production medium for maximising the growth and productivity of isolate 717 by applying a statistical design of experiments where PB was used for screening and CCD for optimising the medium components. As with isolate 66, a chemically defined medium was developed for isolate 717 to test the effect of C/N ratio on the growth and productivity of isolate of interest.

As an important stage to produce a robust process on the bioreactor level a CCD experiment was applied to optimise the culture conditions for isolate 717 as it was found to be the highest and most promising producer in chapter 7 (fifth chapter in results and discussion). Three variables were optimised including temperature, pH and dissolved oxygen (DO). The effect of the growth rate in addition to the effect of temperature at constant growth rate was achieved using continuous culture. In addition, stability of the produced oil and the purity were investigated as a part of testing the product quality.

Chapter1- Introduction and literature review

Lipids are a broad group of naturally occurring organic molecules. This group of molecules includes fats and oils, waxes, phospholipids, steroids (like cholesterol), and some other related compounds which are characterised by their solubility in non-polar organic solvents (e.g. ether, chloroform, acetone and benzene) and general insolubility in water (Fahy *et al.*, 2005). Lipids play a number of vital biological functions including energy storage, structural components of cell membranes and important signaling molecules (Gurr, 1991).

The nutritionally important lipids are fats (solid) and oils (liquids) that consist of fatty acids with 12-20 carbons. Amongst the various foodstuffs, fats provide the body with the highest amount of energy (9 kcal/gram), approximately twice that for an equal amount of protein or carbohydrates. Fats and oils are made from two kinds of molecules: glycerol (an alcohol with a hydroxyl group on each of its three carbons) and fatty acids to form esters. There are many types of fatty acids (FAs), but they can be grouped into three divisions: saturated fats, monounsaturated fats and polyunsaturated fats. Saturated fats have the maximum number of hydrogen atoms on the carbon chain (no double bonds), they are solid at room temperature (above a chain length of 9-10 carbon atoms), have a high melting point and are mainly found in animal products (butter, cheese and meat), while coconut and palm oils are common vegetable sources. Saturated fat raises blood cholesterol levels more than anything else in the diet, even more than dietary cholesterol (Steinberg, 2007). Unsaturated FAs are those containing one double bond, so called monounsaturated FAs, or two or more double bonds, so called polyunsaturated FAs. Monounsaturated FAs are found in olive, peanut and canola oils, nuts, avocados and olives, while polyunsaturated FAs are found in corn, sunflower seed oils and fish.

The human body is able to synthesise both saturated and monounsaturated fatty acids hence they are referred to as non-essential fatty acids, while it is unable to synthesise polyunsaturated fatty acids and hence these are termed as essential fatty acids and must be obtained from food, which could be due to the inability of the human body to synthesise linoleic acid from oleic acid via a de-saturase step (Bajpai and Bajpai, 1993).

1.1 Long chain polyunsaturated fatty acids (PUFAs)

Polyunsaturated fatty acids (PUFAs; long-chain fatty acids with 18 or more carbon atoms containing two or more non-conjugated *cis* double bonds) have recently enjoyed extensive interest and ever-increasing consumption from the general public. This is due to the fact that lipids (oils and fatty acids) are indispensable for the growth and survival of all living organisms ((Gill and Valivety, 1997; Sijtsma and Swaaf, 2004) This fact is better understood when the functions lipids play in humans and animals are critically analysed. Physiologically, they are important structural components of cell membranes, they are crucial in energy storage in many organisms and most recently, they are used in pharmaceutical, nutraceuticals and food supplements (Okuyama *et al.*, 2007a). In fact, PUFAs form a unique class of food constituents with a very wide range of functions in biomedical and nutraceuticals (i.e., food and nutritional products with specific health-promoting biological activities) especially in relation to certain disease conditions (Gill and Valivety, 1997). In addition, the controversy surrounding the food-sources of biofuels could be addressed by utilising microbial sources of oils that could be used for such purposes. It is therefore not surprising that the area of economically efficient production of PUFAs on a large scale is increasingly attracting the attention of researchers worldwide.

Depending on the position of the first double bond, PUFAs can be classified into two main groups, Omega-3 , in which the first double bond appears at the third carbon atom when counting from and including the methyl terminus, and Omega-6 , in which the first double bond appears at the sixth carbon atom when counting from the methyl terminus (Sijtsma and Swaaf, 2004). Examples of chemical structures of typical PUFAs are shown in Figure 1.1.

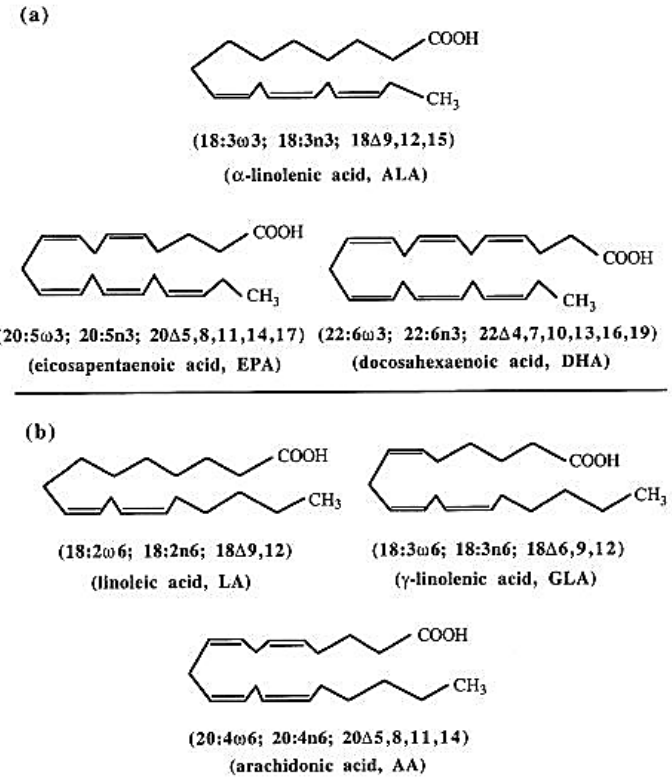

Figure 1.1: Chemical structures of PUFAs, (a) PUFA from ω-3 family, (b) PUFA from ω-6 family (Russell and Nichols, 1999).

1.2 Benefits of PUFAs

Recently PUFAs have attracted extensive attention because of their various physiological functions in the human body. PUFAs are essential components of glycolipids and phospholipids, necessary components of plasma membranes, acting as precursors for many hormones and hormone-like regulatory molecules in addition to their marked effect on human health (Lauritzen *et al.*, 2001; Sauer *et al.*, 2001)

The health benefits of eicosapentaenoic acid (EPA) include the lowering of plasma cholesterol and decreasing the incidence of breast, colon and pancreatic cancers (Kelly, 1991). EPA has a protective function against atherosclerosis and this explains the low percentage of cardiovascular diseases in human populations with high fish utilisation (Nordoy *et al.*, 2001; Metherel *et al.*, 2009). The ability of EPA to prevent and cure most of the blood-circulatory diseases indicates that EPA has an anti-aggregatory character and is essential in maintaining homeostasis (Lagarde *et al.*, 1983; Phang *et al.*, 2009).

Similarly to EPA, docosahexaenoic acid (DHA) plays an important role in human health as it has a critical role in proper visual and neurological development in infants (Das, 2003) and reduces the effect of most of the cardiovascular diseases (Nordoy *et al.*, 2001). Usually 60% of the total fatty acids in the outer segment of the retina consist of DHA (Giusto *et al.*, 2000). Balanced amounts of omega-3 and omega-6 PUFAs in the human body lead to a normal performance of organs (Wu and Lin, 2003).

In addition γ-linolenic acid (GLA) was reported to have a selective anti-cancer property especially against malignant glioma cells (Das, 2004) and breast cancer (Kenny *et al.*, 2000).

Arachidonic acid possesses various physiological roles as it is a biogenetic precursor of the biologically active prostaglandins, thromboxanes, prostacyclins and leukotrienes (Saelao *et al.*, 2011). The addition of arachidonic acid to infant milk was recommended by Food and Agriculture Organisation/World Health Organisation (FAO/WHO) ('WHO and FAO Joint Consultation: Fats and Oils in Human Nutrition,' 1995).

The presence of PUFAs in human diet facilitate the absorption of the fat soluble vitamins, A, D, E, and K , in addition PUFAs regulate the cholesterol metabolism (Das, 2004).

A novel antibacterial role of PUFAs was identified recently, where PUFAs were found to inhibit the growth of different foodborne and food spoilage pathogens such as *Bacillus subtilis* ATCC 6633, *Listeria monocytogenes* ATCC 19166, *Staphylococcus aureus* ATCC 6538, *S. aureus* KCTC 1916 and *Pseudomonas aeruginosa* KCTC 2004. The lethal effect of PUFAs could be due to targeting the enoyl-acyl carrier protein reductase (FabI) which is an essential component of bacterial fatty acid synthesis (Zheng *et al.*, 2005). The addition of the EPA to the growth media of *Pseudomonas aeruginosa and Staphylococcus aureus* lead to a remarkable decrease of their viability after 15 minutes of exposure. Examination by electron-microscope revealed severe damage to the outer membrane of the bacterial cell which could be the reason for the significant bacteriostatic and bactericidal effect of EPA (Shin *et al.*, 2007).

DHA was proven to inhibit the growth and virulence effect, both *in vitro* and in a mouse model, of the drug resistant isolate *Helicobacter pylori* (Correia *et al.*, 2012).

The addition of DHA not only reduced pathogen viability but also reduced its ability to colonise mouse stomach.

α-linolenic acid can also be used as an antimicrobial agent against some food poisoning bacteria like *Staphylococcus aureus* (Sado-Kamdem *et al.*, 2009). Fernandez-Lopez *et al.* (2005) observed that, PUFAs may supress the conjugation process between bacterial cells leading to a reduction in the possibility of transferring the antibiotic resistance gene among bacterial cells making the antibiotic treatment more efficient when supplied in conjugation with a PUFA.

These results suggest that, the microbial PUFAs could be added to food as a food preservative with a dual action.

1.3 Current sources of PUFAs

PUFAs are considered to be a natural product due to the difficulty of producing double bond synthetically (Meyer *et al.*, 1999). Fish, such as mackerel, sardines, and herring are basic sources for the commercial production of omega-3 PUFAs including EPA and DHA (Gunstone, 1996). However, the introduction of fish oil PUFAs into food for dietary and health purposes is fraught with problems due to the unpleasant odour and taste, resource sustainability of fish supply in addition to the expensive purification steps required to separate PUFAs from the complex mixture of fatty acids in the fish oil (Barclay *et al.*, 1994; Ratledge, 2004). Fish oil as the main source of PUFA in the market is not expected to meet the ever-growing global demand due to the overfishing problem (Tocher, 2009). Another concern regarding the use of fish oil is the environmental pollution of marine ecosystems especially with heavy metal and dioxins which accumulate in fish leading to a high hazard to human health (Domingo *et al.*, 2007). Also, both the composition and quantity of PUFAs in fish rely on the species, season and geographical location of the capture. In addition, the refining conditions can affect the quality of PUFAs (Shene *et al.*, 2010).

Recently, fish oil was found to interfere with chemotherapy, causing cancer cells to become less sensitive to such treatments this is due to the presence of 12-oxo-5, 8, 10-heptadecatrienoic acid and hexadeca-4, 7, 10, 13-tetraenoic acid within the fish oil mixture which, even in minute quantities, induces resistance to chemotherapeutic agents. (Roodhart *et al.*, 2011).

As a result, scientists are trying to find alternative ways of obtaining PUFAs in order to satisfy the increasing demand for omega-3 products, and microbial sources were identified as the most attractive potential sources (Ratledge, 2004)

Higher plants are unable to synthesise PUFAs with more than 18 carbons, due to the lack of the enzymes required for such process (Wallis *et al.*, 2002). Recently transgenic plants, where genes responsible for the production of PUFAs were inserted into a plant, were found to have the potential to become a good source of PUFA production as genetically-engineered plants were found to be able to produce and synthesise non-native fatty acids, especially long chain polyunsaturated fatty acids normally found in fish oils and marine microorganisms (Napier, 2007).

Abbadi A (2004) succeeded in obtaining a series of plant transformation constructs containing different combinations of Δ 6-desaturases from different microorganisms. The experiments achieved a low concentration of ARA (1.5% of the total fatty acid) in tobacco and EPA (0.9% of the total fatty acid) in linseed transgenic seeds.

Although promising results were obtained with the transgenic plant approach, as a source of PUFAs, it is still economically non-viable in competition with fish oil (Ruiz-López *et al.*, 2012)

Also this method may face a number of challenges including difficulties with the gene cloning process and the proscription of the use of transgenic plants in the nutraceutical industry in many countries (Certik and Adamechova, 2009).

1.4 Microbial PUFAs

Recently the microbial production of PUFAs, especially EPA and DHA, has gained more attention and a number of companies started to produce them commercially and introduce them to the market. Photonz, (http://www.photonzcorp.com/) established in 2002 in New-Zealand, is the first company to produce microbial EPA commercially.

Microorganisms producing PUFAs through fermentation provide a good and renewable source of PUFAs as the high omega-3 levels in fish are due to the intake of marine microorganisms since the microorganisms are the only *de-novo* source of PUFAs. The main sources of PUFAs in the microbial world are marine algae, fungi and bacteria (Gonzalez-Baro and Pollero ., 1998).

Microorganisms can be used to convert low-value compounds into value-added products for example, oleaginous microorganisms are able to convert agro-industrial

raw materials into valuable lipids as γ-linolenic acid via fermentation either as a solid state or submerged process (Čertík *et al.*, 1997; Gema *et al.*, 2002).

Microbial oils, also called single cell oils (SCOs), have been manufactured on an industrial scale since the 1980s. Many microbial species able to produce and accumulate SCOs were identified in scientific studies. Uunderstanding of the biochemistry of microbial oil production and process development is required in order for large scale production to result in high quantities of marketable products.

Microorganisms tend to produce SCOs under certain set of conditions and divert the metabolism towards lipid formation and accumulation. Although microalgae are considered to be the main source of microbial PUFAs, the growth requirements of the algae, namely the strictly controlled conditions in terms of nutrients, light quantity and quality, oxygenation and carbon dioxide levels represent significant challenges and increase the costs of manufacture (Nichols *et al.*, 1993).

Until the 1990s, bacteria, except for cyanobacteria, were thought to be unable to produce PUFAs in their membranes. This may be because the bacteria that were studied most extensively until that time were mesophiles, such as *E. coli*, which lacks the ability to produce PUFAs. Any results supporting the presence of PUFAs in bacteria were ignored and considered as an analytical error or media contamination (Russell and Nichols, 1999a).

Bacterial EPA and DHA accumulate only within the cellular membrane phospholipids (Nishida *et al.*, 2007). Bacterial EPA is present in the bacterial cell in the form of phospholipids, as cardiolipin, phosphatidyl glycerol and phosphatidyl ethanolamine (Freese *et al.*, 2009).

The discovery of EPA- and DHA-producing bacteria from deep sea water and sediments attracted significant scientific attention (Delong and Yayanos, 1986), opening a new era of using bacteria as an alternative microbial source for PUFA production. This progress was achieved due to the invention of more accurate analytical techniques, including GC and GC-MS, which establish that, bacteria also belong to the pool of EPA-producers.

Microbial PUFAs show advantages compared to the fish oil as,

- Microorganisms can grow on a wide variety of substrates including wastes and by-products.
- Microorganisms can be cultivated under controlled conditions.

- Climatic independent oil production.
- Absence of bad odour and taste.
- Due to the structural simplicity of microorganisms, they can be used as a model to study the metabolic pathway of PUFAs leading to more understanding of the process.
- Easily manipulated to maximise productivity.

(Gill and Valivety, 1997; Certik and Shimizu, 1999)

Certik *et al.* (1998) summarised the strategy to optimise the microbial PUFAs production in Figure 1.2.

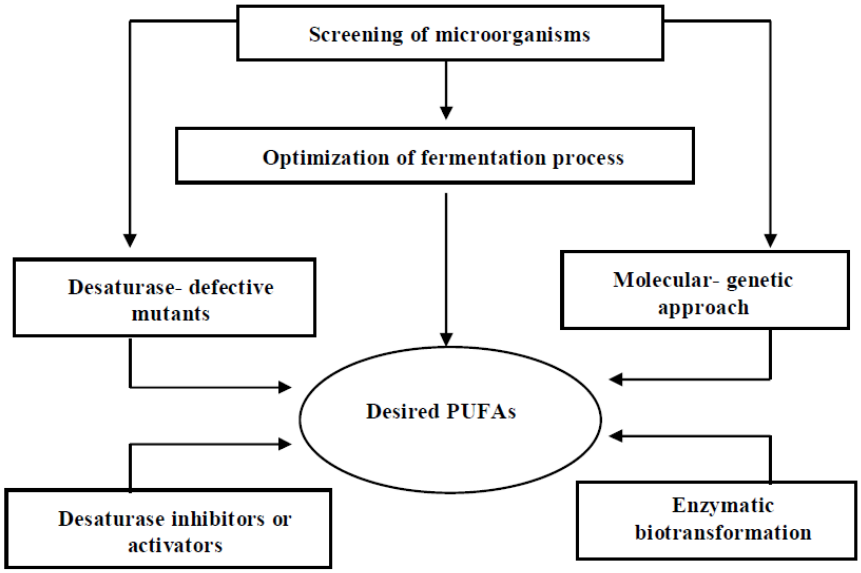

Figure 1.2: The optimisation strategy for maximising microbial PUFA production (Certik *et al.*, 1998).

As described in Figure 1.2, the strategies for maximising microbial PUFA production include three major routes including, optimising the microbial fermentation process, genetic engineered producers and mutants.

1.4.1 Physiological role of PUFAs

PUFA-producing bacteria identified to date mainly belong to *Shewanela, Photobacterium, Colwellia, Vibrio and Psychromonas* (Allen *et al.*, 1999; Fang *et al.*, 2004; Nogi *et al.*, 2007). Generally most of these PUFA producers are psychrophilic and piezophilic and originate from the polar regions and the deep sea (Delong and Yayanos, 1986; Bowman *et al.*, 1997; Nogi *et al.*, 1998; Nichols *et al.*, 1999; Gentile

et al., 2003b). The high PUFA percentage in such microorganisms plays a critical role in their adaptation to these extreme environments, where the high PUFA percentage gives the plasma membrane the ability to remain fluid under low temperatures due to the very low freezing point of these fatty acids (Delong and Yayanos, 1986; Allen *et al.*, 1999; Skerratt *et al.*, 2002b). This theory was confirmed via producing an EPA-deficient mutant of *Shewanella* sp. that became cold-sensitive while the wild type was cold resistant (Sato *et al.*, 2008). The presence of PUFAs provides an appropriate membrane fluidity, which is crucial for life, under low temperature and high pressure conditions.

Another physiological role was revealed by growing the EPA-deficient mutant *Shewanella violacea* DSS12^{pfaA} under relatively high pressure (30 MPa). The mutant showed a remarkable delay in growth and cells became filamentous when exposed to high pressure but not to low pressure. These results indicated that EPA is required for the cells to be adapted when exposed to high hydrostatic pressure (Kawamoto *et al.*, 2011).

EPA protects bacterial cells not only against the extreme cold environments but also against the high pressure stress, which explains the abundance of EPA among the psychrophilic and piezophilic bacteria. Isolate *Shewanella violacea* DSS12 is an EPA producer that can grow at low temperature and relatively high pressure (30 MPa). The EPA-less mutant has an unstable and disordered membrane compared to the wild type over a wide range of pressures. These results suggest that EPA preserves the bacterial membrane stability when exposed to significant changes in pressure (Usui *et al.*, 2012).

Although the regulation of EPA synthesis remains to be fully elucidated, it has been suggested that the enzymes required for EPA synthesis may exhibit higher activity at low temperature and/or high pressure (Wang *et al.*, 2009).

Although most of the known microbial PUFA producers were isolated from the deep sea and cold environments, recently some mesophilic and shallow water bacteria were found to produce EPA, two *Shewanella* strains (ACEM 6 and ACEM 9), isolated from a temperate, humus-rich river estuary in Tasmania Australia, were able to produce a high level of PUFAs at relatively high incubation temperatures (10.2% at 24°C) (Skerratt *et al.*, 2002b). In addition a number of mesophilic strains of gamma-proteobacteria were identified as PUFA producers indicating that they may not be restricted to psychrophilic and piezophilic species (Skerratt *et al.*, 2002b; Ivanova *et*

al., 2003; Frolova *et al.*, 2005; Freese *et al.*, 2009). Also some algae isolated from tropical and subtropical marine habitats were reported to be good DHA producers (Perveen *et al.*, 2006; Okuyama *et al.*, 2007a).

EPA was also found to play a critical role in the bacterial membrane organisation and cell division especially at low temperature. The EPA-less mutant of *Shewanella livingstonensis* Ac10 showed a significant delay in growth at 4°C but not at 18°C. The microscopic examination of the mutant showed that cells became multi-nucleoid filament and several membrane proteins were affected. The important role of EPA in membrane organisation and cell division could be due to specific interaction between EPA and proteins involved in such cellular processes (Kawamoto *et al.*, 2009)

In addition to its role in low temperature adaptation, some PUFAs, especially EPA, were found to have a protective role as an antioxidant. An EPA-deficient mutant of Shewanella *marinintestina* IK-1 was found to be more sensitive to the exogenous addition of H_2O_2 and the mutant showed a remarkable decrease in the amount of cells recovered from the cultures treated with H_2O_2. Protein carbonylation was also enhanced, only in EPA-deficient cells, when treating the cells with 0.01 mM H_2O_2 under bacteriostatic conditions. These results confirm that EPA has a shielding effect protecting the bacterial cells (Nishida *et al.*, 2006a; Nishida *et al.*, 2007). In addition a genetically engineered *E. coli* cells carrying EPA gene cluster showed a remarkable resistance to the addition of exogenous H_2O_2 molecules (Nishida *et al.*, 2006b). The authors reported a constant level of catalase activity after the addition of exogenous H_2O_2 in both the sensitive wild type and the resistant mutant indicating that EPA was the reason for the mutant's resistance perhaps by blocking the entrance of harmful oxygen molecules.

Recently, the anti-oxidant role of PUFA was determined not only in microorganisms but also in rats. Khan *et al.* (2012) reported an important role for PUFA in protecting rat kidneys against oxidative damage.

The presence of EPA in the bacterial membrane could affect the movement of compounds across the membrane, as it was reported that EPA in *Shewanella marinintestina* IK-1 can shield the entry of hydrophilic compounds and facilitate the entry of hydrophobic ones (Nishida *et al.*, 2010). *Shewanella marinintestina* IK-1 was found to be more resistant to water soluble antibiotics and more sensitive to hydrophobic inhibitory compounds than its EPA-deficient mutant which displayed contrasting behaviour.

Two mutants of microalga *Nannochloropsis oculata* ST-6 with overproduction of EPA showed a significant increase in resistance to slightly water soluble antibiotics cerulenin and erythromycin (Chaturvedi and Fujita, 2006).

It was suggested that EPA may increase the resistance of bacterial cells, to such harmful compounds, by enhancing the biosynthesis of proteins such as porins and TolC family proteins which are involved in the efflux of these harmful compounds (Blair and Piddock, 2009; Kawamoto *et al.*, 2009).

Also the presence, the type and the absence of PUFAs inside the bacterial cell have been used for reclassification and chemotaxonomy for a number of bacterial species (Nichols and McMeekin, 2002).

1.4.2 Manufacturing potentials of microbial PUFAs

Bacteria are not fastidious microorganisms and can be grown on the waste products of other agricultural or industrial processes leading to lower production costs, bioremediation and production of valuable compounds in the same process. For example, some psychrophilic bacteria were able to produce PUFAs and to degrade oil and hydrocarbons. These organisms were identified to be 'cold-adapted cleaners with particular importance'(Gentile *et al.*, 2003b)

Bacteria mainly produce one specific type of PUFA rather than a mixture, so there is no need for further purification thus reducing production costs. Such a concentrate was favoured over "whole" fish oil because it keeps the daily amount of fatty acids ingested as low as possible and other fatty acids, which might be physiologically active, are then present only in small amounts or are completely absent. In addition, there are no issues with any unpleasant odour and the metabolic pathways can be studied leading to more extensive knowledge about the biochemical pathways and the genetics in order to develop interesting production systems. Bacterial PUFAs could be used as a direct dietary supplement, or indirectly via introducing them into the food web by using them for food stock for organisms such as rotifers (Russell and Nichols, 1999a). Microbial PUFAs could be extracted and supplied directly in food and pharmaceuticals, or the microbial biomass could be introduced into poultry feed or as a fish supplement in aquaculture (Harel *et al.*, 2002). Arachidonic acid produced by the fungus *Mortierella alpina* is using in the baby formula industry (Sakuradani *et al.*, 2009).

Recently, Certik and Adamechova (2009) succeeded in enriching cereals with a high quantity of microbial PUFAs (γ-linolenic acid yield10 g/kg fermented cereals, 23.4 g EPA/kg product and 36.3 g Arachidonic acid /kg) by performing solid state fermentation.

1.5 Biosynthesis of PUFAs

1.5.1 Conventional biosynthetic pathway

Biosynthesis of PUFAs depends on the genetic sequence responsible for their production in the organism. The fatty acid biosynthetic pathway in all organisms terminates in formation of C16 or C18 saturated fatty acids, then via a sequence of desaturase and elongase enzymes these fatty acids are modified into PUFAs (Figure 1.3).

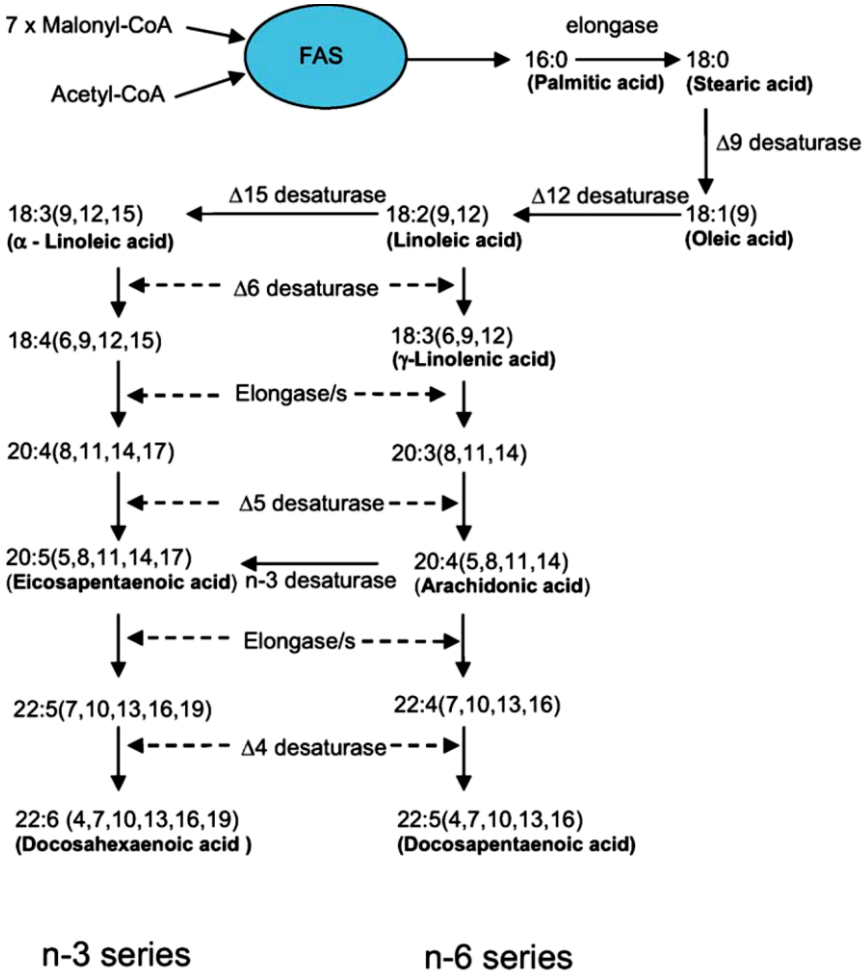

Figure 1.3: Conventional biosynthetic pathway of the formation of PUFAs (Ratledge, 2004)

Figure 1.3 summarises the formation of PUFAs in microorganisms via the conventional fatty acid synthase (FAS) route, where fatty acids are synthesised from acetyl-CoA and malonyl-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 PUFAs (Magnuson *et al.*, 1993).

1.5.2 Novel polyketide synthase biosynthetic pathway

Metz *et al.* (2001) suggested a novel metabolic pathway for PUFA production via the polyketide synthase (PKS) pathway using multi-enzyme complexes and requiring a series of reactions catalysed by keto-synthase, keto-reductase, dehydratase and enoyl-reductase. In PKS-like systems acetyl-CoA and malonyl-CoA are still essential building blocks but this pathway does not involve *in situ* reduction of the intermediates.

Five open reading frames (ORFs) were identified using the analysis of a genomic fragment (cloned as plasmid pEPA) from *Shewanella* sp. strain SCRC2738, that are necessary and sufficient for EPA production (Yazawa, 1996). Since numerous predicted protein domains were homologues of FAS enzymes, it was suggested that PUFA synthesis in *Shewanella* sp. includes the elongation of 16- or 18-carbon fatty acids produced by FAS and the incorporation of double bonds by undefined aerobic desaturases (Yano *et al.*, 1997).

Eleven regions within the five ORFs were identified as putative enzyme domains. When sequences were compared in the non-redundant database, eight of these were more convincingly related to PKS proteins than to FAS proteins. Nevertheless, three regions were homologs of bacterial FAS proteins (Metz *et al.*, 2001; Napier, 2002). The eight PKS domains identified were: 3-ketoacyl synthase, malonyl-CoA:ACP acyl transferase, acyl carrier protein, 3-ketoacyl-ACP reductase, acyltransferase, chain length factor, enoyl reductase, and dehydrase. This theory is supported by the fact that in other PUFA-producing marine bacteria there were genes with homology to the *Shewanella* EPA gene cluster found, indicating that the PKS pathway could be common in these organisms (Allen and Bartlett, 2002).

Metz *et al.* (2009) used radiolabelled ^{14}C-malonyl-CoA in a triacylglycerol fraction and indicated that the PUFAs are released from the enzyme as free fatty acids (FFAs).

In the same work the cultivation of recombinant *E. coli*, carrying the *Schizochytrium* PUFA synthase, resulted in the products of the enzyme accumulating as FFAs.

The PKS has also been detected in some eukaryotes that are able to produce PUFAs. The examination of the genetic makeup of species like *Schizochytrium* sp. revealed that these organisms contain a polyketide synthase (PKS) system which involves acetyl-CoA and malonyl-CoA as precursors for biosynthesis of DHA (Ratledge, 2004). PUFA biosynthesis via the PKS pathway in *Schizochytrium* can be summarised as depicted in Figure 1.4.

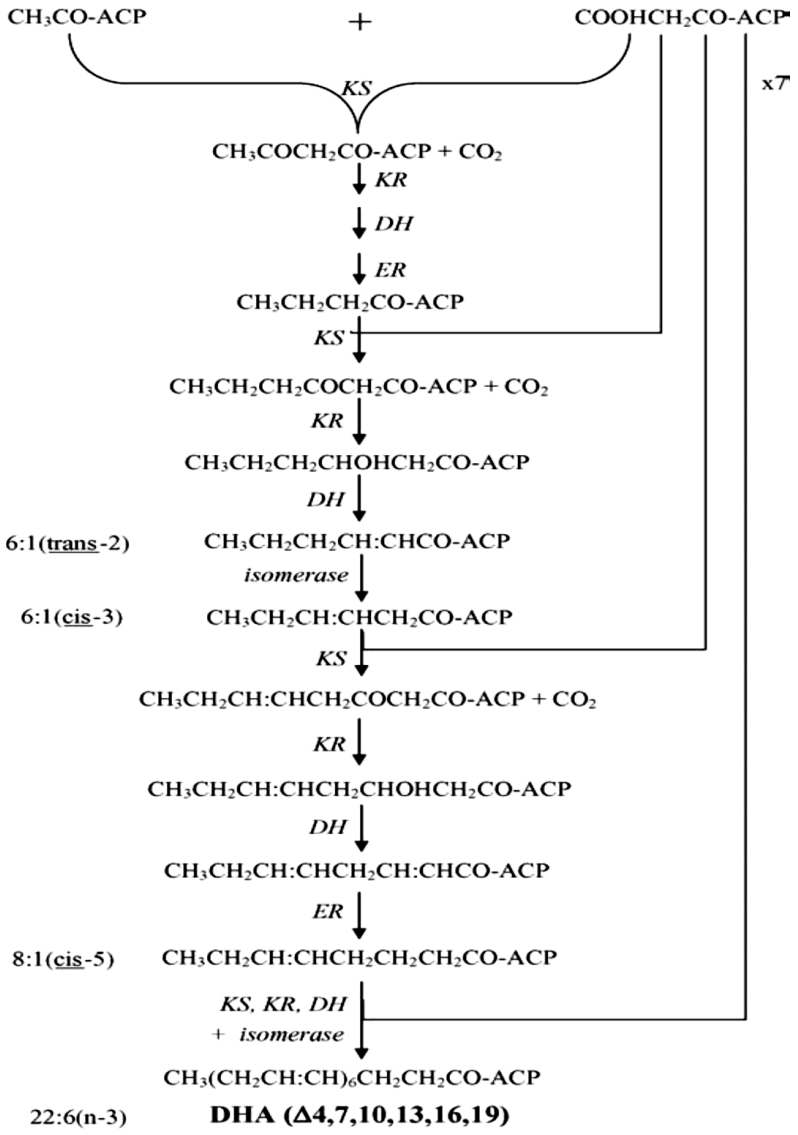

Figure1.4: Polyketide Synthase (PKS) route of synthesis of DHA (Ratledge, 2004) .

Although it has not been completely proven, many reports suggest that, the conventional biosynthetic pathway occurs in eukaryotes, especially fungi and

nematodes, while the unique PKS systems, operating independently of the conventional fatty acid synthesis system, take place in prokaryotes (Sato *et al.*, 2008). There is a long way to go for the exact biosynthetic mechanism of PUFAs to be completely unravelled and there is much speculation about the intermediates involved.

1.6 PUFA genes

The biosynthesis of EPA and DHA via a bacterial polyketide synthase-type multi-enzyme complex is controlled by proteins encoded by five *pfa* genes including *pfaA, pfaB, pfaC pfaD* and *pfaE* (Okuyama *et al.*, 2007b).

The successful cloning of the genes involved in the biosynthesis of EPA from *Shewanella* sp. strain SCRC-2738 by Yazawa (1996) 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 the 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; Lopanik *et al.*, 2006; Nishida *et al.*, 2006b; Hidetoshi *et al.*, 2007) as illustrated in Figure 1.5. The organisation of the gene clusters is divided into three types. Type I, which is present in *Shewanella pneumatophori* SCRC-2738, is a gene cluster including all five *pfa* genes in close vicinity. Type II consists of a cluster of the four genes *pfaABCD,* with *pfaE* separated from the other genes. This type of cluster is present in *Moriella marina* MP-1. The relative direction of *pfaE* has not been determined for this organism. In type III, *pfaE* is integrated into *pfaC/E,* and the cluster is considered to consist of four genes (Orikasa *et al.*, 2004).

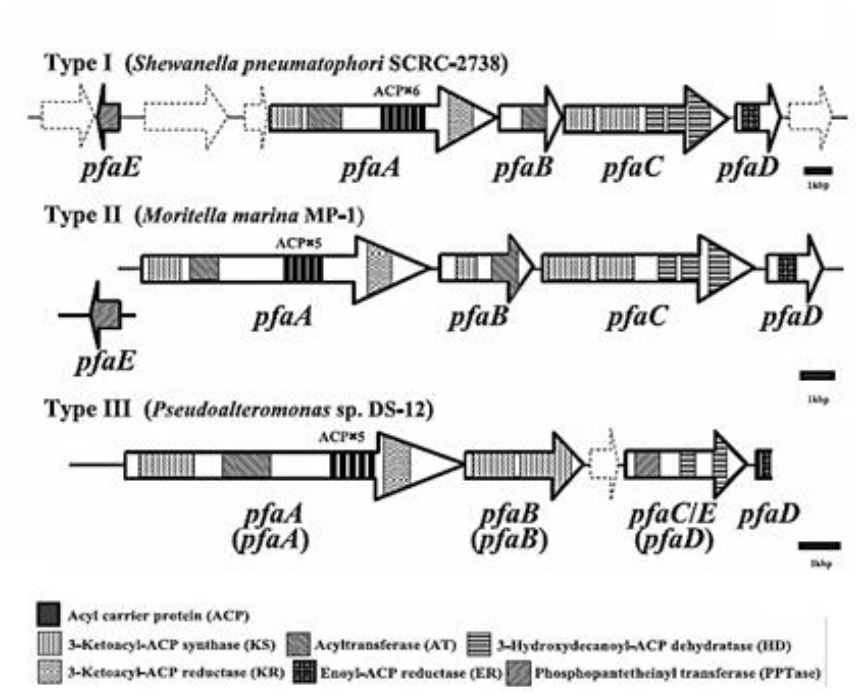

Figure 1.5: Organisation of genes responsible for bacterial EPA and DHA biosynthesis and domain structures of individual genes (Orikasa *et al.*, 2004).

The 38 kb EPA biosynthesis gene cluster of *Shewanella* sp. strain SCRC-2738 was cloned into the cosmid vector (pEPA), and it was found that only five ORFs out of nine in this sequence are required for EPA production. *E. coli* JM109 produced high levels of EPA (16% of the total fatty acids) although it was transformed with a multi-copy pNEB vector carrying only the five essential ORFs (Orikasa *et al.*, 2004).

Lee *et al.* (2008a) successfully cloned the EPA biosynthesis gene cluster from *Shewanella oneidensis* MR-1 under the lacZ promoter on a high-copy number plasmid pBlue-script SK (+) and were able to enhance the productivity of EPA in recombinant *E. coli* up to 7.5% of the total fatty acid content. This takes place via the substitution of promoter sequences within the biosynthesis gene cluster.

The EPA gene cluster from *Shewanella* sp. SCRC-2738 was also cloned into a broad-host range vector, pJRD215, then by conjugation the gene was introduced into a marine cyanobacterium, *Synechococcus* sp. NKBG15041c, producing 7.5 mg/l of EPA (Yu *et al.*, 2000).

Applying the genetic engineering and mutation approaches to enhance the ability of EPA production by microorganisms did not always lead to satisfactory results. Doan and Obbard (2012) succeeded in obtaining a mutant of the microalga *Nannochloropsis* sp. which was able to produce large quantities of intercellular lipids

especially palmitoleic acid (16:1), but the amount of EPA was reduced by 45% compared to the wild type.

1.7 Factors affecting PUFA production

The key controlling factor in PUFA synthesis, in many organisms, is the temperature. The variation in the incubation temperature, as the sole tested variable, significantly affects the PUFA production in bacteria with a high productivity of PUFAs achieved between 5-25°C while there is no production reported at temperatures higher than 25°C (Nichols and McMeekin, 2002; Yang *et al.*, 2007). The same effect of the temperature on the EPA production can be observed, in a one-factor-at-a-time (OFAT) approach, in *Shewanella olleyana* sp. nov. as decreasing the temperature from 24°C to 4°C doubled the production of EPA (from 10.2% to 23.6% of the total fatty acids) while the concentration of mono-saturated fatty acids was reduced (Skerratt *et al.*, 2002b).

Even for DHA the temperature was found to be the most significant factor with lower temperatures preferred for higher DHA production (maximum 3 g/l) by the microalga *Schizochytrium limacinum* (Chi *et al.*, 2007).

Michinaka *et al.* (2003) observed 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 (up to 30% of the total fatty acid).

Yazawa *et al.* (1988) screened 5,000 strains of marine microorganisms for EPA production finding that 88 of them showed a positive result with SCRC-8132 showing the highest yield of EPA of 15 mg/g dry cells. The main factor affecting the productivity was pH with the highest production of EPA obtained at pH 7 and 20-25°C while no productivity was observed at pH 5 and pH 10 at the same temperature, the experiment were performed using the OFAT approach.

A new bacterial strain *Shewanella* sp. KMG427, identified as a psychrophilic EPA producer, was discovered by Lee *et al.* (2008b). Factors affecting EPA productivity in this organism such as temperature, pH and salinity were tested by the OFAT approach and resulted in the maximum EPA production which was achieved at 4°C, in a pH range of 8-9 and a salinity of 1% to 5% (w/v NaCl). At 25^{o}C the EPA production was at the lowest value (2% of total fatty acid), while it progressively increased by

lowering the culture temperature till it achieved its maximum value (11% of the total fatty acids).

In addition to the effect of physical factors, the effect of media composition on the ability to produce PUFAs was studied in a range of organisms. Optimising the culture conditions of the fungus *Thraustochytrium aureum* enhanced the ability to produce DHA leading to a doubling of the productivity (460 mg/l) compared to previous reports (Iida *et al.*, 1996). The main problem was observed when trying to grow the cells in a bioreactor as a dramatic decrease in growth and productivity was reported as cells showed a tendency to coagulate due to the mechanical stirrer in the reactor.

PUFA production was studied, with the OFAT approach, in *Shewanella sp.* GA-22 by Gentile *et al.* (2003b) demonstrating that it is carbon-temperature dependant. Different carbon sources (crude oil, gasoline, glucose, glycerol pyruvate n-tetradecane and Tween) were used as sole carbon source in the media and showed marked influences on PUFA production. In addition, temperature also showed a significant effect on PUFA production with at least a two-fold increase from 2% (w/v) at 20°C to 5% of the total fatty acids at 4°C.

One of the cheapest carbon sources used for the production of PUFAs is sugarcane molasses. Li *et al.* (2008b) studied, applying the OFAT approach, the production of PUFAs from *Mucor recurvus* on an industrial scale. The authors used sugarcane molasses as a carbon source and achieved PUFA concentrations up to 5.74 g/l. Further factors affecting PUFA productivity were also investigated and the optimum conditions were identified as 15% sugarcane molasses, pH 6.0, 28°C, 5 days growth, stirring at 160 rpm and a C/N ratio of 35 using urea as a nitrogen source (0.4 g/l EPA and 0.3 g/l DHA). The authors also found that agitation speed was the most significant factor affecting the ability of *Mucor recurvus* to produce both EPA and DHA, compared to temperature and fermentation time, with the highest productivity obtained at 160 rpm and the yield decreasing sharply when the shaker speed exceeded 180 rpm.

Corn steep liquor and marine industrial waste liquid were used as a carbon source for a marine bacteria identified as *Shewanella putrefaciens*, to reduce the overall fermentation costs (Yazawa, 1996), achieving 200 mg of EPA per litre of broth.

The amount of GLA produced by *Cunninghamella echinulata* was maximised, up to 80mg/g biomass, when it was cultivated on orange peel, agro-industrial by-product as a carbon source, enriched with glucose (Gema *et al.*, 2002).

Maximum amount of GLA produced by *Cunninghamella echinulata* CCRC 31840 was 0.9 g/l which obtained when the isolate was grown on 10% soluble starch, 0.5% yeast extract, 0.11% NH_4NO_3, 0.1% KH_2PO_4, and 0.025% $MgSO_4 \cdot 7H_2O$ as a cultivation media. The optimum C/N ratio was found to be in a range of 33-48 (Chen and Chang, 1996). In the same study, urea and ammonium nitrate were better than potassium nitrate, ammonium chloride, and ammonium sulphate for the growth and productivity of the isolate under investigation.

Various metal salts are required not only for the growth of bacteria, but they also affect the PUFA production. This was proven by Akimoto *et al.* (1991) when they studied the effect of metal salts, on the basis of OFAT, upon the production of EPA by marine bacteria, SCRC-2738, isolated from mackerel intestines. The optimum salt concentration was 2% (w/v) NaCl, 0.15% (w/v) KCl and 0.16% (w/v) $MgCl_2$ which lead to the doubling of the amount of EPA (up to 51.9 mg/l) produced by the isolate under investigation.

Hansson and Dostálek (1988) reported that the addition of Cu^{2+} and Zn^{2+} to the cultivation media enhanced the ability of the fungus *Mortierella ramanniana* in producing GLA while the addition of Mg^{2+} did not show any significant effect on productivity. The addition of 5 mg/l of Mn^{2+} to the cultivation media of *Mortierella ramanniana* var. ramanniana resulted in the highest percentage of GLA of the total fatty acids (13.3%) (Dyal *et al.*, 2005).

The addition of antibiotics, inhibiting the biosynthesis of saturated and monounsaturated fatty acid was demonstrated to result in a remarkable increase in the amount of PUFAs. Cerulenin was found to increase the amount of EPA from 1.6 to 8 mg/l in *Shewanella marinintestina* strain IK-1 (Morita *et al.*, 2005). The same effect was reported, in the same work, for DHA production by *Moritella marina* strain MP-1, enhancing its concentration from 4 to 13.7 mg/l.

In addition to optimising the growth conditions, genetic manipulation of the PUFA producers could lead to significant increase in the production. Amiri-Jami *et al.* (2006) successfully obtained three mutants producing 3-5 times more EPA than the wild type at 10°C. These mutants were obtained via random mutation by inserting the Tn5 transposon in *Shewanella baltica.* The marked increase in EPA production indicates that the insertion of mini-Tn5 affected the enzyme activity responsible for the biosynthesis of EPA. These mutants were confirmed to have insertion(s) in their genomic DNA by antibiotic resistance and PCR

Another method to enhance the productivity of EPA in a recombinant *E. coli* DH5 is to co-express the vector *vktA*, carrying the high performance catalase gene. This increased EPA production from 3% to 12% of the total fatty acids (Orikasa *et al.*, 2007).

1.8 PUFA bioprocessing via Design of Experiment

The main aim of this work is to establish a suitable bioprocess for PUFA production via two main optimising steps: screening and optimising potential production media at the shake flask level followed by subsequent optimisation of the culture conditions at the bioreactor level. Traditionally the optimisation studies reported in the literature were performed using one-factor-at-a-time (OFAT) approach. This approach involves changing a single factor at a time, keeping the rest of the investigated factors at a constant level, to study the effect of the factor on a product or a process. OFAT experiments are easy to understand but they do not investigate how a factor affects a product or process in the presence of other factors and fail to detect the area of optimum response in such process (Zar, 2009; Rocky-Salimi *et al.*, 2011). In addition OFAT lacks the ability to predict the response(s) under untested settings of factors (Saelao *et al.*, 2011).

Design of Experiments (DOE), is an alternative experimental approach that maximises learning using the minimum of resources. DOE is widely used in many fields with broad application in particular to improving manufacturing processes by maximising yield and decreasing variability. In addition, it can be used to optimise processes where no scientific theory or principles are available. Experimental design techniques become extremely important in such circumstances to develop new products and processes in an economical and robust manner. DOE is able to identify the significant variables affecting a process in the most efficient way, with subsequent optimisation of the levels of these variables to achieve improved quality and increased productivity. DOE is much more efficient than OFAT experiments, as it can detect the effect of interaction among variables which is completely ignored when performing the OFAT experiments, and in many processes the interaction can be more significant than the main effect of each variable individually (Montgomery, 2012). The methodology of DOE ensures that all variables and their interactions are systematically examined. Thus, information obtained from a DOE analysis is much

more reliable and complete than the results from OFAT experiments that ignore interactions and may lead to misleading conclusions (Anderson and Whitcomb, 2000). For the purpose of optimising the DHA production by *Aurantiochytrium limacinum* SR21, Rosa *et al.* (2010) revealed that, not only the carbon and nitrogen sources were crucial but also the interaction between them, making the OFAT approach impractical for a full understanding of the process.

1.8.1 Factorial designs

Factorial experiments enable the effect of all factors included in the investigation to be tested and the possible interactions between them. When all of the possible treatment combinations associated with the factors and their levels are tested, the design is called a Full Factorial Design (FFD). Due to the large number of trials required, most of FFD test the factors at two levels (minimum/maximum) and are called two level Full Factorial Designs. The number of runs required to test all possible combinations is 2^n, where n is the number of tested variables. The data obtained from the FFD are reliable with the highest level of confidence (high confidence designs are usually called resolution-5 designs) (Montgomery, 2000).

For a small number of variables a Full Factorial Design is the most appropriate choice (Lazic, 2004) however as the number of tested variables increases the design becomes impractical due to the large number of experiments required. In such cases a two level Fractional Factorial design reducing the number of trials is appropriate (Lazic, 2004). A Fractional Factorial Design is derived from the full factorial matrices by substituting the higher order interactions with new factors leading to a reduced number of runs. This type of a design is called a resolution-4 design with a level of confidence lower than that of a FFD. A Fractional Factorial Design is usually a fraction, most commonly ½ or ¼ , of the FFD, and it is able to measure the main effects, two way interactions and some of the three ways interactions that are not aliased with any other effect, while the higher levels of interaction are usually neglected by the design.

Another design with a lower number of trials and a lower level of confidence (usually called a resolution-3 design) can be applied as a screening method. This screening factorial design usually tests the linear effect of each variable and totally ignores the interaction between them (due to the high confounding and the massive aliased

structure). Such designs offer the minimum number of trials (*n* +1) to validate the main effect of the tested variables and to reduce resource consumption. The most frequently used screening factorial design of experiment is the Plackett-Burman (PB) design (Anderson and Whitcomb, 2001; Lazic, 2004; Montgomery, 2004).

1.8.2 Plackett-Burman design of experiment

PB is a two level fractional factorial design that is useful as a screening tool. The main purpose is to identify the factors with a significant effect on a particular process assuming that the interaction effect among them is negligible. PB is able to identify the most significant factors with the minimum number of trials (*n* +1) , where *n* is the number of variables (Plackett and Burman, 1946). The number of trials in a PB design is always a multiple of 4 (12, 20, 24, 28……) and the PB design with 12 runs is able to screen up to 11 variables.

PB design was used recently as the first step in optimising different bioprocesses, by identifying the most significant factors, which were subsequently optimised with a more sophisticated design, to reveal the optimum combination and to detect the interaction between them (Chauhan *et al.*, 2007).

PB was used to screen the media components for a lipase-producing microorganism showing that oil, $MgSO_4$, and $FeSO_4$ were the most significant factors affecting the lipolytic activity of the isolates (Haider and Pakshirajan, 2007).

Ahuja *et al.* (2004) reported a successful implementation of PB to detect the limiting components for growth of the shipworm bacterium, *Teredinobacter turnirae.* The production of commercially manufactured alkaline protease, produced by a newly isolated *Bacillus* sp. RKY3, was optimised using a PB design for batch culture production (Reddy *et al.*, 2008). Other applications of PB in optimisation studies include protease production by a new *Bacillus subtilis* strain (Dettmer *et al.*, 2012), and the production of exo-polysaccharides by co-culture of Microalgae, Cyanobacteria, and Macromycetes (Angelis *et al.*, 2012).

PB was also applied for screening the most significant factors affecting PUFA production, where seven factors including the substrate particle size, moisture content, time, temperature, yeast extract, glucose and glutamate were screened and the main effect of each variable was determined for ARA production by the fungus *Mortierella alpina* CBS 754.68 (Ghobadi *et al.*, 2011). Nine factors were screened to evaluate

their effect on the ability of the moss *Physcomitrella patens* to produce PUFAs using PB. Five factors including pH, temperature, sucrose, $MgSO_4$ and $CaCl_2$ were found to be the most significant factors in either a positive or a negative way (Chodok *et al.*, 2010).

The maximum DHA yield of 4.91 g/l was obtained when PB was applied to optimise the growth conditions of the microalga *Schizochytrium limacinum* using crude glycerol as a feed stock to produce DHA (Chi *et al.*, 2007).

1.8.3 Central Composite Design (CCD)

Following the selection of the most significant factors, a subsequent investigation to determine the optimum values of these factors, in addition to testing the interactions between them, is required for a full optimisation.

A Box-Wilson Central Composite Design, usually known as 'a central composite design', contains an embedded factorial or fractional factorial design with centre points that are expanded with a group of 'star points' that allow the detection of curvature, Figure 1.6 illustrates the distribution of the points used to create the CCD matrix. This design has been widely used to optimise several bioprocesses recently.

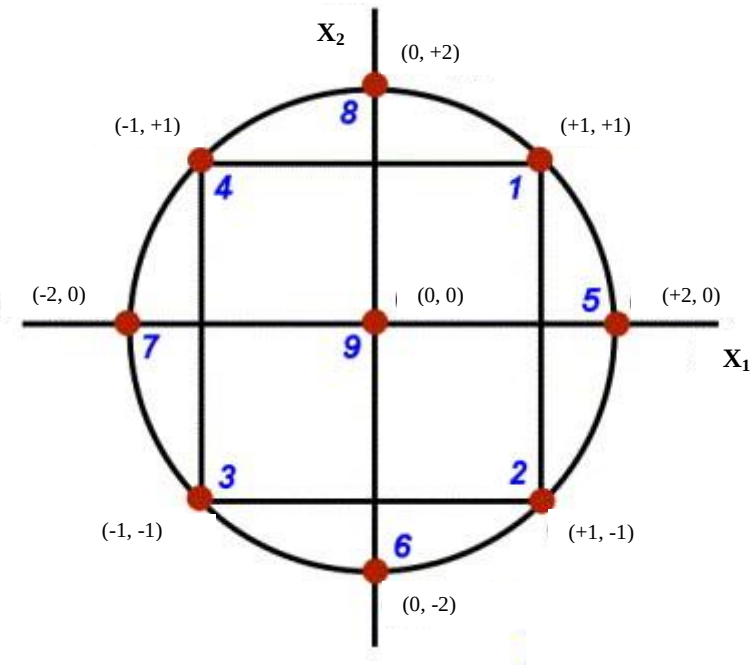

Figure 1.6: Central Composite Design matrix for two factors (Lee *et al.*, 2007).

Response Surface Methodology (RSM) and CCD are powerful tools which can be applied to account for the main effects and the influence of interactions between fermentation variables on the performance of a process. RSM is a sum of statistical techniques for designing experiments, building models, testing the effects of the variables and searching for optimal settings for anticipated responses (Khodaiyan *et al.* (2007). As a result RSM was applied in the optimisation of different fermentation and manufacture processes in the last decade (Park *et al.*, 2005).

CCD was used to generate a selective co-enrichment broth of *Escherichia coli*, *Salmonella* sp. and *Staphylococcus aureus* (Zhang *et al.*, 2012) where, a statistical analysis was carried out to formulate three quadratic polynomial models by computer simulation. These models were then used to investigate the effects of different media components on the composition of bacterial biomass and the PCR amplification yields. Also, CCD was performed to optimise the fermentation variables to attain the maximum extracellular nuclease production by *Bacillus cereus* (Zhou *et al.*, 2010) and the maximum protease production from a novel strain of *Bacillus* sp. (Queiroga *et al.*, 2012).

One of the most important applications of CCD is the optimisation of the cultivation conditions in the bioreactor as a first step towards scaling up the process. The production of glucose oxidase by *Aspergillus niger* was optimised at the bioreactor scale via CCD (Liu *et al.*, 2003).

CCD was recently applied to optimise the production media to maximise PUFA production by different producers although the optimisation of the cultivation conditions has not been performed yet.

Botao *et al.* (2007) studied the effect of several factors affecting EPA production in order to develop an industrial manufacturing bioprocess based on the bacterium *Shewanella* sp. NJ136. These factors included pH, temperature and media composition. A full factorial central composite design was employed and showed that the optimum conditions increased the productivity about 1.7 fold achieving 14 mg/g biomass. The optimum conditions were identified as 15% (w/v) NaCl, 9.98 g/l glucose, 4.42 g/l yeast extract and pH 6.08.

The maximum yield of EPA produced by the diatom *Nitzschia laevis* was 280 mg/l after applying CCD to optimise the pH, temperature, NaCl and $CaCl_2$ (Wen and Chen, 2001).

CCD was applied to optimise the significant variables, including glucose, yeast extract, NaCl, pH and incubation time, affecting the DHA production by *Schizochytrium* sp. s31. The optimum conditions resulted in 0.5 g/l of DHA. Another *Schizochytrium* sp. was found to produce 13.8 g/l of DHA after carrying out a Response Surface Methodology (RSM) optimisation to test the effect of glucose, yeast extract, corn steep liquor and soy peptone (Wu and Lin, 2003; Zhou *et al.*, 2007) Rocky-Salimi *et al.* (2011) optimised five factors, namely glucose, yeast extract, temperature, agitation rate, and fermentation time via CCD in order to maximise the arachidonic acid production by *Mortierella alpina* CBS 754.68 in submerged fermentation. Applying statistical design of experiment lead to increased amount of produced arachidonic acid - 3 g/l representing a 660% increase compared to the amount produced prior to optimisation.

RSM was applied to optimise the culture conditions for maximising the productivity of arachidonic acid (from 0.5 g/l to 21 g/l) by the bacterium *Aureispira maritime* at the shake flask level (Saelao *et al.*, 2011).

To enhance the GLA productivity by the recombinant cells of *Hansenula polymorpha*, a Responses Surface Methodology was applied in a fed-batch fermentation process indicating that dissolved oxygen tension (DOT) was the factor with the most influence (Khongto *et al.*, 2011).

Chapter 2- Materials and methods

All chemicals were purchased from Sigma Aldrich, UK unless otherwise stated

2.1 Sample collections and cultivation conditions

Deep sea core sediments and water samples were collected from three different areas: Mediterranean Sea, Red Sea and Mid Atlantic Ridge. Mid Atlantic Ridge samples were deep sea core sediment and fluff samples collected from the Mid Atlantic ridge by research personnel at the Dove Marine laboratory, Newcastle University and kindly provided for this research, while the Red and Mediterranean Sea samples were collected in Egypt by a diver at 25 meter depth (latitude and longitude are 27.24-33.89 for the Red Sea sampling area and 31.20-29.91 for the Mediterranean Sea sampling area). These samples were diluted in sterile water by serial dilution to 10^{-7}. 100 µl from the last three dilutions were platted out on marine agar plates and were incubated at 20°C for 48hrs. Colonies were collected depending on the morphological variations among them, numbered and cultured on slants for further work.

2.2 Colorimetric screening for PUFAs producers

After 24hrs of growth, 0.1% w/v of the dye 2, 3, 5-triphenyltetrazolium chloride (TTC) was added to the growth broth. Tubes were incubated at (20°C) for one hour. The formation of red colour was considered to be a positive result (Ryan *et al.*, 2010).

2.3 Native gel electrophoresis

The native gel consists of two different layers: a stacking gel (4%) on top of the separating gel (12%). The gel components were monomer solution (30% acrylamide and 2.7% Bisacrylamide, 1.5 M tris HC1 pH 8.8, distilled water, 10% ammonium persulfate, and TEMED, N-Tetramethylethylenediamine,). All components of the separating gel except ammonium persulphate and TEMED were mixed together in a clean beaker and de-gassed under vacuum for several minutes. Then the initiator and cross linking solutions, ammonium persulphate and TEMED, were added and swirled gently to avoid introduction of air bubbles. The separating gel solution was pipetted into the assembled vertical slap gel unit, in the casting mode, to about 1.5-2.0 cm off the top. The surface was levelled off by a thin layer of n-butanol and left to polymerise at a room temperature for at least one hour. The n-butanol

was poured off from the top of the separating gel and washed twice with overlay buffer. Then the stacking gel solution (30% acrylamide and 2.7% bisacrylamide, 0.5 M tris HC1 pH 6.8, distilled water, 10% ammonium persulfate, and TEMED, N-Tetramethylethylenediamine,) was pipetted on top of the separating gel, the comb was inserted into the glass plate sandwich through the stacking gel, and the whole gel was allowed to polymerise for at least two hours at room temperature.

The completely polymerised gel was assembled into the electrophoresis apparatus and submerged with tank buffer (Tris-base, glycine and distilled water), then the comb was slowly removed from the polymerised gel generating the wells. The gel was electrophoresed at 100 Volts for 30 min before loading the protein sample to clean the wells. Equal amounts of proteins were loaded (ranging from 30-40 µl) into the wells using Hamilton syringe. Protein molecular weight marker was loaded into a separate well. The electrophoresis was run at 50 Volts at 4°C in a cold room, until the tracking dye was close to run off the bottom of the gel, about 0.5 cm from bottom. The power supply was then turned off and the glass plate sandwich containing the gel was removed from the electrophoresis tank.

The glass plate sandwich was disassembled to remove the gel. Gel was soaked in a TTC solution and was incubated for 15 minutes.

2.4 Fatty acid methyl ester (FAME) preparation

20 mg of freeze dried cells were suspended in 2 ml of 5% methanolic HCl and heated at 70°C in a water bath for 2 hours in sealed glass tubes. The tubes were cooled 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 in addition to a known volume and concentration of the internal standard (Watanabe *et al.*, 1996). After culturing the bacteria in either a shake flask or a bioreactor with a suitable media, the biomass was harvested and treated to enable further extraction of lipid from the biomass with a suitable solvent. PUFAs were extracted from whole intact cells in order to avoid oxidation resulting from cell disruption. The extract was then methylated and the resulting Fatty Acid Methyl Esters (FAME) was analysed using gas chromatography (GC).

2.5 FAME profiling

The single point internal standard method was used for the determination of EPA concentration. Methyl nonadecanoate (≥ 99.5% GC capillary purity, Sigma-Fluka) was used as an internal standard.

FAMEs were prepared as described in section 2.4 and analysed using a GC with flame ionisation detector (FID) on a Hewlett-Packard 5890 series 2 chromatograph equipped with a DB-23 J&W column; 30 m x 0.25 µm film thickness using helium as the carrier gas. The ideal GC ramp temperature condition (60ºC for 1 min, 140ºC at an increase of 20ºC/min and held for 3 min, 190ºC at increase of 10ºC/min and held for 3 min and then increase of 10ºC/min to 220ºC for 8 min (Jostensen and Landfald, 1997)), was modified and programmed as 220ºC held for 60 minutes as during the preliminary experiment the unmodified and the modified methods were used on the same apparatus, and the results were identical. As a result the modified method was used as it reduced the over all run time. The Supelco-37 was used as a positive control for the determination of the EPA and DHA presence and the peak area was observed and analysed.

.

2.6 DNA extraction

Genomic Kits was used to extract the total genomic DNA from the isolates following the protocol of the manufacturer. To ensure that DNA was successfully extracted, the product was checked via agarose gel electrophoresis.

The specified amount of agarose (1% w/v) was weighted and dissolved in the appropriate buffer (l x TAE: 0.18 M Tris-acetate and 0.002 M EDTA) volume by cooking in the microwave (till complete dissolving of agarose). The melted agarose was cooled to 50°C and ethidium bromide was added (1 µl in 50 ml agarose) before pouring the gel. The agarose was poured into the mould, the well forming comb was placed in position and the gel was left to cool at room temperature for an hour. After solidification the comb was removed and the gel was placed in the electrophoresis apparatus. Sufficient buffer was added to fill the electrode chamber and cover the gel to a depth of about 1 mm. The sample was mixed with the loading buffer, 0.05% bromophenol blue and 0.05% xylene cyanol as tracking dyes and 5% glycerol to increase sample solution density, then the samples were loaded into the previously formed wells. The gel-electrophoresis was performed at a voltage 50 V/cm.

DNA conjugated to ethidium bromide was visualised under long UV light (315 nm) and photographed.

2.7 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 $(NH_4)_2SO_4$, 670 mM Tris-HCl, 0.1% Tween-20), 0.8 µl a mixture of NTPs, 20 µl each of primers 27F (5′-AGAGTTTGATCMTGGCTCAG-3′) and 1525R (5′-AAGGAGGTGATCCAGCC-3′) (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 a Biometra T Gradient Thermocycler according to the following programme:

Table 2.1: The PCR programme for amplifying the gene of interest

Step	Temperature	Time
First Denaturation	95°C	5 Minutes
Step1 Denaturation	95°C	1 Minute
Step2 Annealing	50°C	1 Minute
Step3 Extension	72°C	1 Minute
Final Extension	72°C	10 Minutes

The cycles of steps from 1-3 were repeated 30 times. The PCR products were checked by agarose gel electrophoresis (1% w/v; 30 minutes at 100 V, 0.5 X TBE buffer). The amplified fragments were compared with 100bp molecular size marker (MBI Fermentas, Lithuania). The PCR product was stored at -20^0C.

2.8 Purification and sequencing of 16S rRNA gene

The PCR product was purified before it was sent for sequencing. The purification procedure was as follows: 5 µl of PCR product was mixed with 2 µl of ExoSAP, the mixture was incubated at 37°C for 15 min to remove the excess of primers and dNTPs. For inactivation, the mixture was further incubated at 80°C for 15 minutes and the purified PCR was sequenced at the Census sequencing service at Newcastle University. Sequencing was carried out using BigDye^{TM} version 3.1 Terminator Cycle Sequencing Kit (PE Applied Biosystems, MA). The quality and quantity of sequence obtained was checked with Finch TV version 1.4.0. CAP3 software was used to assemble the gene as a set of contiguous sequences. ClustalX, in addition to Treeview software, was used to construct the evolutionary tree to

identify the isolates. The sequence was identified by BLAST and SeqMatch against Genbank and the Ribosomal database project (RDP).

2.9 Amplification of PUFA-PKS gene specific fragment

Previously extracted genomic DNA was used as a template for the amplification of the specific PUFA PKS gene *pfaB* using the PCR protocol described in section 2.7 with the specific degenerate primers *pfaB* 1240F (GGTGAAGCATCRATGTGGGC) and *pfaB* 1840R (TCSGCRCCAATTTCAACAA) designed on the basis of the known *Shewanella* sp. SCRC-2738 (U73935) sequence (Gentile *et al.*, 2003a) and the PCR product was checked by agarose gel electrophoresis.

2.10 Stability of the bacterial oil

2.10.1 Thermal stability of the bacterial oil

Bacterial total lipid was extracted from the biomass achieved when growing the bacterial cells in bioreactor batch cultures, under optimum conditions to give the large quantities of lipids required to perform the stability experiments, which was unachievable in shake flask cultivation, and to ensure the maximum percentage of EPA within the tested oil.

The total bacterial lipids were extracted using the Bligh and Dyer (1959) method, where for each 1 ml bacterial suspension, 3.75 ml of a mixture chloroform/methanol (1/2) were added and vortexed for 10-15 min, then 1.25 ml chloroform were added with mixing for 1 min followed by adding 1.25 ml of distilled water with mixing for an extra minute before centrifugation. After centrifugation, two layers were obtained. The upper layer was discarded and the lower layer was collected with a Pasteur pipette (proteins were isolated between the two liquid phases). The lower layer (containing the total lipids) was dried under nitrogen gas to avoid any oxidation. After evaporation, the lipid extract was re-dissolved in a known volume of hexane. 1 ml of 1M NaCl was added instead of water to block the binding of some acidic lipids to denatured lipids (Hajra, 1974).

The thermal stability of the total fatty acids, including EPA, was tested via thermo-gravimetric (TG) analysis. TGA was considered as a regular analytical tool to study the thermal behaviour of different materials including oils (Milovanovic *et al.*, 2006). Thermal stability of the oil was monitored as a function of weight loss with respect to temperature. The TGA studies were performed on a Pyris STA 6000 Model under flowing helium at a constant rate of 30 ml/min at a temperature ramp rate of 10^{o}C/min from 30 to1000^{o}C. The

TGA consists of an electronic microbalance and a ceramic container containing a platinum crucible suspended in a furnace. The sample's initial mass, temperature and final mass loss were logged by a computerised control unit. Thermal stability of the bacterial oil was compared to Fish oil from *Menhaden* and fish liver oil from *Gadus morrhua*, while sunflower oil was used as a reference.

2.10.2 Oxidative stability of the bacterial oil

Samples were measured using the ACL Instrument, which measures chemiluminescence arising during the oxidation reaction. Samples underwent a conditioning period of 6 minutes at 30°C, followed by a temperature ramp (heating rate 10°C/minute) until the isothermal temperature, 90°C, was reached. Samples were then held at 90°C. The gas supply was nitrogen until isothermal temperature was reached, upon which the gas supply was switched to bottled synthetic air. Sample masses ranged from 5 - 8.8mg. Samples were tested on 22mm borosilicate glass slides. Data was plotted on Calisto Processing, and smoothed with a 5 point Savitsky Golay peak filter.

2.11 Seed culture in artificial sea water

A loopful of biomass from culture plates was transferred into 250 ml flask containing 50 ml of artificial sea water (peptone 3.5 g/l; yeast extract 3.5 g/l; NaCl 23 g/l; $MgCl_2$ 5.08 g/l; $MgSO_4$ 6.16 g/l; $Fe_2(SO_4)_3$ 0.03 g/l; $CaCl_2$ 1.47 g/l; KCl 0.75 g/l; Na_2HPO_4 0.89 g/l; NH_4Cl 5.0 g/l) (Lang *et al.*, 2005) and incubated at 15°C in an orbital shaking incubator at 160 rpm for 24 hours.

To test the effect of different incubation temperature on the growth and productivity of the isolates under investigations, the isolates were cultivated on 50 ml ASW in a 250 ml flask, subsequently, the flasks were incubated in an orbital shaker incubator at 160 rpm at the tested temperatures (10, 15, 20, 25 and 30°C).

2.12 Growth in production media

The growth was performed in 250 ml sterile flasks with 50 ml of given media at a given temperature for two days in an orbital shaker incubator at 160 rpm. Artificial sea water (ASW) metal ions were used as the basal medium in each experiment. Final biomass from each flask was collected into a 50 ml Falcon centrifuge tube and centrifuged at 6000 rpm for

15 minutes. The cell pellets were transferred into a 1.5 ml screw tube and freeze-dried overnight.

2.13 Screening and optimising the production media and culture conditions

Forty three different potential media components were screened by dividing them into four different Plackett-Burman designs to investigate the most significant factors affecting EPA production. The screening and optimisation experiments were carried out in 250 ml sterile flasks with 50 ml of given media at 15°C as a cultivation temperature for two days in an orbital shaker incubator at 160 rpm

The factors with the most significant positive effect were taken further to a next level of Plackett-Burman designs to compare them.

The third step involved testing the interaction and determining the optimum combination of the potential production media using the Central Composite Design as illustrated in Figure 2.1.

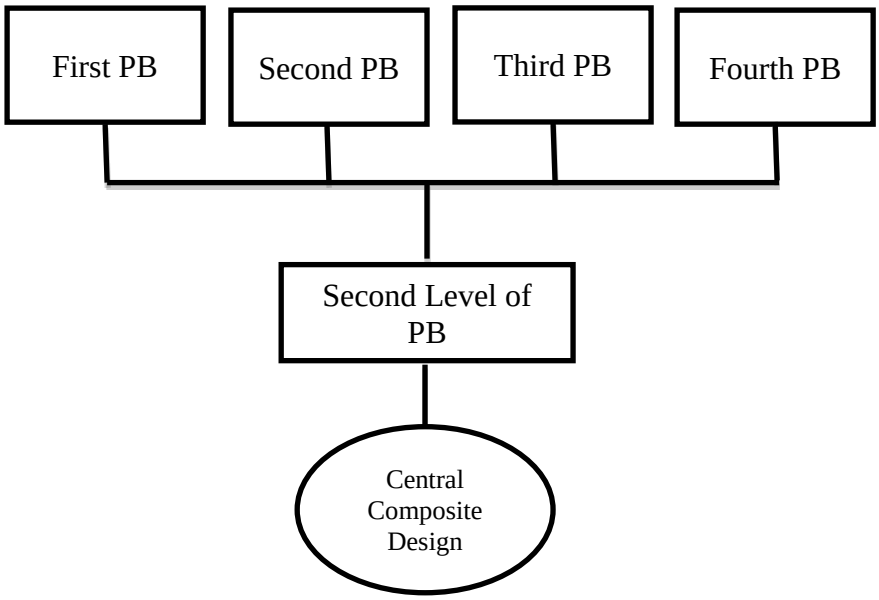

Figure 2.1: Schematic diagram showing the optimisation strategy applied in this research to select and optimise the production media.

In Figure 2.1, the first raw of PB experiments represent the first level of screening, where all tested variables were divided into four different PB designs. The variables with the most significant positive effect were subsequently taken into the second raw, which represent the second level of screening. The CCD experiment represents the final step of the process, where the media components with the most significant positive effect were optimised.

A probability value (P-value) was used to differentiate signals from noise. At 90% level of confidence, any variable with P-value > 0.1 were considered as non-significant. The coefficients with highest probability value were removed by the method of Backward Stepwise Exclusion. Removal of coefficients continued until only coefficients were left with probability values less than 0.1. Some parameters with P-value lager than 0.1 were not removed as they were needed for the design hierarchy (Bosma *et al.*, 2003).

The complete absence of the nutrients would lead to the inability of the tested isolate to grow; however, high concentrations of nutrients could lead to the same result due to substrate inhibition. It is a well established fact that a high concentration of carbon may lead to the repression of one or more of the enzymes responsible for product formation, so called carbon catabolic repression. High concentrations of nutrients may also cause plasmolysis which inhibit the cell growth completely (Stülke and Hillen, 1999). The complete inhibition of growth due to plasmolysis could lead to a confounding result and may mask the significance of the effect of some variables. As a result, preliminary experiments were carried out where five 250 ml flasks, each containing 50 ml of media with all the tested variables, were prepared in different concentrations (5 g/l, 4 g/l, 3 g/l, 2 g/l and 1 g/l for each individual variable). Based on these results the maximum value in each design was set up and calculated.

2.13.1 Initial screening by Plackett-Burman design

In addition to the 43 tested variables a dummy variable was used to evaluate the standard error of the experiments. Each variable was investigated at a high (+) and a low (-) level. The variables were tested on the presence/absence principle, where the low level was always zero except for sodium chloride as the marine isolates tested were unable to grow in the complete absence of it.

The difference between the minimum and maximum values should be neither small, as it may not show the effect, nor large, as it could mask the effect of the others (Ahuja *et al.*, 2004).

Having identified the most positively significant factors within each of the first level PB experiments, a second level of PB design was carried out using these factors.

The variable distribution among the designs and the number of trials in all the PB experiments were set up in a random manner except for the metal ions experiment (fourth PB), as it was performed to test the effect of the metal ions included in the artificial sea water media (Table 2.3).

The main target from this screening experiment was to compare the significance and main effect of each factor on the amount of EPA produced by isolate under investigation. Applying the statistical methods to analyse the data is objective rather than subjective in nature.

The main effects of each variable were calculated using equation 2.1

$$E_{(Xi)} = \frac{2[\sum Y_i^+ - \sum Y_i^-]}{N} \qquad (2.1)$$

where $E_{(Xi)}$ is the effect of the tested variable and Y_i^+ and Y_i^- are the calculated responses, while the significance level (P-value) of each variable was determined using the Student's t test (equation 2.2)

$$t_{(Xi)} = \frac{E_{(Xi)}}{SE} \qquad (2.2)$$

where (SE), the standard error of variables, is calculated as the square root of the variance of an effect. Any variable with ($P < 0.1$) was considered to be significant representing 90% level of confidence.

The relation between the response and the significant variables within Plackett-Burman can be expressed with a first order polynomial order equation (equation 2.3)

$$Y = \beta_0 + \sum \beta_i X_i \qquad (i=1, 2\ldots\ldots, k) \qquad (2.3)$$

where Y is the calculated response, β_0 is the model intercept, β_i is the regression coefficient for each corresponding variable, X_i is the corresponding variable and k is the number of variables (Montgomery, 2000).

For the first three PB designs, the same basal media was used as shown in Table 2.2.

Table 2.2: The basal media composition used for the first three PB designs

Compound	**Amount (g/l)**
Yeast Extract	1
NaCl	10
$MgCl_2$	5
$MgSO_4$	6
$Fe_2(SO_4)_3$	0.03
$CaCl_2$	1.5
KCl	0.75
Na_2HPO_4	0.89
NH_4Cl	5

The basal media for the fourth Plackett-Burman design testing the effect of metal ions was 1 g/l of yeast extract in addition to 10 g/l of sodium chloride.

Table 2.3: The four Plackett-Burman designs included in the first level of the screening step

First Plackett-Burman					**Second Plackett-Burman**					**Third Plackett-Burman**					**Fourth Plackett-Burman**				
Variables	Code	unit	Minimum level (-)	Maximum level (+)	Variables	Code	unit	Minimum level (-)	Maximum level (+)	Variables	Code	unit	Minimum level (-)	Maximum level (+)	Variables	Code	unit	Minimum level (-)	Maximum level (+)
Yeast Extract	X_1	**g/l**	**0**	**1**	**Palmitic acid**	X_1	**g/l**	**0**	**1**	**Meat Peptone**	X_1	**g/l**	**0**	**1**	**NH_4Cl**	X_1	**g/l**	**0**	**5**
Glucose	X_2	**g/l**	**0**	**1**	**Sugar Cane Molases**	X_2	**g/l**	**0**	**1**	**Ammonia**	X_2	**g/l**	**0**	**1**	**Na_2HPO_4**	X_2	**g/l**	**0**	**0.89**
Glycerol	X_3	**g/l**	**0**	**1**	**Linseed Oil**	X_3	**g/l**	**0**	**1**	**Lactose**	X_3	**g/l**	**0**	**1**	**KCl**	X_3	**g/l**	**0**	**0.75**
Soy Bean flour	X_4	**g/l**	**0**	**1**	**Ammonium Nitrate**	X_4	**g/l**	**0**	**1**	**Ground Sesame**	X_4	**g/l**	**0**	**1**	**$CaCl_2$**	X_4	**g/l**	**0**	**0.15**
Corn Step Liquer	X_5	**g/l**	**0**	**1**	**Tryptone**	X_5	**g/l**	**0**	**1**	**Ammonium Sulfate**	X_5	**g/l**	**0**	**1**	**$Fe_2(SO_4)_3$**	X_5	**g/l**	**0**	**0.03**
Glutamic Acid	X_6	**g/l**	**0**	**1**	**Pea Nut Oil**	X_6	**g/l**	**0**	**1**	**Casein**	X_6	**g/l**	**0**	**1**	**$MgCl_2$**	X_6	**g/l**	**0**	**6**
L-proline	X_7	**g/l**	**0**	**1**	**Ethylene Glycol**	X_7	**g/l**	**0**	**1**	**Maltose**	X_7	**g/l**	**0**	**1**	**$MgSO_4$**	X_7	**g/l**	**0**	**5**
Ammonium Acetate	X_8	**g/l**	**0**	**1**	**Hy-Soy**	X_8	**g/l**	**0**	**1**	**Vegetable Peptone**	X_8	**g/l**	**0**	**1**	**NaCl**	X_8	**g/l**	**10**	**30**
Whey Protein	X_9	**g/l**	**0**	**1**	**Fish Peptone**	X_9	**g/l**	**0**	**1**	**Mannitol**	X_9	**g/l**	**0**	**1**	**Dummy**	X_9	**g/l**	**Distilled Water**	**Bi-Distilled Water**
Propanoic Acid	X_{10}	**g/l**	**0**	**1**	**Sodium Acetate**	X_{10}	**g/l**	**0**	**1**	**Dummy**	X_{10}	**g/l**	**Distilled Water**	**Bi-Distilled Water**					
L-Serine	X_{11}	**g/l**	**0**	**1**	**Dummy**	X_{11}	**-**	**Distilled Water**	**Bi-Distilled Water**										
Urea	X_{12}	**g/l**	**0**	**1**															
Sucrose	X_{13}	**g/l**	**0**	**1**															
Glycine	X_{14}	**g/l**	**0**	**1**															
Citric Acid	X_{15}	**g/l**	**0**	**1**															
Fructose	X_{16}	**g/l**	**0**	**1**															
Dummy	X_{17}	**-**	**Distilled Water**	**Bi-Distilled Water**															

The second level of Placket-Burman design for isolate 717 included ten different media components, in addition to one dummy variable. Each variable was investigated at a high (+) and a low (-) level. The variables were tested on the presence/absence principle, where the low levels were always zero except for sodium chloride variable. The used basal medium was 1 g/l yeast extract.

Table 2.4: Variables investigated for EPA production via second level Plackett-Burman design for isolate 717

Variables	**Code**	**unit**	**Minimum level (-)**	**Maximum level (+)**
L-Proline	X_1	g/l	0	2
Casein	X_2	g/l	0	2
Fish Peptone	X_3	g/l	0	2
Na_2HPO_4	X_4	g/l	0	0.89
Ammonium Nitrate	X_5	g/l	0	2
Urea	X_6	g/l	0	2
Hy-Soy	X_7	g/l	0	2
Meat Peptone	X_8	g/l	0	2
Mannitol	X_9	g/l	0	2
NaCl	X_{10}	g/l	10	30
Dummy	X_{11}	-	Distilled Water	Bi-Distilled Water

Seventeen different media components, in addition to one dummy variable to evaluate the standard error of the experiments, were investigated in the second level of Plackett-Burman design for isolate 66 as shown in Table 2.5.

Table 2.5: Variables investigated for EPA production via Plackett-Burman design for isolate 66.

Variables	**Code**	**unit**	**Minimum level (-)**	**Maximum level (+)**
Yeast Extract	X_1	g/l	1	2
Glycerol	X_2	g/l	0	1
Whey	X_3	g/l	0	1
Meat Peptone	X_4	g/l	0	1
Maltose	X_5	g/l	0	1
Mannitol	X_6	g/l	0	1
Urea	X_7	g/l	0	1
Na_2HPO_4	X_8	g/l	0	0.89
Glutamic Acid	X_9	g/l	0	1
Glycine	X_{10}	g/l	0	1
Fish Peptone	X_{11}	g/l	0	1
Hy-Soy	X_{12}	g/l	0	1
Sodium Acetate	X_{13}	g/l	0	1
Casein	X_{14}	g/l	0	1
$MgSO_4$	X_{15}	g/l	0	5
NaCl	X_{16}	g/l	10	30
Lactose	X_{17}	g/l	0	1
Dummy	X_{18}	-	Distilled Water	Bi-Distilled Water

15 different media components were taken further to the 2nd level of PB experiment for the Egyptian isolate Hus-27 as shown in Table 2.6.

Table 2.6: Variables investigated for EPA production via Plackett-Burman design for isolate 66.

Variables	Code	unit	Minimum level (-)	Maximum level (+)
Whey	X_1	g/l	0	1
Na_2HPO_4	X_2	g/l	0	0.89
Casein	X_3	g/l	0	1
Glycine	X_4	g/l	0	1
Ammonium nitrate	X_5	g/l	0	1
Hy-soy	X_6	g/l	0	1
$MgSO_4$	X_7	g/l	0	5
Mannitol	X_8	g/l	0	1
Urea	X_9	g/l	0	1
Fructose	X_{10}	g/l	0	1
Yeast extract	X_{11}	g/l	1	2
Glycerol	X_{12}	g/l	0	1
Ammonium sulphate	X_{13}	g/l	0	1
Meat peptone	X_{14}	g/l	0	1
Sugar cane molasses.	X_{15}	g/l	0	1
Dummy	X_{16}	-	Distilled Water	Bi-Distilled Water

2.13.2 Optimising Central Composite Design

After determining the most significant variables via Plackett-Burman design, a central composite design (CCD) was used to estimate the optimum level of each variable. The CCD matrix included 5 levels for each variable, 6 centre points and star points to estimate the curvature. The CCD provided an indication of the main effect of each factor in addition to the interaction between them. A second order polynomial model was created for mathematical prediction of the optimum growth conditions for EPA production (equation 2.4):

$$Y = \beta_0 + \sum \beta_i X_i + \sum \beta_{ii} X_{ii} + \sum \beta_{ij} X_{ij} \qquad (2.4)$$

where, β_i are the regression coefficients for each factor, β_{ii} are the regression coefficients for square effects and β_{ij} are the regression coefficients for interaction. Analysis of variance (ANOVA) was carried out using Design Expert 8.0 statistical package (StatEase, Inc, Minneapolis, MN, USA) and Minitab 15.

For isolate 717, the most significant components to obtain maximum EPA were casein, Hy-soy and Na_2HPO_4. The variables evaluated are listed in Table 2.7.

Table 2.7: Variables and their levels investigated via CCD for maximum EPA production by isolate 717.

Variables	Levels				
	-2	-1	0	+1	+2
Casein (g/l)	1.0	4	8	12	15
Hy-Soy (g/l)	1.0	4	8	12	15
Na_2HPO_4 (g/l)	0.5	2	4	6	7.5

Four media components were chosen for further study for isolate 66 for the highest possible EPA production. These components were: meat peptone, whey, glycerol and Na_2HPO_4. Table 2.8 summarises the five levels used for each variable in the performed CCD experiment.

Table 2.8: Variables to be investigated and their levels in the CCD for maximum EPA production by isolate 66.

Variables	Levels				
	-2	-1	0	+1	+2
Glycerol(g/l)	1.0	4	8	12	15
Whey (g/l)	1.0	4	8	12	15
Meat Peptone(g/l)	1.0	4	8	12	15
Na_2HPO_4(g/l)	0.5	2	4	6	7.5

An additional investigation was carried out due to the presence of two compounds (whey and glycerol) that were reported as industrial by-products. This experiment target was converting these waste compounds into value-added ones.

A cheap potential production medium, based on glycerol and whey, for EPA production by isolate 66, was investigated.

Table 2.9: A potential cheap production medium components and their levels via CCD for maximum EPA production by isolate 66.

Variables	Levels				
	-2	-1	0	+1	+2
Whey (g/l)	1.0	4	8	12	15
Glycerol (g/l)	1.0	4	8	12	15

The effect of metal ions was tested using a separate CCD experiment to check the significance of their effect on EPA production by isolate 717 and to reveal the interaction between the ions.

Table 2.10: Metal ions and their levels investigated via CCD for maximum EPA production by isolate 717.

Variables	Levels				
	-2	-1	0	+1	+2
Na_2HPO_4 (g/l)	0.05	0.79	1.52	2.26	3
KCl (g/l)	0.05	0.79	1.52	2.26	3
NaCl (g/l)	10	16.25	22.50	28.75	35
$MgSO_4$ (g/l)	1	4.50	8.00	11.50	15

The optimum media components for isolate Hus-27 were peptone, sugar cane molasses and Hy-soy. The tested levels for each individual variable were as shown in Table 2.11.

Table 2.11: Variables to be investigated and their levels in the CCD for maximum EPA production by isolate Hus-27.

Variables	Levels				
	-2	-1	0	+1	+2
Sugar cane molasses (g/l)	1.0	3	4.5	6	8
Hy-soy (g/l)	3	5	7.5	10	12
Meat Peptone(g/l)	3	5	7.5	10	12

For each CCD experiment validation experiments were carried out based on the analysis recommendation(s). 50 ml of the recommended medium composition was prepared in a 250 ml shake flasks and incubated at 15°C in an orbital shaker incubator with 160 rpm.

2.14 Creating chemically defined media

Chemically defined media were developed for both isolates 717 and 66 to simplify the medium component and testing the effect of C/N ratio on the growth and EPA productivity.

The strategy included screening the effect of different amino acids and simple carbon sources in two distinct designs. After determining the amino acid and the simple carbon sources with the most significant positive impact on the growth and productivity of isolates under investigates they were taken further into a separate CCD experiment to optimise and test the interaction between them.

2.14.1 Screening effect of the amino acids

The effect of 20 different amino acids was tested, on the growth and productivity of isolates 66 and 717, via the matrix shown in Table 2.12 producing nine different pools. For each experiment four responses were measured including, dry weight, EPA percentage, EPA concentration (mg/l) and yield (mg/g).

Table 2.12: The amino acid pools tested to investigate the effect of each amino acid on the growth and EPA production by isolates 717 and 66.

Pool	1	2	3	4
5	Phenyla-Alanine	Alanine	Arginine	Leucine
6	Serine	Cysteine	Asparagine	Glycine
7	Tryptophan	Threonine	Aspartic Acid	Iso-Leucine
8	Tyrosine	Glutamine	Proline	Histidine
9	Valine	Methionine	Glutamic Acid	Lysine

A matrix system was used to determine the most significant amino acid from within a mixed pool. Nine different amino acid mixed pools (4 pools vertically and 5 pools horizontally) were tested.

The amino acid that is contained in both the horizontal and the vertical pools which results in the highest responses displays the most significant effect. For example, if the pool number two (of the vertical pools) and the pool number eight (of the horizontal pools) were found to give the highest response, then L-glutamine will be considered as the most significant amino acid on the calculated responses.

The basal media used to test the effect of different amino acids contained the metal ions found to be significant during the screening process for each isolate. The media was autoclaved before adding the amino acid, while the amino acids were sterilised via filtration method.

2.14.2 Resolution-4 fractional factorial design

Resolution four DOE is a two factorial design, PB is a resolution-3 design with less level of confidence, with a number of experiments sufficient to test the main effect, two way interactions and some three way interactions without confounding and in analysis only the non aliased variables were analysed.

Table 2.13: Variables and their levels investigated via level four DOE for EPA production for isolates 66 and 717

Variables	**Code**	**unit**	**Minimum level (-)**	**Maximum level (+)**
Glycerol	*A*	g/l	0	1
Fructose	*B*	g/l	0	1
Arabinose	*C*	g/l	0	1
Mannitol	*D*	g/l	0	1
Sorbitol	*E*	g/l	0	1
Sodium Acetate	*F*	g/l	0	1
Glucose	*G*	g/l	0	1
Maltose	*H*	g/l	0	1
Xylose	*J*	g/l	0	1
Lactose	*K*	g/l	0	1
Sucrose	*L*	g/l	0	1

The design was applied for both isolates 66 and 717. All the tested carbon sources were filter sterilised to avoid any negative effect of temperature on the structure of the carbon source. The basal medium used was the amino acid with the most positive significant effect on isolate under investigation (section 2.14.1).

2.14.3 CCD for the chemically defined media

After determining the best amino acid(s) and the best simple carbon source(s) for isolates 66 and 717, the optimum composition of the medium was estimated via a CCD experiment. The variables and their levels are summerised in Table 2.14 and Table 2.15.

In order to develop a chemically defined media for isolate 717, two amino acids (L-proline and L-histidine) and two carbon sources (glycerol and mannitol) were used, tested and evaluated. To achieve the optimum recipe for the desired medium, a CCD experiment was performed.

Table 2.14: Variables and their levels investigated in the CCD for maximum EPA production by isolate 717 using chemically defined media

Variables	Levels				
	-2	-1	0	+1	+2
Mannitol (g/l)	0.2	1.6	3	4.4	6
L-Histidine (g/l)	0.1	0.8	1.6	2.3	3
Glycerol (g/l)	0.2	1.6	3	4.4	6
L-Proline (g/l)	0.1	0.8	1.6	2.3	3

One amino acid (L-histidine), in addition to one carbon source (arabinose), was used to create an optimised and chemically defined medium for maximum EPA production by isolate 66.

Table 2.15: Variables and their levels investigated via CCD for maximum EPA production by isolate 66 using chemically defined media

Variables	Levels				
	-2	-1	0	+1	+2
Arabinose	3	4.5	7.5	10.5	12
L-Histidine	1.5	2.2	4	5.8	6.5

For each CCD experiment a validation experiment was carried out based on the analysis recommendation(s). 50 ml of the recommended medium composition was prepared in a 250 ml shake flasks and incubated at 15°C in an orbital shaker incubator shaking at 160 rpm.

2.14.4 Effect of C/N ratio on the growth and EPA productivity

The C/N ratios in each tested CCD experiments for isolates 66 and 717 (section 2.14.3) were theoretically calculated and relationships between these ratios and the growth and EPA productivity were investigated.

2.14.5 Effect of additional supplements on EPA production

The effect of different compounds including, α-ketoglutarate, oxaloacetate and malate which are potential substrates for the Krebs cycle, and thiamine (vitamin B1), cobalamin (vitamin B12) and Riboflavin (vitamin B2) which are vitamins, were tested on the ability of isolate 717 to produce EPA. These compounds were added to the previously developed chemically defined medium. They were added to the medium in minute quantities (0.1 g/l) after filter sterilisation. The added amounts were estimated depending on results obtained by the preliminary experiments.

In addition to these supplements, different concentrations of H_2O_2 were added to the developed chemically defined medium to test the effect of the exogenous supply of H_2O_2 on the growth and productivity of isolate 717. The amounts added were (0, 10, 20, 30, 35, 40, 45 50, 60, 100 and 200 µl) for each shake flask containing 50 ml of the chemically defined medium. The H_2O_2 was added to the culture medium in the mid exponential growth phase of isolate 717 (after 24hrs of the first inoculation) and then samples were harvested for FAME preparation at the early stationary phase of growth (36hrs after inoculation).

2.15 Gas chromatography/mass spectroscopy (GC/MS)

GC-MS analysis was performed on an Agilent 7890A GC in split mode, injector at (280°C) linked to a Agilent 5975C MSD with the electron voltage 70 eV, source temperature 230°C, quad temperature 150°C multiplier voltage 1800 V, interface temperature 310°C, controlled by a HP Compaq computer using Chemstation software. The sample (1µl) in hexane was injected using HP7683B auto sampler with the split open. After the main solvent peak had passed the GC temperature programme and data acquisition commenced. Separation was performed on an Agilent fused silica capillary column (30 m x 0.25 mm i.d) coated with 0.25 µm dimethyl poly-siloxane (HP-5) phase. The GC was temperature programmed from 30-130°C at 5°C/ min then to 300°C at 20°C/min and held at final temperature for 5 minutes with helium as the

carrier gas (flow rate of 1 ml/min, initial pressure of 50 kPa, split at 10 ml/min). Peaks were identified and labelled after comparison of their mass spectra with those of the NIST05 library using > 90% fit or from their elution order from the literature.

Figures 2.2 and 2.3 show the EPA and DHA library spectra respectively.

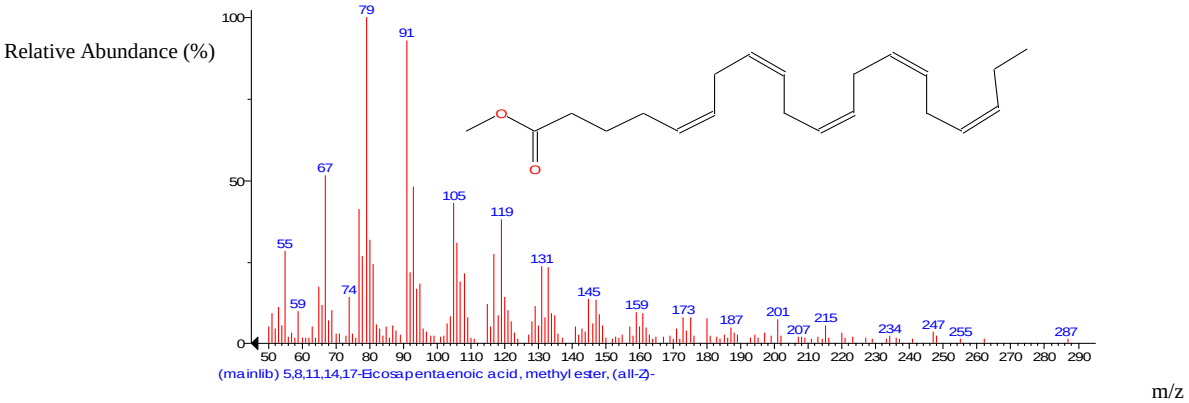

Figure 2.2: Standard EPA mass spectrum from the library.

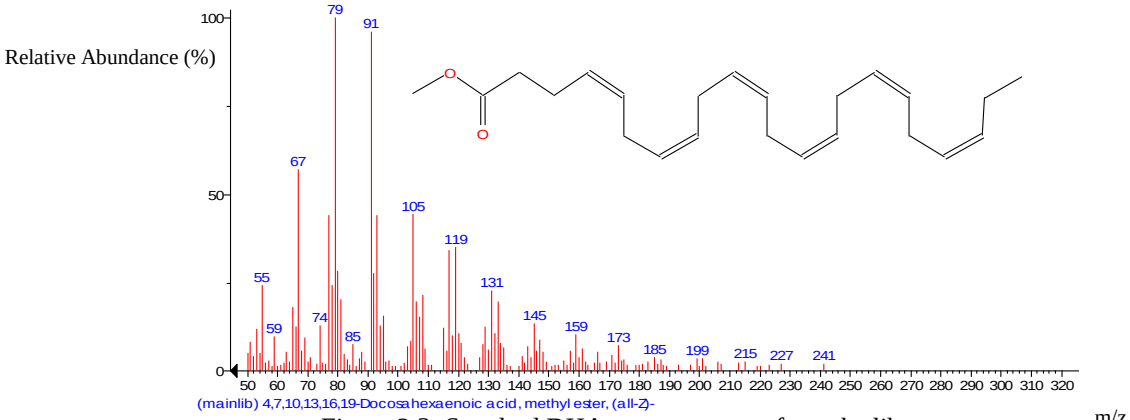

Figure 2.3: Standard DHA mass spectrum from the library.

2.16 Bioreactor Cultivation

An Ez-Control Applicon bioreactor (working volume 2 liter), was used to perform either batch or Chemostat cultures. Figure 2.4 illustrates the experimental setup identifying the major parts of the equipment.

Figure 2.4: The Ez-Control Applicon bioreactor used to optimise the cultivation conditions for maximum PUFAs production.

The bioreactor was equipped with pH, temperature and dissolved oxygen probes to control these factors. Air flow rate was kept constant (2 l/m) for all runs, while the machine varied the agitation speed to keep the dissolved oxygen concentration at the set point. Samples were taken at intervals for offline determination of biomass, OD and amount of EPA.

2.16.1 Batch cultivation

For the batch cultures, 1 litre was used as the working volume; six samples were collected from each batch. The temperature, pH and DO were controlled by the station and monitored via Bioexpert software.

Response surface methodology was applied to optimise the bioreactor cultivation conditions. The main target was to study the effect of pH, temperature and dissolved oxygen (DO) at the bioreactor scale on the EPA production.

For optimising the reactor conditions for isolate 717, using either ASW medium or the developed and optimised production media, a CCD experiment with 20 runs, with six centre points and five levels for each variable was carried out to cover a wide range of the variables and to test the interactions between them. The levels of each individual variable are shown in Table 2.16

Table 2.16: Variables and their levels investigated via CCD for maximum EPA production by isolate 717 when growing in a bioreactor.

Variables	Levels				
	-2	-1	0	+1	+2
Dissolved Oxygen	10%	20%	40%	60%	70%
Temperature	10°C	15°C	20°C	25°C	30°C
pH	5.5	6	7	8	8.5

For each CCD experiment a validation experiment was carried out based on the analysis recommendation(s). 1 l of the medium (ASW or production medium) was prepared and the cultivation conditions were the recommended conditions. Lipid and growth profiles over time were obtained by growing isolate 717 under the recommended optimum cultivation conditions.

The optimum cultivation growth conditions previously determined for isolate 717 were applied on bioreactor cultivation for the other isolates under investigation (66 and Hus-27).

In order to reduce the expected cooling cost, a temperature shock experiment was carried out for isolate 717. On the previously developed production medium, isolate was cultivated in a bioreactor at 25°C till the med-exponential phase. Subsequently the temperature was reduced till it reached 10°C. Samples were collected frequently till the maximum EPA was obtained.

To test the ability of isolate 717 to grow and produce EPA under totally anaerobic conditions, a batch culture was prepared in a bioreactor with the developed production medium as the cultivation substrate. The medium was sparged with nitrogen to eliminate any traces of oxygen within the medium. The complete absence of oxygen all over the cultivation time was confirmed via monitoring the oxygen concentration levels by a dissolved oxygen probe.

2.16.2 Chemostat cultivation

Chemostat studies were carried out at a number of discrete dilution rates. After the analysis of each dilution rate was completed, the dilution rate was changed. Three different growth rates were tested (0.0437, 0.1093 and 0.1530 hr^{-1} representing 20%, 50% and 70% of the maximum specific growth rate μ_{max} of isolate 717 respectively). To achieve the tested growth rates, three medium dilution rates were applied, 30.604,

76.51 and 107.114 ml/h by using calibrated pumps and tubes. Samples were collected over time both at steady states and during the transition phases. Three volume changes were required to attain the new steady state. The incubation temperature was 15°C, pH 7 and the DO was 30%. The chemostat set up is illustrated in Figure 2.5.

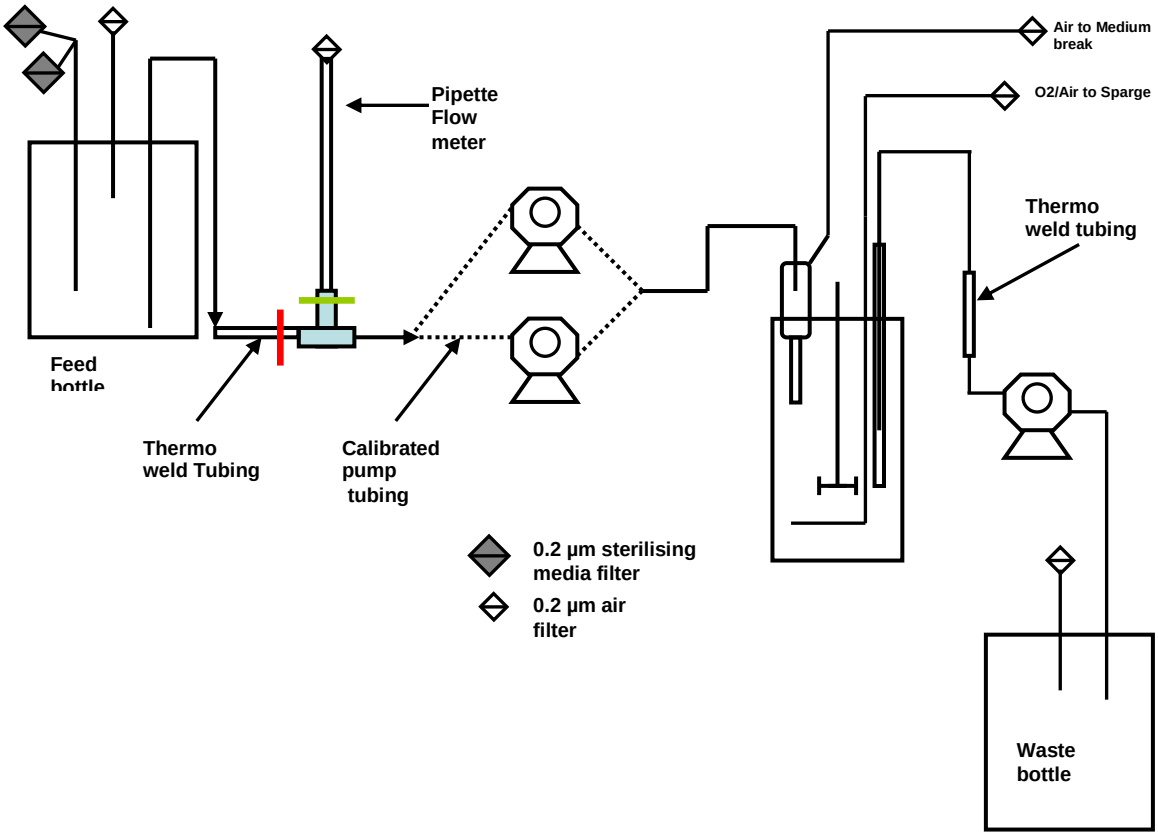

Figure 2.5: A schematic representation of the Chemostat system.

As temperature was found to be the most significant factor affecting the EPA productivity by isolate 717, a separate chemostat cultivation was set up to test the effect of temperature as a sole variable under constant growth rate. Six different temperatures were tested including (5, 10, 15, 20, 25 and 30°C). The growth rate was kept constant (50% of the calculated μ_{max}) and the isolate was exposed to the tested temperature in a random way to avoid any systematic error in the experiment. The cultivation conditions were pH 7, DO 30% and 76.51 ml/h feed rate to achieve a growth rate of 0.1093 hr^{-1}.

2.17 PID control

In order to achieve a robust and reliable control of cultivation variables, in particular the dissolved oxygen, the bioreactor controller was tuned using a combination of Best-Guess approach and Ziegler and Nichols methods. In Ziegler and Nichols method, the proportional (P) gain, K_p, was increased from zero until it achieved the ultimate gain, K_u, at which the output of the control loop oscillated with a constant amplitude. To achieve this, the values of the integral (*I*) and derivative (*D*) gains were set to zero. The oscillation period T_u, was also recorded and using the Ziegler-Nichols Tuning Chart (Table 2.17), the values of the *I* and *D* were determined (Ziegler and Nichols, 1993).

Table 2.17: Ziegler-Nichols tuning chart

	Kp	***Ki***	***Kd***
P control	$K_u / 2$		
PI control	$K_u / 2.2$	$1.2K_p / T_u$	
PID control	$0.60K_u$	$2Kp / T_u$	$K_pT_u / 8$

2.18 Monitoring the uptake of elements by isolate 717

In bioreactor batch cultivation, the uptake of elements by isolate 717 was monitored and investigated. The growth conditions were 10°C and 35% DO while pH was monitored but not controlled. The cultivation medium contained 2.5 g/l Na_2HPO_4, 1.2 g/l KCl, 23 g/l NaCl,1.8 g/l $MgSO_4$, 3.5 g/l peptone and 3.5 g/l yeast extract.

Samples were collected at intervals, centrifuged and the supernatants were freeze dried. The amount of each element was quantified in the freeze dried elements representing the concentration remaining after the uptake process. To quantify the elements, two techniques were used for such purpose.

2.18.1 Electron microscopy

The freeze dried media supernatants, after cultivation, were examined using a Scanning Electron Microscopy [XL30 ESEM-FEG (ESEM)], (Electron Microscopy Services, Newcastle University) which is equipped with an EDX® , energy-dispersive X-ray spectrometer, (RONTEC system with Quantax software) with a liquid nitrogen-cooled anti-contamination device in place at all times. The electron microscope was operated at 25 KV in low vacuum mode. This technique was used to monitor the uptake of sodium, sulphur, chlorine, magnesium, potassium and phosphate elements.
Three readings from different regions were collected showing no change in the readings indicating the homologous distribution of the elements across the samples.
The elemental profile and the presence of heavy metals in the isolate 717 biomass were tested using the same technique.

2.18.2 Elemental Analyser

This technique was used to quantify the amount of hydrogen, nitrogen and carbon in the freeze dried supernatant to test the uptake of carbon and nitrogen over isolate growth. All samples were tested on a Carlo Erba 1108 Elemental Analyser controlled with CE Eager 200 software, run in accordance with the manufacturer's instructions and weighed using a Mettler MX5 Microbalance. Two readings were taken for each sample with a maximum standard deviation less than 0.3%.
The analyser was also used to determine the C/N ratio within the isolate 717 biomass.

2.19 Online monitoring for EPA production

To monitor the amount of EPA produced by isolate 717 in the bioreactor near infra red (NIR) spectroscopy was applied. NIR spectra were obtained on a Perkin-Elmer (Norwalk, CT) Spectrum 2000 spectrometer equipped with a Galileo (Sturbridge, MA) transmission-type fibre-optic probe.

Chapter 3- Screening and Characterisation of PUFA producers

Water samples from different environmental conditions were screened for potential high PUFA producers. Isolated PUFA producers were screened by the TTC colourimetric method and then confirmed as a PUFA producer by GC/MS. The highest producer were characterised and identified on the basis of 16S rRNA.

3.1 Screening and characterisation of the marine isolates

The Mid Atlantic Ridge samples represents the cold environment, where the ability to produce PUFAs was found to be related as an adaptation to low temperature (Nichols *et al.*, 1999). Because most PUFA producers were isolated from the Antarctic regions, large scale production could require the extra cost of cooling to the natural temperature of the production strain. To avoid potential cooling costs, water samples from temperate regions (Red Sea and Mediterranean Sea) were screened. Skerratt *et al.* (2002a) isolated two PUFA producing strains namely ACEM 6 and ACEM 9T from a temperate river estuary in Tasmania, Australia, which were able to produce relatively high levels at high temperature (10% at 24°C).

The screened isolates included 68 samples from Mid Atlantic Ridge, 102 samples from the Red Sea and 81 samples from the Mediterranean Sea. The screening strategy was divided into two main steps. The first step was applying the TTC colourimetric method (as described in section 2.2) and the second step was a gas chromatography of the prepared FAMEs. The ability to reduce the 2,3,5-triphenyltetrazolium chloride (TTC) (colourless) to a red-coloured triphenyl formazan (TF) was found to be related to the ability to produce EPA (Ryan *et al.*, 2010).

The formation of red colour was considered to be a positive result (Figure 3.1.).

a b

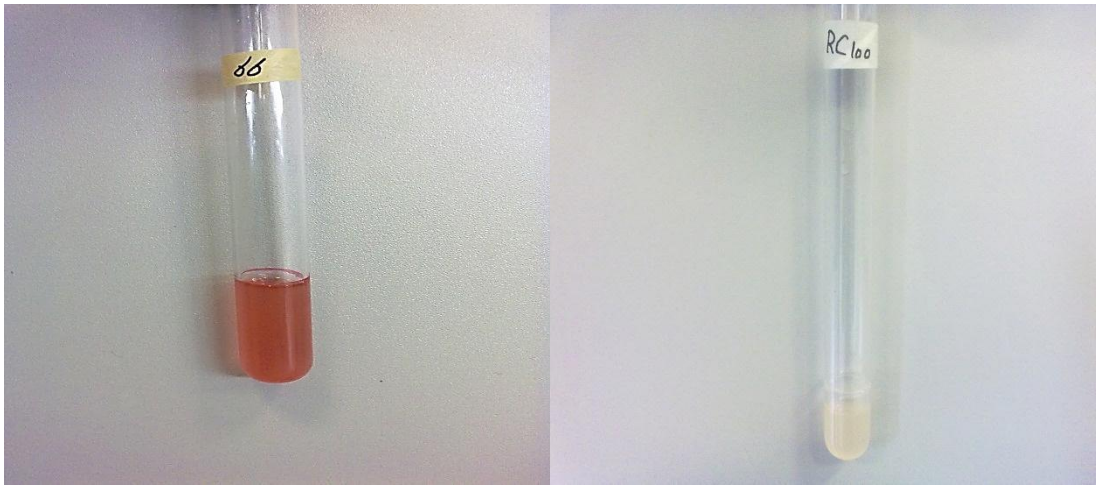

Figure 3.1: The difference between (a) positive (Red) and (b) negative (colourless) sample in the TTC analysis.

The isolates unable to produce the red colour were not investigated further. All the positive TTC isolates were taken to the second step. Table 3.1 shows the PUFA producing isolates and the characterisation of these isolates. The ability to reduce colourless TTC to red coloured TF could be due to the effect of Δ5-desaturase, an enzyme required in the metabolic biosynthesis of PUFAs, where it is required to convert eicosatetraenoic acid (ETA) to EPA (Ryan *et al.*, 2010).

Fourteen different isolates were found to be PUFA producers, either producing EPA or DHA or both. Eleven of them were isolated from the Mid Atlantic Ridge sample while the rest were isolated from the Red Sea. Mediterranean Sea isolates showed no sign of the ability to produce PUFAs. The inability of the Mediterranean Sea isolates to produce PUFAs could be due to the high level of pollution compared to the Red Sea. The United Nations Environment Programme has estimated that 650,000,000 tons of sewage are disposed yearly (El-Sikaily *et al.*, 2004), while the Red Sea is treated by the Egyptian government as an environmentally protected area for tourism. In a comparative study between both, El-Sikaily *et al.* (2004) reported that concentration of iron in the Mediterranean Sea was double that in Red Sea. Iron showed a significant negative impact on the ability of the isolates under investigation to produce PUFAs.

Table 3.1: Characterisation of the PUFA producing isolates (MA refers to Mid Atlantic Ridge samples, while R.S. refers to Red Sea samples)

Isolate	Optimum temp.	Optimum NaCl conc. (g/l)	Growth at 0% Nacl	EPA%	EPA yield (mg/g)	DHA %	DHA yield (mg/g)	Origin	TTC result
66	15°C	10	-	3.5	5.44	-	-	M.A.	+
Hus-4	15°C-20°C	30	-	-	-	0.4	0.13	R.S.	+
88	20°C	30	-	0.3	0.12	-	-	M.A.	+
AT-2	15°C	30-45	-	0.7	0.23	-	-	M.A.	+
Hus-27	20°C	10	-	2.1	0.69	-	-	R.S.	+
AT-6	20°C-25°C	10	-	0.4	0.39	-	-	M.A.	+
717	15°C	10	-	7.6	9.41	0.1	0.23	M.A.	+
NDV-48	20°C	10	-	-	-	0.5	0.71	M.A.	+
560	20°C	10	-	0.4	1.22	5.6	4.81	M.A.	+
AT-14	10°C	30	-	0.8	1.47	-	-	M.A.	+
Hus-7	20°C	30-45	-	1.3	0.42	-	-	R.S.	+
NDV-11	10°C	10	-	0.5	0.33	-	-	M.A.	+
NDV-4	15°C	30	-	1.7	1.32	-	-	M.A.	+
NDV-9	10°C	30	-	2.8	1.86	-	-	M.A.	+

The other possibility for the absence of PUFA producers in the Mediterranean Sea could that the salinity of the sea is much lower compared to the Red Sea, as the ability to produce PUFAs is reported to be a response to the exposure to environmental stress. The Red Sea salinity is 4% higher than the average level of salinity in seas and oceans, Also the Red Sea proved to be populated with a high number of extreme microorganisms due to the high environmental stress (Antunes *et al.*, 2011).

Isolates 717 and 66 were found to be the highest EPA producers. These isolates were therefore investigated within the subsequent optimisation study. Although isolate Hus-27 was not one of the higher, producers, it was also investigated as a representative of a sample isolated from a different environment. Some isolates were able to produce both EPA and DHA to various concentrations (isolates 717 and 560).

The TTC method is a fast and easy method of screening PUFA producers as it reduced the screening time. All the negative TTC results can be excluded from the GC experiments which

require the cultivation and FAME extraction of the isolate under the investigation. During this research, sixty five samples gave positive TTC results. The total fatty acids from all these isolates were extracted and examined by GC (Section 2.5) showing that only fourteen isolates were able to produce PUFAs. This indicates a relatively high false positive rate of this method 79%. In addition, ten samples that resulted in negative TTC result were selected at random and exposed to the GC analysis and none of the tested isolate showed any sign of the ability to produce PUFAs (giving 0% false negative rate). The main disadvantage of using this screening method is the high false positive rate with not all the positive TTC isolates being PUFA producers. Another problem in applying the TTC method is the sensitivity of the result to the incubation time. If the incubation time exceed one hour, all the tubes resulted in a positive response, therefore the incubation time should not exceed 30 minutes.

Regarding the ability of isolate 560 to produce DHA, although the retention time determined by GC suggested that it produced DHA, but the mass spectrum of the product confirmed that isolate 560 was unable to produce DHA but can produce EPA (95% similarity to the standard EPA).

In addition to the potential DHA peak, another three distinct peaks always appeared in relation to that peak, which increased and decreased altogether.

The mass spectrum of these peaks showed low similarity (less than 30%) to compounds such as phthalate and hexanedioic acid though they may be some related derivatives. These unknown compounds, which interfere with the present work, could be new secondary metabolites produced by the isolate, supported by the fact that their mass spectrum did not show any closely related compounds, but further investigations are required.

Control experiments were performed, in addition to the fact that these peaks were not detected with any other isolate, these controls did not yield detectable peaks suggesting that it is of bacterial origin.

Similar bioactive secondary metabolites were previously detected in different microorganisms. *Curvularia senegalensis* was recently found to be a phthalate producer for the first time by Lucas *et al.* (2008). In addition, *Nocardia levis* MK-VL_113 was found to produce two phthalate derivatives with antibacterial activity against *Bacillus cereus* (Kavitha *et al.*, 2009).

A *Streptomyces* strain isolated from the same cold environment, as 560, was found to be able to produce a number of metabolites including phthalic acid diethyl ester, 1, 3-bis (3-phenoxyphenoxy) benzene, hexanedioic acid dioctyl ester and the new substance 2-amino- 9, 13 -dimethyl heptadecanoic acid (Ivanova *et al.*, 2001). These compounds represent various

classes of chemical structures and provide indications for the unexploited biosynthetic potential of marine bacteria from Antarctica.

3.2 Native gel electrophoresis

"Native" or "non-denaturing" gel electrophoresis is run in the absence of SDS, while in SDS-PAGE (Sodium Dodecyl Sulphate Polyacrylamide Gel Electrophoresis) the electrophoretic mobility of proteins depends primarily on their molecular mass, in the native PAGE the mobility depends on both the protein's charge and its hydrodynamic size. The main aim of this experiment (section 2.3) was to detect which protein is responsible for converting TTC to TF producing the characteristic red colour. The intention was to cut out the relevant band from the gel after detecting the corresponding protein and identify the protein using Maldi-TOF mass spectrum via peptide finger printing. After loading the protein sample and running the gel, the gel was incubated with the TTC solution expecting that the protein band responsible of its conversion to TF would turn red providing a visual detection for the corresponding protein.

Unfortunately the whole TTC solution turned red while the gel, including all protein bands, remained colourless leading to the inability to identify such protein. The reason could be due to the high solubility of the coloured product in water, TTC was prepared in water, lead to rapid diffusion of the red colour from the native gel. As a future suggestion, another solvent may be used to prepare the TTC solution to avoid the solubility problem.

3.3 Identification of the PUFA producers

The polymerase chain reaction (PCR) is an *in vitro* method for enzymatic synthesis of specific DNA sequences using two oligonucleotides that hybridise to opposite strands and flank the region of interest in the target DNA. This allows rapid determination of the presence or the absence of a target DNA sequence in any genetic material.

The use of 16S rRNA gene sequences to study bacterial phylogeny and taxonomy has been identified as the most common housekeeping genetic marker used for a number of reasons including: 1) it is present in all bacteria; 2) the function of the 16S rRNA gene over time has not altered; and 3) the 16S rRNA gene (1,500 bp) is large enough for bioinformatics purposes (Janda and Abbott, 2007).

The genomic DNA of each isolate was extracted as described in section 2.6 and used as a template for a PCR reaction to amplify the 16S rRNA. The PCR products were loaded on

agarose gels as described in section 2.6 and the expected band was investigated and detected (Figure 3.2).

To separate the desired band from the non specific PCR products, annealing temperature gradients were applied to increase the specificity between the primer and the target gene. A single band was obtained for each isolate, cut out from the gel, purified and prepared for sequence, as described in section 2.8.

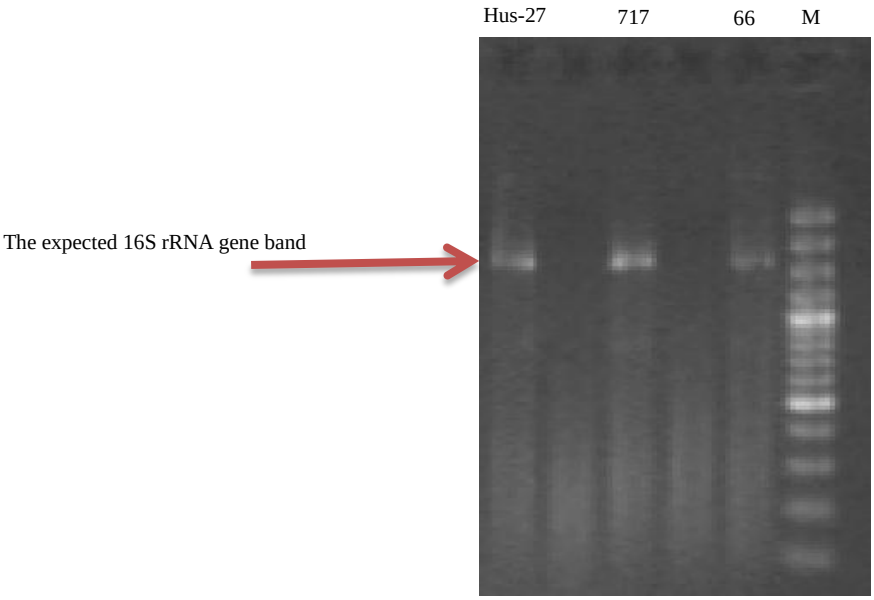

Figure 3.2: Agarose gel (1% w/v) stained with ethidium bromide showing the main 16S rRNA band purified from the non specific PCR products.

The strains were identified on the basis of 16S rRNA sequence data. The identity of the strains was confirmed via Blast sequence against the Ribosomal database project and GenBank database (http://blast.ncbi.nlm.nih.gov/). The sequence obtained was compared with the closest type strains via multiple alignments using ClustalX software followed by the construction of a phylogenetic tree visualised by Tree-view software (Figure 3.3, Figure 3.4, and Figure 3.5).

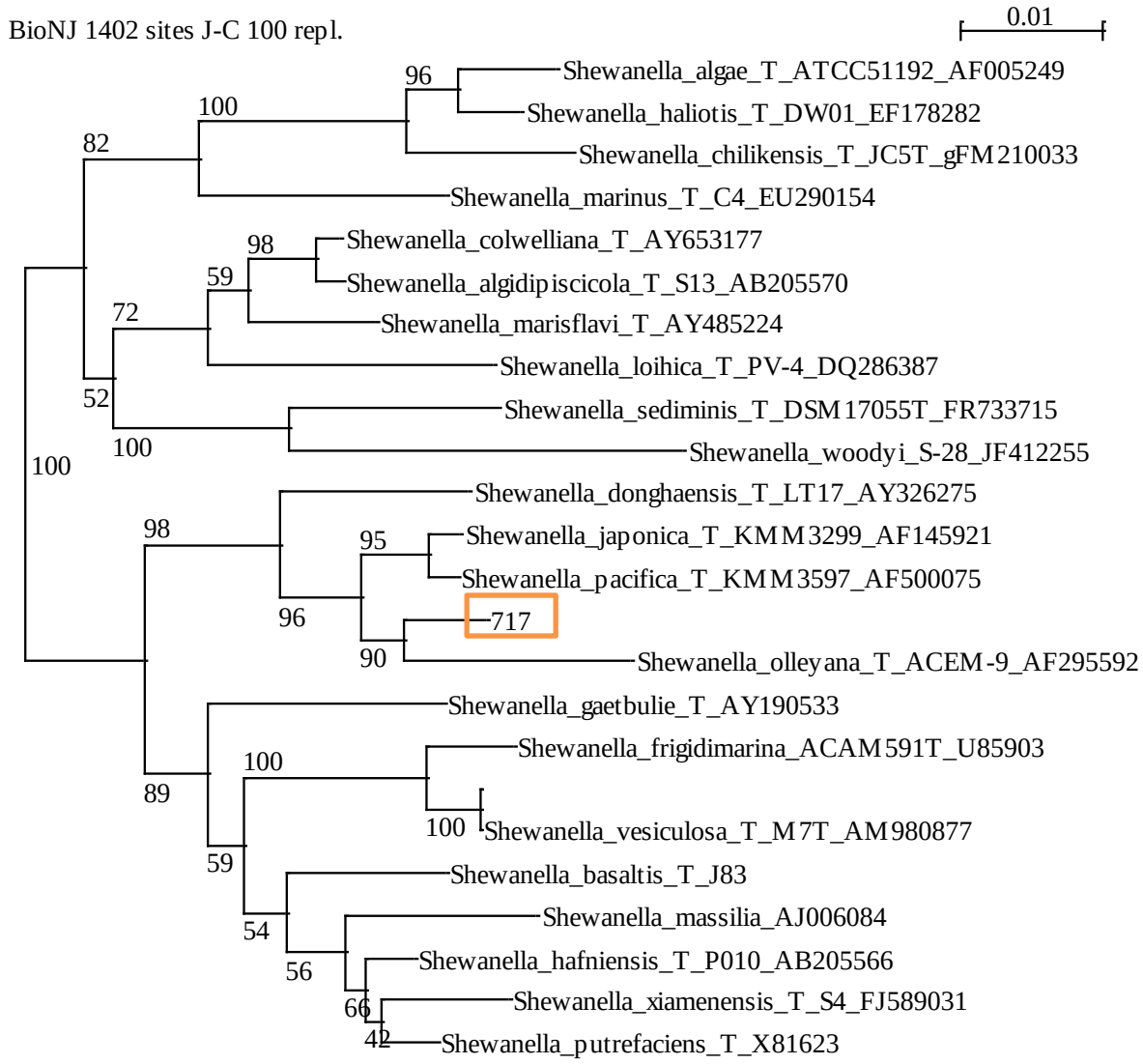

Figure 3.3: Phylogenetic tree based on 16S rRNA gene sequences showing the relationship between isolate 717 and the closest type strains in the same genus. The tree was constructed based on the neighbour-joining method.

The phylogenetic analysis indicated that isolate 717 belongs to *Shewanella sp.* The isolate did not show high similarity to any of the tested type strain suggesting that isolate 717 could be a new strain. However, further work is required to confirm this hypothesis. By aligning the 717 sequence against the *Shewanella olleyana* sequence, 28 basses difference was detected (out of 1478 bases), while the difference between 717 and *Shewanella pacifica* was found to be 17 bases out of the full sequence of 1478 bases. This result indicates that isolate 717 is much closer to *Shewanella pacifica* than to *Shewanella olleyana.* The difference between *Shewanella pacifica* and *Shewanella japonica* is only 11 bases which supports the hypothesis that isolate the 717 could be a new *Shewanella* species.

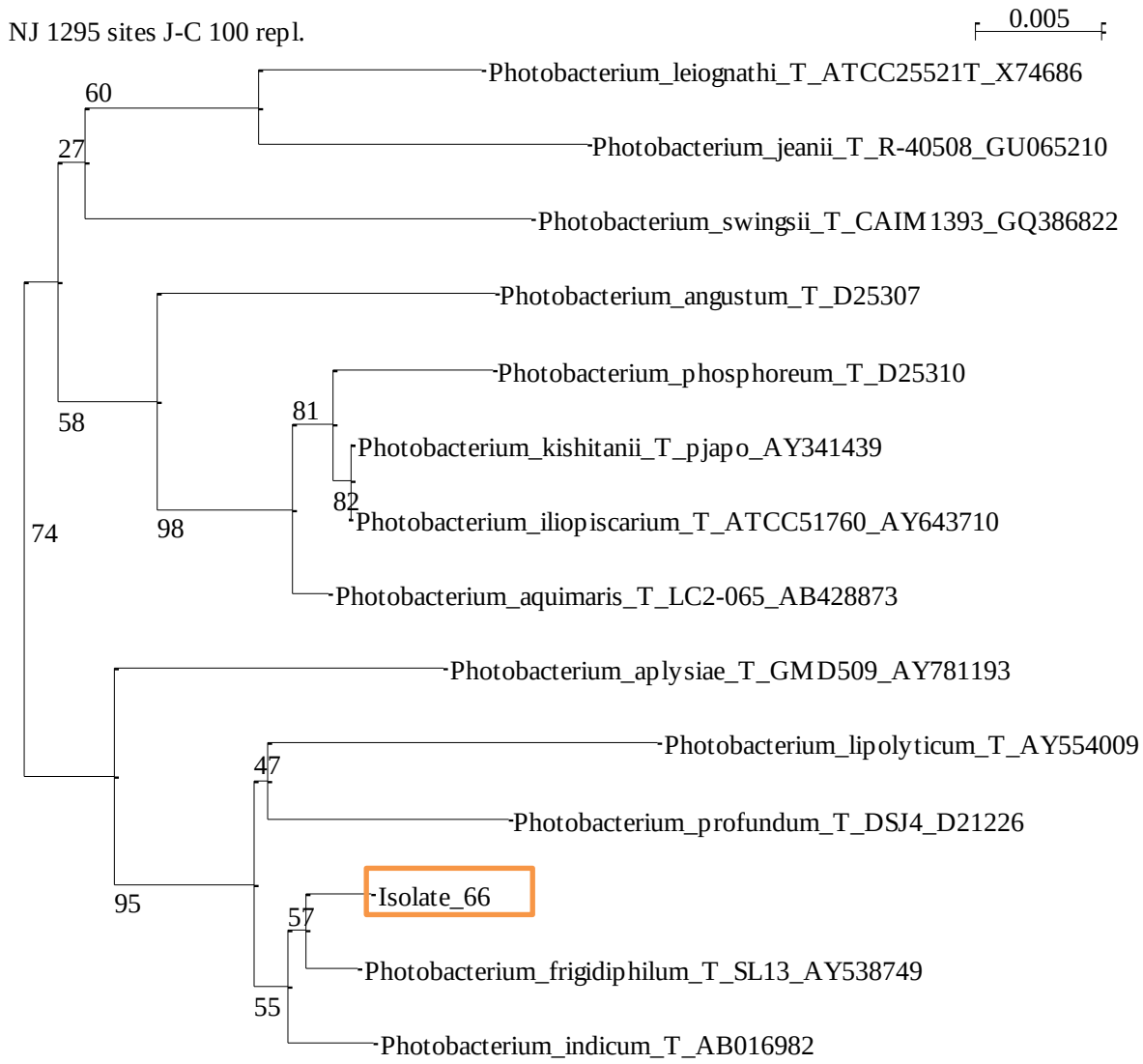

Figure 3.4: Phylogenetic tree based on 16S rRNA gene sequences showing the relationship between isolate 66 and the closest type strains of the same species. The tree was constructed based on the neighbour-joining method.

Isolate 66 was identified as *Photobacterium* sp. with a high similarity to both *Photobacterium frigidiphilum* and *Photobacterium profundum*. Sequence alignment showed that the difference between the isolate 66 sequence and the *Photobacterium frigidiphilum* sequence was 8 bases out of 1437 bases, while the difference between the isolate 66 sequence and the *Photobacterium profundum* sequence was 18 bases out of the same total number of bases. This result indicates that isolate 66 is much closer to *Photobacterium frigidiphilum*.

8 base differences between *Photobacterium kishitani* and *Photobacterium iliopascarium* was enough to separate them into two different species which suggests that isolate 66 could be a new *Photobacterium* species, although further work would be required to confirm this hypothesis.

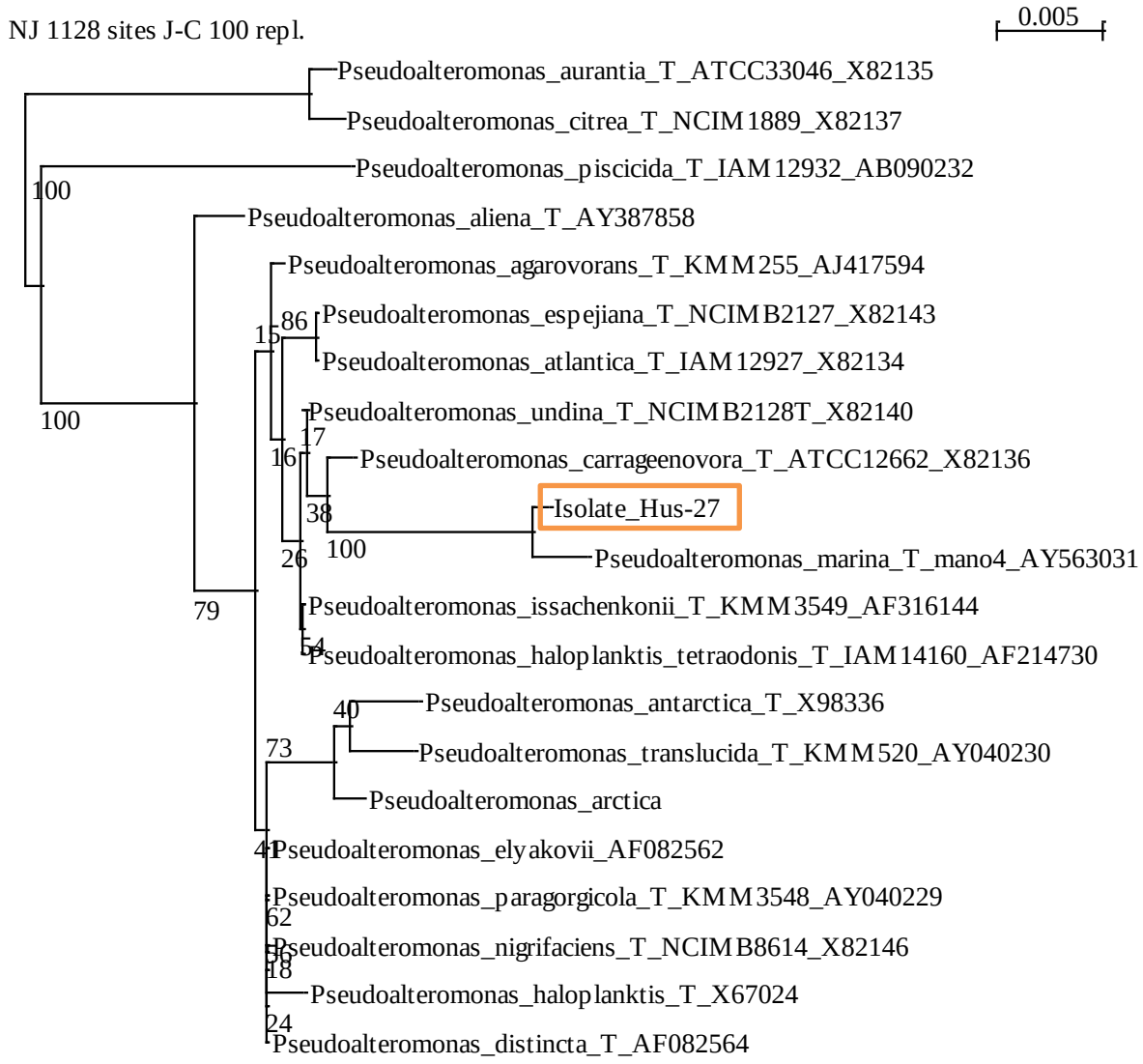

Figure 3.5: Phylogenetic tree based on 16S rRNA gene sequences showing the relationship between isolate Hus-27 and the closest type strains in the same genus. The tree was constructed based on the neighbour-joining method.

The analysis of the 16S rRNA sequence for the Egyptian isolate Hus-27 confirmed that the isolate is a *Pseudoaltermona*s with a high similarity to *Pseudoaltermona*s *marina.* The DNA pairwise alignment showed only 4 base differences between them out of 1355 bases length.

3.4 Amplification of PUFA-PKS gene specific fragment

The amplification of one of the PUFA genes confirms the presence of such gene cluster. The *pfaB* gene is one of five genes responsible for PUFA production via the *PKS* system (Okuyama *et al.*, 2007b). The *pfaB* gene is known to be the key gene determining whether the final product is EPA or DHA (Orikasa *et al.*, 2009).

The optimum annealing temperature was found to be 56°C (Figure 3.6). PCR products were cut out from the gel, purified and prepared for sequence, as described in section 2.8.

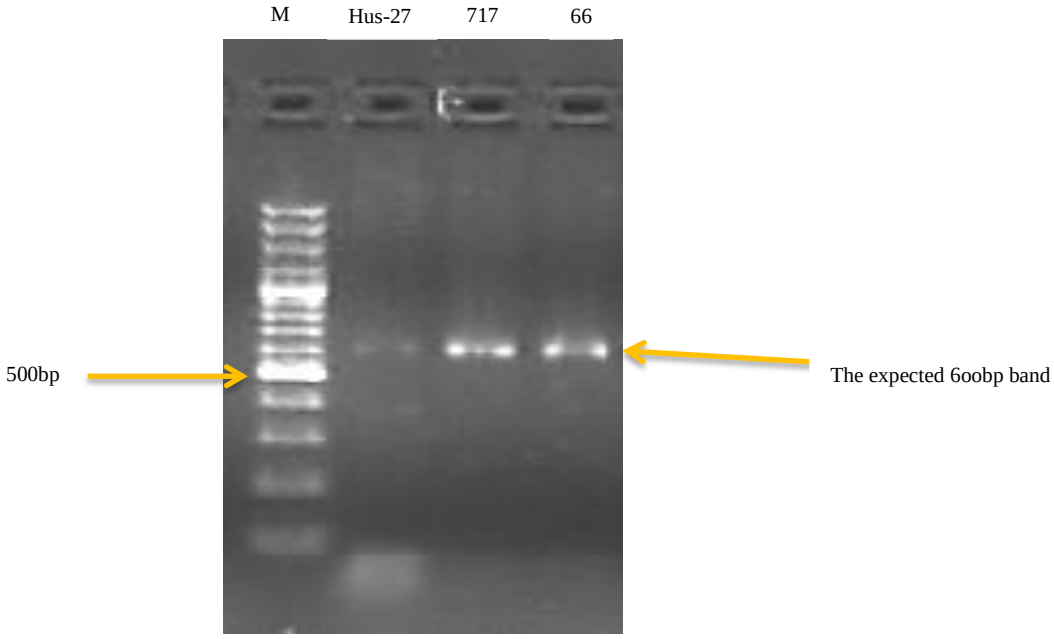

Figure 3.6: Agarose gel (1%w/v) stained with ethidium bromide showing the *pfaB* band at 56°C as annealing temperature, showing the absence of the non specific PCR products. Lane M: 100bp molecular marker, lanes 2-4 containing the PCR product from the isolates (Hus-27, 717 and 66 respectively).

The gene sequence was blasted against the Genbank database confirming that they were the gene of interest. The presence of *pfaB* is confirmatory of the presence of the *pfa* gene cluster which is responsible for PUFA production via the PKS biosynthetic pathway.

3.5 Lipid profile of the PUFA producers

Isolates 66, 717 and Hus-27 were chosen for further work and optimisation. The lipid profiles of the isolates under investigation, represented as percentage of the total fatty acid, are summarised in Table 3.2.

Lipid profiles were obtained from isolates grown in 250 ml shake flasks with 50 ml ASW as the growth medium and 15°C as the incubation temperature in an orbital shaker at 160 rpm (see section 2.11).

Table 3.2: Lipid profiles of isolates under investigation.

Fatty Acids	*Isolate 717*	*Isolate 66*	*Isolate Hus-27*
C 13:0	*2.5*	*2.4*	-
C 14:0	*3.7*	*5.6*	*2.1*
C 15:0	*1.1*	-	*6.8*
C 15:1	-	-	*0.2*
C 16:0	27.9	*23.2*	26.3
C 16:1	*37.1*	*39.4*	*44.7*
C 17:0	*5.8*	-	*0.4*
C 17:1ω8	*8.4*	*4.8*	*5.9*
C 18:0	*3.1*	*3*	*2.6*
C 18:1 ω 9c	*1.9*	*11.8*	*7.1*
C 18:1 ω 9t	-	*6.1*	*1.3*
C 20:1 ω 9	-	-	-
C 20:5 ω 3	*7.6*	*3.5*	*2.1*
C 22:2	-	-	-
C 22:6ω3	*0.3*	-	-

Among the tested isolates five different fatty acids were found to be common in addition to EPA (C 20:5 ω 3). These are C 14:0, C 16:0, C 16:1, C 17:1ω8, C 17:1ω8 and C 18:1 ω 9c.

3.6 Effect of temperature on EPA production

Isolates 66, 717 and Hus-27 were incubated at different temperatures (10, 15, 20, 25 and 30°C) in an orbital shaker as described in section 2.11.

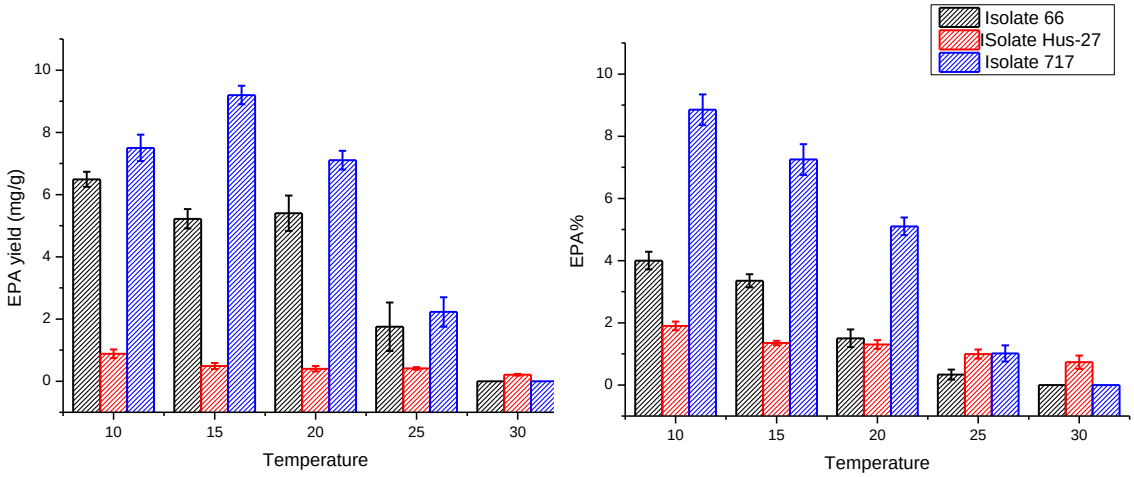

Figure 3.7: Effect of temperature on different EPA responses produced by isolates 717, 66and Hus-27. Black columns represent isolate 66 responses; red columns represent isolate Hus-27 responses while blue columns represent isolate 717 responses.

All isolates showed the same responses toward the temperature variations, where the amount of EPA increased with decreasing the temperature. This inverse relationship between the temperature and the EPA response is especially pronounced in the EPA percentage of the total fatty acids. The EPA totally disappeared when cells were incubated at 30°C except for the Egyptian Red Sea sample.

3.7 Chapter Summary

Bacterial isolates from three different environments were screened for the ability to produce PUFAs especially EPA and DHA. The screening strategy included a rapid screening by the TTC colourimetric method followed by GC and GC/MS for confirmation. Although the TTC screening showed a high percentage of false positives, it was a useful screening tool reducing the number of GC experiments as it could be used to confirm the inability to produce PUFAs. The PCR experiment for the selected isolates confirmed the presence of the *pfaB* gene, which is required for PUFA production by the PKS route.

Three isolates (717, 66 and Hus-27) were chosen for further investigation and optimisation this decision was based on the observation that isolate 717 was found to be the highest EPA producer with the ability to produce traces of DHA, isolate 66 was found to be a potential high EPA producer, producing EPA as the sole PUFA type. Isolate Hus-27 was chosen as a representative of the bacterial isolates isolated from a relatively warm environment.

Isolates were identified by means of 16S rRNA sequence and analysis revealing that two isolates (66 and 717) could be new strains of *Shewanella* and *Photobacterium* respectively. Further investigations, beyond the scope of this project, would be required to confirm this hypothesis.

The effect of temperature confirmed the theory that, PUFAs are produced when bacterial cells are exposed to extremely low temperatures in order to sustain the membrane fluidity under such conditions.

In the upcoming chapter, different media components were screened and optimised for isolate 66 via statistical design of experiment to achieve the maximum EPA productivity. In addition, a chemically defined media was created

Chapter 4-Screening and optimising the media components for isolate 66

In this chapter a sequential methodology combining a screening level by Plackett-Burman fractional factorial design and an optimisation level by Central Composite Design was employed to screen different media components to enhance EPA production by isolate 66.
A chemically defined medium was created to explore the effect of C/N ratio on the growth and productivity of isolate 66.

4.1 Productivity of isolate 66

The results of isolate screening identified isolate 66 as a potential high EPA producer. It was able to produce approximately 5.5 mg/g dry weight and 14 mg/l media of EPA representing 3.5 % of the total fatty acid content under non-optimised conditions (when cultivated on ASW medium as described in section 2.11). Isolate 66 was able to produce EPA as the sole type of PUFA which can be an advantage at the manufacturing scale, as the separation of EPA from other PUFAs, especially in fish oil, introduces a further processing complexity (Yongmanitchai and Ward, 1991).
The mass spectrum of the extracted EPA displayed in Figure 4.1.

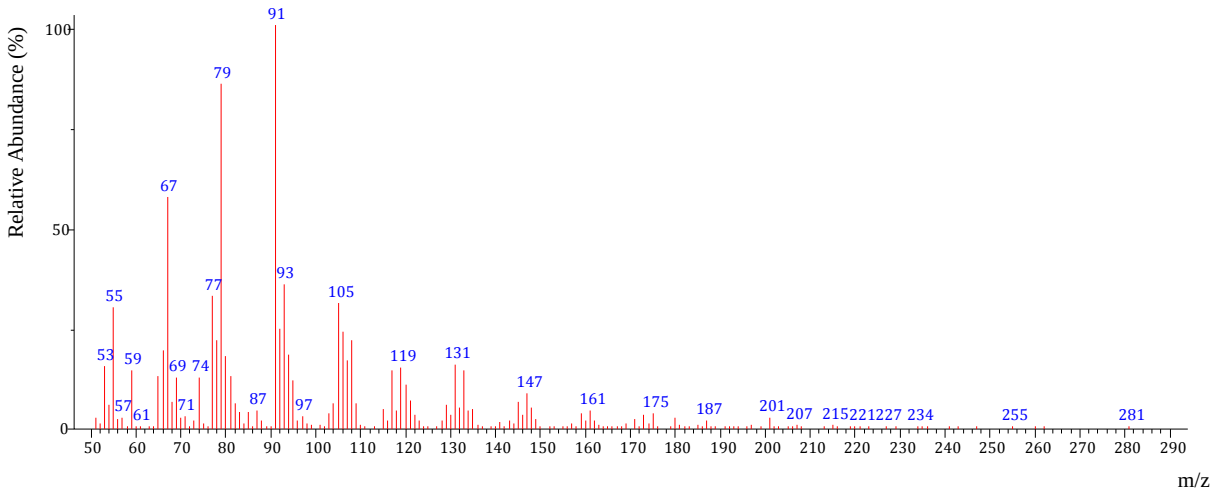

Figure 4.1: Mass spectrum for the produced EPA.

The mass spectrum of the EPA produced by isolate 66 showed more than 95% similarity to the standard EPA mass spectrum (Section 2.15).

4.2 Screening the media components for isolate 66

The media component optimisation screening strategy consisted of dividing all forty three different potential media components investigated into four separate PB designs (as described in section 2.13).

4.2.1 First Plackett-Burman Design

Media components including carbon and nitrogen sources commonly play a major role in a bio-reaction because they are directly linked with biomass and metabolite production.
The experimental runs were performed in a randomised order as indicated in Table 4.1. The maximum and minimum levels used for each variable are indicated as (+) and (-) respectively. Each column in the variables section stands for an independent variable, while each column in the responses section stands for a calculated response and each row represents an experimental trial. Table 4.1 also shows the outcomes of each of the experimental trials in terms of EPA yield, concentration and percentage.

Table 4.1: The matrix and the responses for the 1st Plackett-Burman design for isolate 66

Run	Variables																Responses		
	X_1	X_2	X_3	X_4	X_5	X_6	X_7	X_8	X_9	X_{10}	X_{11}	X_{12}	X_{13}	X_{14}	X_{15}	X_{16}	EPA yield (mg/g)	EPA conc. (mg/l)	EPA %
1	-	+	-	+	-	-	-	-	+	-	+	-	+	+	+	+	1.07	1.42	1.23
2	+	+	-	+	+	+	-	-	+	+	-	+	+	-	-	-	1.22	1.01	0.89
3	+	-	+	+	-	-	-	+	-	+	-	+	+	+	+	-	0.00	0.00	0.00
4	-	-	+	-	-	+	+	+	+	-	-	+	+	-	+	+	1.94	10.35	1.39
5	+	-	-	-	-	+	-	+	+	+	+	-	-	+	+	-	1.17	0.89	1.02
6	+	+	+	+	-	-	+	+	-	+	+	-	-	-	-	+	0.00	0.00	0.00
7	+	-	-	-	+	-	+	-	+	+	+	+	-	-	+	+	0.45	0.59	0.45
8	+	+	+	-	-	-	+	-	+	-	+	+	+	+	-	-	3.91	15.99	2.21
9	+	-	+	+	+	+	-	-	-	-	+	-	+	-	+	+	0.32	0.16	0.41
10	-	-	+	-	+	+	+	-	-	+	+	-	+	+	-	-	0.21	0.07	0.62
11	-	-	+	+	+	-	-	+	+	-	+	+	-	-	-	-	2.31	6.78	1.08
12	-	-	-	+	+	-	+	+	+	+	-	-	+	+	-	+	1.26	1.18	0.82
13	+	+	-	-	+	-	+	+	-	-	-	-	+	-	+	-	0.00	0.00	0.00
14	+	+	+	-	+	+	-	+	+	-	-	-	-	+	-	+	3.18	23.81	1.57
15	-	+	-	-	-	+	-	+	-	+	+	+	+	-	-	+	0.00	0.00	0.00
16	-	+	+	-	+	-	-	-	-	+	-	+	-	+	+	+	0.00	0.00	0.00
17	+	-	-	+	-	+	+	-	-	-	-	+	-	+	-	+	1.36	5.29	1.86
18	-	+	-	+	+	+	+	+	-	-	+	+	-	+	+	-	0.00	0.00	0.00
19	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	0.87	1.51	1.07
20	-	+	+	+	-	+	+	-	+	+	-	-	-	-	+	-	0.32	0.42	0.36

where, X_1 represents yeast extract , X_2 represents glucose , X_3 represents glycerol, X_4 represents soy bean flour, X_5 represents corn step liquor, X_6 glutamic acid, X_7 represents L-proline , X_8 represents ammonium acetate, X_9 represents whey, X_{10} is propanoic acid, X_{11} represents L-serine, X_{12} represents urea , X_{13} represents sucrose, X_{14} represents glycine, X_{15} represents citric acid and X_{16} represents fructose.

The experimental responses were subjected to the analysis of variance and parameter estimates and results are summarised in Table 4.2. The P-value designates a statistical confidence of a factor estimate. A P-value of < 0.1 was used as a cut-off point indicating the statistical significance of a factor.

Table.4.2: Estimated effects, coefficients and P-values of the tested variables for the 1st PB experiment for isolate 66.

Variables	EPA yield (mg/g)				EPA conc. (mg/l)				EPA %			
	Effect	**Coefficient**	**T-Value**	**P-Value**	**Effect**	**Coefficient**	**T-Value**	**P-Value**	**Effect**	**Coefficient**	**T-Value**	**P-Value**
Yeast Extract *(X_1)*	0.32	0.16	1.91	0.152	2.59	1.30	2.16	0.120	0.18	0.09	2.17	0.119
Glucose *(X_2)*	-0.06	-0.03	-0.36	0.742	1.58	0.79	1.31	0.281	-0.25	-0.12	-2.97	**0.059**
Glycerol *(X_3)*	0.43	0.21	2.57	**0.083**	4.56	2.28	3.79	**0.032**	0.02	0.01	0.32	0.767
Soy bean flour *(X_4)*	-0.42	-0.21	-2.51	**0.087**	-3.69	-1.84	-3.07	**0.055**	-0.16	-0.08	-2.00	0.139
Corn Step Liquer *(X_5)*	-0.21	-0.10	-1.24	0.302	-0.23	-0.11	-0.19	0.861	-0.32	-0.16	-3.90	**0.030**
Glutamic Acid *(X_6)*	-0.05	-0.02	-0.32	0.772	1.45	0.72	1.21	0.314	0.12	0.06	1.52	0.225
L-proline(X_7)	-0.11	-0.05	-0.65	0.560	-0.16	-0.08	-0.14	0.898	0.04	0.02	0.51	0.648
Ammonium Acetate*(X_8)*	-0.02	-0.01	-0.16	0.886	1.65	0.82	1.37	0.263	-0.32	-0.16	-3.84	**0.031**
Whey Protein *(X_9)*	1.37	0.685	8.09	**0.004**	5.54	2.77	4.60	**0.019**	0.71	0.35	8.38	**0.004**
Propanoic Acid *(X_{10})*	-1.07	-0.53	-6.34	**0.008**	-6.11	-3.05	-5.08	**0.015**	-0.66	-0.33	-7.89	**0.004**
L-Serine *(X_{11})*	-0.11	-0.057	-0.67	0.549	-1.76	-0.88	-1.47	0.239	-0.09	-0.04	-1.12	0.344
Urea *(X_{12})*	0.23	0.118	1.40	0.257	1.05	0.52	0.87	0.446	0.07	0.03	0.89	0.441
Sucrose *(X_{13})*	-0.01	-0.03	-0.09	0.932	-0.92	-0.45	-0.76	0.504	0.01	0.01	0.18	0.865
Glycine *(X_{14})*	0.43	0.21	2.55	**0.084**	2.78	1.39	2.31	0.104	0.36	0.18	4.35	**0.022**
Citric Acid *(X_{15})*	-0.94	-0.47	-5.56	**0.011**	-4.18	-2.09	-3.47	**0.040**	-0.52	-0.26	-6.22	**0.008**
Fructose *(X_{16})*	-0.07	-0.03	-0.47	0.672	1.61	0.80	1.34	0.273	0.05	0.02	0.59	0.595

In DOE it is common practice to pool non significant variables, as they can be treated as noise (Montgomery, 2012). The non significant factors (P-value > 0.1) were subtracted stepwise in descending order until all non significant variables were removed. Some non significant variables were not removed as the removal of such variables did not lead to an improvement in the statistical model. The resulting reduced set of independent variables is shown in Table 4.3.

Table.4.3: Modified estimated effects, coefficients and P-values of the tested variables for the 1st PB experiment for isolate 66.

Variables	EPA yield (mg/g)				EPA conc. (mg/l)				EPA %			
	Effect	**Coefficient**	**T-Value**	**P-Value**	**Effect**	**Coefficient**	**T-Value**	**P-Value**	**Effect**	**Coefficient**	**T-Value**	**P-Value**
Yeast Extract *(X_1)*	0.32	0.16	2.89	**0.020**	2.59	1.30	2.52	**0.036**	0.18	0.09	2.96	**0.021**
Glucose *(X_2)*	-	-	-	-	1.58	0.79	1.53	0.164	-0.25	-0.12	-4.06	**0.005**
Glycerol *(X_3)*	0.43	0.21	3.89	**0.005**	4.56	2.28	4.43	**0.002**	-	-	-	-
Soy bean flour *(X_4)*	-0.42	-0.21	-3.80	**0.005**	-3.69	-1.84	-3.59	**0.007**	-0.16	-0.08	-2.74	**0.029**
Corn Step Liquer *(X_5)*	-0.21	-0.11	-1.89	**0.096**	-	-	-	-	-0.32	-0.16	-5.33	**0.001**
Glutamic Acid *(X_6)*	-	-	-	-	-	-	-	-	0.12	0.06	2.08	**0.076**
L-proline(X_7)	-0.11	-0.05	-0.99	0.351	-	-	-	-	-	-	-	-
Ammonium Acetate *(X_8)*	-	-	-	-	1.65	0.82	1.61	0.147	-0.32	-0.16	-5.24	**0.001**
Whey *(X_9)*	1.37	0.68	12.27	**0.000**	5.54	2.77	5.38	**0.001**	0.71	0.35	11.45	**0.000**
Propanoic Acid *(X_{10})*	-1.07	-0.53	-9.61	**0.000**	-6.11	-3.05	-5.94	**0.000**	-0.66	-0.33	-10.78	**0.000**
L-Serine *(X_{11})*	-0.11	-0.05	-1.02	0.338	-1.76	-0.88	-1.71	0.125	-0.09	-0.04	-1.53	0.169
Urea *(X_{12})*	0.23	0.11	2.12	**0.067**	-	-	-	-	0.07	0.03	1.21	0.265
Sucrose *(X_{13})*	-	-	-	-	-	-	-	-	-	-	-	-
Glycine *(X_{14})*	0.43	0.21	3.87	**0.005**	2.78	1.39	2.70	**0.027**	0.36	0.18	5.95	**0.001**
Citric Acid *(X_{15})*	-0.94	-0.47	-8.43	**0.000**	-4.18	-2.09	-4.06	**0.004**	-0.52	-0.26	50	**0.000**
Fructose *(X_{16})*	-	-	-	-	1.61	0.81	1.57	0.156	-	-	-	-

A normal probability plot for the standardised effect was plotted (Figure 4.2) indicating whether a factor is significant or not by representing factors with positive effect to the right of the line, representing statistical significance, and those with negative effect to the left of the line. The further the factor lies from the line, the more significant and the greater the effect of this factor for the given responses.

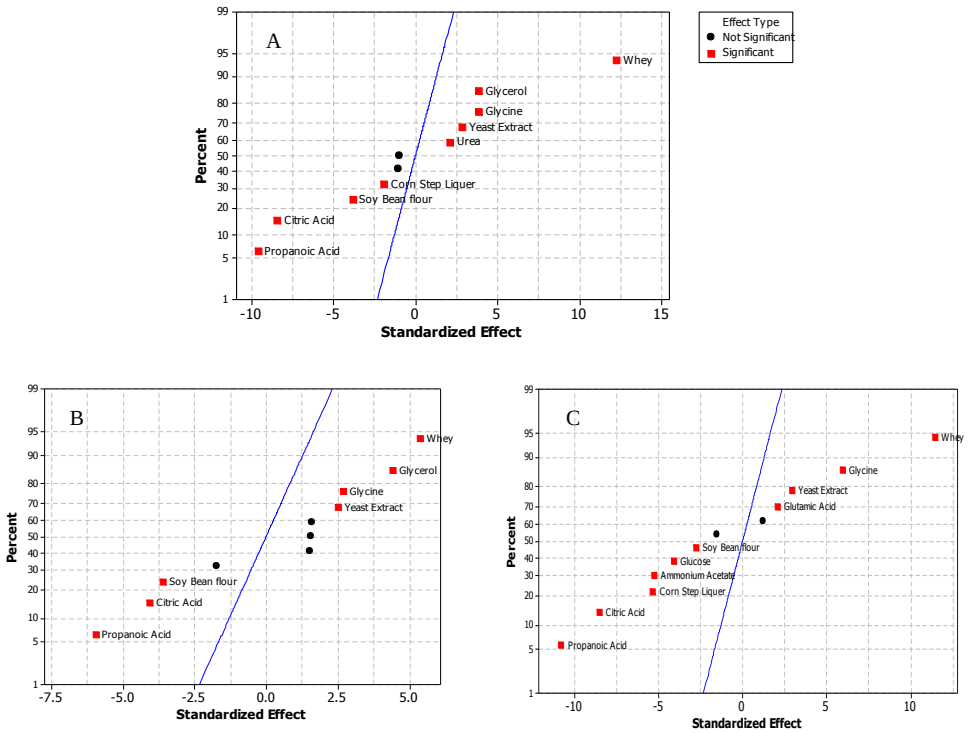

Figure 4.2: Normal probability plot of the standardised effect to show the significance of each factor on each EPA response of isolate 66 (A) EPA yield (mg/g), (B) EPA concentration (mg/l) and (C) EPA %.

A relatively large number of factors were found to have a significant positive effect on EPA production including yeast extract, urea, glycine, glycerol, glutamic acid and whey.

Although glucose showed a non-significant effect on EPA in terms of concentration and yield, it demonstrated a statistically significant negative impact on EPA in term of percentage (P-value, 0.005). In addition to glucose, citric acid, propionic acid and ammonium acetate were also found to have a negative impact on the ability of isolate 66 to produce EPA.

These results contradict some of the reported observations in the literature for different strains. Glucose was previously reported as the optimum carbon source for maximum PUFA production by *Schizochytrium limacinum* (Zhu *et al.*, 2008).

Propionic acid was found to have the most significant negative effect on all EPA as calculated responses produced by isolate 66 which contradict with previously reported effect of the same variable on the ability of *Shewanella gelidimarina* ACAM 456T to produce

EPA, as the addition of propionic acid as sole carbon source led to the maximum EPA of any change (15 mg/g) (Nichols *et al.*, 1997).

Corn steep liquor was found to have a significant negative impact on EPA production, as yield and percentage of total fatty acids, by isolate 66, which conflict with the result observed by Yazawa (1996), when he reported that *Shewanella putrefaciens* achieved 200 mg/l of EPA when grown on corn steep liquor as a carbon source.

Urea showed a significant positive effect on EPA yield (mg/g) produced by isolate 66 while no significant effect was detected on the ability to produce EPA as percentage of total fatty acid or as concentration. The positive effect of urea on EPA was in accordance with the observations obtained by Yongmanitchai and Ward (1991) when they tested different concentrations of urea, as a nitrogen source for maximum EPA production by *Phaeodactylum tricornutum*, and they achieved a significant increase in EPA yield ranging from 66 to 103 mg/l.

Whey was found to be the most statistically significant factor affecting the productivity of isolate 66 in the positive direction.

Based on the results six different media components were chosen for the next level of PB screening design, namely: yeast extract; glycerol; whey; urea; glutamic acid and glycine. Yeast extract, glycine and whey showed a significant positive effect on all calculated responses, glycerol was significant on EPA yield and concentration; urea was significant on EPA yield while glutamic acid showed a significant positive impact on the ability to synthesis EPA only.

4.2.2 Second Plackett-Burman Design

The matrix and achieved responses are summarised in Table 4.4.

Table.4.4: The matrix and the responses for the 2nd Plackett-Burman design for isolate 66

Run	Variables										Responses	
	X_1	X_2	X_3	X_4	X_5	X_6	X_7	X_8	X_9	X_{10}	EPA yield (mg/g)	EPA conc. (mg/l)
1	+	+	+	-	+	+	-	+	-	-	0.148	0.544
2	-	-	+	-	+	-	+	+	+	-	0.000	0.000
3	-	+	-	-	+	+	+	-	+	+	0.748	0.660
4	-	-	-	-	-	-	-	-	-	-	0.617	1.105
5	+	-	+	+	-	+	+	-	+	-	0.000	0.000
6	+	+	+	-	-	-	+	-	-	+	0.000	0.000
7	-	+	+	+	-	-	-	+	+	+	1.091	3.782
8	+	-	-	-	-	+	-	+	+	+	0.973	2.651
9	-	-	+	+	+	+	-	-	-	+	0.421	0.832
10	+	+	-	+	+	-	-	-	+	-	0.573	0.903
11	-	+	-	+	-	+	+	+	-	-	0.646	2.811
12	+	-	-	+	+	-	+	+	-	+	0.424	1.198

where, X_1 represents palmitic acid , X_2 represents sugar cane molases, X_3 represents linseed oil, X_4 represents ammonium nitrate ,X_5 tryptone, X_6 represents pea nut oil, X_7 represents ethylene glycol , X_8 represents Hy-soy , X_9 represents fish peptone and X_{10} represents sodium acetate.

The complete statistical analysis of the design is given in Appendix A.1.

Hy-soy, sodium acetate and fish peptone were found to have a statistically significant positive effect, while linseed oil, palmitic acid, tryptone and ethylene glycol showed a negative impact on EPA production by isolate 66.

Palmitic acid was incorporated into the growth media of isolate 66 as a possible precursor for the biosynthesis of highly unsaturated fatty acids, as palmitic acid was reported to be a potential precursor in the biosynthesis of PUFAs (Ratledge, 2004). The negative effect of adding palmitic acid on the ability of isolate 66 to produce EPA confirms the observation that media containing free fatty acids inhibit the biosynthesis of other fatty acids in microorganisms (Lees and Korn, 1966). However some exceptions were previously reported in the literature where the addition of linoleic acid to cultures of *Euglena gracilis* amplified the EPA production (Bajpai and Bajpai, 1993), and the addition of oils containing individual fatty acid precursors (such as sunflower, rapeseed, corn, soybean and linseed oils) to cereals resulted in a rapid enhancement in PUFA yield (Certik and Adamechova, 2009).

Linseed oil was added as a source of linoleic acid, a potential precursor, its effect was adverse in this case and linseed oil was found to inhibit the biosynthesis of EPA by isolate 66. The positive effect of linseed was reported with the fungus isolate *Mortierella alpine* 1S-4, where the maximum amount of EPA (0.8 mg/ml media) was achieved in a combination of 3% (w/v)

linseed oil and 1% (w/v) glucose (Jareonkitmongkol *et al.*, 1993). These two media components showed a significant inhibitory effect on the ability of isolate 66 to synthesise EPA.

Tryptone showed a statistically significant negative effect on EPA production (P-value, 0.004) by isolate 66 which is in line with the effect of tryptone on the fungus *Mortierella* sp. (Shimiziu *et al.*, 1988). The authors reported that, although tryptone yielded a high biomass for *Mortierella* sp, the amount of EPA produced was dramatically decreased. The inhibitory effect of tryptone could be related to its high content of the aromatic amino acid tryptophan. Tryptophan was reported to activate repressor proteins when present in bacterial culture media, resulting in the prevention of transcription of DNA coding for the enzymes required for the biosynthesis of tryptophan (Gollnick *et al.*, 2005). These repressor proteins may interfere with the biosynthesis of EPA (further research is required to confirm this hypothesis).

Nitrate (with P-values for EPA yield and concentration of 0.435 and 0.353, respectively) did not show any statistically significant effect on the EPA production by isolate 66, although it was found to enhance the ability of isolate *Phaeodactylum tricornutum* to produce EPA , resulting in a four fold increase from 20 to 90 mg/l (Yongmanitchai and Ward, 1991).

Three variables were chosen for the next screening step: Sodium acetate (P- value, 0.039) and Fish Peptone (P-value, 0.098) as they showed a significant positive effect on EPA yield while Hy-soy (P-value, 0.017) showed a significant effect on EPA concentration produced by isolate 66.

4.2.3 Third Plackett-Burman Design

The matrix and responses are summarised in Table 4.5.

Table 4.5: The matrix and the responses for the 3rd Plackett-Burman design for isolate 66

Run	Variables									Responses		
	X_1	X_2	X_3	X_4	X_5	X_6	X_7	X_8	X_9	EPA yield (mg/g)	EPA conc. (mg/l)	EPA %
1	+	+	+	-	+	+	-	+	-	1.72	4.54	2.24
2	+	+	-	+	+	-	+	-	-	0.91	3.11	0.15
3	-	-	+	+	+	-	+	+	-	1.43	4.11	0.37
4	-	+	-	-	-	+	+	+	-	0.15	0.42	0.49
5	-	-	-	+	+	+	-	+	+	0.94	3.89	0.22
6	+	-	-	-	+	+	+	-	+	9.36	26.15	5.77
7	-	-	-	-	-	-	-	-	-	0.46	1.55	1.77
8	-	+	+	-	+	-	-	-	+	0.47	1.04	3.22
9	+	-	+	+	-	+	-	-	-	1.42	5.57	4.41
10	-	+	+	+	-	+	+	-	+	2.21	7.93	0.31
11	+	-	+	-	-	-	+	+	+	11.48	28.71	6.31
12	+	+	-	+	-	-	-	+	+	0.48	1.48	0.11

where, X_1 represents meat peptone, X_2 represents Ammonia, X_3 represents lactose, X_4 represents ground sesame , X_5 represents ammonium sulphate, X_6 represents casein, X_7 represents maltose , X_8 represents vegetable peptone and X_9 represents mannitol.

The P-values, coefficients and effect of each variable are summarised in Appendix A.2.

Meat peptone, lactose, maltose and mannitol were found to have a statistically significant positive effect on EPA production, while the ground sesame seed and ammonia were found to have a significant negative effect on EPA production, for all calculated responses.

The significant negative effect of ground sesame seed could be due to the presence of sesamin analogues that inhibit the Δ5–desaturase which is required in PUFA biosynthesis (Certik and Adamechova, 2009). The other explanation could be due to the presence of tryptophan in high quantities as it has a negative impact on the ability to produce EPA as reported in sections 4.2.2.

For isolate 66, maltose was found to be a potential carbon source for maximising the EPA productivity as it showed a statistical significant positive impact on the EPA yield and concentration. This result contradicts a previous report in the literature where maltose was previously proven to inhibit the PUFA biosynthesis in isolate *Mortierella alpine*, where among five different carbon sources; maltose yielded the lowest amount of PUFAs by isolate under investigation (Jang *et al.*, 2005).

Four different media components (lactose, maltose, meat peptone and mannitol) were selected for the next level of PB design screening experiment, as they were found to significantly enhance at least two of the calculated responses. Maltose was found to enhance

the EPA yield (P-value of 0.00) and EPA concentration (P-value of 0.002), in addition meat peptone showed the same effect on the same variables (P-values of 0.00 and 0.002 for EPA yield and concentration respectively). The carbon source mannitol was found to enhance the ability of isolate 66 to produce EPA on all calculated responses (P-values of 0.00, 0.002 and 0.049 on EPA yield, concentration and percentage respectively). Lactose was found to stimulate EPA production by isolate 66 in terms of yield (P-value of 0.032) and percentage (P-value of 0.018).

4.2.4 Fourth Plackett-Burman Design

The main aim of this set of experiments was to test the effect of the metal ions included in ASW media in the complete absence of confounding effects of various carbon and nitrogen sources. As the effect of the nitrogen and carbon could mask the effect of such ions, the basal media used in this design was 1g/l of yeast extract.

The maximum value for each tested variable was the value added to the ASW media (section 2.11) while the minimum value was the complete absence of the ion, where the ion that showed no significant effect on the EPA production could be removed from the media making the media simpler. The matrix and achieved responses are summarised in Table 4.6.

Table.4.6: The matrix and the responses for the 4th Plackett-Burman design for isolate 66

Run	Variables								Responses		
	X_1	X_2	X_3	X_4	X_5	X_6	X_7	X_8	EPA yield (mg/g)	EPA conc. (mg/l)	EPA %
1	+	-	+	+	-	+	+	+	2.10	1.39	2.67
2	+	+	-	-	-	-	+	+	1.70	1.53	4.90
3	+	+	-	+	+	-	+	-	1.80	1.82	3.20
4	-	+	+	-	+	+	+	-	1.80	1.47	2.50
5	-	-	+	-	+	-	+	+	2.40	1.12	4.70
6	-	-	-	+	-	+	+	-	1.40	0.19	4.40
7	-	+	-	+	+	+	-	+	1.90	1.78	3.80
8	-	-	-	-	-	-	-	-	0.52	0.32	1.10
9	+	-	+	+	+	-	-	-	0.08	0.01	1.00
10	+	-	-	-	+	+	-	+	0.00	0.00	0.00
11	+	+	+	-	-	+	-	-	2.10	1.64	3.40
12	-	+	+	+	-	-	-	+	1.30	1.24	3.50

where, X_1 represents NH_4Cl, X_2 represents Na_2HPO_4, X_3 represents KCl , X_4 represents $CaCl_2$, X_5 represents $Fe_2(SO_4)_3$, X_6 represents $MgCl_2$, X_7 represents $MgSO_4$, X_8 represents NaCl.

Full statistical analysis showed in Appendix A.3.

None of the tested metal ions showed any statistically significant negative effect on the ability of isolate 66 to produce EPA either as concentration or yield, although two variables ($Fe_2(SO_4)_3$, with a P-value of 0.091, and NH_4Cl, with a P-value of 0.077) showed a statistically negative effect on EPA produced as a percentage of the total fatty acids.

Although none of the variables showed a statistically significant positive effect on the ability of isolate 66 to synthesise only EPA rather than total fatty acids, Na_2HPO_4 was shown to have a statistically significant positive effect on the ability of isolate 66 to produce EPA in terms of yield (P-value of 0.090) and concentration (P-value of 0.002), which could be due to the effect of phosphate. Increasing the amount of KH_2PO_4, as a source of inorganic phosphate in the culture media of *Thraustochytrium* sp., from 0.1 g/l to 0.2 g/l led to a 40% increase in the DHA yield (Shene *et al.*, 2010).

$MgSO_4$ showed a significant positive effect on the EPA concentration (P-value of 0.037) produced by isolate 66, which contradicts the observations on the ability of *Phaeodactylum tricornutum* to produce EPA, where it showed a positive effect on the amount of biomass produced without any significant effect on the amount of EPA (Yongmanitchai and Ward, 1991).

Iron ions were found to significantly inhibit the biosynthesis of EPA in isolate 66 (P-value, 0.091). This result was not completely consistent with the result obtained for the fungus *Marchantia polymorpha* where the addition of iron in excess, 30% higher than that used in the regular media, were found to enhance the EPA production by the investigated organism (Chiou *et al.*, 2001).

Ammonium chloride was reported to have a negative effect on the ability of the microalga *Schizochytrium limacinum* to produce DHA (Chi *et al.*, 2007). Even though isolate 66 is producing EPA the effect of ammonium chloride was found to be similar to that on DHA.

In addition to Na_2HPO_4 and $MgSO_4$, NaCl was taken further to the next level of screening as isolate was unable to grow in the complete absence of it (section 3.1) due to the marine nature.

4.2.5 Second level of Plackett-Burman Design

In the first level of Plackett-Burman design of experiment, the effects of a wide range of media components on the ability to produce EPA were tested to choose the optimum production media composition. 17 different factors were found to have a significant positive

effect on EPA production and they were taken to a further comparison to determine the final recipe for the production media.

Some factors could be affected by the presence or absence of others, where the presence of one variable could inhibit or enhance the effect of other variables. The Plackett-Burman as a screening design is neglecting the interaction among the variables which could be significant. For these reasons, another round of Plackett-Burman was required for more robust indication of the effect of each variable. Among different variables, a Plackett-Burman design was applied to identify the most significant ones (full statistical analysis displayed in Appendix A.4) which result in high production of EPA. The matrix and achieved responses are summarised in Table 4.7.

Table.4.7: The matrix and the responses for the 2nd level of Plackett-Burman design for isolate 66

Run	Variables																	Responses		
	X_1	X_2	X_3	X_4	X_5	X_6	X_7	X_8	X_9	X_{10}	X_{11}	X_{12}	X_{13}	X_{14}	X_{15}	X_{16}	X_{17}	EPA yield (mg/g)	EPA conc. (mg/l)	EPA %
1	+	-	+	-	+	+	+	+	-	-	+	-	+	+	-	-	-	6.23	19.88	5.01
2	+	+	-	+	+	-	-	-	-	+	-	-	+	+	+	+	-	0.47	1.46	0.58
3	-	+	-	+	-	+	+	+	+	-	-	+	-	+	+	-	-	12.16	35.47	7.21
4	-	+	+	-	+	+	-	-	-	-	+	+	-	+	+	+	+	4.61	13.33	2.35
5	+	+	-	-	-	-	+	-	+	-	+	+	+	-	-	+	+	9.75	19.32	4.99
6	+	+	+	+	-	-	+	+	-	+	+	-	-	-	+	-	+	18.45	50.41	7.17
7	-	+	-	+	+	+	+	-	-	+	+	+	+	-	-	-	-	7.59	15.52	8.367
8	-	-	+	+	-	+	+	-	-	-	-	-	+	-	+	+	+	2.64	6.13	1.87
9	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	0.95	0.99	1.85
10	-	-	+	-	+	-	+	+	+	+	-	+	+	-	+	+	-	1.58	3.97	2.29
11	-	+	+	+	+	-	-	+	+	-	+	-	-	-	-	+	-	11.83	38.07	5.54
12	+	-	-	+	+	-	+	+	-	-	-	+	-	+	-	+	+	8.04	18.77	4.62
13	+	-	+	+	-	-	-	-	+	-	+	+	+	+	+	-	-	14.08	36.51	6.12
14	-	+	+	-	-	-	-	+	-	+	-	+	+	+	-	-	+	15.16	47.75	4.89
15	+	-	+	+	+	+	-	-	+	+	-	+	-	-	-	-	+	8.91	19.46	3.63
16	+	-	-	-	-	+	-	+	-	+	+	+	-	-	+	+	-	3.71	9.87	1.33
17	-	-	-	+	-	+	-	+	+	+	+	-	+	+	-	+	+	4.39	12.53	2.17
18	+	+	-	-	+	+	-	+	+	-	-	-	+	-	+	-	+	7.17	23.14	1.83
19	-	-	-	-	+	-	+	-	+	+	+	-	-	+	+	-	+	1.46	3.16	1.66
20	+	+	+	-	-	+	+	-	+	+	-	-	-	+	-	+	-	8.17	24.67	2.42

where, X_1 represents yeast extract, X_2 represents glycerol, X_3 represents whey , X_4 represents meat peptone, X_5 represents maltose , X_6 represents mannitol , X_7 represents urea, X_8 represents Na_2HPO_4 , X_9 represents glutamic acid, X_{10} represents glycine, X_{11} represents fish peptone , X_{12} represents Hy-soy, X_{13} represents sodium acetate, X_{14} represents casein, X_{15} represents $MgSO_4$, X_{16} represents NaCl and X_{17} represents lactose .

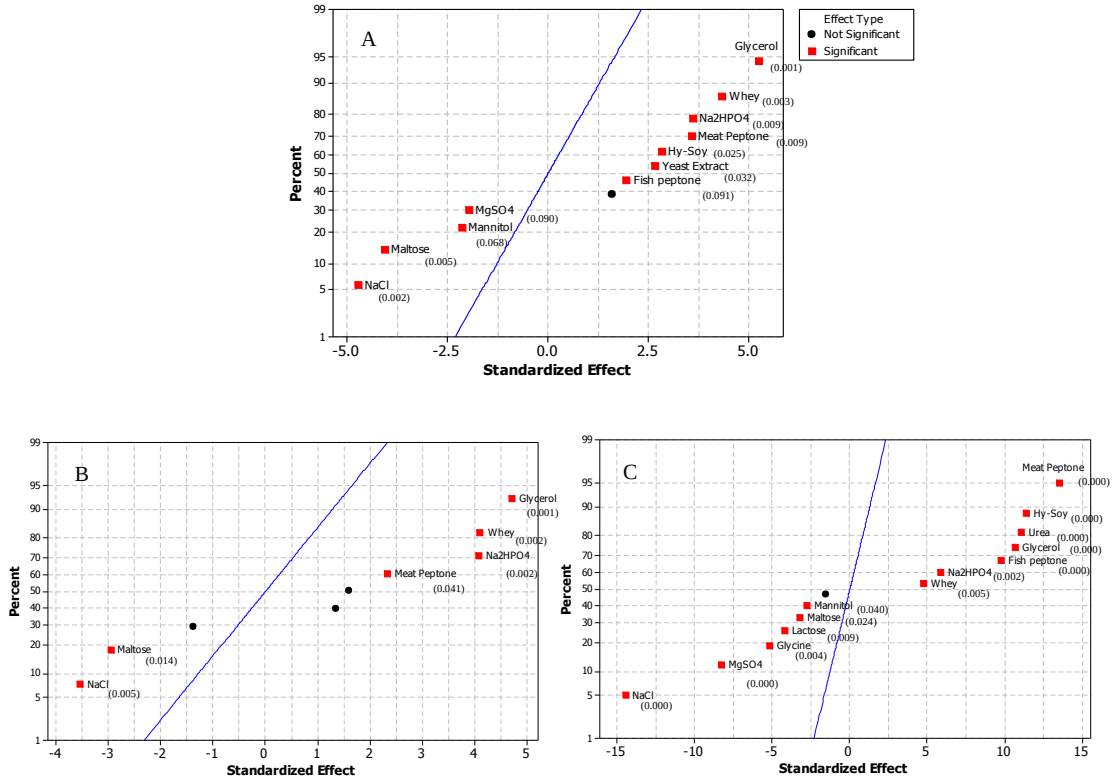

Figure 4.3: Normal probability plot of the standardised effect to show the significant of each factor were tested in the 2nd level of screening for isolate 66 on each response (A) EPA yield (mg/g), (B) EPA concentration (mg/l) and (C) EPA %. Variable with red squares are considered to be significant.

The relatively large number of significant factors with a positive impact on the EPA production (Figure 4.3) made the choice more complicated. Some variables, e.g. Hy-soy, urea, yeast extract and fish peptone showed a statistically significant positive effect on EPA yield (mg/g) and percentage of total fatty acid, but they showed a non significant effect on EPA concentration (mg/l).

Sodium chloride was found to have a statistically significant influence on the EPA production (P-values of 0.002, 0.005 and 0.000 on EPA yield, concentration and percentage respectively) by isolate 66 with the minimum concentration of NaCl resulting in higher production. This result leads to the addition of sodium chloride to the cultivation media with the minimum value (10 g/l). The addition of the low concentration of sodium chloride to the production media could be advantageous to avoid the potential corrosion in the large scale production. Negative effect of high concentration of sodium chloride was previously reported on the DHA producer *Crythecodinium cohnii* ATCC 30556, where a significant decrease in the amount of produced DHA when doubling the concentration of sodium chloride from 15 to 30 g/l in the cultivation media (Jiang and Chen, 1999).

As with glucose, the tested sugars including maltose, lactose and mannitol showed either no significant effect or a statistically significant negative effect, compared to the other tested factors, on the ability to produce EPA. This result contradicts the results obtained by Dai *et al.* (2001) as they reported maltose as the best carbon source for increasing PUFA production by *Cephalosporium* sp. and glucose as the best carbon source to obtain the highest percentage of PUFAs as a fraction of total fatty acids for the same organism.

Four variables were found consistently with a significant positive effect on all terms of EPA production by isolate 66. These variables were glycerol, meat peptone, whey and Na_2HPO_4. These four variables were therefore investigated further in a central composite design to determine the optimum combination and to test the interactions between them.

4.3 Optimising the production media for isolate 66

As described in section 2.13.2 and 4.2.5, after determining the most significant variables via Plackett-Burman design a central composite design (CCD) was performed to estimate the optimum level of each variable.

Four media components were chosen for further study and to create the potential optimum production media to isolate 66 for the highest possible EPA production. These components were meat peptone, whey, glycerol and Na_2HPO_4. A CCD experiment with 30 trials was performed to optimise the media and evaluate the effect of each factor in addition to the interaction between them.

Table 4.8: Central Composite Design of variables (in coded units) with EPA productions as responses for isolate 66

Run	Variables				Responses		
	Glycerol	Whey	Meat Peptone	Na_2HPO_4	EPA yield (mg/g)	EPA conc. (mg/l)	EPA %
1	+2	0	0	0	8.83	3.31	1.79
2	0	0	0	-2	15.48	47.63	5.89
3	0	0	+2	0	2.20	5.98	2.66
4	0	0	0	+2	6.03	8.27	3.72
5	0	0	0	0	13.59	40.77	5.98
6	-2	0	0	0	12.42	36.96	6.76
7	0	-2	0	0	1.37	0.99	0.83
8	0	+2	0	0	7.71	13.29	2.27
9	0	0	0	0	15.11	45.95	5.21
10	0	0	-2	0	18.51	62.02	10.55
11	-1	+1	-1	-1	4.99	17.34	9.87
12	0	0	0	0	13.24	46.41	5.50
13	0	0	0	0	15.32	45.75	5.14
14	+1	-1	-1	+1	11.11	23.61	6.44
15	-1	-1	+1	+1	7.76	27.77	8.94
16	+1	+1	+1	+1	10.75	27.42	5.92
17	0	0	0	0	14.89	43.94	4.98
18	-1	-1	-1	-1	4.33	10.51	7.73
19	-1	-1	+1	-1	11.99	35.97	4.56
20	+1	+1	-1	+1	15.11	22.66	4.26
21	+1	-1	+1	+1	11.55	19.93	6.97
22	-1	-1	-1	+1	7.03	15.47	6.84
23	+1	-1	-1	-1	7.63	5.91	7.06
24	+1	+1	+1	-1	17.14	33.24	5.34
25	+1	+1	-1	-1	8.65	15.35	7.44
26	-1	+1	-1	+1	17.57	83.05	4.87
27	-1	+1	+1	-1	15.48	78.19	5.98
28	+1	-1	+1	-1	5.52	10.49	3.27
29	0	0	0	0	15.02	45.43	5.69
30	-1	+1	+1	+1	11.87	34.12	6.41

To determine the main effect and the significance of each factor, the estimated effects, coefficients and the P-values for the main effect, interactions among the variables and the quadratic effect of each variable, are shown in Table 4.9. The variable with P-value < 0.1 were considered as significant at 90% level of confidence.

Table 4.9: ANOVA table for the CCD experiment to optimise the production medium of isolate 66

Variables	EPA yield (mg/g)			EPA conc. (mg/l)			EPA %		
	Sum of Squares	F Value	p-value Prob > F	Sum of Squares	F Value	p-value Prob > F	Sum of Squares	F Value	p-value Prob > F
A-Glycerol	0.18	7.084E-003	0.9340	1804.86	4.79	**0.0449**	13.56	3.87	**0.0679**
B-Whey	119.02	4.70	**0.0467**	1762.47	4.67	**0.0472**	0.044	0.012	0.9125
C-Peptone	1.46	0.058	0.8133	8.02	0.021	0.8860	21.21	6.06	**0.0265**
D-Na_2HPO_4	0.094	3.727E-003	0.9521	0.13	0.004	0.9854	1.43	0.41	0.5325
AB	1.000E-004	3.948E-006	0.9984	443.63	1.18	0.2952	0.003	0.004	0.9832
AC	3.72	0.15	0.7068	42.67	0.11	0.7412	0.003	0.003	0.9707
AD	0.046	1.825E-003	0.9665	6.54	0.017	0.8970	0.15	0.043	0.8377
BC	1.69	0.067	0.7997	1.05	0.003	0.9587	0.15	0.042	0.8398
BD	0.24	9.286E-003	0.9245	0.037	0.005	0.9922	11.80	3.37	**0.0863**
CD	82.90	3.27	**0.0905**	1301.95	3.45	**0.0829**	22.04	6.29	**0.0241**
A^2	1.11	0.044	0.8370	130.95	0.35	0.5644	0.94	0.27	0.6127
B^2	82.95	3.27	**0.0904**	1335.50	3.54	**0.0794**	6.73	1.92	0.1860
C^2	5.41	0.21	0.6505	1.51	0.003	0.9504	16.28	4.65	**0.0477**
D^2	14.34	0.57	0.4635	320.81	0.85	0.3709	0.55	0.16	0.6977

Table 4.9 displays the values for all of the variables included in the design, but to reduce the noise within the design, the non significant factors (unless they would affect the hierarchy) (P-value > 0.1) were subtracted sequentially in descending order till no observed difference in the analysis.

Table 4.10: Modified ANOVA table for the CCD experiment to optimise the production medium of isolate 66

Variables	EPA yield (mg/g)			EPA conc. (mg/l)			EPA %		
	Sum of Squares	F Value	p-value Prob > F	Sum of Squares	F Value	p-value Prob > F	Sum of Squares	F Value	p-value Prob > F
A-Glycerol	-	-	-	1950.36	6.77	**0.0160**	12.71	4.63	**0.0427**
B-Whey	123.24	6.94	**0.0148**	1828.67	6.35	**0.0192**	0.022	0.003	0.9290
C-Peptone	2.17	0.12	0.7298	7.22	0.025	0.8756	20.86	7.60	**0.0115**
D-Na_2HPO_4	0.97	0.055	0.8175	45.05	0.16	0.6962	0.89	0.32	0.5749
AB	-	-	-	-	-	-	-	-	-
AC	-	-	-	-	-	-	-	-	-
AD	-	-	-	-	-	-	-	-	-
BC	-	-	-	-	-	-	-	-	-
BD	-	-	-	-	-	-	11.80	4.30	**0.0501**
CD	82.90	4.67	**0.0414**	1301.95	4.52	**0.0445**	22.04	8.03	**0.0097**
A^2	-	-	-	-	-	-	-	-	-
B^2	100.22	5.64	**0.0262**	1612.14	5.59	**0.0268**	-	-	-
C^2	-	-	-	-	-	-	15.21	5.54	**0.0279**
D^2	-	-	-	-	-	-	-	-	-

The calculated P-values for each response revealed that in addition to linear effects of some of the variables two ways interaction and quadratic effects had a significant effect on the ability of isolate 66 to produce EPA.

Although Na_2HPO_4 was found to be insignificant either as a linear or a quadratic effect, its interaction with peptone was found to be significant on all EPA responses. In addition the interaction between Na_2HPO_4 and whey was found to be significant on the EPA percentage of the total fatty acids, while the rest of the two way interactions were found to be statistically insignificant.

The main and quadratic effects of whey were found to be significant on EPA concentration and EPA yield, while the significant effect on the EPA percentage came via its interaction with Na_2HPO_4. Even though glycerol did not show any significant effect on EPA yield, it showed a significant main effect on EPA concentration and percentage as calculated responses. Peptone was determined as the most significant factor on EPA percentage either as a main effect, a two way interaction or even as a quadratic effect.

For optimisation, second order polynomial order models were created, using coded units, to predict the optimum combination for each response, equations 4.1-4.3,

$$EPA\ yield = 12.92 + 0.079A + 2.47B - 0.33C - 0.21D - 2.28CD - 2.82B^2 \quad \text{(eq. 4.1)}$$

$$EPA\ conc. = 38.07 - 9.74A + 9.51B - 0.59C - 1.41D - 9.02CD - 11.33B^2 \quad \text{(eq. 4.2)}$$

$$EPA\ \% = 4.90 - 0.79A - 0.033B - 1.02C - 0.20D - 0.86BD + 1.17CD + 1.10C^2 \quad \text{(eq. 4.3)}$$

Where A, B, C and D were glycerol, whey, peptone and Na_2HPO_4

The models were verified experimentally (see section 2.13.2). The actual EPA results were then compared with the values predicted by the models shown in equations 4.1- 4.3 (Table 4.11).

Table 4.11: The predicted and the actual values when applying the optimum media combination for each response for isolate 66

Target	**Optimised media concentrations**				**Predicted Values**	**Actual Values**
	Glycerol (g/l)	Whey (g/l)	Meat Peptone (g/l)	Na_2HPO_4 (g/l)		
Maximise EPA yield (mg/g)	3.95	9.52	4.30	0.50	17.86	21.34 (±1.2)
Maximise EPA conc. (mg/l)	4.54	10.87	1.50	5.30	68.87	89.47 (±4.3)
Maximise EPA %	1.67	7.50	1.81	0.89	10.74	11.8 (± 0.6)

These optimum compositions were estimated using the models with the aid of the 3D surface response plots (Appendix A.5).

As the optimum ranges of the growth and the EPA productivity diverged, an additional optimisation media was suggested by the software to maximise all the calculated responses simultaneously (Table 4.12),

Table 4.12: The predicted and the actual values when applying the optimum medium combination for maximum EPA production by isolate 66

Optimised medium concentrations				Predicted Values			Actual Values		
Glycerol (g/l)	Whey (g/l)	Meat Peptone (g/l)	Na_2HPO_4 (g/l)	EPA yield	EPA conc.	EPA %	EPA yield (mg/g)	EPA conc. (mg/l)	EPA %
1.90	9.41	2.50	1.30	12.8	50.15	9.987	15.6 (±0.98)	72.11 (±5)	10.48 (±0.3)

The actual values were obtained by applying the suggested media combination in triplicate (standard deviation of the reading displayed in brackets). The model estimated lower response values for all terms.

4.4 Optimising a potential industrial medium for isolate 66

A potential economically viable medium for EPA production was investigated in a separate CCD experiment. Glycerol and whey were considered as components of this potential production medium, as they are by-products produced during other industrial processes. A production medium consisting of such cheap components, which would otherwise have to be disposed of, offers a number of advantages from the point of view of economically viable manufacture of valuable products, such as PUFAs.

The main by-product of the biodiesel industry is glycerol, which usually requires costly purification steps. Glycerol has been used as a carbon source for a range of microbial fermentation processes to produce different products including lipids (Papanikolaou and Aggelis, 2002; Narayan *et al.*, 2005). The usage of glycerol as a potential medium component for PUFAs production was previously reported by Chi *et al.* (2007) as the biodiesel waste glycerol was used by microalgae to produce 4.9 g/l of DHA.Using glycerol as a substrate to produce microbial EPA lead to convert a low economic value waste into a value added product.

Whey is considered as the main by-product of the dairy industry and is produced in massive quantities (Peters, 2007). Recently, cheese whey was successfully used to support the growth and γ-linolenic acid (GLA) production by *Mortierella isabellina* achieving 301 mg/l (Vamvakaki *et al.*, 2010).

Supporting evidence for choosing these two substrates as a potential cheap production medium was obtained in the first round of the statistical analysis for the second level PB design, prior of removing the non significant variables (Appendix A.4, Table A.4.1), only four variables were found to have a statistically significant effect on the EPA in terms of all

calculated responses. These variables were NaCl, Na_2HPO_4, whey and glycerol. The amount of NaCl in the potential cheap production medium was kept at minimum concentration (10 g/l) as this value was found to be preferable for maximum EPA production (as in section 4.2.5). The concentration of Na_2HPO_4 was kept constant at the maximum value (0.89 g/l). The concentrations of glycerol and whey were varied according to a CCD as indicated in Table 4.13.

Table 4.13: Central Composite Design of variables (in coded units) for a potential cheap production medium composition with EPA productions as responses for isolate 66

Run	**Variables**		**Responses**		
	Whey	Glycerol	EPA yield mg/g	EPA conc. (mg/l)	EPA %
1	0	0	12.68	35.98	9.42
2	-1	+1	7.02	24.38	6.54
3	+1	+1	8.03	48.81	8.16
4	-2	0	3.61	5.25	2.61
5	0	0	14.22	32.43	9.83
6	0	0	13.55	33.21	10.52
7	0	0	15.78	38.12	9.41
8	0	+2	5.21	35.23	2.34
9	-1	-1	5.17	10.11	3.63
10	0	0	14.95	32.55	10.25
11	+1	-1	17.11	57.43	7.87
12	+2	0	5.48	47.44	4.67
13	0	-2	4.61	4.39	6.41

A full statistical analysis of the CCD experiment is shown in Appendix A.6. The ANOVA table (Table A.6.1) shows that the quadratic effects of the variables were significant on EPA production in terms of yield and percentage (whey quadratic effect P-values were 0.0176 and 0.0105, while glycerol quadratic effect P-values were 0.0218 and 0.0212 for EPA yield and percentage, respectively). The main effects were more significant in term of EPA concentration (with P-values of 0.0010 and 0.0821 for whey and glycerol, respectively). Although the interaction between the variables was insignificant, the P-value of the interaction between glycerol and whey on the EPA yield was 0.1270 indicating that it is significant at a lower level of confidence and thus its effect should not be ignored.

CCD was augmented to allow a second order polynomial model that predicted the amount of EPA as a function of the experimental variable, equations 4.4-4.6,

$$EPA\ yield = 14.24 + 1.95A - 0.80B - 2.73AB - 3.69A^2 - 3.51B^2 \text{---} \quad \text{(eq. 4.4)}$$

$$EPA\ conc. = 34.46 + 16.43A + 6.16B - 5.72AB - 1.04A^2 - 4.30B^2 \text{---} \quad \text{(eq. 4.5)}$$

$$EPA\ \% = 9.89 + 1.10A - 0.32B - 0.65AB - 2.48A^2 - 2.12B^2 \text{---} \quad \text{(eq. 4.6)}$$

Where A and B were whey and glycerol respectively.

The colour contours, Figure 4.4, in the response surface plots had obvious maximum points betweeen the tested variables on each calculated response.

The colour contour plots showed that the maximum amount of EPA yield (mg/g biomass) could be obtained when growing isolate 66 in a medium contain a range of 4.5-7.5 g/l glycerol and 9-11 g/l whey. The optimum medium combination for maximum EPA percentage of total fatty acids was found to be in a range of 8.5 to 10 g/l of whey and a range of 6-8 g/l of glycerol. The model and with the help of the contour predicted that the maximum concentration of EPA to be achieved requires the addition of more whey to the production media (the area of maximum concentration being placed at the extreme right of Figure 4.4 B). However adding higher concentrations of whey to the media would significantly affect the other calculated EPA responses as Figures 4.4 A and C indicate.

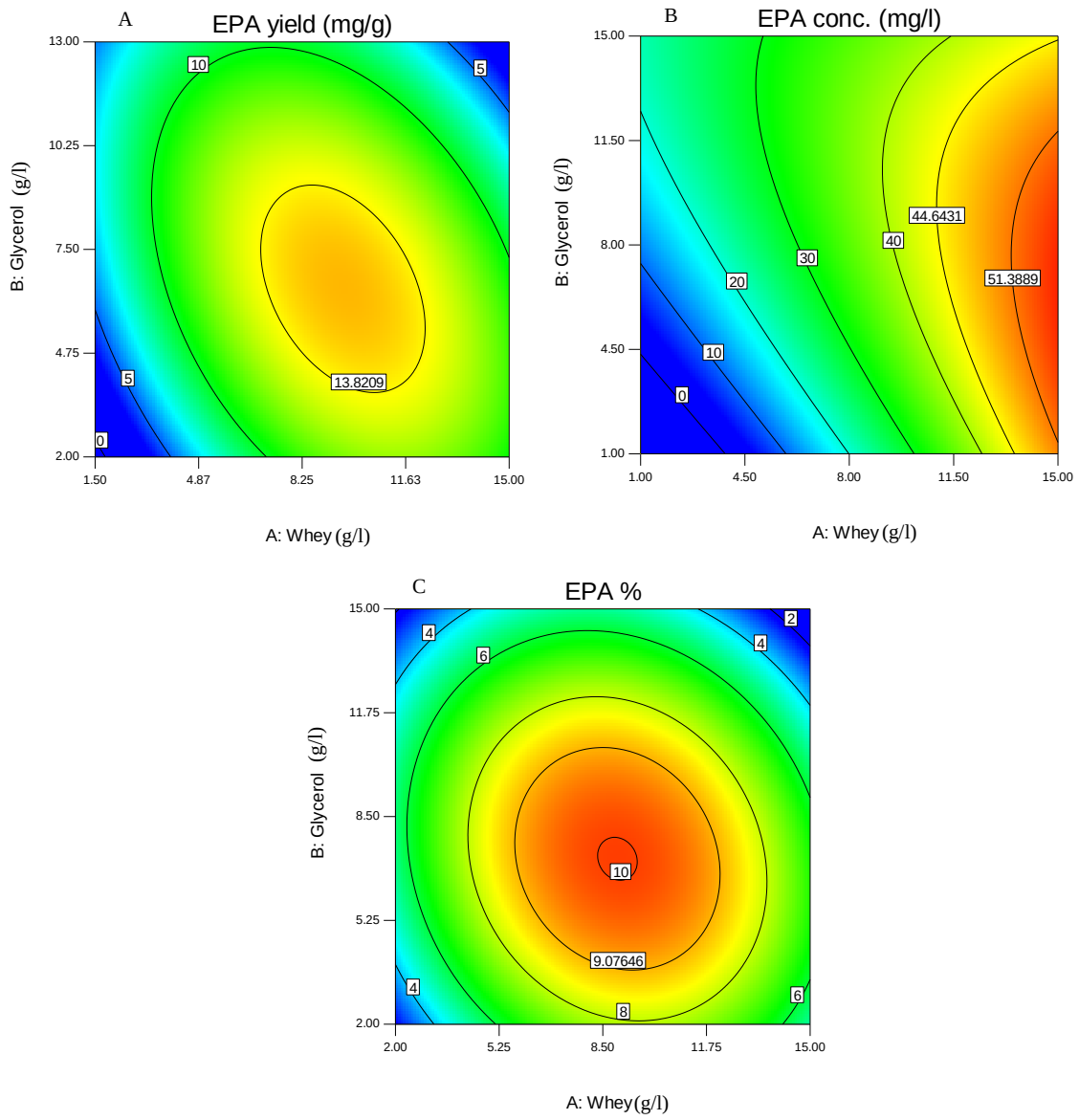

Figure 4.4: Colour contour plot showing the optimum the interaction between the variables for maximum EPA production.

The optimum medium composition that is required for optimum performance in terms of all EPA calculated responses is shown in Figure 4.5.

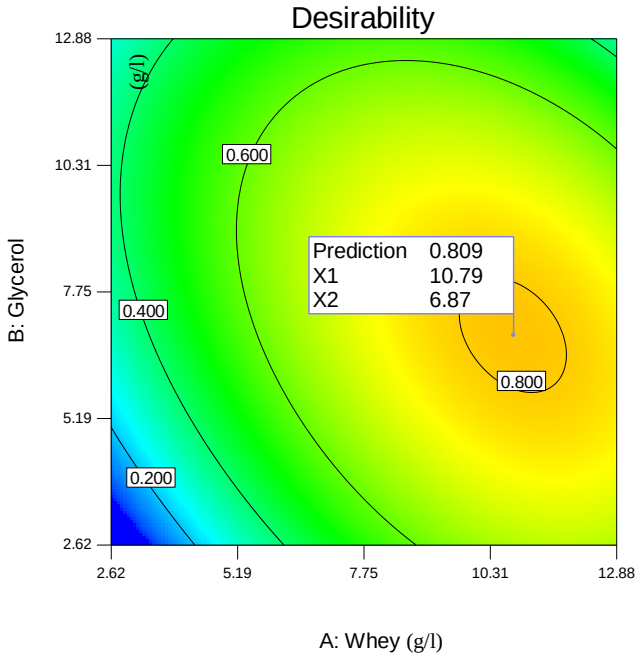

Figure 4.5: Colour contour plot showing the optimum combination of the variables that give 80% desirability of isolate 66 calculated responses.

Figure 4.5 shows the desirability (predicted values divided by the target values) for each contour together with a flag stating the optimum values for each variable leading to a maximum. According to the model predictions the maximum EPA production (25 mg/g, 57 mg/l representing 13.5% of the total fatty acids) will be achieved when the concentration of whey (X1) in the media is 10.79 g/l and the glycerol (X2) concentration is 6.87 g/l. This prediction was also verified experimentally (as described in section 2.11) and the actual EPA production achieved was 20 mg/g (±1.5), 45 mg/l (±3.2) representing 11% (±0.98) of the total fatty acids with an over prediction by the model.

4.5 Creating chemically defined medium for isolate 66

Although there is a growing trend of customising a chemically defined soluble medium for a particular bio-product, efforts to generate satisfactory chemically defined media for different fermentation processes have been inadequate (Rehm and Reed, 1993). Applying a chemically defined media allows a tight control of the cell environment during the fermentation process and avoids any complicated interactions due to complex media composition.

Applying chemically defined media at laboratory scale facilitate the studying of the effects of nutrients on cell growth and biosynthesis of specific metabolite of interest. In addition, it facilitate collecting certain biochemical data, that are essential for rationally and

systematically improving of fermentation media, including information on precursors, specific nutrients requirements, biosynthetic pathways, regulation of primary and secondary metabolite production especially by carbon, nitrogen, phosphate and trace elements.

Applying a chemically defined media to continuous culture fermentation may enable the determination of the limiting media components that limit the growth and productivity of the isolate under investigation.

Even though the chemically defined media is desired in the laboratory scale, it is not desired in the bioreactor scale as it is too expensive and usually gives a sub-maximal productivity. So, complex media are the most frequently used media in the fermentation process especially for new metabolites as the information on the biosynthetic pathway is too limited.

The main goal of this section is to create a production medium with chemically defined recipe. The Artificial sea water media consists of two main parts; first part includes the inorganic metal ions while the second part consists of yeast extract and peptone as a source of carbon and nitrogen. In this chapter the main target was reducing the numbers of metal ions included within the media then replacing the yeast extract with a simple and chemically defined amino acids and carbohydrates.

4.5.1 Reducing metal ions

In section 4.2.4, the effect of the metal ions present in ASW was tested and only two of the eight salts tested were found to have a statistically significant positive effect on EPA production. These ions were Na_2HPO_4 and $MgSO_4$. Although sodium chloride did not show a statistically significant effect on EPA production as the organism cannot grow in the complete absence of sodium chloride (as described in section 3.1), it was added at the minimum level required for growth (10 g/l) in all experiments.

Thus following these experiments the production medium composition was simplified by reducing the number of metal ions to three and reducing the sodium chloride concentration to a minimum concentration to avoid potential salinity problems in large scale production.

4.5.2 Screening the amino acids

The main aim of this experiment was to replace the yeast extract with one or more amino acids as the second step in building a chemically defined media. The basal media used

contained only the metal ions identified in the previous section and the experiment was carried out as described in section 2.14.1.

The responses obtained are summarised in Table 4.14

Table 4.14: The responses of isolate 66 grown in different amino acid pools.

Pool	EPA yield.(mg/g)	EPA conc. (mg/l)	EPA %	Dry Weight (g/l)
1	1.64	0.83	1.16	0.49
2	1.87	2.46	1.44	**1.32**
3	2.05	2.27	**4.12**	**1.11**
4	**3.32**	**6.24**	**5.92**	**1.88**
5	1.84	1.52	1.64	0.83
6	1.86	1.43	2.08	0.77
7	1.72	1.13	**4.28**	0.66
8	**4.98**	**5.77**	**6.93**	**1.16**
9	1.51	2.02	2.04	**1.34**

For the highest EPA response as yield (mg/g dry weight) and concentration (mg/), L-histidine was found to be the most important amino acid as it is the common amino acid in the pools number 4 and 8. L-asparagine, L-isoleucine, L-histidine and L-proline were the most important amino acids which induce the EPA production, expressed as the percentage of total fatty acids. For the growth of isolate 66 a relatively large number of different amino acids were found to enhance growth including, L- glutamine, L-methionine, L-proline, glutamic acid, L-lysine and L-histidine.

To simplify the medium, only L-histidine was taken further as it was the common variable enhancing all the calculated responses.

4.5.3 Screening different carbon sources

The main aim of this experiment was to screen for the effect of different carbon sources on the growth and the productivity of isolate 66. The basal media used for this experiment consists of Na_2HPO_4, $MgSO_4$, and NaCl in addition to L-histidine as the main nitrogen source (see section 4.6.2). Two Plackett-Burman designs were applied to screen a large number of potential carbon sources, but in the two designs, surprisingly the organism under investigation did not grow in most of the trial media. These unexpected results made the statistical analysis unusable and indicated that there were significant interactions among the tested variables. In addition this result indicated that some of the carbon sources used may have a detrimental effect on the isolate. The design provided insufficient data for the full statistical analysis due to the absence of growth in more than 80% of the design. However the analysis revealed that, malic acid, ascorbic acid and citric acid could have a detrimental effect

on isolate 66. This effect could be due to the effect of each carbon source individually or due to an interaction between them. In the subsequent design of experiment these three carbon sources were discarded as they masked the effect of the rest of the variables.

As PUFA production is a carbon-source dependant process (Gentile *et al.*, 2003b) the unexpected results obtained in the Plackett-Burman design of experiments, meant the rest of the tested different carbon sources were investigated further using a resolution-4 Design Of Experiment (DOE). This type of the DOE is able to reveal any two and some three way interactions which could have a significant effect on the growth and EPA production of isolate 66.

A set of 32 experiments carried out as described in section 2.14.2 and summarised in Table 4.15.

Table 4.15: The matrix and the responses for the resolution-4 DOE to test the effect of different carbon sources on isolate 66.

Run	Variables											Responses			
	A	*B*	*C*	*D*	*E*	*F*	*G*	*H*	*J*	*K*	*L*	**EPA yield (mg/g)**	**EPA conc. (mg/l)**	**EPA %**	**Dry Weight (g/l)**
1	+	+	-	-	-	+	+	-	-	-	-	0.19	0.24	1.19	1.28
2	+	+	-	-	+	-	+	-	+	+	+	2.15	4.56	6.68	2.11
3	+	-	-	-	-	+	+	+	+	-	+	0.00	0.00	0.00	0.00
4	+	-	+	-	-	-	-	+	+	+	-	3.68	6.39	11.59	1.73
5	-	-	+	+	-	+	-	+	-	-	-	0.42	0.33	2.16	0.79
6	-	+	-	+	+	+	+	-	-	-	+	0.00	0.00	0.00	0.00
7	+	+	-	+	+	-	-	+	+	-	-	2.67	3.21	8.65	1.19
8	+	+	+	+	+	+	+	+	+	+	+	0.00	0.00	0.0	0.00
9	+	-	-	+	+	-	-	-	-	-	+	2.99	6.86	7.37	2.29
10	-	-	+	+	+	-	-	+	+	+	+	3.86	6.79	9.21	1.75
11	-	+	-	+	-	-	+	-	+	+	-	2.45	4.03	9.71	1.63
12	-	+	-	-	-	-	-	+	+	-	+	3.35	7.61	8.78	2.27
13	+	+	-	+	-	+	-	+	-	+	+	0.00	0.00	0.00	0.00
14	+	+	+	-	-	-	-	-	-	+	+	4.08	9.33	8.25	2.28
15	-	+	+	+	+	-	-	-	-	+	-	3.71	6.94	9.09	1.87
16	+	-	+	+	+	+	+	-	-	+	-	0.83	0.46	6.04	0.56
17	+	+	+	-	+	+	-	-	+	-	-	3.02	0.66	1.23	0.22
18	+	-	+	+	-	-	+	-	+	-	+	1.91	3.23	7.07	1.71
19	-	+	-	-	+	+	-	+	-	+	-	0.14	0.05	0.79	0.39
20	-	-	+	-	+	-	+	-	+	-	-	3.95	7.79	9.71	1.97
21	+	-	-	+	-	+	-	-	+	+	-	0.00	0.00	0.00	0.00
22	+	+	+	+	-	-	+	+	-	-	-	1.63	2.03	8.46	1.24
23	-	+	+	-	+	-	+	+	-	-	+	1.21	1.06	5.35	0.87
24	-	-	-	-	+	+	-	-	+	+	+	0.25	0.18	0.89	0.74
25	-	+	+	-	-	+	+	+	+	+	-	0.00	0.00	0.00	0.00
26	-	-	-	+	+	+	+	+	+	-	-	1.76	0.57	2.03	0.32
27	-	-	+	-	-	+	+	-	-	+	+	0.63	0.43	2.71	0.69
28	-	-	-	-	-	-	-	-	-	-	-	1.32	0.28	1.22	0.21
29	-	-	-	+	-	-	+	+	-	+	+	1.13	0.84	2.85	0.74
30	+	-	-	-	+	-	+	+	-	+	-	0.71	0.76	3.61	1.08
31	-	+	+	+	-	+	-	-	+	-	+	0.17	0.09	0.55	0.52
32	+	-	+	-	+	+	-	+	-	-	+	0.19	0.24	0.00	0.00

A represents glycerol, B represents fructose, C represents arabinose, D represents mannitol, E represents sorbitol, F represents sodium acetate, G represents glucose, H represents maltose, J represents xylose, K represents lactose, and L represents sucrose

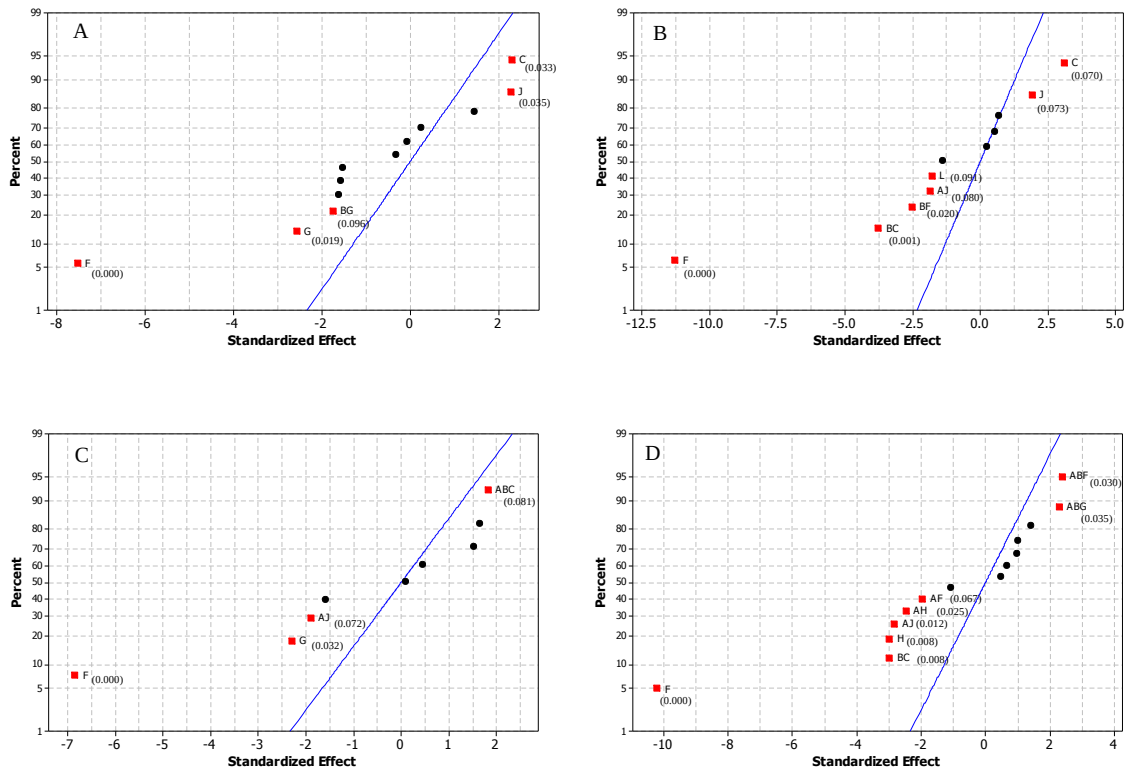

Figure 4.6: Normal probability plot of the standardised effect to show the significant effect of each carbon source and their interactions on each response for isolate 66 (A) EPA yield (mg/g), (B) EPA percentage of total fatty acids, (C) EPA concentration (mg/l) and (D) Dry weight (g/l).

As shown in Figure 4.6 acetate (F) was found to be the most statistically significant factor negatively affecting all the calculated responses, while arabinose (C) was found to be the most statistically significant variable positively affecting EPA production in terms of yield (mg/g) and percentage of EPA in total fatty acids. This indicates that arabinose may have a significant role in EPA biosynthesis by isolate under investigation. Even for EPA concentration (mg/l) arabinose was found to be involved in a three way interaction which enhances the productivity. In addition, the P-value for arabinose on the EPA concentration was 0.110, which means that even if it is not statistically significant at the 90% level of confidence it was significant at a slightly lower level of confidence. However arabinose was not found to have a significant effect on the biomass concentration, on the contrary, it showed a negative effect in the interaction with fructose (B). This result confirms that the variables significantly enhancing bacterial EPA production can have an adverse impact on growth or no significant effect that could be detected.

Xylose (J) was identified as a significant factor affecting the ability to produce EPA by isolate 66, but the interaction between xylose and glycerol (A) was found to have a statistically significant negative effect on the EPA concentration (mg/l).

As expected, the interactions (two way and three way interactions) between the variables were significant either in a negative or positive direction, especially for growth, explaining the lethal effect of these compounds on isolate 66 which lead to growth inhibition in the PB experiment that were carried out before the resolution-4 experiment.

4.5.4 Chemically defined media

Due to the large number of potential amino acids that affect the EPA production (see section 4.6.2), only L-histidine was investigated further as it was the common amino acids which enhanced EPA production in terms of all calculated responses. Arabinose was used as a carbon source during these experiments as described in section 4.6.3. To achieve the optimum recipe for the desired media, a CCD experiment was performed.

The matrix and responses are summarised in Table 4.16 and the full statistical analysis in Appendix A.7.

Table 4.16: The matrix and responses for the CCD experiment to create the chemically defined media for isolate 66

Run	**Variables**		**Responses**		
	Arabinose	Histidine	EPA yield. (mg/g)	EPA conc. (mg/l)	EPA %
1	0	0	8.81	21.58	9.14
2	0	+2	1.45	1.29	1.34
3	0	0	7.93	19.82	9.33
4	-1	+1	4.44	8.39	3.46
5	-1	-1	3.14	2.73	1.22
6	0	0	8.29	23.2	9.22
7	+2	0	0.05	0.01	0.34
8	0	0	8.41	17.68	8.89
9	-2	0	2.34	1.28	2.98
10	+1	+1	2.83	1.69	6.29
11	+1	-1	4.91	5.45	5.68
12	0	-2	1.81	2.22	1.65
13	0	0	7.62	16.76	9.6

The statistical analysis revealed that the quadratic effect of the two variables had the most significant effect on EPA production in terms of all calculated responses. The main effect of the amino acid histidine was statistically significantt, at 90% level of confidence, on EPA concentration (P-value of 0.0715) and EPA perecentage (P-value of 0.0653). The P-value of the effect of histidine on EPA yield was caclucalted as 0.1230, indicating tha the variable effect is not significant at the 90% level of confidence but signifcant at a lower level of confidence. The main effect of arabinose on all EPA responses were statistically insignifcant.

The effect of two way interaction (interaction between the two tested variables) was not significant at the 90% level of confidence but at a lower level of confidence it showed a significant effect on the EPA yield (P-value of 0.1546) and EPA concentration (P-value of 0.2081).

The main effects, interactions and quadratic effects are mathematically described for the coded units using equations 4.7- 4.9,

$$EPA\ conc. = 8.05 - 0.38A + 0.65B - 0.84AB - 2.11A^2 - 2.14B^2 \text{---} \quad \text{(eq. 4.7)}$$

$$EPA\ yield = 18.90 - 0.60A + 2.54B - 2.35AB - 6.01A^2 - 6.38B^2 \text{---} \quad \text{(eq. 4.8)}$$

$$EPA\ \% = 9.13 + 0.24A + 1.27B - 0.41AB - 2.30A^2 - 2.69B^2 \text{---} \quad \text{(eq. 4.9)}$$

Where A represents arabinose and B represents L-histidine.

The 3D response surfaces displayed in Figure 4.7 are the graphical representation of the regression equation and explore the optimum area in addition to determining the interaction between the investigated variables.

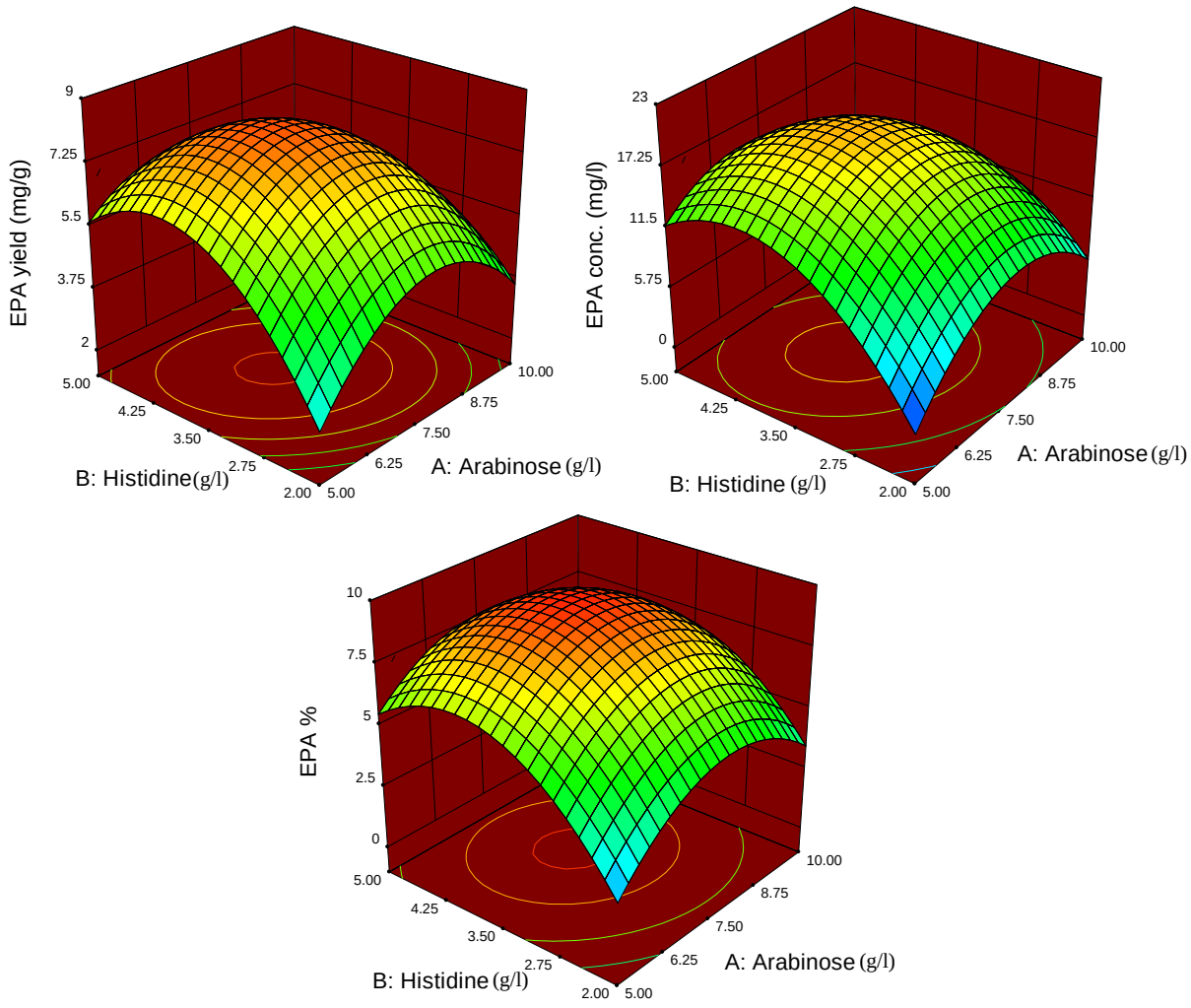

Figure 4.7: Three –dimensional surface plot of EPA responses as a function of arabinose (g/l) and histidine (g/l) showing the interaction between them on different EPA production responses by isolate 66.

The surface plot in Figure 4.7 shows that the optimum concentrations of arabinose and histidine for maximum EPA are approximately the same for all the calculated responses. For arabinose, the optimum concentration ranges between 7-8 g/l and for histidine the range is between 3.5 to 4.25 g/l.

The maximum productivity of 8.5 mg/g (± 0.7), 24 mg/l (± 2.5) representing 9.5 (± 1) of the total fatty acids was experimentally obtained using 8 g/l arabinose and 3.8 g/l histidine.

The significant role of histidine in the EPA production, may be due to its vital role in the activity of Ketoacyl-acyl carrier protein (ACP) synthase enzymes which are involved in the PUFAs biosynthetic pathway (Von Wettstein-Knowles *et al.*, 2006).

As the interaction between bacterial cells and their environment is a continuous process, bacterial cells evolve surface-exposed signal transduction systems to monitor environmental parameters, including osmotic activity and ionic strength, pH, temperature, and the

concentrations of nutrients and harmful compounds. These transduction systems are usually transmembrane (TM) proteins, one of them is histidine Kinase (Mascher *et al.*, 2006).
Histidine Kinase was proven to control the transcription of the genes included in the stress response by *Vibrio parahaemolyticus* (Kwon *et al.*, 2012). In addition, auto-phosphorylation of the histidine kinase thermo-sensor DesK at low temperatures activates the expression of the acyl lipid desaturase, which promotes membrane fluidity to overcome rigidity induced by low temperature in the bacteria *Bacillus subtilis* (Wootton, 2010).
The presence of L-histidine in the cultivation media may enhance the stimulant response of the bacterial cells leading to higher production of EPA as a response to environmental stress especially low temperature and oxygen stress.

4.5.5 Effect of carbon to nitrogen ratio (C/N)

One of the advantages of using a chemically defined medium, the effect of C/N on EPA production can be more easily tested. The C/N values were calculated theoretically from the CCD experiment performed to optimise the chemically defined media (Section 4.6.4), the design contain 13 different trials with 13 different C/N values. The relation between the ratio values and each response was plotted in Figure 4.8 and a best fit line was estimated and the P-values for each response was calculated in order to test the effect of the C/N values on growth and productivity of isolate 66.

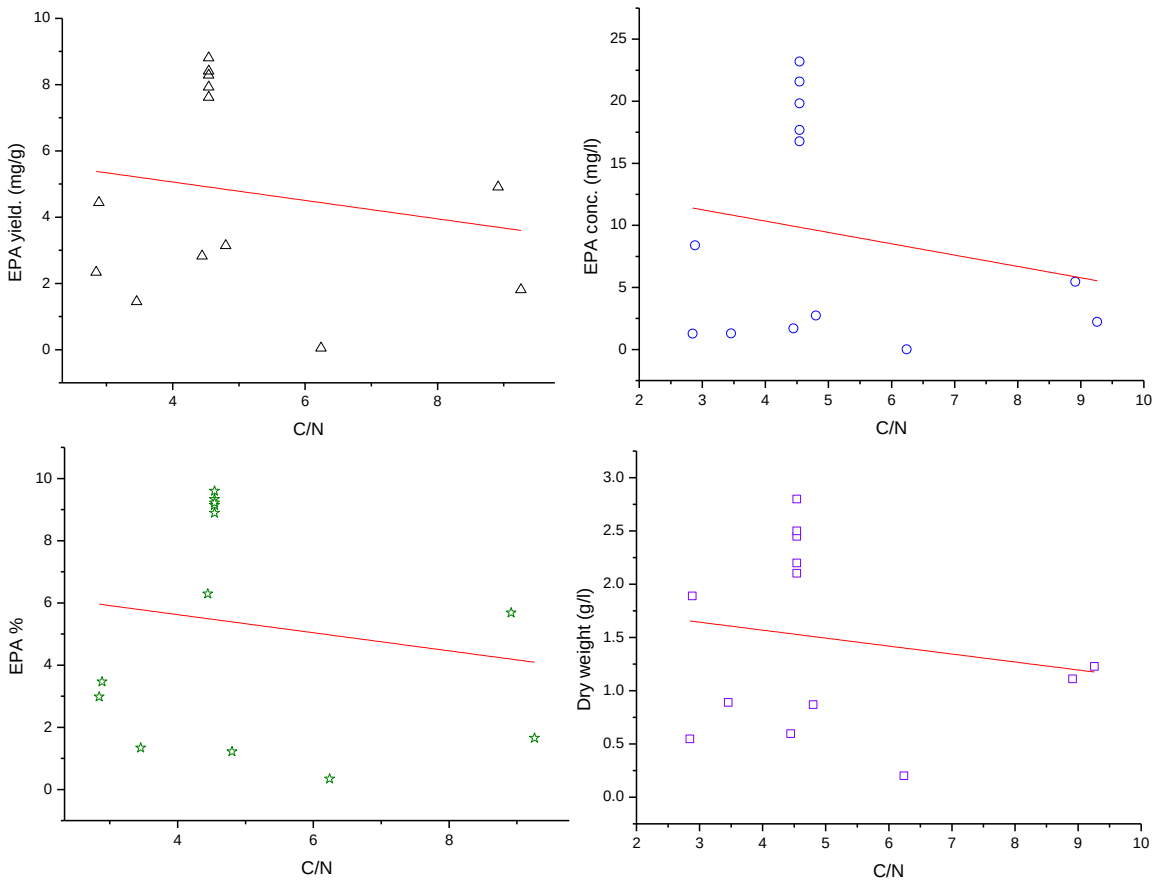

Figure 4.8: the relation between the C/N ratio and the growth and the EPA production by isolate 66 with the best fit line.

The effect of the C/N ratio was negative on growth and productivity of isolate 66. Lower C/N values were preferred indicating that a higher nitrogen was required for better growth and EPA production.

The P-value for the effect of C/N ratio on EPA yield (mg/g) was 0.558, indicating that the effect of C/N could only be significant at the 45% level of confidence. The effect of the C/N on the EPA concentration (mg/l) and EPA% was nearly the same, as the P-values were 0.504 and 0.608 respectively. Even for growth the effect of C/N was statistically insignificant with P-value 0.573.

4.6 Chapter Summary

In this chapter, different media components were investigated and screened to determine the significance of their effect on the growth and productivity of isolate 66.

To achieve this rapidly and reliably, a statistical design of experiments (resolution-3 PB design) was applied. The most statistically significant positive media components included glycerol, meat peptone, Na_2HPO_4 and whey.

Another statistical design of experiment (Central Composite Design) was applied to determine the optimum concentration of the components and to assess the interaction between them via response surface methodology. The maximum amount of EPA achieved was 21 mg/g EPA yield and 90 mg/l EPA concentration representing 12% of the total fatty acids.

Glycerol and whey were tested as potential cheap production medium components by applying a separate CCD experiment. The amount of produced EPA was 20 mg/g EPA yield and 45 mg/l EPA concentration representing 11% of the total fatty acids. The amount of percentage and yield was competitive to the previous media but the reduction in the EPA concentration indicates that the removal of peptone reduced the growth significantly.

A chemically defined medium was created for isolate 66 through a series of four main steps, 1) reducing the metal ion content of the media, 2) replacing yeast extract with a simple amino acid (L-histidine), 3) screening different carbohydrates as potential carbon sources identifying arabinose as the best source, 4) identifying the optimum combination of the selected compounds to obtain the maximum EPA productivity (8.5 mg/g, 24 mg/l, representing 9.5% of the total fatty acids).

In this chapter, four different media were tested and Figure 4.9 shows the comparison between them for all the calculated responses.

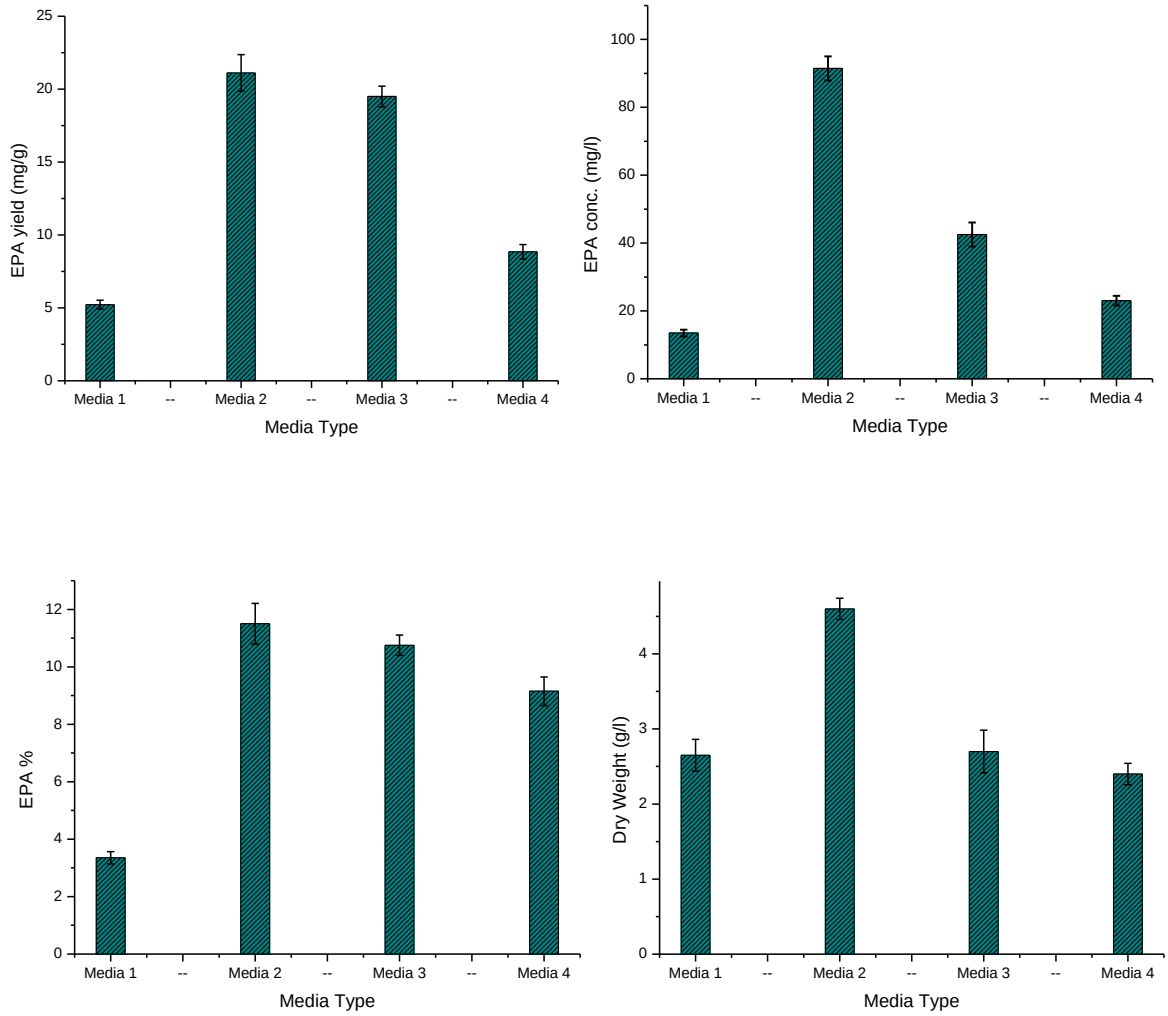

Figure 4.9: A comparison between different media on the productivity and growth of isolate 66
Media 1: Artificial Sea Water (ASW), Media 2: Optimum production media (glycerol, peptone, whey and Na_2HPO_4), Media 3: Potential cheap production media (whey and glycerol), Media 4: chemically defined media (arabinose and histidine).

The highest growth and productivity was achieved in the production medium (medium 2), while the lowest growth and productivity was obtained from ASW media (medium 1). Even the chemically defined medium (medium 4) showed a higher productivity than ASW, while the growth in both media was approximately the same.

The amount of EPA (as yield and as percentage) was achieved by growing isolate 66 in the potential cheap production medium (medium 3) showed a competitive result compared to the more complex production medium (medium 2), but the amount of growth significantly decreased in medium 3 compared to medium 2 (optimum medium).

For large scale production, even though growth was reduced but the medium using waste by-products would be preferable as discarding the expensive substrate, peptone, would be advantageous.

Chemically defined media can be used to determine the growth limiting substrate which may lead to a successful fed-batch process.

In the upcoming chapter, the main effect of different media components was screened and the production medium developed for the Egyptian isolate Hus-27.

Chapter 5-Screening and optimising the media components for isolate Hus-27

In this chapter a simplified four separate two level fractional factorial designs (PB) were used for initial screening to explore the effect of different media components and to screen the effect of variables on the ability of isolate Hus-27 to produce EPA. Subsequently a CCD experiment with five coded levels was applied to determine the optimum concentration of nutrients.

Isolate Hus-27 is a bacterial strain isolated from the Red Sea water sample, and it was identified as an EPA producer in the preliminary screening (section 3.1). The isolate was identified, by 16S rDNA sequencing and phylogenetic analysis, to be *Pseudoaltermona*s with high similarity to *Pseudoaltermonas marina.* (Section 3.3).

The amount of EPA produced by isolate Hus-27 was calculated as 0.6 mg/g, 3 mg/l representing 1.2% of the total fatty acids

The mass spectrum of the extracted EPA is displayed in Figure 5.1.

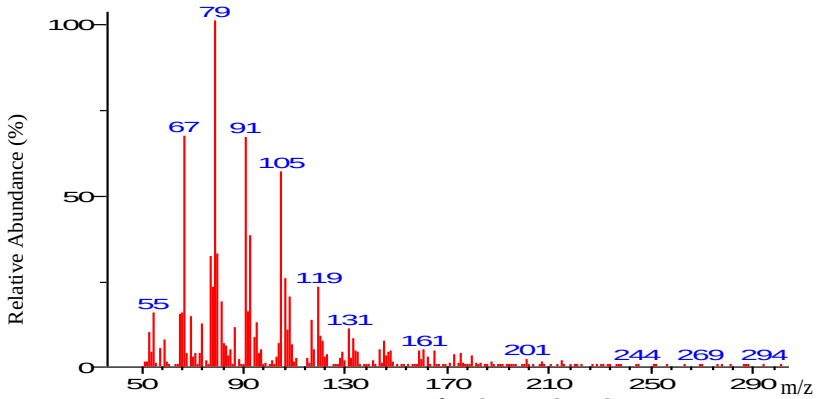

Figure 5.1: Mass spectrum for the produced EPA.

The mass spectrum of the EPA produced by isolate Hus-27 showed more than 92% similarity to the standard EPA mass spectrum (section 2.15)..

The optimisation stratgey included dividing all the media components into four separate PB designs as described in section 2.13.

5.1 Screening the media components for isolate Hus-27

As described in section 2.13, forty three different potential media components were screened by dividing them into four different Plackett-Burman designs to investigate the most significant factors affecting EPA production.

5.1.1 First Plackett-Burman Design

The experiment matrix and responses are summarised in Table 5.1 and the full statistical analysis is presented in Appendix B.1.

Table.5.1: The matrix and the responses for the 1st Plackett-Burman design for isolate Hus-27

Run	Variables																Responses		
	X_1	X_2	X_3	X_4	X_5	X_6	X_7	X_8	X_9	X_{10}	X_{11}	X_{12}	X_{13}	X_{14}	X_{15}	X_{16}	EPA yield (mg/g)	EPA conc. (mg/l)	EPA %
1	-	+	-	+	-	-	-	-	+	-	+	-	+	+	+	+	0.574	0.761	0.659
2	+	+	-	+	+	+	-	-	+	+	-	+	+	-	-	-	0.683	0.563	0.501
3	+	-	+	+	-	-	-	+	-	+	-	+	+	+	+	-	0.021	0.065	0.140
4	-	-	+	-	-	+	+	+	+	-	-	+	+	-	+	+	0.970	5.166	0.697
5	+	-	-	-	-	+	-	+	+	+	+	-	-	+	+	-	0.639	0.487	0.556
6	+	+	+	+	-	-	+	+	-	+	+	-	-	-	-	+	0.043	0.098	0.032
7	+	-	-	-	+	-	+	-	+	+	+	+	-	-	+	+	0.244	0.317	0.245
8	+	+	+	-	-	-	+	-	+	-	+	+	+	+	-	-	2.233	6.153	1.259
9	+	-	+	+	+	+	-	-	-	-	+	-	+	-	+	+	0.201	0.102	0.258
10	-	-	+	-	+	+	+	-	-	+	+	-	+	+	-	-	0.107	0.040	0.335
11	-	-	+	+	+	-	-	+	+	-	+	+	-	-	-	-	1.257	3.693	0.587
12	-	-	-	+	+	-	+	+	+	+	-	-	+	+	-	+	0.669	0.624	0.435
13	+	+	-	-	+	-	+	+	-	-	-	-	+	-	+	-	0.000	0.000	0.000
14	+	+	+	-	+	+	-	+	+	-	-	-	-	+	-	+	2.593	9.385	1.281
15	-	+	-	-	-	+	-	+	-	+	+	+	+	-	-	+	0.030	0.032	0.012
16	-	+	+	-	+	-	-	-	-	+	-	+	-	+	+	+	0.011	0.210	0.123
17	+	-	-	+	-	+	+	-	-	-	-	+	-	+	-	+	0.781	3.027	1.065
18	-	+	-	+	+	+	+	+	-	-	+	+	-	+	+	-	0.093	0.043	0.100
19	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	0.160	0.508	0.533
20	-	+	+	+	-	+	+	-	+	+	-	-	-	-	+	-	0.188	0.245	0.207

where, X_1 represents yeast extract , X_2 represents glucose , X_3 represents glycerol, X_4 represents soy bean flour, X_5 represents corn step liquor, X_6 glutamic acid, X_7 represents L-proline , X_8 represents ammonium acetate, X_9 represents whey, X_{10} is propanoic acid, X_{11} represents L-serine, X_{12} represents urea , X_{13} represents sucrose, X_{14} represents glycine, X_{15} represents citric acid and X_{16} represents fructose.

Whey, glycine, yeast extract, glycerol, urea and fructose were found to significantly enhance the ability of isolate under investigation to produce EPA, either as EPA yield, concentration or percentage of the total fatty acids. As a result, these variables were taken further to the second level of screening.

Compared to the result obtained for isolate 66, the same variables were found to have the same effect on the productivity, except that fructose, in addition to glutamic acid, showed a positive effect on all EPA calculated responses for isolate 66 while for isolate Hus-27 glutamic acid was found to be significant on EPA concentration and percentage but not the yield.

Another similarity between the two isolates, Hus-27 and 66 was the significant negative effect of some variables, such as citric acid, propionic acid, soy bean flour and corn steep liquor.

Surprisingly glucose showed a significant inhibition of EPA biosynthesis which contradicts the effect of the same variable on PUFA production reported by Funtikova *et al.* (2002). These authors reported that glucose was the most promising carbon source to enhance the level of unsaturation in the fatty acid profile of *Mucor lusitanicus* 306D over the other tested carbon sources such as sunflower oil and maize extract.

Ammonium acetate showed a statistically significant negative impact on EPA percentage produced by isolate Hus-27, similarly to isolate 66, indicating that it may inhibit the biosynthesis of EPA. However this variable showed a significant positive impact on EPA concentration and yield in case of Hus-27 (non significant effect on isolate 66 for the same responses) indicating that it may enhance growth and total fatty acid biosynthesis.

Corn steep liquor showed a statistical significant negative impact on the biosynthesis of EPA by isolate Hus-27. The same effect was previously reported for the addition of corn steep liquor to the cultivation media of *Aurantiochytrium limacinum* SR21 in which it yielded a high biomass but a significant decrease in the total lipids resulting in a reduction of the amount of DHA produced by the isolate (Rosa *et al.*, 2010).

Although L-serine showed a significant negative effect on EPA concentration and EPA percentage produced by isolate Hus-27, no significant effect on the ability to produce EPA by *Shewanella gelidimarina* ACAM 456T was detected (Nichols *et al.*, 1997).

Glutamic acid was found to significantly enhance EPA production by isolate Hus-27 in terms of concentration (mg/l) and percentage of the total fatty acids, while for isolate 66 the positive significant effect was detected on percentage only. The same observation was reported for *Mortierella alpine*, where the addition of 0·8 g/l of glutamic acid resulted in 1.4 g/l arachidonic acid produced representing a 170% increase compared to the control cultivation (Lan *et al.*, 2002). Also, the addition of glutamic acid to the cultivation media of the fungus *Choanephora cucurbitarum* resulted in the formation of a number of long-chain fatty acids longer than γ-linolenic acid. These additional fatty acids were not detected when the isolate was grown in the cultivation medium in the absence of glutamic acid (Deven and Manocha, 1975). The

significant effect of glutamic acid may be due to the fact that glutamate is required to activate acetyl-CoA carboxylase, an enzyme involved in the formation of malonyl-CoA, an essential substrate for fatty-acid synthase and for fatty acyl chain elongation systems, (Kowluru *et al.*, 2001).

Glycerol enhanced the EPA productivity by isolate Hus-27. Glycerol was previously reported to be a preferred carbon source, following glucose, for PUFA production among five different tested carbon sources for the fungus *Mortierella alpine* (Jang *et al.*, 2005).

5.1.2 Second Plackett-Burman Design

The matrix and responses are summarised in Table 5.2 and the full statistical analysis is presented in Appendix B.2.

Table 5.2: The matrix and the responses for the 2nd Plackett-Burman design for isolate Hus-27

Run	Variables										Responses	
	X_1	X_2	X_3	X_4	X_5	X_6	X_7	X_8	X_9	X_{10}	EPA yield (mg/g)	EPA conc. (mg/l)
1	+	+	+	-	+	+	-	+	-	-	0.047	0.162
2	-	-	+	-	+	-	+	+	+	-	0.004	0.003
3	-	+	-	-	+	+	+	-	+	+	0.094	0.047
4	-	-	-	-	-	-	-	-	-	-	0.190	0.708
5	+	-	+	+	-	+	+	-	+	-	0.987	3.121
6	+	+	+	-	-	-	+	-	-	+	1.637	3.603
7	-	+	+	+	-	-	-	+	+	+	3.271	18.231
8	+	-	-	-	-	+	-	+	+	+	0.132	0.162
9	-	-	+	+	+	+	-	-	-	+	0.862	1.815
10	+	+	-	+	+	-	-	-	+	-	2.363	1.299
11	-	+	-	+	-	+	+	+	-	-	2.883	11.173
12	+	-	-	+	+	-	+	+	-	+	1.462	6.399

where, X_1 represents palmitic acid , X_2 represents sugar cane molases, X_3 represents linseed oil, X_4 represents ammonium nitrate ,X_5 tryptone, X_6 represents pea nut oil, X_7 represents ethylene glycol , X_8 represents Hy-soy , X_9 represents fish peptone and X_{10} represents sodium acetate.

Unlike the effect on EPA productivity by isolate 66, ammonium nitrate and sugar cane molasses were found to be the factors with the most positive significant effect on EPA productivity by isolate Hus-27. In addition tryptone showed the same negative effect on both isolates.

The significant negative effect of peanut oil on the EPA production by isolate Hus-27 may be due to the presence of the high percentage of oleic acid, linoleic acid and palmitic acid which may inhibit the EPA biosynthetic pathways. The same observation was reported for *Phaeodactylum tricornutum* upon the addition of oleic acid to the production media (Yongmanitchai and Ward, 1991). Also, the addition of oleic acid to potato dextrose media reduced the ability of soil fungi to produce PUFAs (Gayathri *et al.*, 2010).

Hy-soy showed a significant positive effect on EPA concentration produced by isolate Hus-27 which is similar to the observations for isolate 66. The positive effect of Hy-soy probably due to the relatively high quantities of glutamic acid (120 mg/g) compared to the other amino acids in its composition (information provided by the supplier).

Sugar cane molasses was found to be the most potential beneficial carbon source for the maximum production of EPA by isolate Hus-27. Sugar cane molasses was previously suggested to be a superior medium component for the industrial production of PUFAs by the fungus *Mucor recurvus* (Li *et al.*, 2008b).

Ammonium nitrate, Hy-soy and sugar cane molasses were taken to the next step of screening.

5.1.3 Third Plackett-Burman Design

The matrix and the responses obtained are summarised in Table 5.3 and the full statistical analysis is presented in Appendix B.3.

Table 5.3: The matrix and the responses for the 3rd Plackett-Burman design for isolate Hus-27

Run	Variables									Responses		
	X_1	X_2	X_3	X_4	X_5	X_6	X_7	X_8	X_9	EPA yield (mg/g)	EPA conc. (mg/l)	EPA %
1	+	+	+	-	+	+	-	+	-	2.483	4.34	2.046
2	+	+	-	+	+	-	+	-	-	1.673	5.77	0.543
3	-	-	+	+	+	-	+	+	-	0.195	0.47	0.760
4	-	+	-	-	-	+	+	+	-	2.450	5.22	1.842
5	-	-	-	+	+	+	-	+	+	3.031	8.29	0.973
6	+	-	-	-	+	+	+	-	+	3.611	9.78	2.320
7	-	-	-	-	-	-	-	-	-	0.184	0.95	1.428
8	-	+	+	-	+	-	-	-	+	1.681	2.50	2.027
9	+	-	+	+	-	+	-	-	-	2.644	9.33	0.811
10	-	+	+	+	-	+	+	-	+	3.012	10.49	0.807
11	+	-	+	-	-	-	+	+	+	3.257	7.59	2.082
12	+	+	-	+	-	-	-	+	+	2.504	6.96	0.661

where, X_1 represents meat peptone, X_2 represents Ammonia, X_3 represents lactose, X_4 represents ground sesame , X_5 represents ammonium sulphate, X_6 represents casein, X_7 represents maltose , X_8 represents vegetable peptone and X_9 represents mannitol.

None of the variables tested showed a statistically significant negative effect on any of the EPA response variables, except the effect of ground sesame on EPA percentage. Ground sesame showed a significant positive effect on EPA concentration (mg/l), but a significant negative effect on EPA as a fraction of the total fatty acids indicating that ground sesame may enhance the growth of isolate but supresses the biosynthesis of EPA.

The significant positive impact of meat peptone and mannitol on EPA production by isolate Hus-27 was found to be similar to the effect of these compounds on the ability of isolate 66 to produce EPA.

In addition, casein was found to have a significant positive effect on all EPA terms. Therefore casein, meat peptone, ammonium sulphate and mannitol were chosen for the next level of screening.

5.1.4 Fourth Plackett-Burman Design

The matrix and responses are summarised in Table 5.4 and the full statistical analysis is presented in Appendix B.4.

Table 5.4: The matrix and the responses for the 4th Plackett-Burman design for isolate Hus-27

Run	Variables								Responses		
	X_1	X_2	X_3	X_4	X_5	X_6	X_7	X_8	EPA yield (mg/g)	EPA conc. (mg/l)	EPA %
1	+	-	+	-	-	-	+	+	0.588	1.501	1.93
2	+	+	-	+	-	-	-	+	1.460	0.886	1.01
3	-	+	+	+	-	+	+	-	1.253	1.655	1.49
4	+	+	-	+	+	-	+	-	0.611	1.208	0.84
5	-	-	-	-	-	-	-	-	0.084	0.435	0.25
6	-	+	-	-	-	+	+	+	0.895	2.173	1.05
7	+	-	+	+	-	+	-	-	0.000	0.000	0.00
8	-	-	+	+	+	-	+	+	0.036	0.523	0.66
9	-	+	+	-	+	-	-	-	0.625	0.158	0.88
10	+	+	+	-	+	+	-	+	0.000	0.000	0.00
11	+	-	-	-	+	+	+	-	0.043	1.085	0.18
12	-	-	-	+	+	+	-	+	0.000	0.000	0.00

where, X_1 represents NH_4Cl, X_2 represents Na_2HPO_4, X_3 represents KCl , X_4 represents $CaCl_2$, X_5 represents $Fe_2(SO_4)_3$, X_6 represents $MgCl_2$, X_7 represents $MgSO_4$, X_8 represents NaCl.

$MgSO_4$ and Na_2HPO_4 were identified as the most significant variables enhancing EPA production, in all terms, by isolate Hus-27. These two variables showed a similar significant positive effect on isolate 66.

Iron was found to be the most significant variable inhibiting EPA biosynthesis in isolate Hus-27. The same result was reported for isolate 66 especially on the EPA percentage of the total fatty acids. The negative impact of iron on the ability to produce EPA contradicts the result obtained by Chiou *et al.* (2001), where the addition of iron to the growth medium of the bryophyte *Marchantia polymorpha* showed a significant increase in the amount of ARA and EPA produced.

$CaCl_2$ and KCl showed a statistically significant negative influence on EPA concentration. The statistical significant negative effect of $CaCl_2$ on PUFA production was previously reported on the moss *Physcomitrella patens* (Chodok *et al.*, 2010).

The fact that, $MgSO_4$ showed a positive effect on EPA percentage, while $MgCl_2$ showed a negative effect on the same response variable suggests that the positive effect of $MgSO_4$ was due to the sulphate ion (SO_4^{2-}). Another possible explanation for

this observation is that the negative effect of $MgCl_2$ was due to chloride ion as all the chloride metal ions, except NaCl which showed a non-statistically significant effect, showed a significant negative impact on EPA production (KCl, $CaCl_2$ and $MgCl_2$).

The positive effect of Na_2HPO_4 could be due to the effect of inorganic phosphate as inorganic phosphate has been reported to have a significant effect on microbial fatty acid profiles. Khozin-Goldberg and Cohen (2006) reported that, decreasing the concentration of inorganic phosphate (K_2HPO_4) from 175 to 17.5 µM reduced the amount of EPA produced by the algal isolate *Monodus subterraneus*, from 28.2 to 19.4 mol% (of total fatty acids), respectively.

$MgSO_4$ and Na_2HPO_4 were taken further to the next level of screening.

5.1.5 Second level of Plackett-Burman Design

In this section, the previously chosen media components which had demonstrated a significant positive effect on EPA production by isolate Hus-27 were tested via a PB experiment. 15 different variables were tested to choose the most promising media components to create a potential optimum production medium for maximum EPA production.

The matrix and responses are summarised in Table 5.5 the full statistical analysis is presented in Appendix B.5.

Table 5.5: The matrix and the responses for the 2nd level of Plackett-Burman design for isolate Hus-27

Run	Variables															Responses		
	X_1	X_2	X_3	X_4	X_5	X_6	X_7	X_8	X_9	X_{10}	X_{11}	X_{12}	X_{13}	X_{14}	X_{15}	EPA yield (mg/g)	EPA conc. (mg/l)	EPA %
1	+	+	+	-	-	+	+	-	+	+	-	-	-	-	+	1.55	4.970	2.04
2	+	-	-	-	-	+	-	+	-	+	+	+	+	-	-	0.11	0.365	0.23
3	-	+	+	-	+	+	-	-	-	-	+	-	+	-	+	3.04	8.867	2.88
4	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	0.21	0.865	0.91
5	+	+	-	+	+	-	-	-	-	+	-	+	-	+	+	2.43	4.830	1.99
6	-	-	+	+	-	+	+	-	-	-	-	+	-	+	-	4.61	12.602	2.86
7	-	-	-	-	+	-	+	-	+	+	+	+	-	-	+	1.89	3.880	3.34
8	-	+	-	+	+	+	+	-	-	+	+	-	+	+	-	1.15	3.335	0.94
9	-	-	+	-	+	-	+	+	+	+	-	-	+	+	-	0.23	0.245	0.74
10	-	+	-	+	-	+	+	+	+	-	-	+	+	-	+	0.39	0.995	0.91
11	+	+	-	-	+	+	-	+	+	-	-	-	-	+	-	2.95	9.517	2.21
12	+	+	-	-	-	-	+	-	+	-	+	+	+	+	-	2.01	4.692	1.84
13	-	-	-	+	-	+	-	+	+	+	+	-	-	+	+	3.52	9.127	2.44
14	+	-	+	-	+	+	+	+	-	-	+	+	-	+	+	3.79	11.937	1.95
15	+	+	+	+	-	-	+	+	-	+	+	-	-	-	-	2.22	4.865	1.45
16	-	+	+	-	-	-	-	+	-	+	-	+	+	+	+	0.92	2.467	0.53
17	-	+	+	+	+	-	-	+	+	-	+	+	-	-	-	1.09	3.132	0.86
18	+	-	-	+	+	-	+	+	-	-	-	-	+	-	+	1.79	5.785	0.73
19	+	-	+	+	+	+	-	-	+	+	-	+	+	-	-	0.36	0.790	0.66
20	+	-	+	+	-	-	-	-	+	-	+	-	+	+	+	2.04	6.167	0.96

where, X_1 represents whey, X_2 represents Na_2HPO_4, X_3 represents casein , X_4 represents glycine, X_5 represents ammonium nitrate, X_6 represents Hy-soy, X_7 represents $MgSO_4$, X_8 represents mannitol, X_9 represents urea , X_{10} represents fructose, X_{11} represents yeast extract, X_{12} represents glycerol, X_{13} represents ammonium sulphate, X_{14} represents meat peptone and X_{15} represents sugar cane molasses.

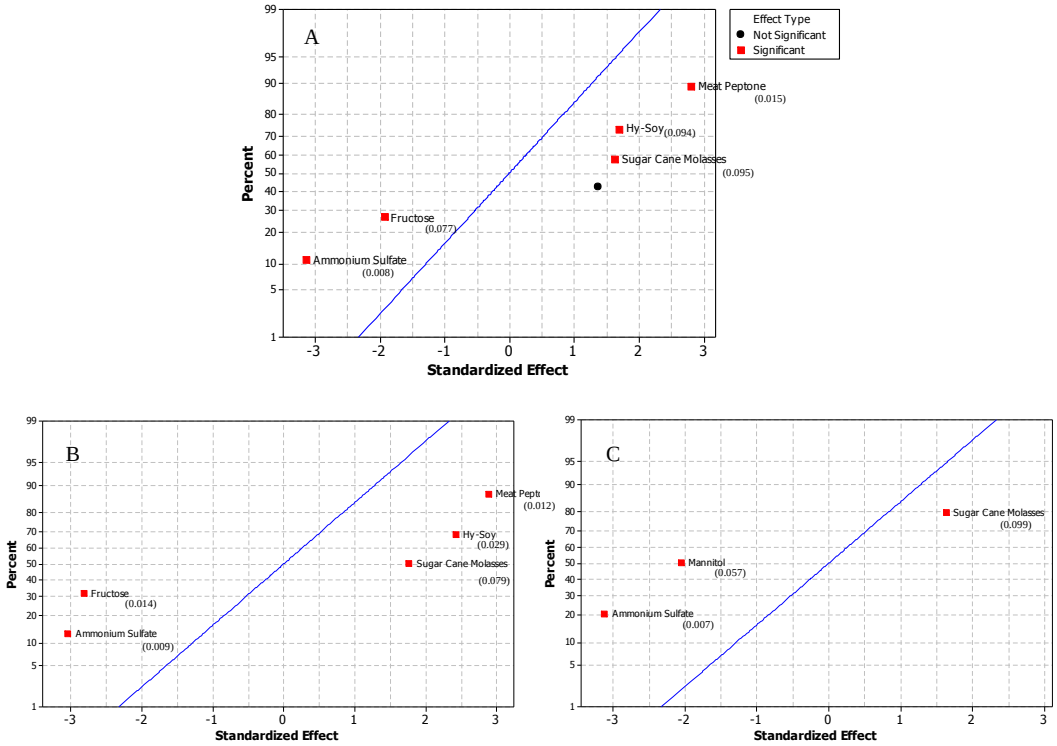

Figure 5.2: Normal probability plot of the standardised effect to show the significant of each factor on each response for the 2nd level PB experiment for isolate Hus-27 (A) EPA yield (mg/g), (B) EPA concentration (mg/l) and (C) EPA %.

Sugar cane molasses, meat peptone and Hy-soy were found to be the most significant factors augmenting the ability of isolate Hus-27 to produce EPA in all calculated responses (Figure 5.2) and they were used to build a potential production medium for maximising the productivity of isolate Hus-27.

The significant positive effect of peptone on the ability of isolates Hus-27 and 66 to produce EPA, the significant positive effect of Hy-soy on EPA yield and the significant inhibition of EPA percentage by mannitol were common features between the two tested isolates.

Ammonium sulphate showed a significant negative effect on all calculated responses for isolate Hus-27.

5.2 Optimising the production media for isolate Hus-27

Three media components were chosen as a production medium recipe including, Hy-soy, sugar cane molasses and peptone as they showed the most positive significant effect on the EPA production.

The statistical model was constructed by applying a Central Composite Design (CCD) matrix (Table 5.6). CCD reveals the interactions and the optimum medium composition for EPA production.

Table 5.6: Central Composite Design of variables (in coded units) with EPA production responses

Run	Variables			Responses		
	Peptone	Sugar cane molasses	Hy-soy	EPA yield (mg/g)	EPA conc. (mg/l)	EPA %
1	-1	1	1	1.155	7.16	2.99
2	0	0	0	1.043	1.54	1.32
3	1	1	-1	2.836	12.40	6.35
4	0	0	0	0.912	0.65	1.45
5	0	2	0	2.751	9.78	2.54
6	0	0	2	1.094	7.83	2.02
7	0	0	0	1.254	1.35	0.98
8	-1	-1	1	0.641	3.74	1.65
9	-2	0	0	0.707	2.12	1.67
10	0	0	0	0.876	1.12	1.54
11	0	0	0	0.876	1.32	1.21
12	-1	1	-1	0.559	2.93	1.30
13	1	1	1	4.256	4.05	5.75
14	1	-1	1	0.665	0.68	1.69
15	0	0	0	0.960	0.51	1.73
16	-1	-1	-1	1.449	1.84	2.69
17	0	-2	0	0.281	1.15	0.49
18	1	0	0	0.771	3.64	1.55
19	1	-1	-1	0.831	3.54	2.69
20	0	0	-2	0	0	0

With 90% level of confidence any factor with a P-value < 0.1 is considered as significant (the full ANOVA table is displayed in Appendix B.6).

The main effect of sugar cane molasses was found to be statistically significant on all EPA calculated responses, where the P-values were 0.0005, 0.0018 and 0.0453 for EPA yield, concentration and percentage, respectively. In addition, sugar cane molasses was involved in a number of significant interactions as its interaction with peptone was found to be significant on EPA yield (P-value 0.0022) and EPA percentage (P-value 0.0447). Also its interaction with Hy-soy was significant on EPA

yield (P-value 0.0827). The quadratic effect of sugar cane molasses was found to be significant on EPA yield only (P-value 0.0317).

Peptone showed a significant effect on EPA yield (P-value 0.0390), in term of main effect, but it was not significant for the other calculated responses at a 90% level of confidence. At a slightly lower level of confidence the linear effect of peptone could be significant (P-value, 0.1302). Peptone was involved in a significant interaction but its quadratic effect was found to have a negligible effect on all the calculated responses.

The main effect of Hy-soy had an insignificant effect on all EPA calculated responses at the 90% level of confidence, but its main effect on EPA yield was significant at a lower level of confidence (P-value, 0.1834). In addition to a significant interaction with sugar cane molasses on EPA yield (P-value 0.0827), its quadratic effect on EPA concentration was also found to be statistically significant (P-value, 0.0934).

The ANOVA showed that the multiple correlation coefficients (R^2) was 0.943. This is an estimate of the fraction of overall variation in the data accounted by the model and so the model is capable of explaining 94.3%

The Model F-value of 7.47 and P-value of 0.0013 when the response was EPA yield (mg/g) implies the model is significant. There is only a 0.13% chance that a "Model F-Value" this large could occur due to noise. Even thought the model was significant the lack of fit was significant with a P-value of 0.0014.

This statistical analysis indicated that, the produced ANOVA table was trustable and the significant term was significant due to its effect not due to noise. Unfortunately, the significant lack of fit indicated that, the produced polynomial model could not be used to predict the optimum combination, and implies that the overall mean is a better predictor of the response than the current model.

The same observation was found with the other calculated responses, EPA concentration and percentage. As a result the optimum media combination for each responses was estimated from the CCD table (Table 5.6). As the main target of this research was optimising the EPA production, the production medium for isolate Hus-27 was determined to be the medium combination in trial number 13 in Table 5.6 with 6 g/l sugar cane molasses, 10 g/l Hy-soy and 10g/l peptone achieving 4.25 mg/g EPA yield and 4 mg/l representing 6% of the total fatty acids.

5.3 Chapter summary

In this chapter a large number of different media components were investigated and tested to choose the most promising compounds which may lead to the development of a potential production medium to maximise EPA production by the Egyptian isolate Hus-27.

Hus-27 was isolated from a temperate region (Red Sea) and was identified as *Pseudoaltermona*s with a high similarity to *Pseudoaltermona*s *marina* on the basis of 16S phylogenetic analysis.

The potential production medium contained sugar cane molasses which is a by-product of sugar cane processing. As the result showed the feasibility of using sugar cane molasses as a substrate for a bacterial fermentation process to produce PUFA, the next step beyond this research would be testing the productivity of isolate when grown on sugar cane molasses to find the optimum cultivation procedure for maximising the productivity on such a cheap substrate.

Prior to optimisation the isolate was capable of producing 0.6 mg/g of EPA representing 1.2% of the total fatty acids. After subjecting the isolate to the suggested three step optimisation strategy, where PB was applied as a powerful tool to identify the significant effect of different variables, while a subsequent CCD was able to determine the optimal values for the individual variables, the amount increased to 4.25 mg/g EPA representing 6% of the total fatty acids (more than 3-times increase in the calculated responses). This increase was obtained via manipulating the medium composition using statistical DOE techniques as a useful tool for process optimisation, although the underlying mechanisms by which these media components affected EPA production still remain unrevealed.

In the next chapter, the effect of different media components on the ability of isolate 717 to produce EPA was addressed.

Chapter 6-Screening and optimising the media components for isolate 717

In this chapter a sequential methodology combining three levels of DOE to enhance EPA production by isolate 717. A screening level by Plackett-Burman fractional factorial designs to screen different media components. A Plackett-Burman design of experiment was applied for the initial screening to test the significant effect of each variable on the calculated response, and allowed the fitting of a first-order model to the data. Subsequently a Central Composite Design was employed to optimise the level of each chosen variables and allowed the fitting of a second-order model to the data

A chemically defined medium was created to explore the effect of C/N ratio on the growth and productivity of isolate 717.

6.1 Productivity of isolate 717

Isolate 717 was considered to be the highest EPA producer based on initial screening (section 3.1), with the ability to produce approximately 9 mg EPA/g dry weight representing 7.6 % of the total fatty acid content, prior to optimisation. In addition to EPA, isolate 717 was able to produce traces of DHA. To confirm the ability to produce these compounds, the mass spectra of the extracted EPA and DHA were examined (Figure 6.1 and Figure 6.2).

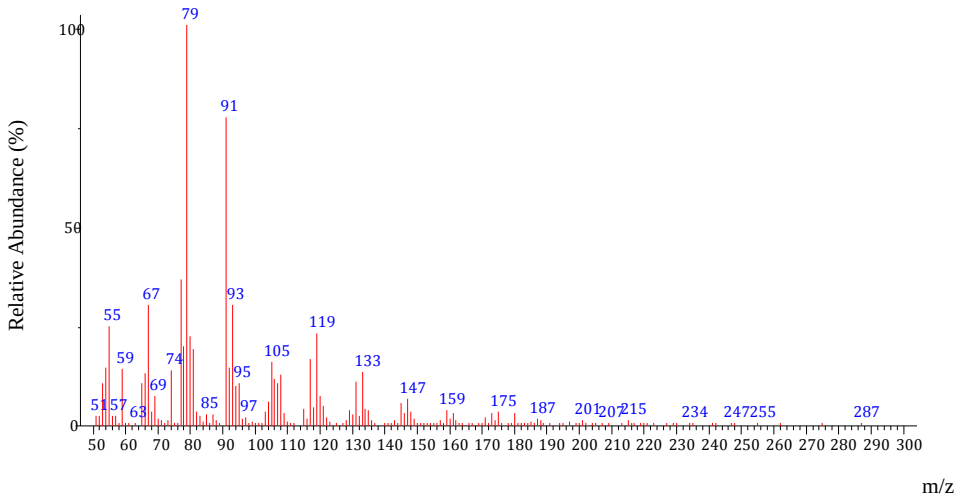

Figure 6.1: Mass spectrum for the produced EPA by isolate 717

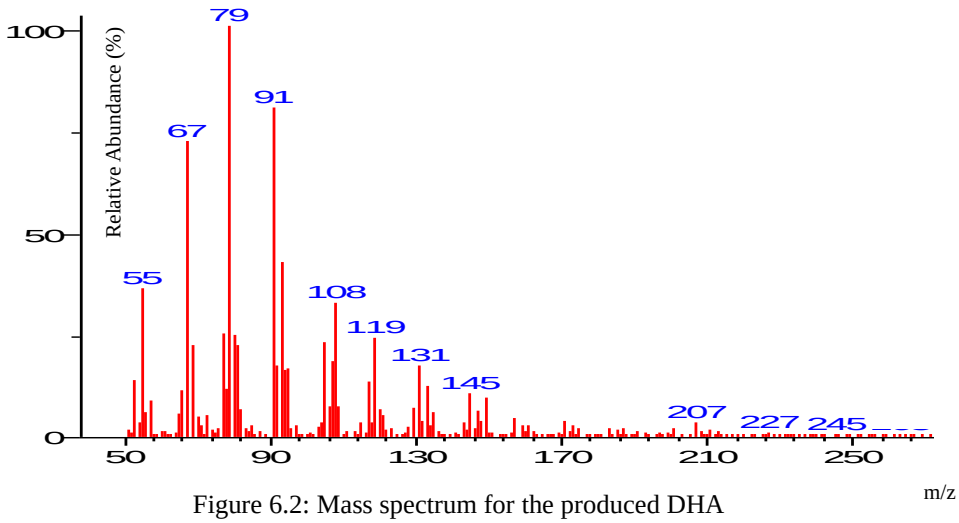

Figure 6.2: Mass spectrum for the produced DHA

For a long time, it was believed that microorganisms are unable to produce EPA and DHA at the same time as isolated PUFA producers were found to be either EPA (Bowman *et al.*, 1997; Nichols *et al.*, 1997) or DHA producers (Delong and Yayanos, 1986; Hamamoto *et al.*, 1995). Even if some bacteria were found to be able to produce both PUFAs at the same time, the DHA was considered as the dominant one, while EPA was the minor one (Yano *et al.*, 1998). Isolate 717 was confirmed to be an EPA and DHA producer with EPA as the dominant product.

The fact that isolate 717 is producing only traces of DHA could be considered an advantage from the downstream processing perspective as the minute quantities of DHA produced by the isolate could avoid the potential problem of separating DHA from EPA (Cao *et al.*, 2008).

6.2 Screening the medium components for isolate 717

As described in section 2.13, forty three different potential media components were screened by dividing them into four different Plackett-Burman designs to investigate the most significant factors affecting EPA production.

6.2.1 First Plackett-Burman Design

The experiment matrix and responses are summarised in Table 6.1 and the full statistical analysis is presented in Appendix C.1.

Table.6.1: The matrix and the responses for the 1st Plackett-Burman design for isolate 717

Run	Variables																Responses		
	X_1	X_2	X_3	X_4	X_5	X_6	X_7	X_8	X_9	X_{10}	X_{11}	X_{12}	X_{13}	X_{14}	X_{15}	X_{16}	EPA yield (mg/g)	EPA conc. (mg/l)	EPA %
1	-	+	-	+	-	-	-	-	+	-	+	-	+	+	+	+	0.93	1.41	1.35
2	+	+	-	+	+	+	-	-	+	+	-	+	+	-	-	-	0.93	1.01	1.17
3	+	-	+	+	-	-	-	+	-	+	-	+	+	+	+	-	0	0	0
4	-	-	+	-	-	+	+	+	+	-	-	+	+	-	+	+	1.53	3.18	2.16
5	+	-	-	-	-	+	-	+	+	+	+	-	-	+	+	-	1.27	1.42	1.33
6	+	+	+	+	-	-	+	+	-	+	+	-	-	-	-	+	0.13	0.04	0.91
7	+	-	-	-	+	-	+	-	+	+	+	+	-	-	+	+	1.81	1.83	1.95
8	+	+	+	-	-	-	+	-	+	-	+	+	+	+	-	-	4.11	10.03	6.04
9	+	-	+	+	+	+	-	-	-	-	+	-	+	-	+	+	0.77	0.34	2.36
10	-	-	+	-	+	+	+	-	-	+	+	-	+	+	-	-	0.29	0.08	1.23
11	-	-	+	+	+	-	-	+	+	-	+	+	-	-	-	-	5.99	28.32	5.51
12	-	-	-	+	+	-	+	+	+	+	-	-	+	+	-	+	1.05	1.07	0.99
13	+	+	-	-	+	-	+	+	-	-	-	-	+	-	+	-	0.64	0.17	1.31
14	+	+	+	-	+	+	-	+	+	-	-	-	-	+	-	+	2.26	3.65	2.45
15	-	+	-	-	-	+	-	+	-	+	+	+	+	-	-	+	0	0	0
16	-	+	+	-	+	-	-	-	-	+	-	+	-	+	+	+	0.11	0.04	0.46
17	+	-	-	+	-	+	+	-	-	-	-	+	-	+	-	+	8.07	22.19	7.85
18	-	+	-	+	+	+	+	+	-	-	+	+	-	+	+	-	1.09	0.59	2.26
19	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	2.52	2.52	4.62
20	-	+	+	+	-	+	+	-	+	+	-	-	-	-	+	-	1.05	1.31	2.51

where, X_1 represents yeast extract , X_2 represents glucose , X_3 represents glycerol, X_4 represents soy bean flour, X_5 represents corn step liquor, X_6 glutamic acid, X_7 represents L-proline , X_8 represents ammonium acetate, X_9 represents whey, X_{10} is propanoic acid, X_{11} represents L-serine, X_{12} represents urea , X_{13} represents sucrose, X_{14} represents glycine, X_{15} represents citric acid and X_{16} represents fructose.

Although glucose is generally one of the most popular carbon sources, it showed a statistically significant negative effect on EPA productivity (P-values were 0.043, 0.083 and 0.040 on EPA yield, EPA concentration and percentage respectively) of the marine isolate 717. A similar effect of glucose was reported for isolate 66. This

observation is in line with the observation on an EPA producing recombinant *E. coli* which lost its ability to produce EPA when glucose was added to the medium. This was argued to be due to suppression of promoter activity (Lee *et al.*, 2008a).

Glucose was found to suppress growth and productivity of the isolates under investigation. This negative impact could be due to the so called "glucose-shock" phenomenon. The glucose-shock phenomenon is the reduction of the growth and productivity of microorganisms from the addition of glucose to the culture medium especially in the presence of low nitrogen and phosphorus. This phenomenon was previously reported for some marine isolates belonging to the genus *Vibrio* and was also reported on the addition of sucrose, fructose, and maltose to starvation media. This could be due to the fact that these sugars are assimilated via a common metabolic pathway (Shiba *et al.*, 1995). This phenomenon was not detected with arabinose and xylose (Bag, 1974).

Shiba *et al.* (1995) suggested that, the reason of the phenomenon arises from a nutritional imbalance between available carbohydrate and nitrogen and phosphate in the cultivation media. In addition they stated that "The difference in sensitivity to glucose shock may be ecologically significant with respect to distribution and/or selection of strains of bacterial species in the marine environment".

As with isolates 66 and Hus-27, citric acid and propionic acid showed a statistically negative effect on EPA production by isolate 717, indicating that they may suppress the metabolic pathway for EPA biosynthesis.

For isolate 717, propionic acid was found to be the most significant factor affecting all EPA responses in a negative way. This result contradicted the result observed for *Shewanella gelidimarina* ACAM 456T where it was found to be the best carbon source, significantly enhancing the EPA yield for the investigated isolate (Nichols *et al.*, 1997).

The negative effect of ammonium acetate on EPA production by isolate 717 is in line with the observation reported by Chi *et al.* (2007), where ammonium acetate was found to have a negative effect on DHA production by the microalga *Schizochytrium limacinum*. The same negative effect of ammonium acetate, especially on EPA percentage, was also seen on isolates 66 and Hus-27.

Yeast extract showed a non-significant effect on all the tested responses for isolate 717 which was in line with the effect of the same variable on the ability of Shewanella sp. NJ136 to produce EPA (Botao *et al.*, 2007). This result could be

advantageous due to the high cost of using yeast extract as a nitrogen source in production media.

Although sucrose showed a significant negative effect on the ability of 717 to synthesise EPA and a non-significant effect on the previously tested isolates 66 and Hus-27, it was reported to enhance the ability of the moss *Physcomitrella patens* in producing EPA (Chodok *et al.*, 2010).

Urea was found to be a promising nitrogen source for EPA production for isolates 717, Hus-27, 66 as well as for the fungus *Mucor recurvus* (Li *et al.*, 2008a), where urea was identified as the best nitrogen source of the five different nitrogen sources tested. The same effect of urea was reported for *Phaeodactylum tricornutum* (Yongmanitchai and Ward, 1991).

L-proline was found to have a significant positive effect not only for the EPA percentage but also for EPA yield by isolate 717. This is in line with the result obtained *Shewanella gelidimarina* ACAM 456T, where Nichols *et al.* (1997) tested the effect of L-proline, reporting that L-proline was the most significant factor affecting the EPA percentage in the total fatty acids.

L-serine showed no significant effect on the ability of isolate 717 to produce EPA which is the same observation for *Shewanella gelidimarina* ACAM 456T (Nichols *et al.*, 1997).

For isolate 717, glucose showed a significant negative impact on EPA production while urea showed the opposite effect on the same response. A contrary result was reported for the same variables on the ability of *Shewanella* sp. NJ136 to produce EPA, where glucose resulted in the highest amount of EPA when used as a carbon source while urea resulted in the lowest amount of EPA when used as a nitrogen source (Botao *et al.*, 2007). This contradictory result was obtained even though both isolates belong to the same genus (*Shewanella).*

Only two of the tested factors were considered further in the screening based on these results, namely urea and L-proline.

6.2.2 Second Plackett-Burman Design

A second Plackett-Burman experimental design was used to screen the significant effect of the individual variables (ten variables) that affect EPA production by isolate

717. The matrix and responses achieved are summarised in Table 6.2 and the full statistical analysis is presented in Appendix C.2.

Table 6.2: The matrix and the responses for the 2nd Plackett-Burman design for isolate 717

Run	Variables										Responses	
	X_1	X_2	X_3	X_4	X_5	X_6	X_7	X_8	X_9	X_{10}	EPA yield (mg/g)	EPA conc. (mg/l)
1	-	-	-	+	+	+	-	+	+	-	14.45	29.26
2	+	+	-	+	+	-	+	-	-	-	2.25	1.35
3	-	+	+	+	-	+	+	-	+	-	0.00	0.00
4	-	-	-	-	-	-	-	-	-	-	2.17	3.64
5	-	+	-	-	-	+	+	+	-	+	1.87	0.84
6	+	-	+	-	-	-	+	+	+	-	1.52	0.68
7	-	+	+	-	+	-	-	-	+	+	0.67	0.16
8	+	+	+	-	+	+	-	+	-	-	0.67	0.48
9	+	-	+	+	-	+	-	-	-	+	0.00	0.00
10	+	+	-	+	-	-	-	+	+	+	17.07	23.90
11	+	-	-	-	+	+	+	-	+	+	0.87	0.46
12	-	-	+	+	+	-	+	+	-	+	1.89	2.46

where, X_1 represents palmitic acid , X_2 represents sugar cane molases, X_3 represents linseed oil, X_4 represents ammonium nitrate ,X_5 tryptone, X_6 represents pea nut oil, X_7 represents ethylene glycol , X_8 represents Hy-soy , X_9 represents fish peptone and X_{10} represents sodium acetate.

The positive effect of ammonium nitrate on EPA production by isolate 717 was previously spotted on isolate Hus-27 and similar to the effect of nitrate on Mortierella alpine as it was preferred for maximum PUFA production over urea and ammonium (Jang *et al.*, 2005).

Also, the positive effect of Hy-soy was previously detected on isolates 66 and Hus-27. Peptone, from enzymatic digestion of salmon, was found to enhance the EPA productivity of isolates 717 and 66 but not isolate Hus-27.

The significant negative effect on EPA production upon the addition of palmitic acid, ethylene glycol and linseed oil to the cultivation medium of isolates 717 and 66 was a common feature.

The incorporation of linseed oil in the cultivation media of isolate 717 was found to have a statistically significant inhibitory effect on EPA production (P-value, 0.000 and 0.001). Linseed oil stimulated EPA production when used as a carbon source in the growth media for *Mortierella alpine*. The enhancement of EPA production by using linseed oil may be due to the ability of *Mortierella alpine* to convert arachidonic

acid (via the n-6 route) and α-linolenic acid (via the n-3 route) to EPA at the same time (Shimizu *et al.*, 1989), while isolate 717 may lack this conversion ability.

Jang *et al.* (2005) found that, the addition of different oils, such as linseed, peanut and sunflower oils, enhanced the ability of microorganisms for PUFAs production. This observation contradicts with the result obtained not only with isolate 717, but also with isolates 66 and Hus-27.

Adding a free fatty acid as a potential precursor for EPA production was shown to inhibit its biosynthesis by isolate 717 where adding palmitic acid to the media, as a potential precursor for EPA production, significantly suppressed the EPA concentration (P-value, 0.017), while its effect on EPA yield was not statistically significant (P-value, 0.928). The addition of oleic acid to the production media of *Phaeodactylum tricornutum* showed a marked suppression on the biomass and the amount of EPA produced (Yongmanitchai and Ward, 1991). Adding free fatty acid to the cultivation media, as a potential precursor for PUFA biosynthesis, proved to have an inhibitory effect on all the investigated isolates, 66, Hus-27 and 717.

Although sugar cane molasses is rich in vitamins and nutrients, it did not show a significant effect on EPA yield (P-value, 0.864), and showed a significant negative effect on EPA concentration (P-value, 0.016) in the case of isolate 717. This carbon source was previously reported as a promising production media for industrial PUFA production (Li *et al.*, 2008a).

Nitrate was found to be the most positive significant factor affecting EPA production by isolate 717 and it was also reported to enhance the ability of *Phaeodactylum tricornutum* to produce EPA, increase from 20 to 90 mg/l (Yongmanitchai and Ward, 1991).

Three variables were chosen for the next screening step based on their statistically significant positive effects upon EPA production at the 90% level of confidence. These factors were ammonium nitrate (P-values 0.000 and 0.001), Hy-soy (P-values 0.000 and 0.001) and fish peptone (P-Values 0.000 and 0.001).

6.2.3 Third Plackett-Burman Design

The matrix and responses are summarised in Table 6.3 and the full statistical analysis is presented in Appendix C.3.

Table 6.3: The matrix and the responses for the 3rd Plackett-Burman design for isolate 717

Run	Variables									Responses		
	X_1	X_2	X_3	X_4	X_5	X_6	X_7	X_8	X_9	EPA yield (mg/g)	EPA conc. (mg/l)	EPA %
1	+	+	+	-	+	+	-	+	-	7.96	28.89	14.12
2	+	+	-	+	+	-	+	-	-	0.51	2.35	0.33
3	-	-	+	+	+	-	+	+	-	5.24	39.23	2.54
4	-	+	-	-	-	+	+	+	-	12.61	38.52	1.55
5	-	-	-	+	+	+	-	+	+	10.66	92.48	2.56
6	+	-	-	-	+	+	+	-	+	10.52	38.08	14.65
7	-	-	-	-	-	-	-	-	-	1.93	3.06	4.49
8	-	+	+	-	+	-	-	-	+	6.04	13.25	9.18
9	+	-	+	+	-	+	-	-	-	11.25	65.35	2.51
10	-	+	+	+	-	+	+	-	+	11.52	77.28	3.02
11	+	-	+	-	-	-	+	+	+	10.21	33.14	8.88
12	+	+	-	+	-	-	-	+	+	6.99	29.34	2.42

where, X_1 represents meat peptone, X_2 represents Ammonia, X_3 represents lactose, X_4 represents ground sesame , X_5 represents ammonium sulphate, X_6 represents casein, X_7 represents maltose , X_8 represents vegetable peptone and X_9 represents mannitol.

Casein, mannitol and vegetable peptone were found to have a statistically significant positive effect on the amount of EPA in terms of yield (mg/g) (P-values, 0.002, 0.034 and 0.090, respectively) and concentration (mg/l) (P-values, 0.001, 0.012 and 0.051, respectively). Two of these variables were chosen for the next step of optimisation, casein and mannitol, as they proved to be more significant than vegetable peptone.

Meat peptone and ammonium sulphate were found to have a significant positive effect on the EPA percentage of the total fatty acids (P-values 0.067 and 0.077, respectively), ammonium sulphate showed no effect on the ability of the fungus *Thraustochytrium aureum* to produce DHA (Iida *et al.*, 1996). Meat peptone was chosen for the next step of optimisation, but not ammonium sulphate as it showed a significant negative effect on EPA yield (mg/g). The significant positive effect of ammonium sulphate on only EPA percentage was previously reported for isolate Hus-27.

Ground sesame was found to have a significant negative effect on EPA percentage of total fatty acids but a positive effect on EPA concentration (mg/l). This result indicates that ground sesame may inhibit the biosynthesis of EPA, but enhance the amount of biomass. This effect was similar to that for isolate Hus-27 on the same calculated responses. The significant negative effect of ground sesame on the ability to synthesise EPA, could be due to the presence of sesamin analogues that inhibit the Δ5–desaturase which is required in PUFA biosynthesis (Certik and Adamechova, 2009).

Casein, a phosphorus-protein usually found in milk, supplies amino acids, carbohydrates, and two inorganic elements (calcium and phosphorus). In addition it is rich in L-proline residues. These components, especially phosphorus and L-proline could be the reason for the significant positive effect on EPA production. Similar to isolate 717, casein was found to be the most significant variable enhancing EPA productivity by isolate Hus-27.

The positive effect of mannitol and meat peptone was similar to that observed for isolates 66 and Hus-27.In addition, the significant negative effect of ammonia and ground sesame on EPA production was found to be similar for both isolates 66 and 717.

6.2.4 Fourth Plackett-Burman Design

The matrix and responses are summarised in Table 6.4 and the full statistical analysis is presented in Appendix C.4.

Table 6.4: The matrix and the responses for the 4th Plackett-Burman design for isolate 717

Run	Variables								Responses		
	X_1	X_2	X_3	X_4	X_5	X_6	X_7	X_8	EPA yield (mg/g)	EPA conc. (mg/l)	EPA %
1	+	-	+	-	-	-	+	+	3.53	9.01	13.55
2	+	+	-	+	-	-	-	+	8.76	5.32	7.06
3	-	+	+	+	-	+	+	-	7.52	9.93	10.43
4	+	+	-	+	+	-	+	-	3.67	7.25	5.91
5	-	-	-	-	-	-	-	-	0.48	2.61	1.80
6	-	+	-	-	-	+	+	+	5.37	13.04	7.36
7	+	-	+	+	-	+	-	-	0.94	0.69	9.34
8	-	-	+	+	+	-	+	+	0.22	3.14	4.65
9	-	+	+	-	+	-	-	-	3.75	0.95	6.21
10	+	+	+	-	+	+	-	+	4.03	5.77	6.45
11	+	-	-	-	+	+	+	-	0.26	6.51	1.26
12	-	-	-	+	+	+	-	+	1.54	1.17	2.09

where, X_1 represents NH_4Cl, X_2 represents Na_2HPO_4, X_3 represents KCl , X_4 represents $CaCl_2$, X_5 represents $Fe_2(SO_4)_3$, X_6 represents $MgCl_2$, X_7 represents $MgSO_4$, X_8 represents NaCl.

Although isolate 717 was able to grow well in the complete absence of all of the tested metal ions, i.e. to grow in the presence of sodium chloride and yeast extract only, the amount of EPA produced decreased dramatically (trial number 5), suggesting that these metal ions could have a significant role in the biosynthesis of EPA.

The effect of $CaCl_2$ on EPA produced by isolate 717 was negative and statistically significant especially on EPA yield (mg/l), which is similar to the effect of the same variable on the isolate SCRC-2738, where Akimoto *et al.* (1991) reported that the addition of $CaCl_2$ to SCRC-2738 media sharply decreased the amount and the percentage of EPA although it showed a positive effect on the amount of biomass produced.

Iron showed a significant negative effect on all EPA calculated responses for isolate 717. This result was not completely consistent with the result obtained by Chiou *et al.* (2001) as iron ions were found to enhance PUFA production when added to the cultivation media of *Marchantia polymorpha*. The negative effect of iron on EPA production was found to be consistent among all the tested isolates.

Ammonium chloride showed no significant effect on any of the EPA calculated responses for isolate 717 and the same effect was reported on *Phaeodactylum tricornutum* (Yongmanitchai and Ward, 1991). The same lack of a significant effect

of ammonium chloride was also reported on isolates Hus-27 and 66. For the diatom *Nitzschia laevis*, the addition of ammonium chloride in the growth media led to a remarkable decrease in the amount of EPA produced (Cao *et al.*, 2008).

The significant effect of $MgSO_4$ on isolate 717 was found to be in terms of EPA concentration (mg/l) and is mostly related to increasing biomass rather than increasing the EPA itself within the cells. $MgSO_4$ showed a positive effect on the amount of biomass produced without any significant effect on the amount of EPA produced by *Phaeodactylum tricornutum* (Yongmanitchai and Ward, 1991).

Effect of Na_2HPO_4 on the ability of isolates 717, Hus-27 and 66 to produce EPA was in line with the same positive effect of Na_2HPO_4 on *Phaeodactylum tricornutum* to produce EPA (Yongmanitchai and Ward, 1991). This positive effect was suggested to be due to the effect of the inorganic phosphate.

The main difference between 717 on one side and 66 and Hus-27 from the other side, in response to effect of metal ions, was the effect of some metal chlorides. Chloride was found to have a statistically significant negative effect on EPA concentration and percentage produced by isolate Hus-27 ($Cacl_2$, KCl and $MgCl_2$), and EPA percentage of the total fatty acid produced by isolate 66 (NH_4Cl). But for isolate 717, NaCl and $MgCl_2$ had a positive significant effect on EPA concentration and KCl was the most significant variable enhancing the percentage of EPA. This difference could be due to the fact that, 66 and Hus-27 were EPA producers, while 717 was able to produce EPA and DHA (even though as traces).

Two metal ions were investigated further based on the results reported in this section: Na_2HPO_4 as it demonstrated a significant positive effect on EPA production, and NaCl as isolate 717 cannot grow in its complete absence. The same effect of sodium chloride was previously reported by Yoon *et al.* (2004), as *Shewanella gaetbuli* could not grow in the complete absence of sodium chloride.

The effect of the other metal ions, which were found to affect the ability to produce EPA, was also tested in a separate CCD experiment, in the presence of the minimal amount of yeast extract used as a basal medium, The low concentration of yeast extract would eliminate the potential masking of the effect of these ions due to high carbon and/or nitrogen concentrations in the medium (Section 6.4).

6.2.5 Second level of Plackett-Burman Design

In this section the media components with a statistically significant positive effect on EPA production by isolate 717 in the first level of PB were investigated. 10 different variables, namely L-proline, casein, fish peptone, Na_2HPO_4, ammonium nitrate, urea, Hy-soy, meat peptone, mannitol and NaCl were tested.

The matrix and responses are summarised in Table 6.5 and the full statistical analysis is presented in Appendix C.5.

Table 6.5: The matrix and the responses for the 2nd level of Plackett-Burman design

Run	Variables										Responses		
	X_1	X_2	X_3	X_4	X_5	X_6	X_7	X_8	X_9	X_{10}	EPA yield (mg/g)	EPA conc. (mg/l)	EPA %
1	-	+	+	+	-	+	+	-	+	-	26.47	15.28	11.62
2	+	-	-	-	+	+	+	-	+	+	1.21	2.11	6.64
3	-	+	+	-	+	-	-	-	+	+	5.13	9.11	7.91
4	+	-	+	+	-	+	-	-	-	+	1.19	2.34	7.12
5	+	-	+	-	-	-	+	+	+	-	3.02	13.71	11.47
6	-	-	-	+	+	+	-	+	+	-	1.55	6.21	5.53
7	-	-	+	+	+	-	+	+	-	+	3.97	9.89	13.43
8	-	-	-	-	-	-	-	-	-	-	2.23	2.35	5.18
9	+	+	+	-	+	+	-	+	-	-	4.63	5.97	9.78
10	+	+	-	+	+	-	+	-	-	-	26.17	16.35	15.39
11	-	+	-	-	-	+	+	+	-	+	2.41	7.94	14.44
12	+	+	-	+	-	-	-	+	+	+	3.14	12.41	10.44

where, X_1 represents L-proline, X_2 represents casein, X_3 represents fish peptone, X_4 represents Na_2HPO_4, X_5 represents ammonium nitrate, X_6 represents urea, X_7 represents Hy-soy, X_8 represents meat peptone, X_9 represents mannitol and X_{10} represents NaCl.

The conditions and medium composition required for the maximum EPA production by isolate 717 appears to be dissimilar to that required for maximum growth. The highest amount of EPA (mg/g) was obtained in trials number 1 and 10 whereas the growth was at its minimum (approximately 0.6 g/l), while trials number 5 and 6 showed relatively low amounts of EPA with the biomass reaching its maximum (approximately 4 g/l). As a fraction of the total fatty acids, the highest EPA% was found to be independent of the biomass concentration. The highest EPA percentage

values were obtained in trials 10 and 11 with high biomass in trial number 11 (approximately 3.2 g/l) and low biomass in trial number 10 (approximately 0.6 g/l). These results indicated that the optimum conditions for maximum growth could be non optimum for EPA production and vice versa,

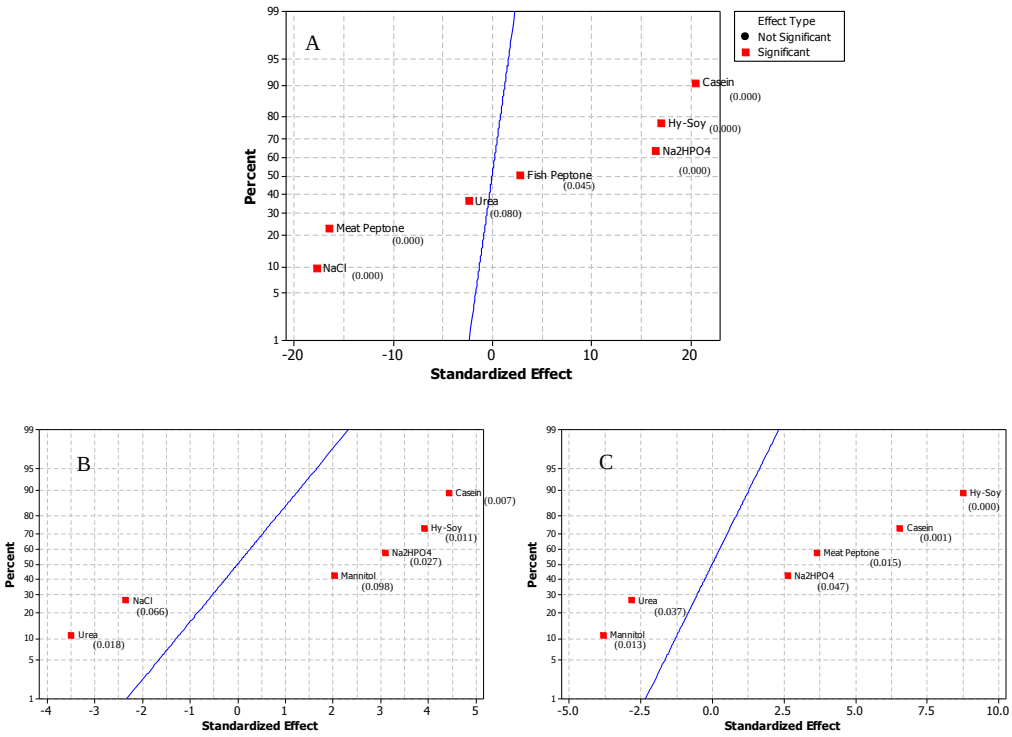

Figure 6.3: Normal probability plot of the standardised effect to show the significant of each factor on each response for isolate 717 where, (A) EPA yield (mg/g), (B) EPA concentration (mg/l) and (C) EPA %.

Casein, Hy-soy and Na_2HPO_4 were found to have a statistically significant positive effect on the ability of isolate 717 to produce EPA either expressed as concentration, yield or percentage of total fatty acid.

L-proline and ammonium nitrate showed no significant effect on the calculated responses in this PB design of experiments. Casein (which is rich in L-proline residues) could be masking the effect of L-proline on EPA production.

A low concentration of NaCl (10 g/l) was preferred by isolate 717 for EPA production. A similar observation was reported with the EPA producer diatom *Phaeodactylum tricornutum* where low sodium chloride concentration (1 g/l) was preferred for higher EPA percentage although, a relatively higher concentration of NaCl (12 g/l) was required to give the highest EPA concentration (Yongmanitchai and

Ward, 1991). The enhancement of EPA production by lowering the concentration of NaCl contradicts the results previously reported by Lee *et al.* (2008b), as increasing the amount of NaCl from 1 to 5% (w/v) lead to a remarkable increase in cellular EPA content in *Shewanella* sp KMG427.

For isolate 717, 10 g/l of sodium chloride was determined as the lowest concentration which supports growth as lower concentrations led to a dramatic decrease in growth, to the extent that the amount of growth was insufficient for EPA extraction. Total growth inhibition was reached when the sodium chloride concentration dropped to 6 g/l.

Mannitol, urea and meat peptone were found to have a negative impact on at least one of the calculated responses. The significant inhibition of mannitol on EPA percentage was found to be a common feature among all the investigated isolates (717, 66 and Hus-27).

After two levels of screening, casein, Hy-soy and Na_2HPO_4 were selected as production medium components for optimum EPA production by isolate 717. Casein and Hy-soy were found to be the preferred sources of carbon and nitrogen for EPA production while Na_2HPO_4 was added as a source of phosphate required for EPA production. Low concentrations of NaCl used in the production medium are also beneficial from the large scale manufacturing point of view as it limits any potential processing issues due to high salinity.

The three production medium components were investigated further using another statistical design of experiment to determine the optimum concentrations, testing the interactions among them and to create a predictive mathematical model.

6.3 Optimising the production medium for isolate 717

So far, complex media are most frequently used in the fermentation industry. Usually, complex media for production of new metabolites are used, since in most cases little is known about the microbe or the biosynthesis of the desired compounds. Creating production media is considered to be a significant step in product evaluation studies.

Three media components were chosen to create a production medium recipe including, Hy-soy, casein and Na_2HPO_4 as they showed the most significant effect on EPA production.

Plackett-Burman design of experiment is a useful screening technique which can be used to compare the relative effect of different variables for a specific response with a minimum number of trials, but ignoring the interaction among these variables. To reveal the interactions and the optimum combination for the EPA production media, a Central Composite Design (CCD) experiment was performed (Table 6.6).

Table 6.6: Central Composite Design of variables (in coded units) with EPA productions as responses for isolate 717

Run	Variables			Responses		
	Casein	Hy-soy	Na_2HPO_4	EPA yield mg/g	EPA conc. (mg/l)	EPA %
1	0	-2	0	29.714	72.47	17.13
2	+1	+1	+1	18.26	65.38	16.12
3	-1	-1	-1	26.32	49.03	18.71
4	-1	+1	-1	19.76	42.63	15.19
5	-1	+1	+1	27.01	74.86	17.21
6	0	0	-2	32.37	86.74	18.07
7	+1	-1	-1	15.04	66.46	17.16
8	0	0	0	34.11	72.61	15.02
9	-1	-1	+1	12.46	43.62	18.06
10	0	+2	0	0	0	0
11	0	0	0	30.03	70.12	14.21
12	0	0	0	28.22	65.99	15.87
13	+1	-1	+1	32.71	88.73	15.02
14	0	0	0	29.91	69.22	13.56
15	+2	0	0	3.89	5.457	9.68
16	-2	0	0	18.14	44.53	17.71
17	+1	+1	-1	4.45	13.11	9.11
18	0	0	0	31.92	68.01	14.05
19	0	0	0	31.47	70.65	14.27
20	0	0	+2	23.12	61.21	11.57

With a 90% level of confidence any factor with a P-value < 0.1 was considered significant (ANOVA table displayed in Appendix C.6). The calculated P-values for each response reveal that in addition to the main effects, two way interactions and quadratic effects could have a significant effect on the ability to produce EPA.

For EPA yield (mg/g biomass), the main effect of all the variables was statistically significant with P-values for casein 0.0338, for Hy-soy 0.0532 and for Na_2HPO_4

0.0920. In addition to the main effect, the quadratic effect showed a statistically significant effect especially for casein (P-value of 0.0022) and Hy-soy (P-value of 0.0069), however the quadratic effect for Na_2HPO_4 was not significant (P-value of 0.4398).

The interactions between the variable were identified as statistically significant with the P-value of the interaction between casein and Hy-soy 0.0715 and the interaction between casein and Na_2HPO_4 0.0692, while the interaction between Hy-soy and Na_2HPO_4 was estimated as non-significant (P-value 0.4225).

The effect of casein on the amount of EPA as (mg/l media) was statistically significant both as a main effect (P-value 0.0065) and as quadratic effect (P-value 0.0131). In addition to casein, Hy-soy showed the same effect with the main effect P-value 0.0848 and the quadratic P-value, 0.0715. Also, the interaction between casein and Hy-soy showed a significant effect on EPA concentration (P-value 0.0470). Na_2HPO_4 did not show any significant effect on EPA concentration (mg/l) neither as a main effect, quadratic effect nor as two way interaction, suggesting that Na_2HPO_4 is involved in enhancing the amount of EPA production rather than the biomass.

This hypothesis was supported by the observation that Na_2HPO_4 was found to have a significant effect on EPA percentage as main effect (P-value, 0.0167) and quadratic effect (P-value, 0.0635). The same significant main effect was detected for casein where, the P-value of the main effect of 0.0497, while the quadratic effect was not significant (P-value 0.6938). Although Hy-soy did not show a statistically significant effect on the EPA percentage (P-value 0.8534), its quadratic effect was statistically significant (P-value 0.0200). None of the interactions between the tested variables were determined as significant.

These results indicated that casein has a significant effect in increasing the amount of EPA either as a fraction of total fatty acids (EPA %) or as yield (EPA mg/g). In addition, casein enhances growth leading to a high biomass and EPA at the same time (EPA mg/l).

Hy-soy was detected as significant in EPA enhancement as a part of the biomass (EPA mg/g) and the total amount of EPA (mg/l), but was not involved in the biosynthesis of EPA rather than the total fatty acids (EPA %).

Na_2HPO_4 was more significant, which was due to the effect of phosphate in EPA biosynthesis, on EPA responses (EPA % and mg/g) rather than enhancing the biomass.

For numerical optimisation, a second order polynomial order model was generated, using coded units, to predict the optimum combination for each response, equations 6.1 – 6.3.

$$EPA\ yield\ (mg/g).\ = 19.65 + 3.51\ A + 3.67\ B - 0.5\ 6\ C - 0.21\ AB + 0.098\ AC - 0.37\ A^2 - 0.31\ B^2 \text{---} \quad \text{(eq.6.1)}$$

$$EPA\ conc\ (mg/l).\ = 29.82 + 12.02\ A + 6.99\ B - 0.67AB - 0.78\ A^2 - 0.53\ B^2 \text{---} \quad \text{(eq.6.2)}$$

$$EPA\ \% = 22.80 - 0.44\ A + 0.1\ B - 0.5\ C + 0.031\ BC - 0.10\ B^2 + 0.012\ C^2 \text{---} \quad \text{(eq.6.3)}$$

Where A, B and C were casein, Hy-soy and Na_2HPO_4 respectively. By using the mathematical model and with the aid of the 3D surface response plots (Appendix C.7), the optimum combination for maximum EPA production was estimated and experimentally tested as summarised in the Table 6.7.

Table 6.7: The predicted and the actual values under the optimum medium component concentration for each process response

Target	**Media**			**Predicted**	**Actual**
	Casein (g/l)	Hy-Soy (g/l)	Na_2HPO_4 (g/l)	**Values**	**Values**
Maximise EPA yield (mg/g)	8.34	6.52	4.78	29.71	34.44 (± 1.1)
Maximise EPA conc. (mg/l)	9.08	7.83	3.95	80.41	95.32 (± 3)
Maximise EPA %	1.76	3.35	1.2	20.83	19.25 (±0.65)

The optimum medium composition for EPA production in terms of concentration and yield is nearly the same. However the medium required to increasie EPA percentage is different. Thus an additional optimisation was carried out to achieve a maximum productivity on terms of all (Table 6.8).

Table 6.8: The predicted and the actual values under the optimum medium combination for maximising EPA productivity in all terms for isolate 717

Media			Predicted Values			Actual Values		
Casein (g/l)	Hy-Soy (g/l)	Na_2HPO_4 (g/l)	EPA yield (mg/g)	EPA conc. (mg/l)	EPA %	EPA yield (mg/g)	EPA conc. (mg/l)	EPA %
6.31	4.25	2.2	26.65	71.72	16.6	32.51	75.45	18.2

6.4 Testing the effect of metal ions on the growth and productivity of isolate 717

Many of the metal ions were previously reported to have a significant effect on the biosynthesis of several metabolites (Schrader and Blevins, 2001). The main aim of this experiment was to test the effect of metal ions on growth and the ability to produce EPA by isolate 717. During the screening by Plackett-Burman, the effect of metal ions was tested and three of them were found to have a significant effect on EPA production. These three ions, in addition to sodium chloride, were taken further to an optimisation step via CCD (as described in section 2.13.2). These metals were Na_2HPO_4, $MgSO_4$ and KCl (the same effect was previously seen with isolate 66).

This experiment was carried out with the minimum amount of yeast extract as a basal medium as the presence of carbon or nitrogen may mask the effect of the metal ions either on growth or EPA productivity.

Table 6.9: Central Composite Design of variables (in coded units) with EPA productions as responses to test the effect of metal ions on EPA productivity by isolate 717

Run	**Variables**				**Responses**		
	Na_2HPO_4	KCl	NaCl	$MgSO_4$	EPA yield (mg/g)	EPA conc. (mg/l)	EPA %
1	0	0	0	-2	11.83	9.41	12.94
2	-1	-1	+1	-1	10.28	12.35	13.18
3	-1	+1	+1	+1	8.19	11.88	14.32
4	+1	-1	-1	+1	6.11	12.63	10.68
5	+1	+1	-1	+1	4.81	11.84	9.45
6	0	0	-2	0	2.76	1.52	4.98
7	-1	+1	-1	+1	9.49	13.25	13.49
8	-2	0	0	0	2.11	4.35	3.02
9	0	0	0	0	13.46	15.88	15.25
10	-1	-1	-1	+1	15.61	18.14	14.19
11	-1	-1	+1	+1	8.13	11.04	13.56
12	0	+2	0	0	6.82	9.11	10.36
13	+2	0	0	0	9.82	21.56	8.38
14	0	0	0	0	12.21	16.46	14.65
15	0	0	0	0	12.57	14.93	14.11
16	-1	+1	+1	-1	8.46	9.69	12.82
17	0	0	+2	0	3.99	8.86	2.76
18	+1	-1	+1	-1	7.31	13.43	14.04
19	0	0	0	+2	2.38	5.71	3.11
20	0	-2	0	0	8.71	14.42	14.59
21	+1	-1	+1	+1	6.65	17.72	15.58
22	0	0	0	0	12.71	15.37	15.35
23	+1	+1	-1	-1	9.91	10.75	16.88
24	+1	-1	-1	-1	8.056	6.42	13.65
25	0	0	0	0	13.28	14.91	14.61
26	0	0	0	0	13.53	13.73	15.28
27	-1	-1	-1	-1	6.98	19.44	13.31
28	-1	+1	-1	-1	9.29	9.12	11.36
29	+1	+1	+1	+1	3.51	10.79	9.13
30	+1	+1	+1	-1	9.76	16.45	12.55

The ANOVA table (Appendix C.8), indicated that Na_2HPO_4 is the only variable with main effect statistically significant on all EPA responses (P-values for EPA yield, concentration and percentage were 0.0285, 0.0001 and 0.0943, respectively) indicating that it could be the most significant ion affecting the biosynthesis of EPA by isolate 717. This hypothesis is supported by the fact that it was the only ion with a significant main effect on the EPA percentage of the total acid suggesting that Na_2HPO_4 significantly induces the synthesis of EPA more than the other ions tested. In addition to the main effect, the Na_2HPO_4 quadratic effect was also found to have a significant effect on the EPA yield (P-value 0.0465) and EPA% (P-value 0.0211) but not on EPA concentration.

KCl was found to have a significant effect in terms of the main effect on EPA concentration (mg/l) only (P-value, 0.0791) while its main effect on the other responses was found to be insignificant. Neither the KCl quadratic effect, nor any of its two ways interactions were found to have any significant effect on any of the EPA responses.

The main effect of NaCl, which was estimated as non significant on all EPA responses, but the quadratic effect of NaCl was estimated as significant on all EPA responses (for EPA yield the P-value was 0.0073, for EPA concentration P-value was 0.0274 and for EPA% P-value was 0.0179). Also, NaCl was found to be involved in a significant interaction with Na_2HPO_4 (P-value, 0.0253) on EPA concentration.

$MgSO_4$ showed a significant main effect (P-value, 0.0604) on EPA yield. In addition to the significant main effect, its interaction with Na_2HPO_4 was significant (P-value, 0.0840) on the same response.

In a one factor at a time study testing the effect of metal ions on EPA production by isolate SCRC-2738, Akimoto *et al.* (1991) reported that $MgSO_4$ was found to be insignificant on the EPA percentage and thus it was discarded from the optimum medium. The same result obtained from the CCD experiment confirmed that the main effect of that salt is insignificant (P-value, 0.2771). But the interaction between $MgSO_4$ and Na_2HPO_4 (P-value, 0.0977) and the quadratic effect (P- value, 0.0947) were significant, indicating that its effect should not be ignored. Detecting these effects was not possible in the one factor at a time approach.

Significant effect of Mg^{2+} ions on the ability of the microorganisms to produce PUFAs could be due to its effect on ATP citrate lyase and Malic enzyme which

influence the Acetyl CoA and NADPH for lipogenesis in microbial cells (Muhid *et al.*, 2008)

Second order polynomial order models were created, using coded units, to predict the optimum metal ion concentration for each response, with the aid of the colour contour (Appendix C.9) to visualise the optimum area, equations 6.4 – 6.6.

$$EPA\ yield. = 11.21 + 2.70A - 0.49B - 0.30C - 1.22D - 1.28AD - 1.19A^2 - 2.41C^2 \text{---(eq.6.4)}$$

$$EPA\ conc. = 14.28 + 6.44A - 1.32B + 0.53C - 0.003D + 2.11AC - 2.39C^2 - 6.87AB^2 \text{---(eq. 6.5)}$$

$$EPA\ \% = 14.14 + 2.20A - 0.74B - 0.058C - 0.78D - 1.07AD - 1.54A^2 - 2.30C^2 + 1.28D^2 - 2.47AB^2 \text{---(eq.6.6)}$$

Where A, B, C and D were Na_2HPO_4, KCl, NaCl and $MgSO_4$ respectively.

Table 6.10: The predicted and the actual values under the optimum metal ions concentrations for maximum EPA production by isolate 717

Media				Predicted Values			Actual Values		
Na_2HPO_4 (g/l)	KCl (g/l)	NaCl (g/l)	$MgSO_4$ (g/l)	EPA yield (mg/g)	EPA conc. (mg/l)	EPA %	EPA yield (mg/g)	EPA conc. (mg/l)	EPA %
2.56	1.21	22.92	1.80	15.61	19.44	16.57	14.6 (±0.98)	17.11 (±3.2)	17.48 (±0.3)

For isolate 717, the optimum sodium chloride concentration for the highest EPA production was approximately 20 g/l which is approximately the same optimum concentration for EPA production by isolate SCRC-2738 (Akimoto *et al.*, 1991). Growing the isolate SCRC-2738 in the bioreactor on the modified media gave 1.2 times higher than growing under the same conditions on ASW media. Relatively low salinity was also preferred by *Nitzschia laevis* for maximum EPA production (Cao *et al.*, 2008).

The effect of NaCl and KCl on EPA content could be due to controlling the fluidity of the cellular membrane (Akimoto *et al.*, 1991). Also, the variation in the cellular membrane composition could be an adaptation response towards the salt concentration (Watanabe and Takakuwa, 1987).

Bivalent metal ions such as Mg^{2+} are usually required as cofactors. Acetyl coenzyme A carboxylase, catalysing the conversion of acetyl coenzyme A to malonyl coenzyme A via a carboxylation step, generally requires such bivalent metal ions; however exceeding the optimum concentration could have an inhibiting effect on the process. (Guchhait *et al.*, 1974).

6.6 Creating chemically defined media for isolate 717

The strategy used to create a chemically defined medium for isolate 717 included a number of steps. In the first step the number of metal ions included in the ASW medium was reduced via screening the main effect of each ion and discarding all other ions except those showing a significant positive effect on EPA responses. The next step was replacing the yeast extract with a amino acid(s). To achieve this replacement, the organism was grown on nine different amino acid pools with different amino acid combinations as described in section 2.14.1).

At this stage, the yeast extract was replaced and the metal ions were reduced to the minimum number in the medium. The third step included testing different chemically defined carbon sources via a resolution-4 design of experiment (section 2.14.2).

The last step was optimising and determining the optimum concentration of each of the medium compounds and testing the interactions between the chosen carbohydrate(s) and amino acid(s) by performing a CCD experiment with the chosen compounds (section 2.14.3).

6.6.1 Reducing the metal ions contents

In section 6.2.4, the effect of the ASW metal ions were tested and only three of eight tested salts were identified to have positive significant effect on EPA as a calculated responses. These ions were Na_2HPO_4, KCl and $MgSO_4$ in addition to sodium chloride, although it did not show any significant effect but due to the fact that the organism could not grow in the complete absence of sodium chloride (section 3.1), it was added with its minimum level (the same result was obtained with isolate 66).

After this experiment, the number of metal ions within the medium were reduced from eight to four and the sodium chloride concentration was reduced to its minimum value to avoid any potential salinity problems in large scale production.

6.6.2 Screening the amino acids

The main target was replacing the yeast extract with one or more amino acids as a second step to build a chemically defined medium. The basal medium used was the chosen metal ions from the previous section with no added carbon or nitrogen except for the tested amino acids. The responses obtained are summarised Table 6.11.

Table 6.11: The EPA responses of isolate 717 grown in different amino acid pools (see section 2.14.1 for pools composition).

Pool	EPA yield mg/g	EPA conc. (mg/l)	EPA %	Dry Weight (mg/l)
1	3.68	4.79	7.7	**1.30**
2	4.64	2.7	4.6	0.58
3	**11.595**	**6.52**	**11.5**	0.56
4	**9.73**	**6.64**	**12.8**	0.68
5	4.174	**6.95**	4.7	**1.66**
6	2.31	3.755	5.7	**1.62**
7	5.855	5.475	6.8	0.93
8	**7.03**	4.99	**13.3**	0.71
9	4.68	2.985	8.2	0.63

Cultivation in the media containing amino acids from pools number 3, 4 and 8 resulted in the highest EPA yield and percentage suggesting that L-histidine and L-proline could play a role in the biosynthesis of EPA. The highest concentration of EPA was obtained with cultivation in the medium containing amino acids from pools number 3, 4 and 5 suggesting that the two amino acids L-arginine and L-leucine could enhance the amount of EPA and biomass concentration simultaneously. Cultivation in the media containing amino acids from pools number 1, 5 and 6 resulted in the highest biomass, suggesting that Phenyl-alanine and L-Serine play an important role in the growth of this isolate. This experiment indicated that the requirements for high EPA production are not the same requirements for optimum growth.

The same effect of L-leucine on the amount of EPA and the effect of L-proline on the percentage of EPA was previously reported on the productivity of *Shewanella gelidimarina* ACAM 456. L-proline enhanced the percentage of EPA however this increase was not mirrored by an increase in the quantitative yield due to a lower level of total fatty acids produced in the L-proline culture. On the contrary, L-leucine led to a significant increase in the quantitative levels of EPA with no remarkable effect on the EPA percentage (Nichols *et al.*, 1997). .

Enhancement of EPA production when cultivating the bacterial isolate on L-histidine was a common observation with isolates 66 and 717 confirming its role in the biosynthesis of EPA, either via direct involvement in the metabolic pathway, or via enhancing and inducing the EPA production.

Two amino acids were chosen based on these results to replace yeast extract for further study: L-proline and L-histidine.

6.6.3 Screening different Carbon Sources

The main aim of this experiment was screening the effect of different carbon sources on the growth and productivity of isolate 717. The basal medium used for this experiment contained Na_2HPO_4, KCl, $MgSO_4$, NaCl in addition to L-proline and L-histidine as the main nitrogen sources (see section 2.14.2). Two Plackett-Burman designs were applied to screen a large number of potential carbon sources. However no growth was observed in over 80% of the trials in both PB designs. These unexpected results made the statistical analysis impossible and indicated that there was a significant interaction between the tested variables. This result also suggested that some of the carbon sources used may have a lethal effect on the isolate and by applying statistical analysis; these lethal compounds could be identified and eliminated from subsequent cultivations.

The statistical analysis (at 80% level of confidence) indicated that, malic acid, ascorbic acid and citric acid may have a lethal effect on isolate 717 (the same result was observed on isolate 66). This effect may be due to the effect of each or due to an interaction between them or with different carbon sources included within the design. In the next design these three carbon sources were eliminated as they masked the effect of the rest of the variables.

The lethal effect of malate could be due to the fact that, it affects the osmotic balance in living cells as it has critical role in the movement of water from outside into the stomata guard cells (Wang and Blatt, 2011).

The negative effect of citric acid on microbial growth was previously reported for *Clostridium botulinum* (Graham and Lund, 1986). It was found that the negative effect of citric acid may be due to the chelating property of citric acid as it interacts with the ions as Ca^{2+} and Mg^{2+} making them unavailable to the microbe. The same inhibitory effect of citric acid on the growth of isolates *Pseudomonas* sp. and *Moraxella* sp. was presumed to be due to the acidification of the cell interior and inhibition of nutrient transport (De Leon *et al.*, 1993).

Due to the fact that PUFA production is a carbon-source dependant process (Gentile *et al.*, 2003b) and in view of the unexpected results obtained in the previous Plackett-Burman experiments, these different carbon sources including glycerol, fructose, arabinose, mannitol, sorbitol, sodium acetate, glucose, maltose, xylose, lactose and

sucrose were taken further to a resolution-4 Design Of Experiment (DOE) (as described in section 2.14.2).

Table 6.12: The matrix and the responses for the resolution-4 DOE to test the effect of different carbon sources on isolate 717 (A represents glycerol, B represents fructose, C represents arabinose, D represents mannitol, E represents sorbitol, F represents sodium acetate, G represents glucose, H represents maltose, J represents xylose, K represents lactose, and L represents sucrose).

Run	Variables											Responses			
	A	*B*	*C*	*D*	*E*	*F*	*G*	*H*	*J*	*K*	*L*	EPA yield (mg/g)	EPA conc. (mg/l)	EPA %	Dry Weight (g/l)
1	+	+	-	-	-	+	+	-	-	-	-	7.65	7.98	11.45	1.04
2	+	+	-	-	+	-	+	-	+	+	+	4.61	5.11	8.73	1.08
3	+	-	-	-	-	+	+	+	+	-	+	8.44	7.64	8.61	0.90
4	+	-	+	-	-	-	-	+	+	+	-	7.51	11.85	8.23	1.57
5	-	-	+	+	-	+	-	+	-	-	-	9.36	6.95	7.43	0.74
6	-	+	-	+	+	+	+	-	-	-	+	12.11	10.41	9.9	0.86
7	+	+	-	+	+	-	-	+	+	-	-	13.32	12.38	10.61	0.93
8	+	+	+	+	+	+	+	+	+	+	+	0	0	0.00	0
9	+	-	-	+	+	-	-	-	-	-	+	12.62	15.52	11.18	1.23
10	-	-	+	+	+	-	-	+	+	+	+	10.59	9.81	11.1	0.92
11	-	+	-	+	-	-	+	-	+	+	-	17.73	17.34	10.87	0.97
12	-	+	-	-	-	-	-	+	+	-	+	11.05	12.35	9.46	1.11
13	+	+	-	+	-	+	-	+	-	+	+	8.37	7.64	8.61	0.91
14	+	+	+	-	-	-	-	-	-	+	+	6.45	4.75	8.96	0.73
15	-	+	+	+	+	-	-	-	-	+	-	12.22	13.32	10.75	1.09
16	+	-	+	+	+	+	+	-	-	+	-	11.59	11.31	10.2	0.97
17	+	+	+	-	+	+	-	-	+	-	-	8.75	10.06	10.38	1.15
18	+	-	+	+	-	-	+	-	+	-	+	13.91	15.17	11.04	1.09
19	-	+	-	-	+	+	-	+	-	+	-	8.89	8.53	9.12	0.96
20	-	-	+	-	+	-	+	-	+	-	-	8.27	6.86	8.90	0.83
21	+	-	-	+	-	+	-	-	+	+	-	12.68	17.75	7.43	1.40
22	+	+	+	+	-	-	+	+	-	-	-	3.84	1.94	3.46	0.50
23	-	+	+	-	+	-	+	+	-	-	+	9.69	11.07	5.75	1.14
24	-	-	-	-	+	+	-	-	+	+	+	7.81	6.49	9.08	0.83
25	-	+	+	-	-	+	+	+	+	+	-	0	0	0.00	0
26	-	-	-	+	+	+	+	+	+	-	-	0	0	0.00	0
27	-	-	+	-	-	+	+	-	-	+	+	8.76	7.41	8.3	0.84
28	-	-	-	-	-	-	-	-	-	-	-	3.35	1.86	8.76	0.55
29	-	-	-	+	-	-	+	+	-	+	+	6.77	3.98	5.85	0.58
30	+	-	-	-	+	-	+	+	-	+	-	10.31	11.71	6.97	1.13
31	-	+	+	+	-	+	-	-	+	-	+	0	0	0.00	0
32	+	-	+	-	+	+	-	+	-	-	+	7.76	7.08	8.18	0.91

The inhibition of growth in four trials, including the one containing all the tested variables (trial 8), even after removing the lethal ones supports the idea that the interactions among these variables were significant and may be lethal for the tested isolate. To assemble the effect and the significance of the variables in one graph, a normal probability for standardised effect was plotted showing if the factor is significant or not and whether the factor had a negative or positive impact on the calculated responses.

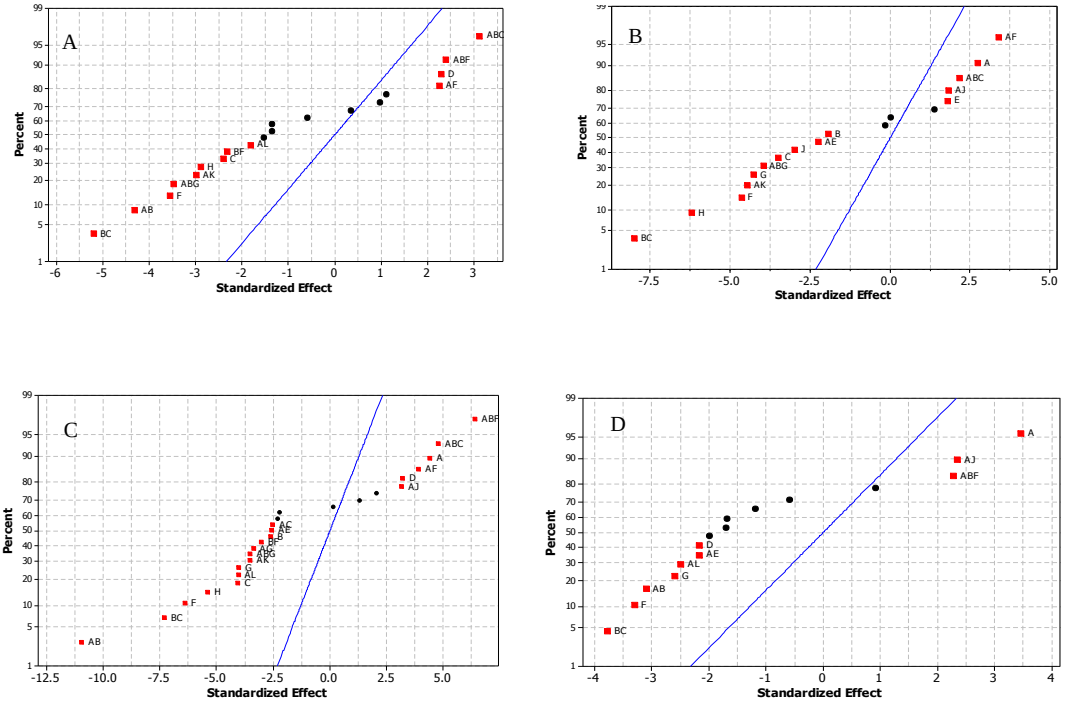

Figure 6.4: Normal probability plot of the standardised effect to show the significant effect of each carbon sources and their interactions on each response for isolate 717 (A) EPA yield (mg/g), (B) EPA percentage of total fatty acids, (C) EPA concentration (mg/l) and (D) Dry weight (g/l).

As shown in Figure 6.4, the two and three way interactions were found to be more significant than the main effect of most of the tested variables; not only on EPA production but also on the growth and the amount of biomass.

Glycerol and mannitol main effects were the only statistical significant ones on the calculated responses. To avoid sever and complicated interaction these two variables (glycerol and mannitol) were taken subsequently to test their ability to form constitute the chemically defined media along with the selected amino acids.

The effect of different carbon sources were also tested on the moss like plant *Marchantia polymorpha* via one factor at a time experiments (Chiou *et al.*, 2001).

The authors stated that glucose, fructose and sucrose were potentially the best carbon sources for EPA production while mannitol and sodium acetate proved to have a negative impact on EPA production these results contradict with results obtained through this study by isolate 717, where mannitol and glycerol was found to be the most statistically significant positive effect on EPA production and growth. Fructose, sucrose and glucose showed a significant negative affect either as main affect or involved in a two or three way interactions.

Based on these result and to avoid any complication due to the effect of the interactions, two carbon sources were chosen for further optimisation namely mannitol and glycerol. These two carbon sources were optimised with the chosen amino acids from the previous section.

6.6.4 Chemically defined medium composition

Two amino acids (L-proline and L-histidine) and two carbon sources (glycerol and mannitol) were used as composition of a chemically defined medium (as described in section 2.14.3). To achieve the optimum medium composition for maximum EPA production, a CCD experiment was performed.

The matrix and responses are summarised in Table 6.13 and the full statistical analysis in Appendix C.10.

Table 6.13: The matrix and responses for the CCD experiment to create the chemically defined media for isolate 717

Run	Variables				Responses		
	Mannitol	Histidine	Glycerol	Proline	EPA yield (mg/g)	EPA conc. (mg/l)	EPA %
1	0	0	0	0	15.42	14.41	10.5
2	+1	-1	+1	+1	8.26	6.71	7.7
3	-1	+1	+1	-1	12.25	16.06	8.9
4	0	-2	0	0	7.41	1.97	2.7
5	+1	-1	+1	-1	3.71	1.13	5.4
6	-1	-1	+1	+1	6.51	4.47	7.8
7	+2	0	0	0	0	0	0
8	+1	+1	-1	+1	3.41	3.26	6.8
9	0	0	0	0	14.34	14.05	10.5
10	-1	+1	-1	-1	3.89	4.31	9.3
11	0	+2	0	0	0.67	1.33	1.3
12	0	0	0	0	14.68	14.06	10.4
13	0	0	0	0	14.41	14.43	11.2
14	0	0	0	-2	4.71	5.34	6.2
15	0	0	0	+2	12.37	10.02	13.2
16	+1	+1	+1	-1	4.46	4.31	5.7
17	+1	+1	+1	+1	10.93	1.91	3.3
18	-1	+1	+1	+1	11.95	12.94	10.2
19	-2	0	0	0	0.32	0.48	0.9
20	-1	-1	-1	+1	7.72	4.41	7.1
21	+1	-1	-1	-1	3.26	2.42	6.6
22	+1	-1	-1	+1	4.01	3.19	4.7
23	0	0	-2	0	10.49	10.53	11.3
24	-1	-1	+1	-1	7.19	6.27	5.6
25	0	0	+2	0	0	0	0
26	-1	+1	-1	+1	14.61	20.32	12.6
27	-1	-1	-1	-1	3.19	2.01	3.4
28	0	0	0	0	14.33	14.05	10.9
29	0	0	0	0	13.63	12.96	9.8
30	+1	+1	-1	-1	10.69	19.12	11.1

The only significant main effect on EPA yield (mg/g), at 90% level of confidence, was the mannitol (P-value 0.0229). In addition the quadratic effects of mannitol (P-value 0.0018), L-histidine (P-value 0.0029) and glycerol (P-value 0.0139) were found to be significant on EPA yield (mg/g). None of the two way interactions were marked

as significant on the studied response. At a lower level of confidence the main effect of the other medium components would be considered as significant indicating that even though the main effect of these variables were not significant at 90% level of confidence but their linear effect should not be ignored.

Except for L-proline the main effect of the rest of variables on EPA concentration was determined as significant (mannitol, 0.0447, L-histidine, 0.0973 and glycerol 0.0856). Although the quadratic effects of glycerol and L-proline were found to be insignificant, the quadratic effect of mannitol (P-value, 0.0449) and L-histidine (0.0077) were significant.

The amount of EPA as a fraction of total fatty acids was significantly affected by the main effect of mannitol (P-value 0.0266), L-histidine (P-value 0.0976) and glycerol (P-value 0.0114), in addition to the quadratic effect of mannitol (P-value 0.0032), L-histidine (P-value, 0.0026) and L-proline (P-value 0.0026). None of the two way interactions effect was detected to be statistically significant on EPA percentage at 90% level of confidence, while performing the analysis at a lower level of confidence; revealed that the interaction between mannitol and L-proline and the interaction between L-histidine and glycerol were significant on the calculated response.

As PUFA is produced when microbial cells exposed to a high stress the positive significant effect of mannitol on EPA productivity may be due to the fact that mannitol is an osmotic diuretic agent exhibiting osmotic stress that may induce the EPA production (Tholakalabavi *et al.*, 1994).

Glycerol, L-prolie and L-histidine were previously also found to be significant for the EPA production by isolate 66. This common significant effect on two different producers indicates that these compounds may play a vital role in the metabolic synthesis of the product.

A second order polynomial order models were generated, using coded units, to predict the optimum medium composition, equations 6.7 – 6.9.

$$EPA\ yield = 11.46 - 1.20A + 1.21B - 0.73C + 1.15D - 0.98A^2 - 2.58B^2 - 0.74C^2 \quad \text{--- (eq. 6.7)}$$

$$EPA\ conc. = 12.37 - 1.59A + 3.04B - 1.29C + 0.27D - 0.98A^2 - 3.59B^2 \quad \text{---(eq. 6.8)}$$

$$EPA\ \% = 9.71 - 1.02A + 1.15B - 1.18C + 0.35D - 0.80A^2 - 2.28B^2 + 0.98D^2 \quad \text{---(e/q. 6.9)}$$

Where A is mannitol, B is L-histidine, C is glycerol and D is L-proline.

The models were verified experimentally and the predicated responses the actual values are shown in Table 6.14.

Table 6.14: The predicted and the actual values of EPA produced by isolate 717 grown on the optimum medium for each response

Target	**Variable**				**Predicted Values**	**Actual Values**
	Mannitol (g/l)	Histidine (g/l)	Glycerol (g/l)	Proline (g/l)		
Maximise EPA yield (mg/g)	2.06	1.76	2.37	1.82	15.7075	18.89 (±1.1)
Maximise EPA conc. (mg/l)	3.62	2.82	2.50	1.45	18.1838	22.02 (± 3.8)
Maximise EPA %	2.22	1.80	1.28	2.80	14.9108	15.67 (± 0.67)

The medium composition required to maximise all these responses simultaneously is shown in Table 6.15.

Table 6.15: The predicted and the actual values of EPA produced by isolate 717 grown on the optimum medium for each response

Variable				Predicted Values			Actual Values		
Mannitol (g/l)	Histidine (g/l)	Glycerol (g/l)	Proline (g/l)	EPA yield (mg/l)	EPA conc. (mg/l)	EPA %	EPA yield (mg/g)	EPA conc. (mg/l)	EPA %
2.21	1.98	1.84	1.80	11.7828	17.3405	11.594	15.34(±1.3)	19.65(± 2.8)	13.67 (± 1)

6.6.5 Effect of carbon to nitrogen ratio (C/N)

One of the advantages of using chemically defined media is that, the effect of C/N on the EPA production could be easily tested. The C/N ratio in each trials of the Table 6.13 was theoretically calculated and tested against each response (as described in section 2.14.4).

Figure 6.5, shows the relationship between the C/N ratio and the growth and EPA production by isolate 717.

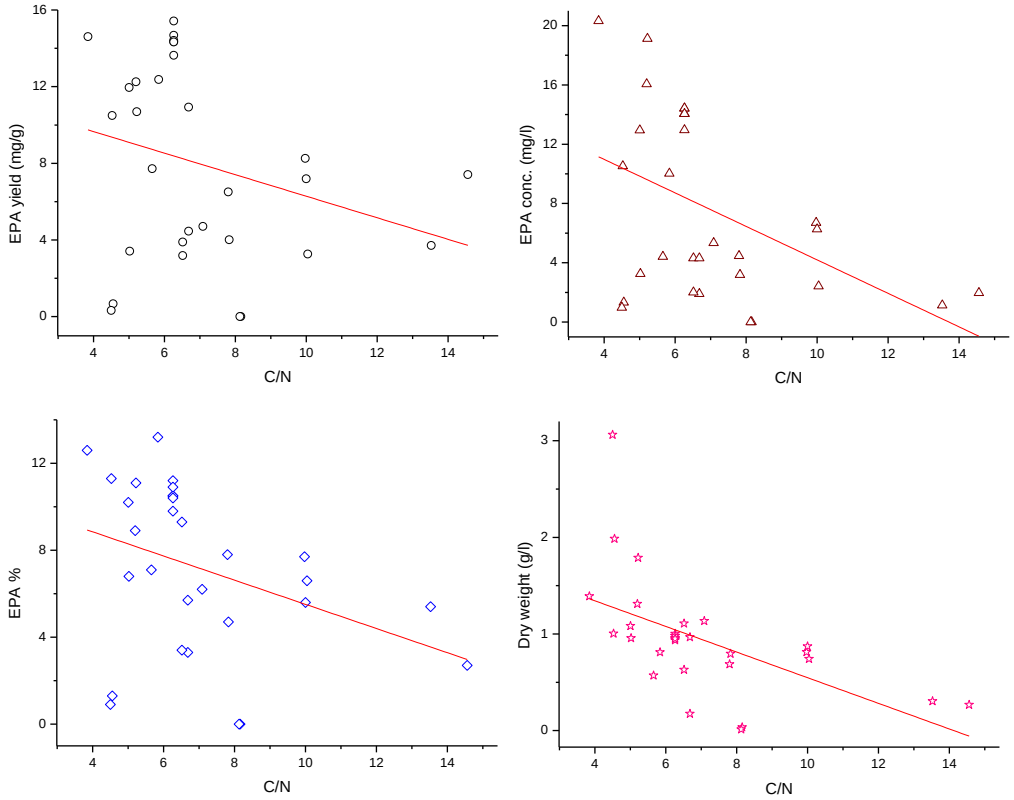

Figure 6.5: The relation between the growth and EPA productivity by isolate 717 and the carbon to nitrogen ratio in the cultivation media listed in Table 6.13. The scatter points represent the response readings while the line represents linear best fit.

Figure 6.5 displays a single symbol of C/N ratio for each cultivation (X-axis) listed in Table 6.13 against one of the responses (Y-axis). The line of best fit is also presented. The C/N ratio inside the microbial cell, when grew on ASW, was found to be 3. The lower C/N ratio was preferred by isolate 717 both for growth and productivity as indicated by the negative slope of all lines of best fit in Figure 6.5. The effect of C/N was found to be statistically significant especially on dry weight (P-value 0.00143), EPA concentration (P-value 0.01129) and EPA percentage (P-value 0.04959) with a level of confidence higher than 90%. The effect of C/N on EPA yield to be significant, the confidence level would have to be lowered to 85%.

For isolate *Mortierella alpina* the optimum C/N ratio for maximum PUFAs production was estimated to be slightly low in a range of 5-9, above which the amount of PUFAs significantly decrease (Jang *et al.*, 2005).

In the diatom *Nitzschia laevis*, the intercellular lipids accumulate under nitrogen-limited conditions, while EPA accumulated at high nitrogen conditions which did not

match with the lipids accumulation conditions (Cao *et al.*, 2008), this result suggested that, EPA biosynthesis could be not accompanied with lipid biosynthesis.

6.6.6 Effect of additional supplements on EPA production

The amount of EPA produced by isolate 717 were relatively low when grown on the developed chemically defined media (section 6.3) compared to the amount produced by the same isolate when grown on the previously developed complex media (section 6.6.4), indicating an absence of a potentially important compound present in the complex media.

The effect of different compounds including, α-ketoglutarate, oxaloacetate and malate which are a potential substrate for Krebs cycle and thiamine (vitamin B1), cobalamin (vitamin B12) and Riboflavin (vitamin B2) which are vitamins were tested, as described in section 2.14.5, on the ability to produce EPA.

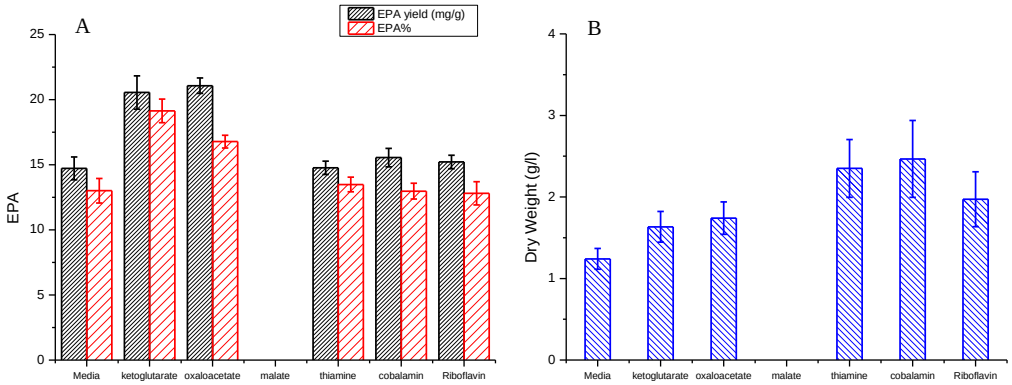

Figure 6.6: Effect of different precursors and additives on the growth and EPA productivity of isolate 717. The columns with the error bars represent the different calculated responses where the black columns represent the EPA yield, red columns represent the EPA percentage and the blue columns represent the biomass concentrations.

The highest amount of EPA and the highest percentage was obtained when α-ketoglutarate and oxaloacetate were supplied, although they were not the optimum for the biomass. The addition of malate to the media led to a complete inhibition of the bacterial growth.

The addition of the vitamins to isolate 717 increased the biomass instead of increasing the EPA productivity. For isolate *Phaeodactylum tricornutum* the addition of vitamin B12, to the production media of isolate, led to 65% increase in EPA yield, but the addition of vitamin B1 did not enhance EPA production (Yongmanitchai and Ward, 1991).

These results indicated that vitamins were not the missing components from the complex medium which enhanced the EPA production over the chemically defined medium. Vitamins may be required for enhancing the growth of the investigated isolate.

Krebs cycle substrates, except for malate, showed a remarkable increase in the EPA productivity may be by providing a high energy which is required to synthesise EPA. The complex media may contain components which provide high energy or enhance Krebs cycle for isolate 717.

6.6.7 Effect of different H_2O_2 concentrations on EPA production

As EPA was identified as an antioxidant, the main target was to create oxidative stress on the producer when growing in shake flasks leading to enhanced EPA production. The experiment was carried out as described in section 2.14.5.

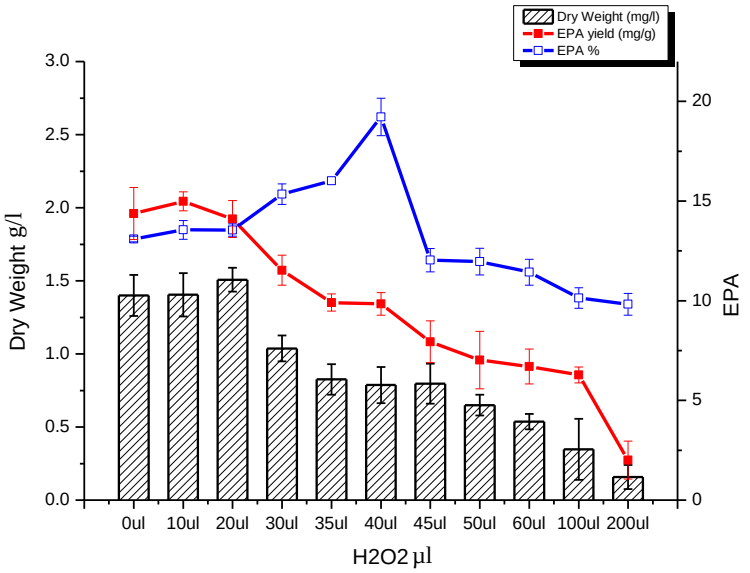

Figure 6.7: Effect of different H_2O_2 on the growth and productivity of isolate 717, where the columns with error bars represent the biomass concentration, the red line represents the EPA yield (mg/g) and the blue line represents the EPA percentage of the total fatty acids.

The addition of H_2O_2 to the previously developed chemically defined media led to a remarkable decrease in the biomass and the yield of EPA as mg/g biomass. The decrease of EPA could be due to an interaction between the added H_2O_2 and the EPA within the membrane that work as a shield, to protect the cell. The addition of H_2O_2 to

the bacterial media triggered the biosynthesis of EPA rather than the total fatty acids for the purpose of protection.

The harmful effect of H_2O_2 on the biomass could be due to the carbonylation of proteins that are vital for the growth and also H_2O_2 may damage the DNA molecules, while the harmful influence on the total lipid could be due to the formation of lipid peroxy radicals (Mostertz and Hecker, 2003).

6.7 Comparison of the growth and EPA productivity of isolate 717 in different media

Isolate 717 was cultivated on three different media, at the same incubation temperature 15°C, namely artificial sea water (medium 1), developed production media, consists of casein, Hy-soy and Na_2HPO_4, (medium 2) and the chemically defined medium, consists of glycerol, mannito, L-proline and L-histidine (medium 3). Figure 6.8 summarise the comparison of the growth and EPA productivity of isolate 717 in these media.

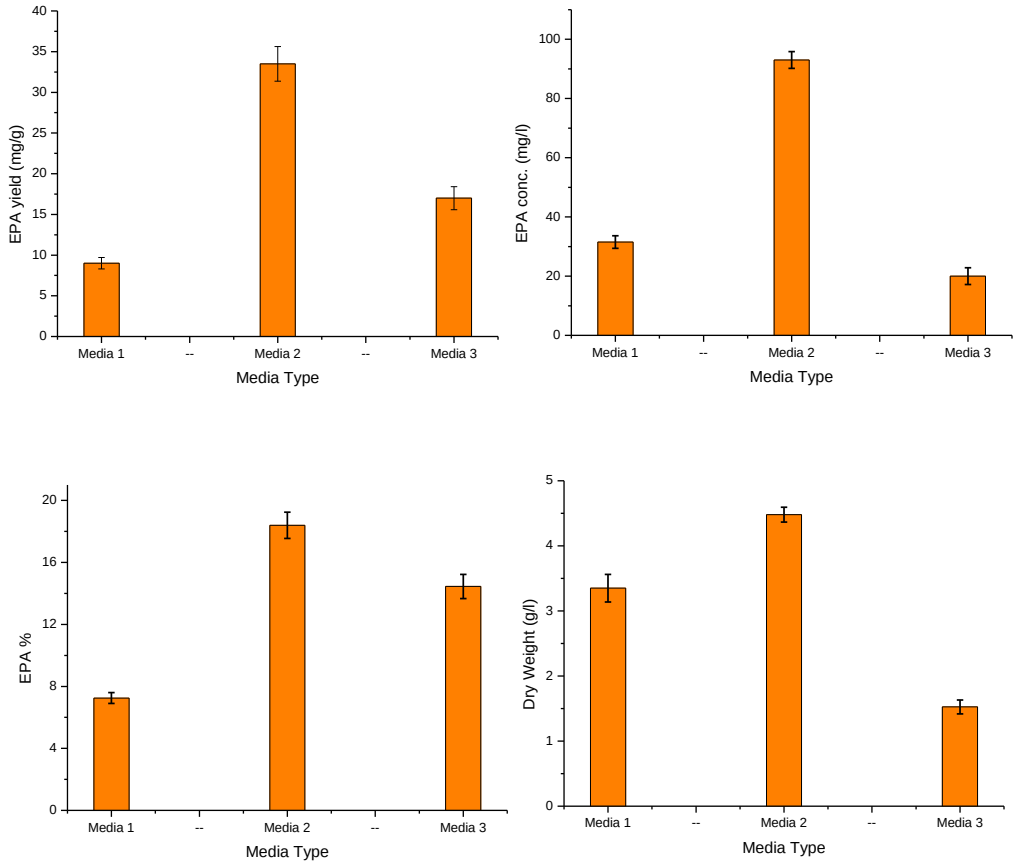

Figure 6.8: A comparison of growth and productivity of isolate 717 growing on artificial sea water (medium 1), developed production medium (medium 2) and the chemically defined medium (medium 3).

The cultivation of isolate 717 on the previously optimised potential production medium resulted in the maximum growth and productivity compared to ASW and chemically defined media.

Chemically defined medium showed a higher EPA productivity (EPA yield and percentage) than the ASW medium while the ASW medium showed a higher growth and EPA-growth related productivity.

6.8 Chapter Summary

Isolate 717, identified as *Shewnella* sp. 717, was found to be the highest EPA producer among the screened isolates. In addition to EPA, isolate 717 is able to produce traces of DHA. The ability to produce EPA and DHA was confirmed via GC/MS method.

In order to develop a potential optimum production medium for maximum EPA productivity and growth, three different components namely Hy-soy, casein and Na_2HPO_4 were selected on the basis of a PB screening design of experiment. Cultivating isolate 717 in this potential production medium resulted in approximately 3-times more EPA yield, concentration and percentage than ASW media.

The effect of metal ions was tested independently of the effect of carbon and nitrogen, and although the amount of growth, EPA yield and concentration were relatively low, the percentage of EPA was relatively high (17% of the total fatty acids). This indicated that the metal ions may not have a significant effect on the growth and total fatty acid production by isolate, but they have a vital role in the biosynthesis of EPA. The significant positive effect of Na_2HPO_4 and $MgSO_4$ on the ability to produce EPA by isolates under investigation (717, Hus-27 and 66) indicated that these elements could be involved directly in the metabolic biosynthesis of EPA. In addition, the negative effect of $Fe_2(SO_4)_3$ on EPA production by all isolates suggest that iron may inhibit the biosynthetic pathway of bacteria EPA synthesis.

A chemically defined medium was developed using simple carbohydrates and amino acids as a recipe for the medium. This medium enabled the effect of C/N ratio on the EPA productivity by isolate under investigation to be tested revealing that a low C/N ratios were preferred for the growth and EPA production.

Adding vitamins to the developed chemically defined media did not show a significant effect on EPA productivity, but their effect on growth was observable. While Krebs cycle substrates significantly enhanced the EPA productivity probably due to providing high energy which may be required for biosynthesis of EPA,

Adding H_2O_2 to the media may enhance the EPA biosynthesis as a fraction of total fatty acids within the bacterial cell via creating a stress leading to enhance the cells to produce more EPA as a protective shield due to its antioxidant effect. The addition of H_2O_2 would be useful from the downstream prospective, where although the yield was less but the high percentage of EPA facilitates its separation and purification. The ability of the EPA producer to protect themselves, against the external addition of H_2O_2, criteria could be used to establish a rapid and reliable method to screen the EPA producers among a large number of potential producers (need more work).

Chapter 7- Optimising the growth conditions for isolates

Microbial fermentations are important sources of biological products used in the food, chemical and pharmaceutical industries. The microbial production of PUFAs is still in the process development and requires better understanding of the microbial physiology and the interaction of the producer strains with the physical environment in the bioreactor.

The main aim of the work reported in this chapter was to establish a suitable industrial bioprocess by testing the effect of some physical factors including pH, temperature and dissolved oxygen level within the medium to optimise the growth and productivity conditions for isolate 717 as it was found to be the highest EPA producer and most promising isolate to be used in the industrial scale.

7.1 PID control

The cultivations were carried out in ASW medium in the bioreactor (see section 2.17). Prior to using the automated control of the process variables, it was necessary to ensure that the controllers were correctly tuned. Controller tuning is the process of establishing the controller parameters which produce the anticipated output, and it is used for optimising the process and minimising the error between the process variables and its set point. Ziegler- method of control tuning was used as this method is often used when the mathematical model of the system is unavailable (Ziegler and Nichols, 1993).

By testing the performance of the bioreactor, the temperature and the pH control were found to be satisfactory, however the DO control was found to be unstable as shown in Figure 7.1.

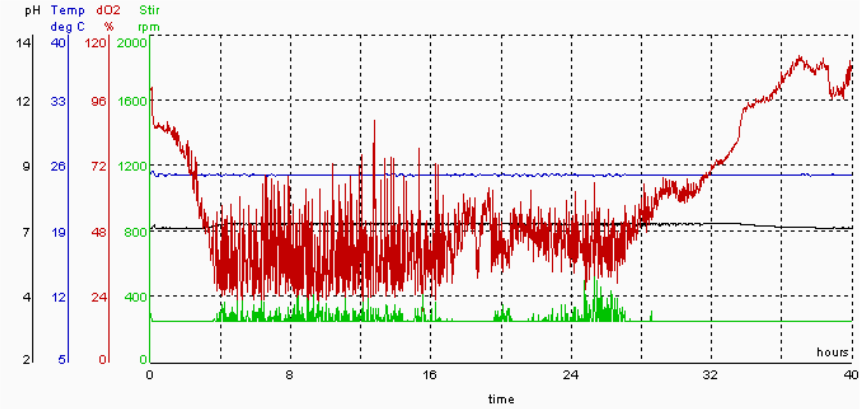

Figure 7.1: Online reading for the temperature, pH and DO control during the cultivation of isolate 717 in ASW medium in a bioreactor. Green line represents agitation speed, red line represents DO%, blue line represents temperature (°C) and black line represents pH.

Figure 7.1 clearly shows unacceptable oscillations in the DO level. Since the optimisation strategy consisted of a CCD design with 20 trials each had a specific DO set point, the presence of oscillations would make the analysis of the results inaccurate as the set point won't be achieved and the effect would be the effect of the oscillation range rather than the exact set point of DO. An accurate control was required to reduce the oscillation and to make the testing of the DO effect reliable.

Dissolved oxygen is controlled by the agitation and the aeration. One of the most difficult challenges in scaling up a bioprocess is controlling these two factors within acceptable operating boundaries (Rehm and Reed, 1993) as illustrated in Figure 7.2.

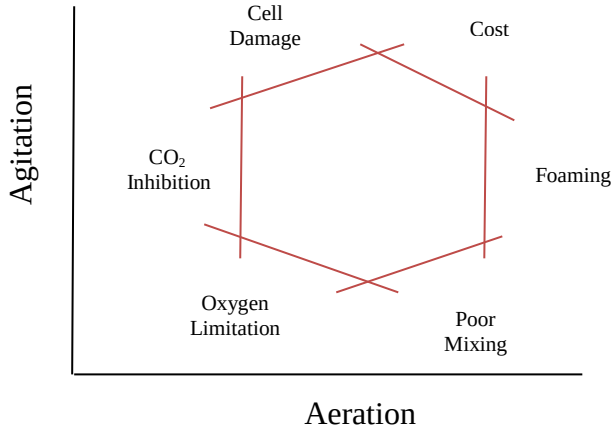

Figure 7.2: Operating boundaries for fermentation scale up (Rehm and Reed, 1993)

Figure 7.2 shows the relation between the agitation and the aeration where crossing the boundaries may lead to a significant inhibition in the process, as example, high agitation and

low aeration may lead to cell damage while low agitation and high aeration may cause a poor mixing.

A combination of Ziegler-Nichols method and best-guess-approach was used to control DO tuning. Using Ziegler-Nichols method did not give an accurate control (i.e. the oscillation was not eliminated but was reduced). Different PID values were tested around the PID values obtained by applying ZN method (best-guess-approach).

The best control was obtained at the following PID values,

P=0.91

I= 160

D= 6

Applying these PID values the DO control was acceptable, as indicated in Figure 7.3 with 30% as a set point for DO.

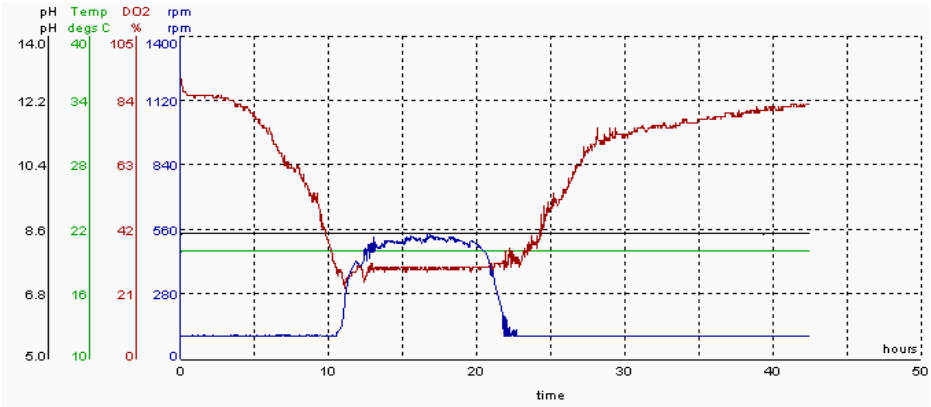

Figure 7.3: The control behaviour of the bioreactor after applying the new PID values for the cultivation process of isolate 717 in ASW media. DO set point = 30%.. Green line represents agitation speed, red line represents DO%, blue line represents temperature (°C) and black line represents pH.

The adjusted PID values resulted in a precise control of the DO concentration during the cultivation of isolate 717 at different DO set points (Appendix D.1). Although different strain often require an appropriate PID tuning due to their different behaviour and oxygen demand in a bioreactor the adjusted PID values were suitable for an accurate DO control for the other isolates investigated in this study (66, 560 and Hus-27) (Appendix D.1).

7.2 Optimisation of bioreactor conditions with ASW medium

A set of 20 bioreactor cultivation, with six centre points and five levels for each variable were applied to cover a wide range of the process variables (pH, temperature and DO) and to test the interactions among them (section 2.16.1). The main aim of this work was to identify

operating conditions for an economically viable industrial production of EPA via structured optimisation procedure within a bioreactor.

Optimising the growth conditions was expected to show a significant increase in the amount of produced EPA, previous literature reports indicated that the EPA biosynthesis is affected more significantly by the environmental parameters than by the nutrient composition and/or availability (Jostensen and Landfald, 1996). This assumption is right when the EPA calculated as a percentage of the total fatty acids, while the amount of EPA either as (mg/g or mg/l) were found to be affected by both the environmental factors and the media composition which can be concluded from the screening and optimisation work that was carried out across this study where the amount of produced EPA increased significantly via manipulating the culture media composition.

Four samples were collected from each batch providing a time trajectory of EPA. The comparison was based on the highest amount of EPA achieved in the batch, regardless of the time required to achieve this. The matrix and responses are summarised in Table 7.1 and a full statistical analysis displays in Appendix D.2.

Table 7.1: The matrix and responses for the CCD experiment performed to optimise the cultivation conditions at bioreactor level for isolate 717 grown on ASW medium

Run	**Variables**			**Responses**		
	Dissolved Oxygen	pH	Temperature	EPA yield (mg/g)	EPA conc. (mg/l)	EPA %
1	-1	-1	-1	32.35	120.58	16.47
2	0	0	0	21.54	101.61	14.6
3	+1	-1	-1	9.52	31.63	18.16
4	0	0	0	22.03	100	14
5	0	0	0	21.88	108	13.8
6	+2	0	0	15.8	57.68	14.1
7	0	0	0	20.29	102	14.5
8	0	0	+2	0	0	0
9	0	+2	0	18.28	60.73	13.29
10	-1	+1	-1	30.58	88.81	16.23
11	+1	-1	+1	8.05	17.86	10.97
12	-2	0	0	26.24	98.56	4.03
13	+1	+1	+1	12.55	77.66	11.06
14	+1	+1	-1	8.76	25.01	14.37
15	0	0	0	21.92	99.57	14.22
16	-1	-1	+1	6.33	25.86	11.07
17	0	-2	0	0.29	0.254	3.46
18	0	0	-2	21.73	87.57	21.41
19	0	0	0	20.88	101.61	14.22
20	-1	+1	+1	14.58	44.07	10.8

By comparing the generated P-values, via ANOVA analysis (Appendix D.2), the main effect of temperature was found to be the most significant growth factor affecting all EPA calculated responses (with P-values of 0.0015 for EPA yield, 0.0011 for EPA concentration and 0.0007 for EPA percentage).

The main effect of DO also showed a statistically significant impact on all EPA responses with P-values of 0.0017, 0.0007 and 0.089 for EPA yield, concentration and percentage of total fatty acid respectively. The main effect of pH was found to be significant on EPA yield (P-value 0.0428) and EPA concentration (P-value 0.0072) but its linear effect on EPA percentage found to be insignificant (P-value 0.5688).

The ANOVA table indicates that at 90% level of confidence, some of the interactions and quadratic effects were statistically significant in terms of EPA yield or concentration, but not in term of EPA percentage.

The quadratic effects of the pH and temperature were evaluated as significant on EPA yield and EPA concentration but not on EPA percentage and the interaction between DO and temperature was located to be statistically significant on the ability of isolate 717 to produce EPA (mg/g) and EPA (mg/l). The interaction between pH and temperature was found to have a statistically significant effect on EPA concentration only.

At a lower level of confidence the interaction between DO and pH was found to have a significant effect on EPA concentration and the interaction between pH and temperature was significant on EPA yield.

Contour plots (Figures 7.4, 7.5 and 7.6) were constructed by plotting the response against each of the two independent variables, while the third variable was maintained at its fixed (zero) level, to identify the optimum range for each variable and illustrate the interaction among the factors in terms of individual response variables. Colour degradation and numbers displayed on each contour were used to show the direction of the optimum regions,

In terms of the optimum values of pH and DO illustrated in Figure 7.4 the optimum pH range for all the calculated responses was between 7-7.5. The optimum DO for the EPA yield and concentration was relatively low (10-20%), and for EPA% it was relatively high (45-55%).

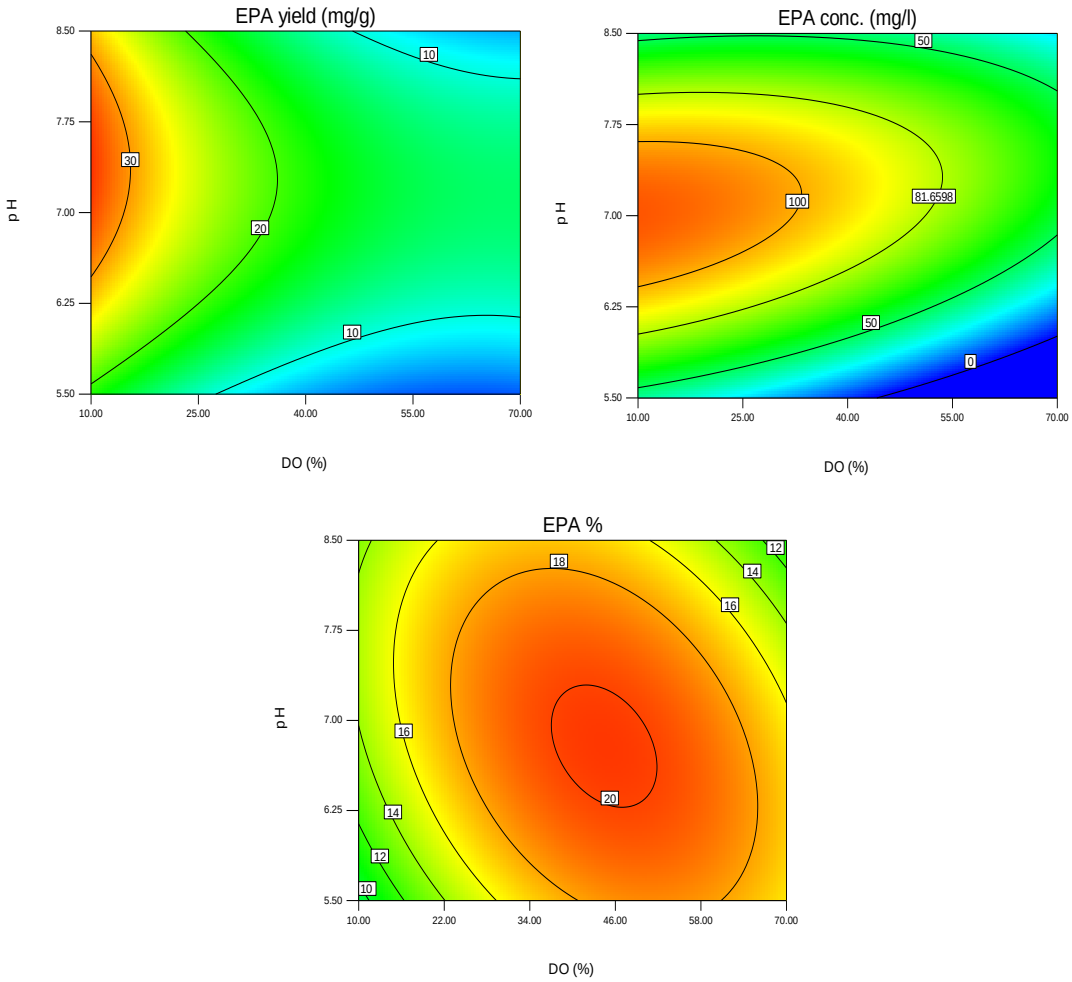

Figure 7.4: Contour plots for EPA production for the CCD experiment performed to optimise the bioreactor cultivation conditions for isolate 717 grown in ASW medium to shows the interaction between pH and DO on: EPA yield (mg/g), EPA concentration (mg/l) and EPA percentage to the total fatty acids.

In terms of interaction between DO and temperature (Figure 7.5) low temperature was desirable for all EPA response variables (10-15°C), with higher DO (40-50%) levels preferable for high EPA percentage compared to EPA yield and concentration (10-20%).

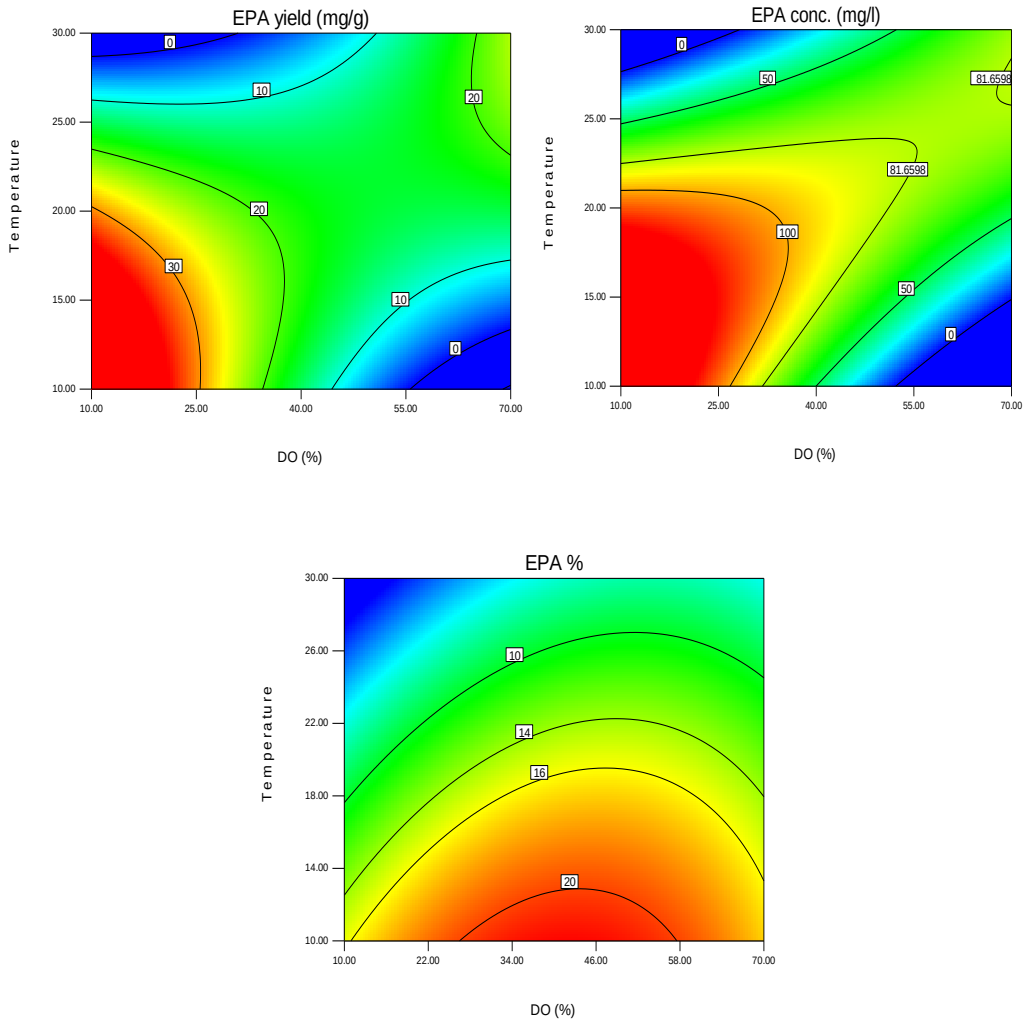

Figure 7.5: Contour plots for EPA production for the CCD experiment performed to optimise the bioreactor cultivation conditions for isolate 717 grown in ASW medium to shows the interaction between temperature and DO on: EPA yield (mg/g), EPA concentration (mg/l) and EPA percentage to the total fatty acids.

Figure 7.6, visualise the interaction between the temperature and pH revealing that the optimum pH was in the neutral range (6.8-7.2), and optimum temperature was relatively low for maximum EPA yield and percentage (10°C) and higher (13-16°C) for maximum EPA concentration (mg/l), which could be an indication that slightly higher temperature was desirable for higher growth and biomass.

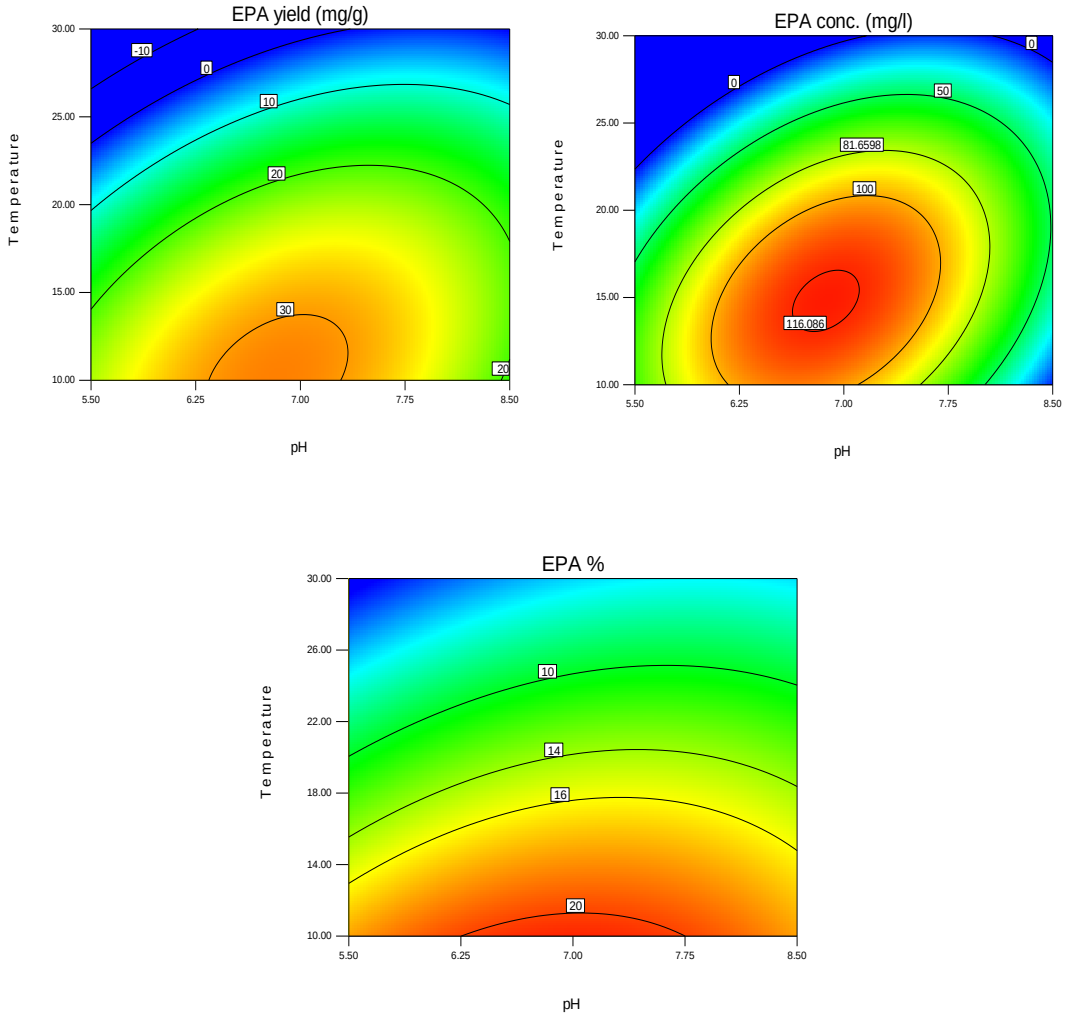

Figure 7.6: Contour plots for EPA production for the CCD experiment performed to optimise the bioreactor cultivation conditions for isolate 717 grown in ASW medium to shows the interaction between temperature and pH on: EPA yield (mg/g), EPA concentration (mg/l) and EPA percentage to the total fatty acids.

The lower temperature resulting in higher EPA production is in line with the reported ability of EPA to maintain the fluidity of the membrane in cold conditions (Sato *et al.*, 2008), also EPA was the main fatty acid required for growth of isolate *Shewanella piezotolerans* WP3 under low temperature and high pressure (Wang *et al.*, 2009).

An interesting observation of low DO levels (10-20%) being preferable for high EPA yield and concentration, compared to the high DO levels (40-50%) required for high EPA percentage, suggests that low dissolved oxygen supports both the biomass and the total fatty acid production leading to high EPA yield and concentration. In the case of high DO levels the cells produce high EPA levels rather than high concentrations of fatty acids and thus the percentage of EPA increases.

The increase in EPA compared to the total fatty acids when cells are exposed to relatively high DO could be due to either the antioxidant capability of EPA (Nishida *et al.*, 2007) or the activation of oxygen-dependant enzymes in the desaturation and elongation of PUFAs (Higashiyama *et al.*, 1999).

For *Marchantia polymorpha* 25°C was preferred for a higher EPA production ,may be due to higher biomass, while 15°C was preferred for higher EPA percentage from the total fatty acids (Chiou *et al.*, 2001). So, even for organisms that preferred higher temperature for growth, lower temperature enhances the EPA production over the total fatty acids.

The significant effect of pH may be by affecting uptake of nutrients and other physiological activities.

The effect of DO, the quadratic effects and the interaction between the investigated environmental factors, upon the EPA production, have not been previously explored in the literature. These were modelled with a second order polynomial (in coded units): equations 7.1 – 7.3. ----7.1

$$\text{EPA yield} = 19.42 - 4.66\,A + 2.67\,B - 4.45\,C - 0.56\,AB + 5.62\,AC + 2.29\,BC + 1.27A^2 - 4.15\,B^2 - 2.21\,C^2 \quad \text{(eq.7.1)}$$

$$\text{EPA conc.} = 97.82 - 12.15\,A + 11.18\,B - 13.47\,C + 6.27\,AB + 22.59\,AC + 14.55\,BC - 2.69\,A^2 - 29.7\,B^2 - 13.85\,C^2 \quad \text{(eq.7.2)}$$

$$\text{EPA \%} = 14.80 + 2.09\,A + 0.74\,B - 3.93\,C - 0.82\,AB + 0.46\,AC + 0.48\,BC - 1.24\,A^2 - 1.17\,B^2 - 0.40\,C^2 \quad \text{(eq.7.3)}$$

Where A represents DO (%), B represents pH and C represents temperature (°C).

These models were experimentally verified and the predicted responses were compared to the actual values (Table 7.2).

Table 7.2: Optimum culture condition combinations for each response for isolate 717 in bioreactor cultivation with ASW medium in addition to the predicted and the actual values for each response variable

Target	**Variable**			**Predicted Values**	**Actual Values**
	Dissolved Oxygen (%)	pH	Temperature (°C)		
Maximum EPA yield (mg/g)	12.52	7.10	14.06	40.38	35.5(±4)
Maximum EPA conc. (mg/l)	16.67	7.13	14.44	139.078	150.8(±7)
Maximum EPA %	50	7.43	10	23.7544	22.5(±1)

The optimum conditions for EPA production in terms of concentration and yield were nearly identical. The conditions required to increase EPA percentage are different therefore the

optimisation was repeated with the objective of maximising all three responses simultaneously (Table 7.3).

Table 7.3: The optimum culture condition combination for EPA production by isolate 717 when cultivated in bioreactor level and ASW as cultivation medium

Variable			Predicted Values			Actual Values		
Dissolved Oxygen (%)	pH	Temperature (°C)	EPA yield (mg/g)	EPA conc. (mg/l)	EPA %	EPA yield (mg/g)	EPA conc. (mg/l)	EPA %
25.28	7.21	10.00	35.3501	120.587	19.2563	29.8(±2.2)	137.4 (±9.8)	18.22(±0.6)

7.3 Optimisation of bioreactor conditions with production medium

The main aim of this work was to identify the optimum operating conditions for an economically viable industrial production of EPA, by using previously created production media (Section 6.3), via structured optimisation procedure within a bioreactor.

The same CCD was applied as for the ASW (Table 7.1) however the cultivation medium was the developed production medium (Hy-soy, casein and Na_2HPO_4).

The matrix and responses are summarised in Table 7.4 and the full statistical analysis displays in Appendix D.3.

Table 7.4: The matrix and responses for the CCD experiment performed to optimise the cultivation conditions at bioreactor level for isolate 717 grown on production medium

Run	**Variables**			**Responses**		
	Dissolved Oxygen	pH	Temperature	EPA yield (mg/g)	EPA conc. (mg/l)	EPA %
1	-1	-1	-1	40.81	283.18	12.19
2	0	0	0	32.07	144.33	14.51
3	+1	-1	-1	7.43	33.23	16.28
4	0	0	0	33.73	148.35	15.45
5	0	0	0	32.19	159.04	14.71
6	+2	0	0	18.24	124.63	20.61
7	0	0	0	31.67	153.14	13.76
8	0	0	+2	0	0	0
9	0	+2	0	13.28	100.22	10.36
10	-1	+1	-1	43.31	220.75	16.09
11	+1	-1	+1	9.97	20.46	9.71
12	-2	0	0	24.36	303.02	2.89
13	+1	+1	+1	13.81	86.32	12.31
14	+1	+1	-1	13.98	80.97	19.12
15	0	0	0	33.26	164.28	13.75
16	-1	-1	+1	3.04	11.08	7.97
17	0	-2	0	1.6	1.9	1.5
18	0	0	-2	39.36	300.62	24.34
19	0	0	0	32.23	161.41	14.44
20	-1	+1	+1	16.97	52.2	11.78

Extra five points were tested (Table 7.5) to check the validity of the model and to test some points that were not included in the CCD design. The dissolved oxygen was tested at extreme levels (90% and 5 %). Also, two pH extreme points were tested including pH 5 and pH 9 to check the behaviour of the organism. These extra points would cover a wider range of conditions and increase the confidence on the produced model.

Table 7.5: Extra trials to validate the CCD model

Run	Variables			Responses		
	Dissolved Oxygen%	pH	Temperature	EPA yield (mg/g)	EPA %	Dry weight (mg/g)
21	90	7	8	10.28	18.23	0.41
22	30	5	20	0	0	0
23	20	7	15	37.52	16.89	293.5
24	5	7.5	12	11.35	13.76	78.3
25	30	9	20	0	0	0

The results confirmed that the EPA production is not directly related to growth. For example, trial number 12 resulted in the maximum biomass (12 g/l) but not the maximum EPA amount expressed as yield and percentage.

By comparing the generated P-values, via ANOVA analysis, the main effect of temperature was found to be the most significant growth factor affecting the growth and the ability to produce EPA (P-values were, 0.0004, 0.0001and 0.0001 for EPA yield, concentration and percentage respectively).

The main effect of DO also showed statistical significant effects on all the EPA calculated responses (P-values were, 0.0074, 0.0619 and 0.0041 for EPA yield, concentration and percentage respectively). The statistical significant effect of DO on microbial EPA productivity could be related to the antioxidant capability of EPA (Nishida *et al.*, 2007).

At 90% level of confidence, the main effect of pH was found to be significant on EPA yield only (P-value, 0.0677) but performing the statistical analysis at slightly lower level of confidence revealed that the main effect of pH was significant on EPA concentration (P-value, 0.1110) and on EPA percentage (P-value, 0.1135) . The significant effect of pH could be due to its influence on the physiology of the organism via influencing enzyme activity, cell membrane morphology, by-product formation or nutrient solubility and uptake (Cromwick *et al.*, 1996).

The interaction between the temperature and the DO was found to have a significant effect on EPA yield (P-value, 0.0017) and EPA concentration (P-value, 0.0011). The significant interaction between the temperature and the DO on the EPA yield could be due to the fact that, the solubility of the oxygen in water significantly increases by lowering the temperature (Geng and Duan, 2010), which may lead to a higher production of EPA as a protection against the potential harmful effect of oxygen.

Unlike the quadratic effect of DO (estimated as insignificant on all EPA calculated responses), and quadratic effect of temperature (estimated to be significant on EPA yield only with P-value of 0.0306), the quadratic effect of pH was found to be significant on all EPA responses (P-values were, 0.0002, 0.0030 and 0.0462 for EPA yield, concentration and percentage respectively).

Significant effect of pH and temperature match with the result obtained by Lee *et al.* (2008b), who reported that temperature and pH have the most significant effect on the EPA productivity by isolate *Shewanella sp.* KMG427.

Compared the statistical analysis for the two CCD experiments (one with ASW and the other with previously created production media) revealed that, the statistical significant effect of DO and temperature was similar on all EPA calculated responses.

As with the ASW media, the interaction between temperature and DO was found to be significant on EPA yield and concentration but not on percentage. Also, the significant effect of the interaction between pH and temperature, when 717 was cultivated on production medium; on EPA concentration did not appear to be similar when isolate was cultivated on the production media.

The DO quadratic effect was found to be insignificant on any of the EPA calculated responses whatever the cultivation media. The quadratic effect of pH showed a significant effect on all EPA responses in the production media experiment, while it was considered as significant on EPA yield and concentration, but not percentage, in the ASW experiment.

The quadratic effect of temperature was found to be significant on EPA yield and concentration when ASW was used as a cultivation media, but when production media was used, the quadratic effect of temperature was estimated as significant on EPA yield only.

The slight change in the significant effect of the different terms on isolate 717 when grown on ASW or optimised cultivation media indicates that, there could be an interaction between the media and the tested operating conditions or the behaviour of the organism may vary with varying the cultivation media components.

The generated 2D contour plots (Figures 7.6, 7.7, 7.8) could be used to investigate the behaviour of interactions between the factors, and to deduce the optimum conditions.

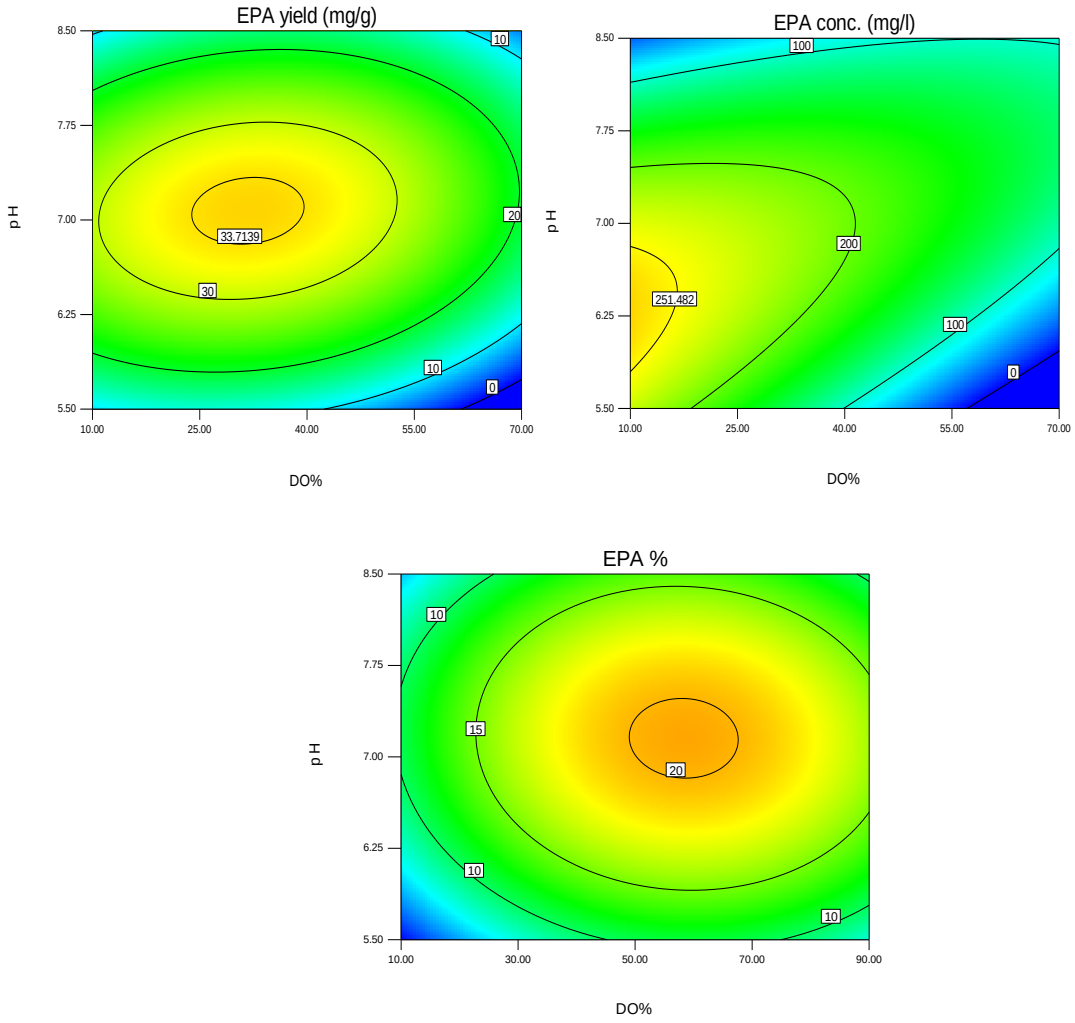

Figure 7.7: Contour plots for EPA production for the CCD experiment performed to optimise the bioreactor cultivation conditions for isolate 717 grown in the developed production medium to shows the interaction between DO and pH on: EPA yield (mg/g), EPA concentration (mg/l) and EPA percentage to the total fatty acids

The interaction between pH and DO variables on EPA productivity (Figure 7.7) showed that, the optimum pH for EPA yield (mg/g) and EPA percentage was the same (pH 7), but slightly lower (6.5-6.8) for EPA concentration (mg/l).

At extreme pH points, pH 5 and 9, only minimal growth and EPA productivity were observed (trials number 22 and 25).

The DO optimum range differs between the responses with the optimum DO for the EPA concentration was relatively low (10-15%), while for the EPA yield (mg/g) it was relatively high (30-40%) and 50-60% for percentage of EPA of the total fatty acids. High DO enhanced the biosynthesis of EPA as the proportion of the total fatty acids. The high level of EPA at high DO could be due to its shielding effect to protect the cells against the harmful oxygen molecules, where the cells enhance the biosynthesis mechanism of EPA as a response to high

dissolved oxygen concentration in the surrounding environment, while the low optimum range for EPA concentration (mg/l) may be due to that low DO% was preferable for isolate growth.

Compared to the optimum ranges obtained for isolate 717 when grown under the same conditions but cultivated on ASW medium, the pH range was similar. Also, the relatively high DO% range for EPA percentage and relatively low DO% range for EPA concentration was identical whatever the cultivation medium. The only differ was detected on the desired range for maximising EPA yield, where when isolate 717 was grown on ASW medium the optimum DO% range was relatively low (10-20%), while when was grown on the developed production medium the optimum DO% range was slightly higher (30-40%). Again this difference could be could be an interaction between the media and the tested operating conditions or the behaviour of the organism may vary with varying the cultivation media components.

In previously reported studies EPA was found to a have a protective role as an antioxidant and an EPA-deficient mutant of isolate *Shewanella marinintestina* IK-1 was found to be more sensitive to the exogenous addition of H_2O_2 and showed a remarkable decrease in the amount of cells recovered from the cultures treated with H_2O_2. In addition protein carbonylation was enhanced only in EPA-deficient cells when treating the cells with 0.01 mM H_2O_2 under bacteriostatic conditions. These results confirmed that EPA has a shielding effect for protecting the bacterial cells (Nishida *et al.*, 2006a; Nishida *et al.*, 2007).

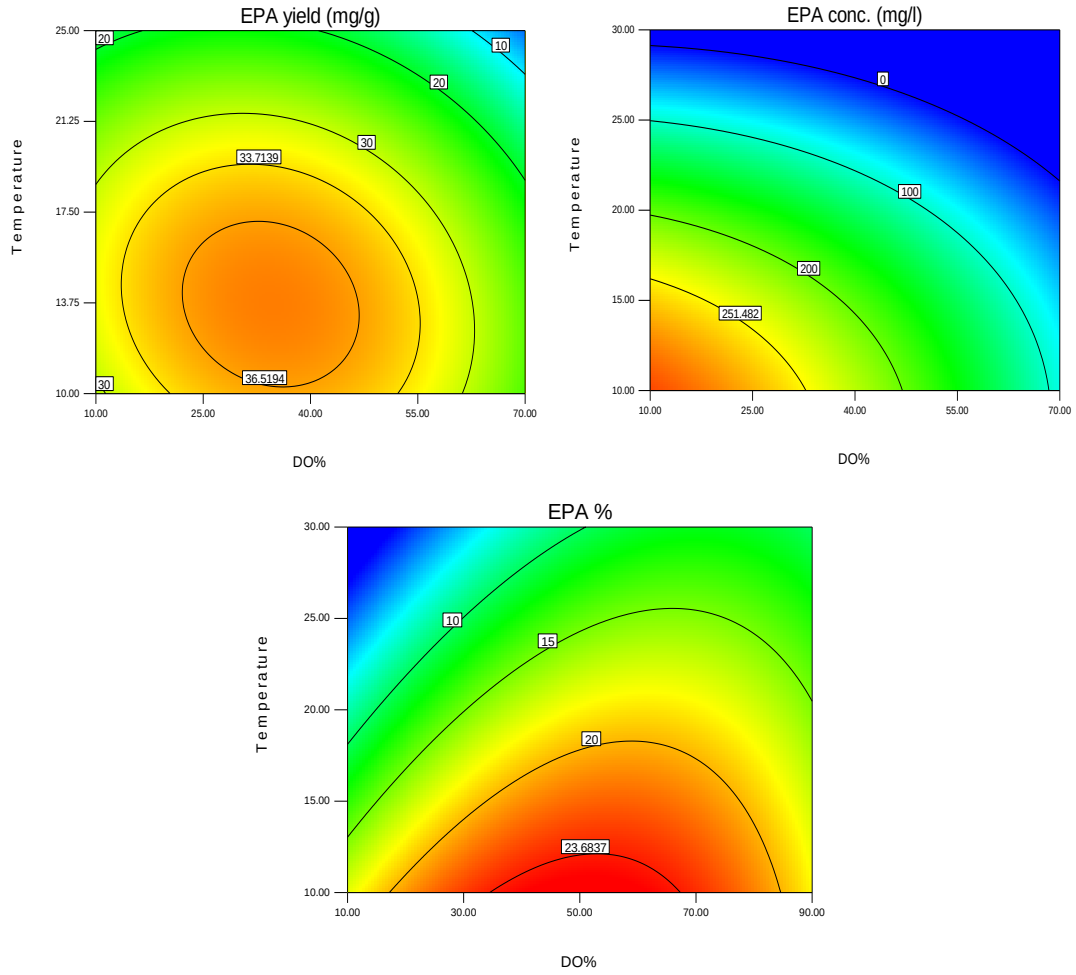

Figure 7.8: Contour plots for EPA production for the CCD experiment performed to optimise the bioreactor cultivation conditions for isolate 717 grown in the developed production medium to shows the interaction between DO and temperature on: EPA yield (mg/g), EPA concentration (mg/l) and EPA percentage to the total fatty acids

The interaction between DO and temperature (Figure 7.8) showed that the optimum range of DO was relatively low for high EPA concentration (mg/l), but relatively high for EPA expressed either as yield or percentage of the total fatty acids.

The optimum temperature range, for EPA yield (mg/g), was between 13-15°C, and for EPA concentration was between 10-12°C, while for the EPA percentage the analysis indicates that the lower the temperature the higher the EPA percentage. This observation is in line with the theory suggesting that EPA is required to sustain the fluidity of the plasma membrane when the organism grows in extreme low temperature environment (Skerratt *et al.*, 2002b). Isolate 717 completely loses the ability to produce EPA when incubated at 30°C (trial number 8).

The same observation was previously reported for different bacterial isolates, as they were unable to produce EPA at temperatures above 25°C (Nichols and McMeekin, 2002).

EPA was also proven to play an important role in bacterial membrane organisation and cell division especially at low temperature. The lack of EPA resulted in the cells of *Shewanella livingstonensis* Ac10 to form multi-nucleoids filaments leading to growth retardation (Kawamoto *et al.*, 2009).

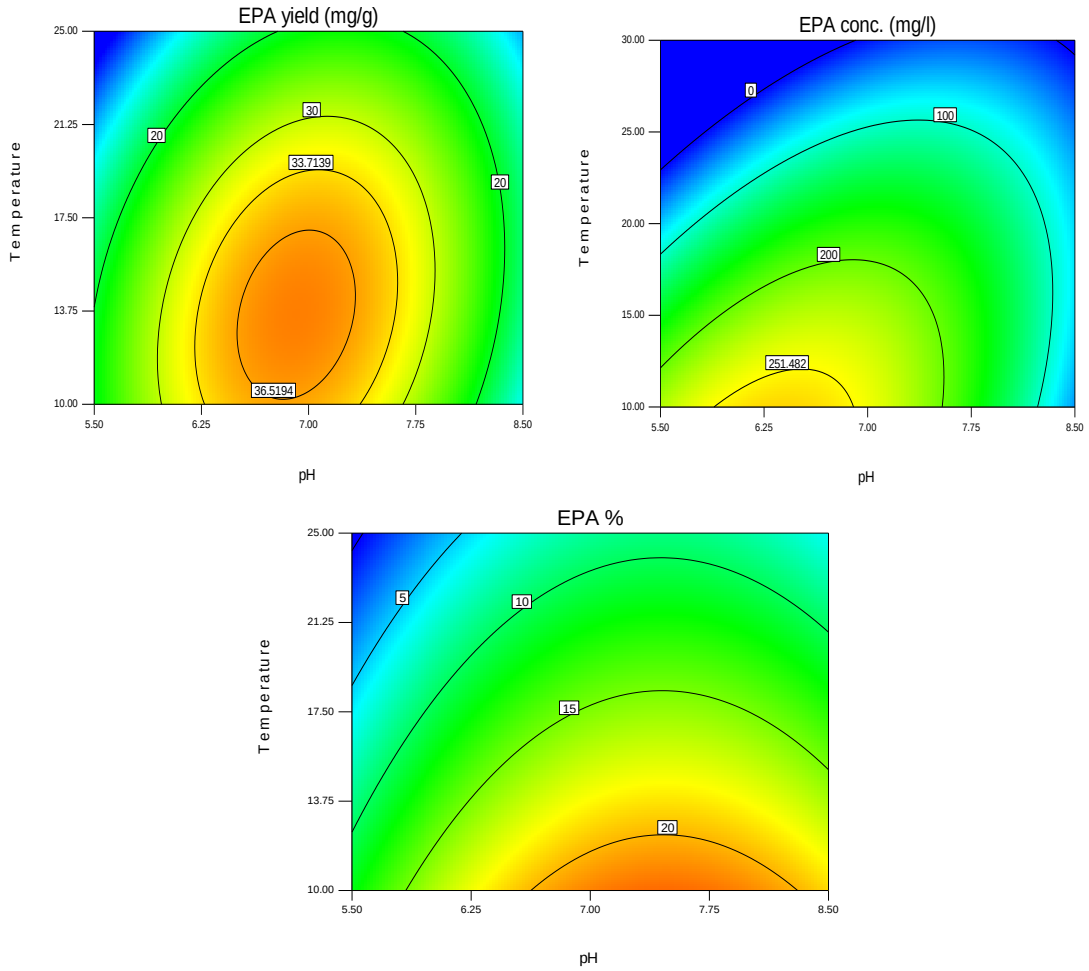

Figure 7.9: Contour plots for EPA production for the CCD experiment performed to optimise the bioreactor cultivation conditions for isolate 717 grown in the developed production medium to shows the interaction between temperature and pH on: EPA yield (mg/g), EPA concentration (mg/l) and EPA percentage to the total fatty acids

The interaction between pH and temperature (Figure 7.9) confirmed the optimum ranges: neutral pH and relatively low temperature were preferred by isolate 717 for maximum growth and productivity.

For numerical optimisation, second order polynomial order models were developed, using coded units, to predict the optimum combination of investigated process variables for each response, equations 7.4-7.6,

$$EPA\ yield\ (mg/g) = 32.99 - 3.52A + 3.34B - 7.41C - 0.75AB + 8.21AC + 1.09BC - 1.97A^2 - 10.48B^2 - 3.23C^2 \quad \text{(eq.7.4)}$$

$$EPA\ conc.\ (mg/l) = 169.96 - 22.02A + 14.50B - 57.78C + 32.00AB + 5.56AC + 30.26\ BC - 6.44A^2 - 48.56B^2 - 12.98C^2 \quad \text{(eq.7.5)}$$

$$EPA\ \% = 15.30 + 3.31A + 2.06B - 4.42C - 0.42AB - 0.046AC - 0.041BC - 0.74A^2 - 2.57B^2 - 0.15C^2 \quad \text{(eq.7.6)}$$

Where A represents DO (%), B represents pH and C represents temperature (°C).

Using the mathematical model and the interaction colour contour graphs, the optimum conditions were estimated. Subsequently, three cultivations were carried out under these conditions and the actual values of response variables were compared to the predicted ones (Table 7.6).

These results confirm that a neutral pH was optimal for both the growth and productivity of isolate 717. A relatively high DO and low temperature were desirable for the highest EPA yield while a relatively low DO was desirable for highest concentration, indicating that low oxygen concentration in the media was desirable for maximum growth.

Table 7.6: Optimum culture condition combinations for each response for isolate 717 in bioreactor cultivation with developed production medium in addition to the predicted and the actual values for each response variable

Target	**Variables**			**Predicted Values**	**Actual Values**
	Dissolved Oxygen (%)	pH	Temperature (°C)		
Maximise EPA yield (mg/g)	33.35	7.00	13.54	39.57	47.45 (± 2)
Maximise EPA conc. (mg/l)	10.66	6.81	10.11	323.33	348.89 (± 5)
Maximise EPA %	55.37	7.13	8.10	23.09	25.6 (± 0.8)

As the optimum ranges of the growth and the EPA productivity diverged, an additional optimisation was carried out to maximise all the calculated responses simultaneously (Table 7.7).

Table 7.7: The optimum culture condition combination for EPA production by isolate 717 when cultivated in bioreactor level and the production medium

Variables			Predicted Values			Actual Values		
Dissolved Oxygen (%)	pH	Temperature (°C)	EPA yield (mg/g)	EPA conc. (mg/l)	EPA %	EPA yield (mg/g)	EPA conc. (mg/l)	EPA %
34.45	7.08	10.00	35.99	283.024	21.52	42.34(± 2)	308.2(± 7)	22.23(± 1.1)

7.4 Comparison of EPA production in ASW and production media

Under the optimum conditions for EPA production in the bioreactor, a comparison was performed between the ASW media and the optimised production medium and the results are shown in Figure 7.10.

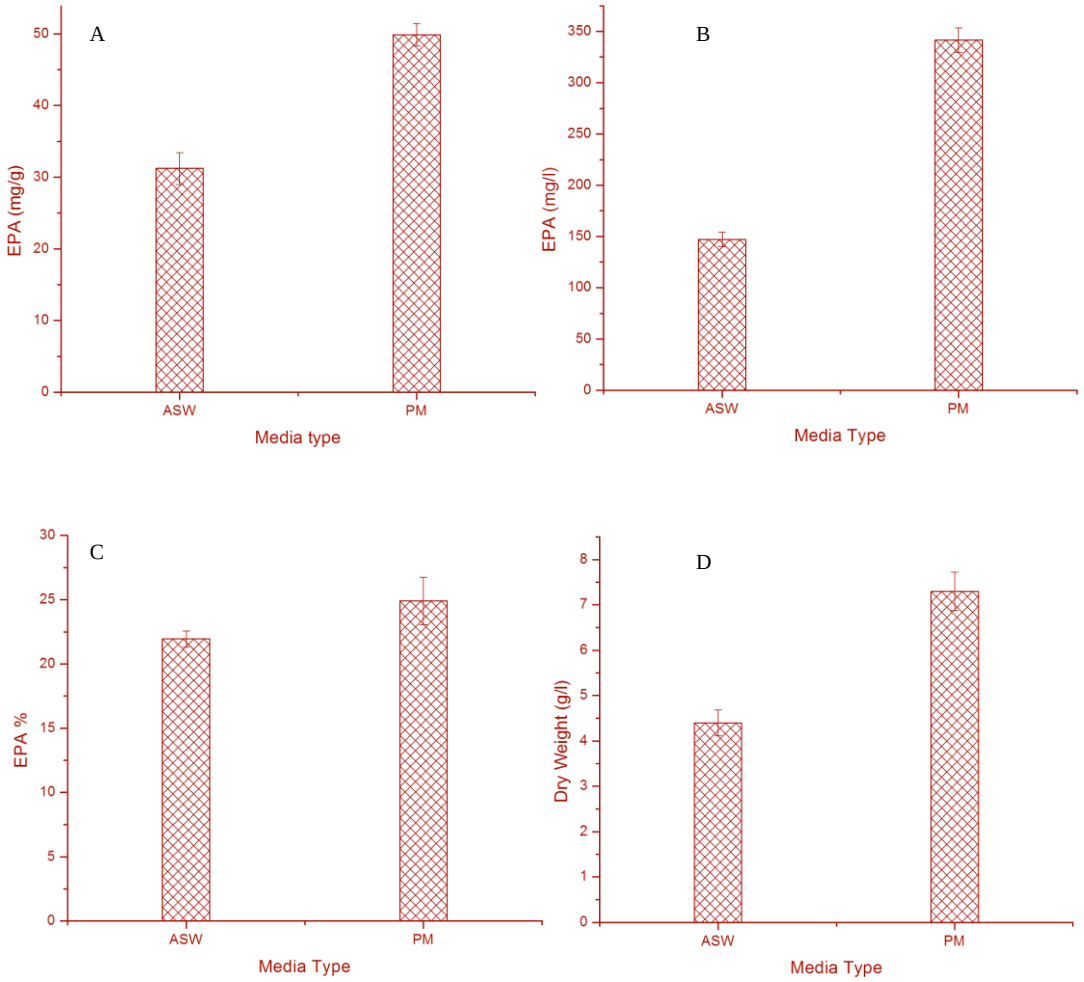

Figure 7.10: Comparison of growth and EPA production in ASW medium and production medium, for isolate 717, under the optimum growth conditions in the bioreactor for each medium. The optimum conditions for ASW medium are summarised in Table 7.2, while for production medium are summarised in Table 7.6.

The difference between the ASW and the production medium (PM) in the ability to produce EPA either as concentration or yield was obvious. The amount of EPA (mg/g) produced when growing in the PM was approximately 46 mg/g while in the ASW it was 32 mg/g. Regarding the concentration, the amount of EPA produced when growing in the PM was approximately 350 mg/l which is more than double of that produced when growing in the ASW media (150 mg/l). The differences in term of EPA% were not as significant. For the ASW medium, the EPA% increased from 8% in shake flak to 23% in bioreactor, while for production medium the EPA% increased from 19% in shake flask to 25% in the bioreactor indicating that 25% may be the maximum possible achievement in terms of percentage of total fatty acids.

The amount of EPA produced by isolate 717 in the bioreactor was significantly increased compared to the amount of EPA produced in shake flasks in all calculated responses both in ASW and production media.

7.5 Chemostat

In order to characterise the physiology of isolate under steady state conditions, a chemostat experiment was performed. The effect of different growth rates on the ability to produce EPA to be studied in more details.

A continuous stirred-tank reactor (CSTR) was set up to test the effect of three different growth rates. Another chemostat was performed at constant growth rate under varying temperature, to test its sole effect on the EPA production as it was identified as the most significant variable affecting the EPA productivity (section 7.2 and 7.3).

In the first set of chemostat cultivations the effect of specific growth rates was assessed. The experiments were carried out as described in section 2.16.2. The growth rate effect was found to be negligible on the ability of isolate 717 to produce EPA (Figure 7.11).

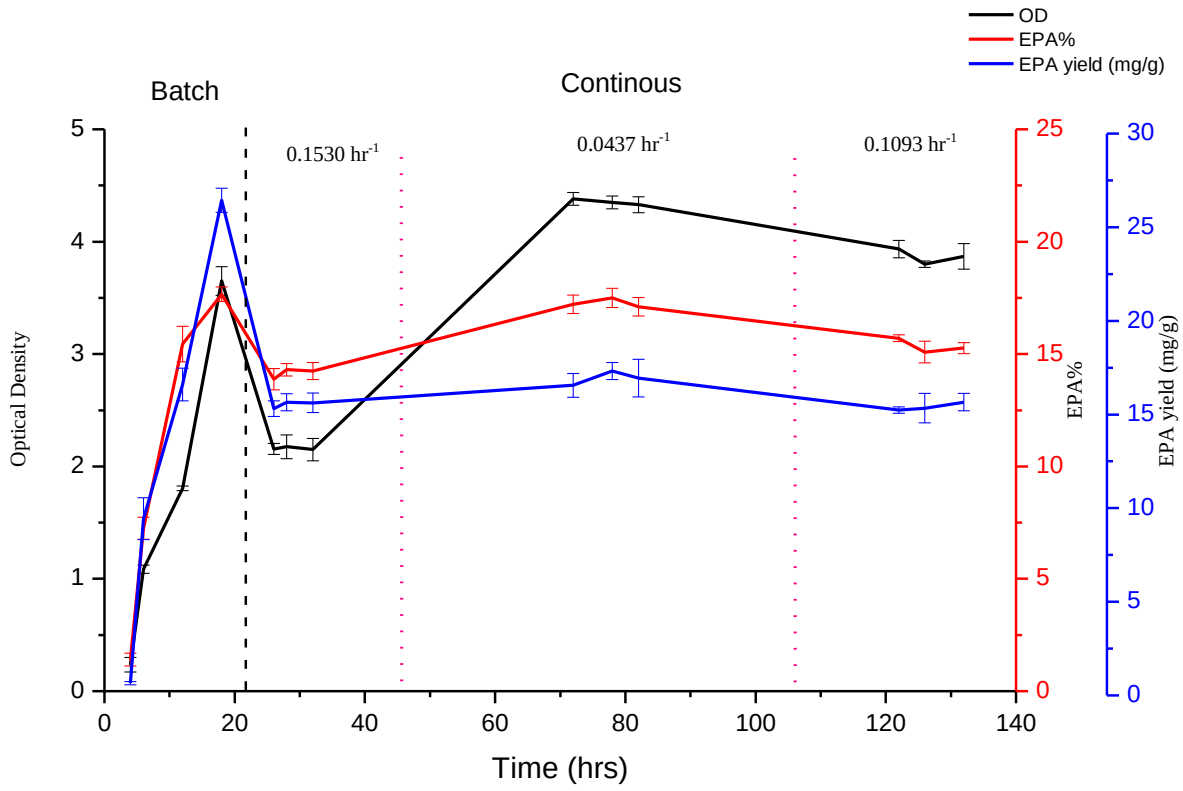

Figure 7.11: Effect of different growth rates on the growth and EPA productivity of isolate 717 in chemostat cultivation in ASW medium with 15°C as the cultivation temperature, pH 7 and 30% DO. The green line represents the optical density, red line represents EPA yield (mg/g), black line represents EPA% of the total fatty acids and the blue line represents biomass concentration (g/l).

The effect of the tested growth rates on the EPA productivity by isolate 717 the differences were insignificant with slightly preference of the lower growth rate.

The insignificant effect of growth rate on the ability of isolate 717 to produce EPA contradict with the effect of dilution rate on the ability of the microalga *Phaeodactylum tricornutum* to produce PUFA, where the high dilution rates lead to maximising the amount of PUFAs produced compared to the low ones (Reis *et al.*, 1996).

The different growth rates were tested in a random way to avoid any consistent or accumulative errors.

As temperature was found to be the most significant factor affecting the EPA production during the bioreactor cultivations, another set of chemostat experiments was performed to test temperature effect under constant growth rate (μ= 0.1093 hr^{-1}), Figure 7.12.

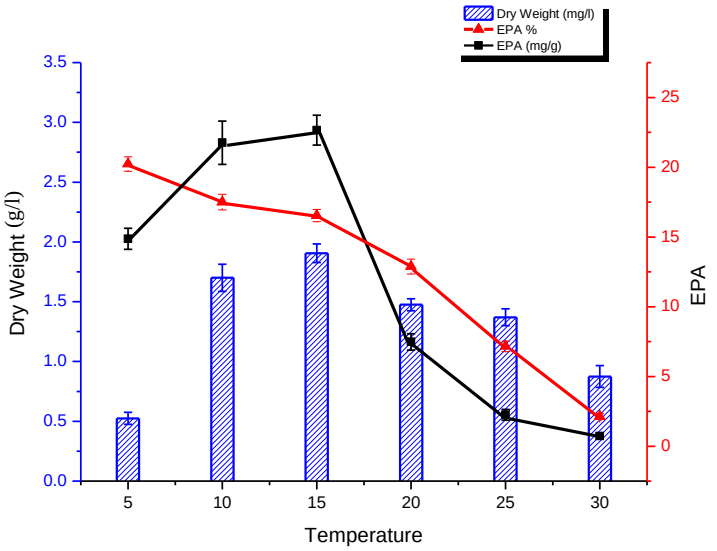

Figure 7.12: Effect of different temperatures, at a constant growth rate(μ= 0.1093 hr^{-1}), within CSTR, on the growth and EPA productivity of isolate 717 growing in bioreactor with ASW as a cultivation medium and 30% DO and pH 7. The red line represents EPA as a fraction of total fatty acids, black line represents EPA yield (mg/g) and blue columns represent biomass concentrations (g/l).

Figure 7.12 shows that the optimum temperature for growth and EPA yield was found to be 15°C. For EPA percentage an inversely proportional relationship was found between temperature and EPA percentage as it decreases with increasing the temperature from the highest percentage (20%) at 5°C to its minimum value (2%) at 30°C.

It was reported that the limited production of EPA from species related to *Shewanella pneumatophore* at 30°C may be due to that temperature-sensitive enzymes are included in EPA biosynthesis (Hirota *et al.*, 2005).

The results showed that lower temperature was desired for higher EPA production particularly for the EPA percentage. This result supports the theory that EPA may play a role in the cold adaptation mechanism by sustaining the membrane fluidity under such extreme conditions (Delong and Yayanos, 1986; Sato *et al.*, 2008).

7.6 Growth and EPA profile for isolate 717 under optimum conditions

To check the EPA profile over time isolate 717 was cultivated in the previously developed production medium (casein, Hy-soy and Na_2HPO_4) in a bioreactor under the cultivation conditions summarised in Table 7.7 which were found to maximise all the calculated responses simultaneously (DO 34%, temperature 10°C and pH 7).

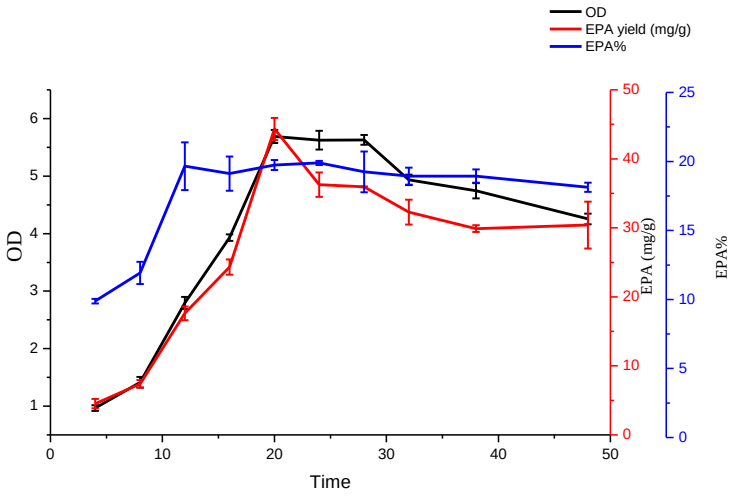

Figure 7.13: The growth and EPA profile for isolate 717 when grown in a bioreactor as batch cultivation under optimal conditions (DO 34%, temperature 10°C and pH 7). Blue line represents EPA%, red line represents EPA yield (mg/g) and black line represents OD.

The maximum amount of EPA expressed as either as percentage or yield was obtained at the late exponential phase and early stationary phase (Figure 7.13). For growth kinetics during the exponential phase it was found that there were two growth phases with two different maximum specific growth rates (μ_{max}). Figure 7.14 shows these two different growth phases.

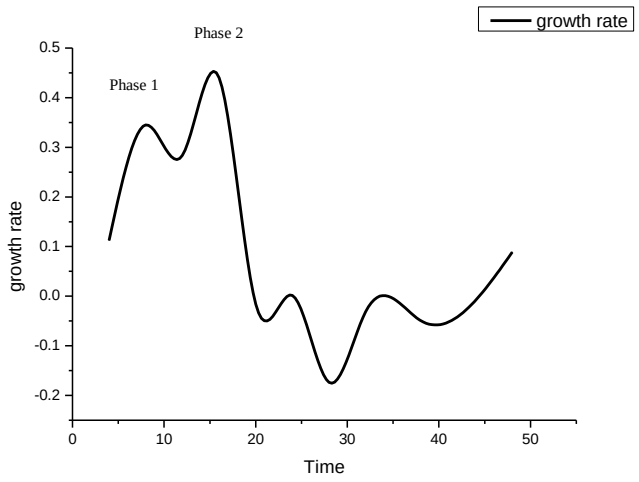

Figure 7.14: The growth rate of isolate 717 when grown in a bioreactor as batch cultivation under optimal conditions ((DO 34%, temperature 10°C and pH 7). Black line represents the growth rate.

In Figure 7.13 it is clear that isolate 717 shows a two distinct growth phases (phase 1 and phase 2) during the exponential phase. This observation suggests that the organism displays diauxic growth behaviour. The production medium contained casein and Hy-soy which could be the reason for the diauxic growth of isolate 717. The phase 1 is characterised by a $\mu_{max.1}$= 0.3726 hr^{-1} and the phase 2 by a $\mu_{max.2}$= 0.3889hr^{-1} showing that the growth rate when utilising the substrate in phase 2 was faster than that in phase 1.

Figure 7.15 shows that a similar trend of two distinct phases in the EPA specific production rate.

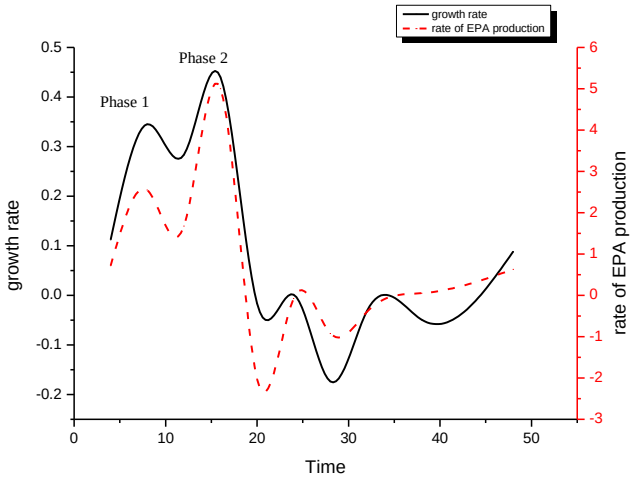

Figure 7.15: The EPA specific rate of production for isolate 717 when grown in a bioreactor as batch cultivation under optimal conditions ((DO 34%, temperature 10°C and pH 7). Black line represents the growth rate. Black line represents growth rate while red line represents EPA specific production rate.

The EPA specific production rate in phase 2 was much higher than that at phase 1. It was not clear if these two phases of EPA production rates were due to the differences in growth rates or due to the switching of the substrate utilisation (substrate utilised at the phase 2 was preferred for EPA production than substrate utilised at phase 1).

The relationship between the growth rate and rate of change in EPA percentage is summarised in Figure 7.16.

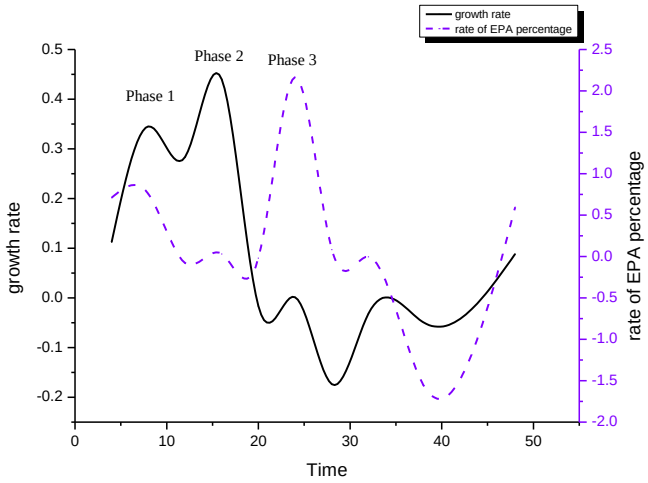

Figure 7.16: The rate of change in EPA percentage for isolate 717 when grown in a bioreactor as batch cultivation under optimal conditions (DO 34%, temperature 10°C and pH 7 Black line represents growth rate and the violet line represents EPA percentage rate.

EPA percentage formation was found not to be related to growth and did not show the two distinct phases as previously found with the growth and EPA specific rate of production. On the contrary, the very low EPA percentage rate during the exponential phase may be due to the high rate of total fatty acid formation during the exponential growth. The maximum EPA percentage rate obtained at the stationary phase probably due to the high stress during this phase where the formation of free radicals is a common feature and the high death rate and low microbial cell activity leads to a high exposure to dissolved oxygen. This stress may trigger increased rate of EPA compared to the total fatty acids to protect the cells against such stressful conditions.

Phase 3 is preferred for harvesting from the downstream prospective where the high EPA parentage facilitates its separation from the fatty acid mixture. However, in terms of large scale manufacture, an important factor to investigate would be the extension of the time window of high EPA percentage synthesis to enable effective processing at this scale.

7.7 Temperature shock

In all previous cultivations the maximum amount of EPA was obtained at the late exponential phase and the start of the stationary phase. This is similar to other organisms such as *Marchantia polymorpha* , a moss like plant, with the maximum amount of PUFAs were produced at the late exponential phase or the early stationary phase (Chiou *et al.*, 2001).

Low temperature proved to enhance the EPA production, thus in order to avoid intensive cooling requirements during the entire cultivation which may become prohibited expensive at manufacturing scale, an experiment was carried out to test the feasibility of a short term temperature shock. This experiment was carried out as described in section 2.16.1. Figure 7.17 shows the temperature profile throughout the cultivation and the resulting EPA% and yield.

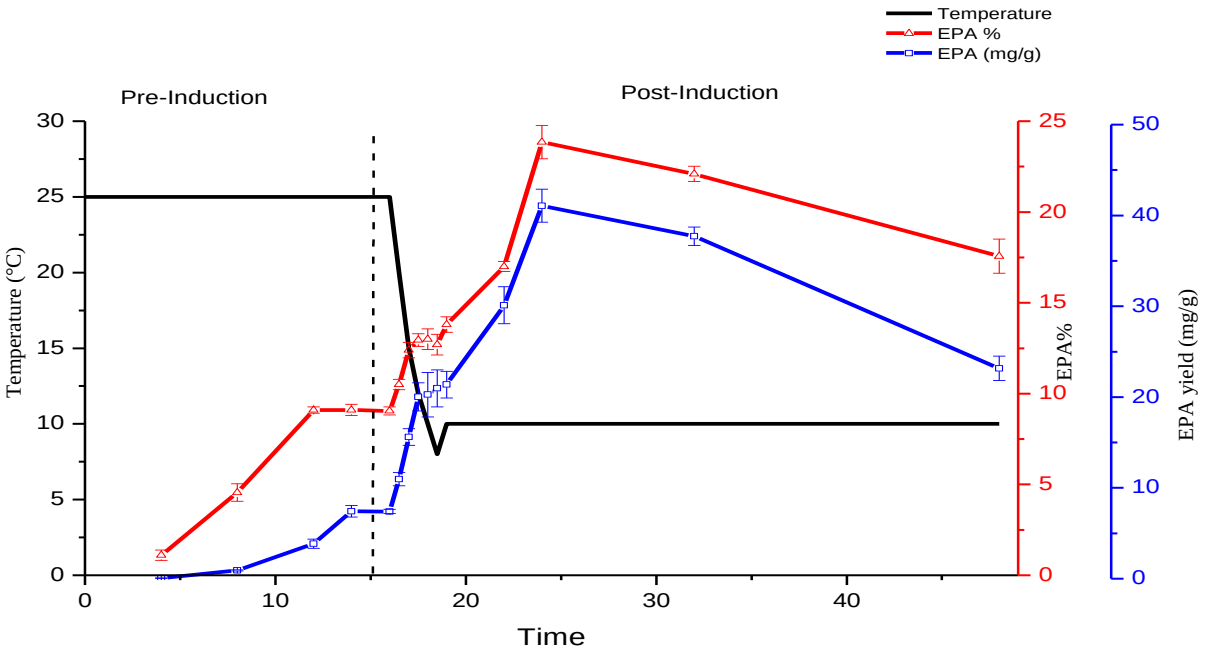

Figure 7.17: The amounts of EPA produced in response to the temperature reduction in bioreactor batch cultivation for isolate 717. Blue line represents EPA%, red line represents EPA yield (mg/g) and black line represents temperature (°C).

The highest amount of EPA was obtained 4 hours after reaching the new temperature set point. Applying this operating policy will limit the requirement of intensive cooling to around four hours prior to harvesting and still resulting in a 40 mg/g EPA yield with representing 22% of the total fatty acids compared to 45 (±2) mg/g EPA and 25 (±1.1) % EPA when applying the low temperature over the whole fermentation process (10 ^{0}C).

7.8 Production under anaerobic conditions

To test the ability of isolate to grow and produce EPA under totally anaerobic conditions (as described in section 2.16.1), batch cultivation was carried out in the complete absence of oxygen. The EPA yield was 11.64 mg/g biomass (compared to 45 mg/g of EPA under the optimum conditions, DO 34%, temperature 10°C and pH 7) representing approximately 8% of the total fatty acids (compared to 25% of the total fatty acids under the optimum conditions).

Although high oxygen levels increased the amount of EPA, the ability of isolate to produce EPA in the complete absence of oxygen suggests that its biosynthesis does not require oxygen, and the role of oxygen may be enhancing the process as an inducer. The fact that, the anaerobic cultivation was carried out at low temperature (10 ^{0}C) suggest that, the amount of EPA produced (11.64 mg/g representing 8% of the total fatty acids) may be the amount of EPA required by isolate to sustain the fluidity of plasma membrane under extreme cold conditions, while the high amount of EPA produced during the aerobic cultivation processes may be due to the interaction between DO and temperature. However this hypothesis requires further experimental confirmation.

7.9 Monitoring the uptake of element by isolate 717

The effect of metal ions on the growth and EPA productivity of isolate 717 was studied using a separate CCD and results are presented in section 6.4. The optimum metal ion combination for maximum productivity was determined to be (2.5 g/l Na_2HPO_4, 1.2 g/l KCl, 23 g/l NaCl and 1.8 g/l $MgSO_4$).

A medium with this suggested concentration in addition to 3.5 g/l peptone and 3.5 g/l yeast extract was used to carry out an experiment at a bioreactor scale, as described in section 2.18).

Isolate 717 was grown in bioreactor 10°C with 35% DO set-point and no pH control to avoid any potential interference of acid/alkali with the tested chemical elements.

Figures 7.18, 7.19 and 7.20 show a time trajectory of each residual elements in the medium (measured as described in section 2.18.1) in relation to the growth (OD) and EPA yield and percentage. Figure 7.18 shows the relationship between the elements present in relatively high concentrations, so called macro-element, (g/l of medium) including Cl and Na.

Elements with relatively low concentrations (g/l), so called micro-elements, were plotted against the growth and EPA productivity in Figure 7.19. Figure 7.20 shows the relationship between the growth and EPA productivity and the concentration of residual carbon and nitrogen (in elemental form) elements in the medium.

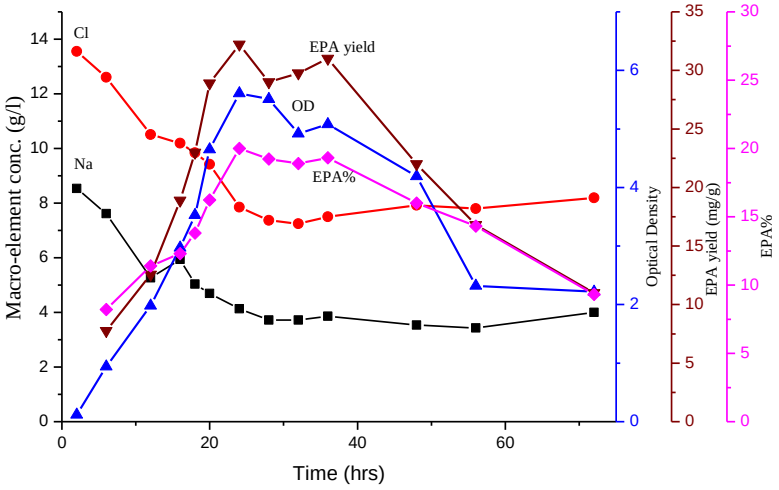

Figure 7.18: Time trajectory of the residual macro-elements (Cl and Na) in relation to the growth (OD) and EPA yield (mg/g) and percentage.

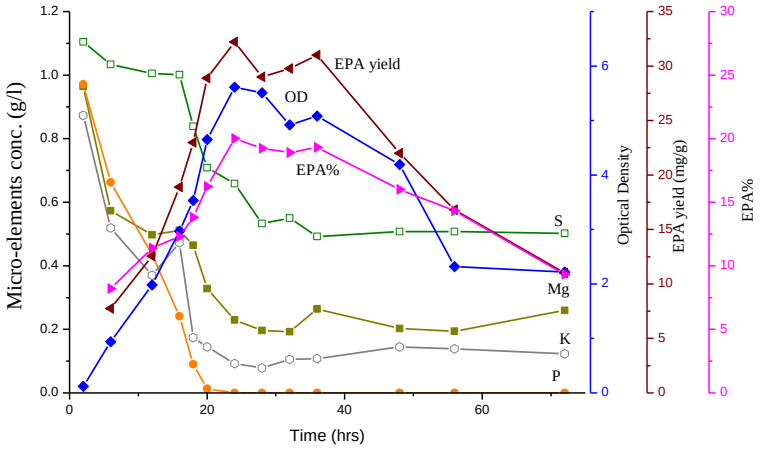

Figure 7.19: Time trajectory of the residual micro-elements (S, Mg, K and P) in relation to the growth (OD) and EPA yield (mg/g) and percentage.

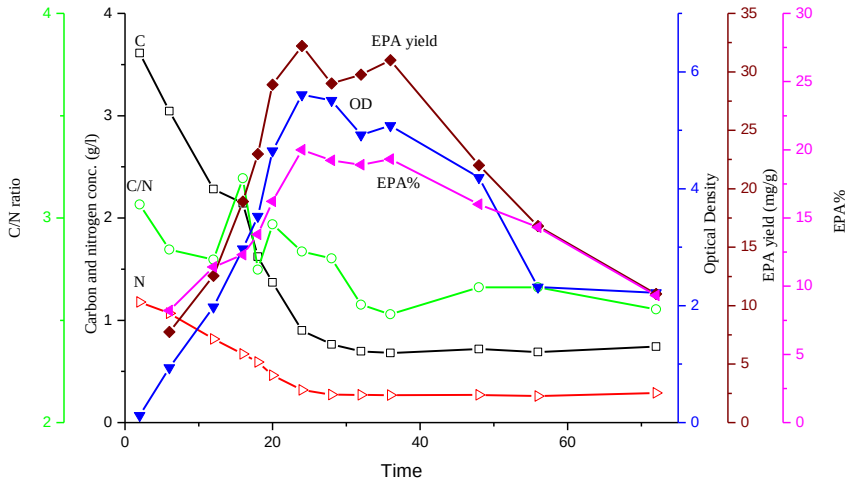

Figure 7.20: Time trajectory of the residual carbon and nitrogen elements and C/N ratios in relation to the growth (OD) and EPA yield (mg/g) and percentage.

As expected all investigated elements showed a remarkable concentration reduction during the exponential growth of isolate 717 due to high uptake. In stationary phase all elements, except phosphate, were present in the cultivation medium in sufficient amount suggesting that phosphate is limiting the growth of isolate 717. Also, the highest amount of EPA was produced following the phosphate depletion suggesting that the phosphate starvation may trigger an increased rate of EPA biosynthesis. However further experimental work is required to confirm the hypothesis.

The C/N ratio profile was relatively low when EPA was at its maximum confirming the results described in section 6.6.5.

7.10 Bacterial oil stability

An important aspect from the point of view of economic viability of PUFA manufacturer from bacterial sources is the stability and the purity of the produced PUFA compared to current sources.

To test the bacterial oil stability, relatively large quantities were required to perform such experiments, so the oil was extracted from the biomass achieved in the bioreactor cultivation to obtain enough biomass. Also, the oil obtained from isolate when it was cultivated in the bioreactor under optimum conditions achieved the highest percentage of EPA as a fraction of total fatty acid.

Two stability tests were performed including thermal stability and oxidative stability as described in section 2.10.

7.10.1 Bacterial oil thermal stability

The thermal stability of the fish oil is an important criterion in terms of oil purification, handling and utilisation operations and in the quality assessment of the oil (Wesolowski, 1987; Wesolowski and Erecinska, 1998). Bacterial oil stability was tested against the fish and fish liver oil showing a competitive stability (Figure 7.21). The bacterial oil, extracted from isolate 717, appears to be more stable than the liver oil and is comparable to the fish oil.

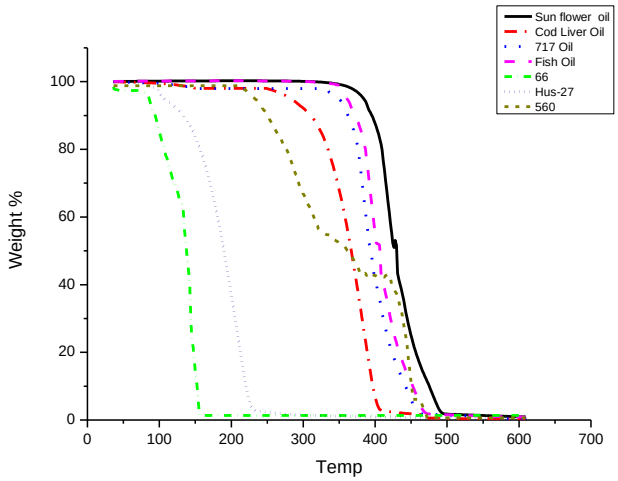

Figure 7.21: Weight loss over time at different temperatures to compare the stability of the bacterial oil extracted from isolates 717(blue dots), Hus-27 (navy dots), 66 (light green short dash dots) and 560 (dark green dashes) against fish (magenta dashes) and fish liver oil (red dash dots), with sunflower oil (black line) used as a reference.

The bacterial oil, including EPA, produced by isolate 717showed a competitive stability compared to fish and fish liver oils. The additional lack of the bad smell means that bacterial PUFA could be used as an omega-3 supplement for applications in particular those requiring high temperature exposures.

The oil extracted from isolate 66 showed the least thermal stability among the tested oils, followed by the oil extracted from the Egyptian isolate Hus-27. The oil extracted from isolate 560 dissociate on two steps indicating that part of the oil was lost before the rest decomposed. The oil extracted from isolate 717 showed stability higher than that of the fish liver oil and nearly the same behaviour as the fish oil.

In order to reduce the risk of the cooking oil, a balanced amount of omega-3 to omega-6 start to be added by different company. The bad smell of the fish oil makes its incorporation to be inapplicable. The bacterial oil, rich in omega-3 and with high thermal stability, could be a potential source of omega-3 supplemented in the cooking oil.

Another application of the bacterial oil is incorporating the oil in dairy product as milk. The incorporation of bacterial oil, with a high thermal stability, into the milk could be a potential good source for the daily requirements of omega-3. Applying fish oil to such application was un-practical.

7.10.2 Bacterial oil oxidative stability

The oxidative stability of the bacterial isolate 717 isolate and cod liver oils and the fatty acid methyl esters (FAME) of the bacterial isolate 717 and cod liver oils was measured using the ACL Instrument. It is clear to see from Figure 7.22 that the FAMEs are less stable than the cod liver oil itself as they are derivatised and extracted from the oil so they were volatile and susceptible to oxidation in this state.

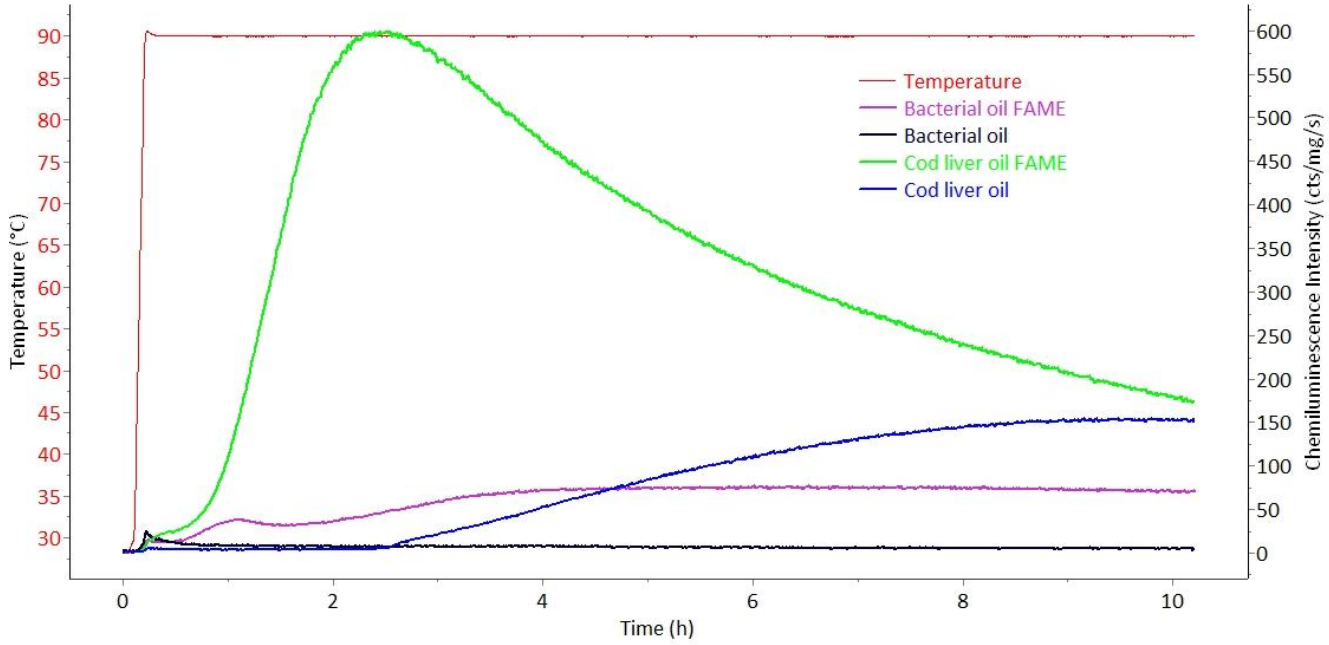

Figure 7.22: CL profiles of cod liver oil, cod liver oil FAME, bacterial isolate 717 oil and bacterial isolate 717 oil FAME, where the red line represents the operating temperature, pink line represents isolate 717 FAME, black line represents isolate 717 oil, green line represents the cod liver oil FAME and blue line represents cod liver oil.

The cod liver oil has a clear induction period (the flat line before the point of inflection on the curve), before the oxidation begins to accelerate to a peak. This is not apparent in the cod liver oil FAME which begins to oxidise as soon as the air supply was changed from nitrogen

to synthetic air which was at the point the temperature reaches 90^0C, which indicated that it was less stable than the cod liver oil.

The same hypothesis applies to the bacterial oil FAME which had a much higher CLI at its peak than the bacterial oil. What is interesting about the comparison between the bacterial oil and bacterial oil FAME curves is that the bacterial oil showed a peak very early on as the gas supply changed from nitrogen to oxygen. This sharp peak may be the main oxidation event. An explanation for observation may be that this peak did not represent oxidation, but the bacterial oil was very stable and may not oxidised during the time course of this experiment, so it may be necessary to run this for longer to see if a peak appears after 10 hours.

The shape of the bacterial oil FAME curve, with two distinct regions may suggest that there are two oxidation events occurring here. e.g. the first part of the curve may correspond to the least stable FAME in the mixture with the second part of the curve being attributable to a more stable set of FAMEs which continue to oxidise as the run comes to an end.

7.11 Testing the presence of heavy metals

One of the main health risks associated with the consumption of fish and fish products is the bioaccumulation of heavy metals within the fish fats (De Gieter *et al.*, 2002), Thus extra purification expenses would be required to purify the product before release to the market. Energy-dispersive X-ray spectroscopy (EDX) was found to be a useful technique for detecting heavy metals (Roach *et al.*, 2009). A preliminary screening for isolate biomass using EDX indicated undetectable levels of heavy metals under the test conditions applied in this experiment (Figure 7.23). This promising initial result indicates that purification costs associated with the removal of heavy metal ions may be avoided during bacterial oil manufacture.

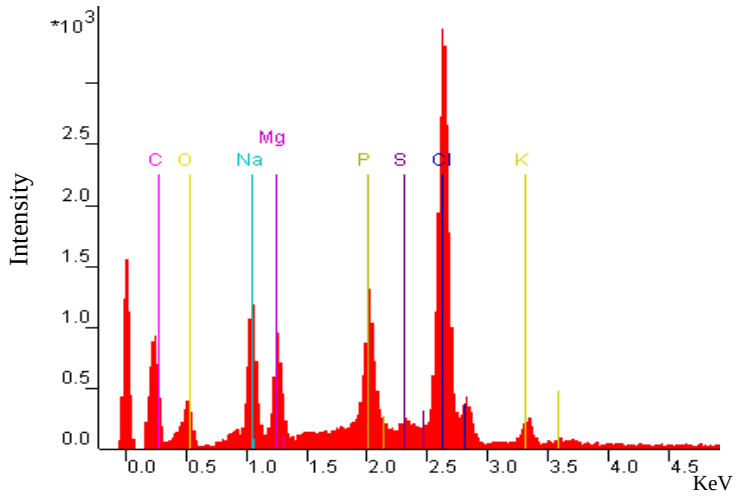

Figure 7.23: EDX spectrum for isolate 717 biomass when cultivated in the previously developed production medium under the optimum cultivation conditions in bioreactor.

The lack of metal ions, unpleasant odour, together with a thermal and oxidative stability competitive to the fish oil make the bacterial oil a promising alternative PUFA provider.

7.12 Comparison of the growth and productivity of isolates under investigation under different cultivation conditions

During this study, the cultivation conditions of three different isolates (Hus-27, 66 and 717) were screened and optimised at two levels, shake flask and bioreactor levels. Potential production media to achieve the maximum productivity were developed for each isolate.

Figure 7.24 shows the comparison between the growth and productivity at shake flask and bioreactor levels for each of isolates. The comparison is based on the growth and EPA productivity in both artificial sea water (ASW) and production media (PM) developed for the given isolates.

The shake flask experiments were carried out at 15°C as a cultivation temperature and 160 rpm in a shake flask orbital incubator. The bioreactor cultivations were carried out at 35% dissolved oxygen, pH 7.08 and 10°C as incubation temperature.

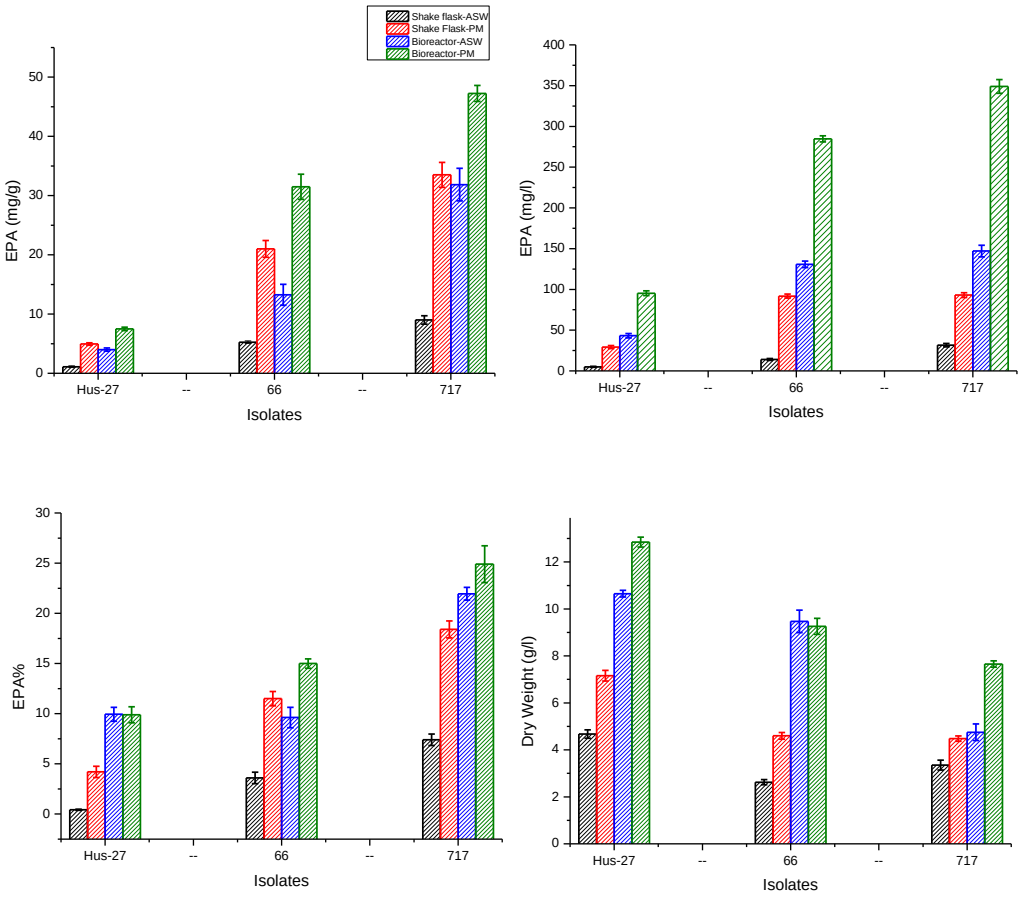

Figure 7.24: Comparing the growth and productivity of isolates under investigation under different cultivation conditions. Black columns represent the growth in ASW medium at shake flask level, red columns represent the growth in PM at shake flask level, blue columns represents the growth in ASW medium at bioreactor level and green columns represent the growth in PM medium at bioreactor level.

The all over pattern for all isolates clearly showed that the production media were preferred for maximum growth and EPA productivity when compared to the ASW media, and the growth and productivity in the bioreactor were much higher than that in shake flask experiments.

However some exceptions were spotted where, the amount of biomass produced by isolate 66 in the bioreactor was approximately the same in both cultivation media. This was also the case for the EPA % of the total fatty acids produced by isolate Hus-27 (10%) and the percentage of EPA produced by isolate 717 in the bioreactor (25% and 22%) indicating that the cultivation conditions were affecting the EPA percentage than the media composition.

Isolate 717 was found to be the highest EPA producer under all treatments but isolate Hus-27 achieved the highest biomass concentration.

7.13 Online monitoring of the produced EPA

The main target of this experiment was to develop a suitable method for online estimation for the produced EPA as described in section 2.19.

Different combinations of different EPA and biomass concentrations of isolate 717 were prepared as in Table 7.8.

Table 7.8: Combinations of different biomass and EPA concentrations to test the online monitoring for the growth and EPA productivity by isolate 717

Cell Density (OD)	EPA concentrations (µg/l)
1.0	5
1.2	45
1.5	15
1.5	45
0.5	45
0.5	15
0.5	5
0.2	35
1.5	25
0.5	25
0.2	5
1.2	5
0.2	15
1.2	25
1.5	5
1.0	35
1.0	5
1.2	45
1.5	15
1.5	45
0.5	45
0.5	15
0.5	5
0.2	35
1.5	25

These different combinations were scanned by the NIR spectroscopy (Figure 7.25). The obtained spectra were analysed by Dr. Ronan O'Kendey and the validation experiments showed an accurate prediction for the biomass but not for the EPA concentrations.

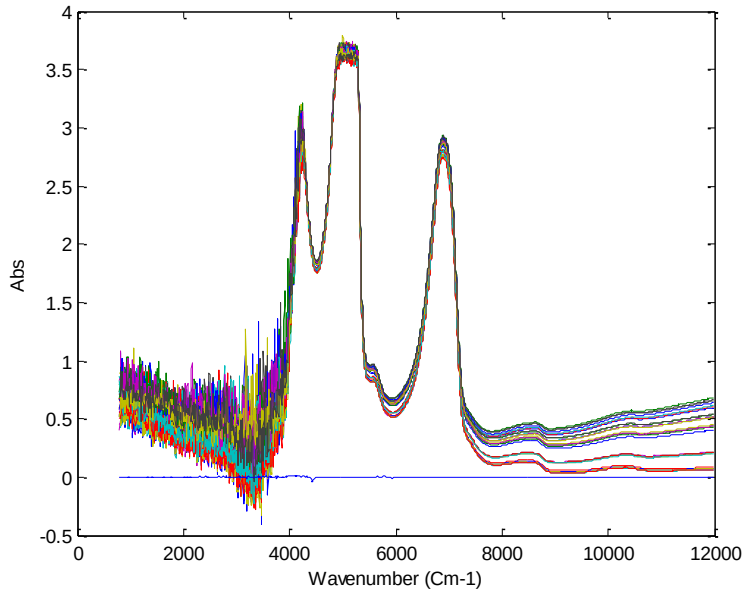

Figure 7.25: The NIR spectra for different concentrations of the biomass and the EPA produced by isolate 717.

Succeeding in online quantification of EPA during the batch cultivation would determine the optimum harvest time for each process, in addition would reduce the time required for GC experiments.

Berzaghi *et al.* (2005) used NIR spectroscopy to predict the physicochemical composition of hens breast meat and the omega-3 composition. In addition Cozzolino *et al.* (2005) used NIR spectroscopy to determine fatty acid contents in fish oil.

Although applying the NIR spectroscopy did not succeed in accurate prediction of the amount of EPA produced by isolate 717, further investigation may lead to improve and develop a robust and reliable technique for online monitoring of EPA.

7.14 Chapter Summary

Response surface methodology is a useful technique to optimise the culture growth conditions for different bioprocesses including EPA production. Culture conditions are a key factor in optimising the EPA production process. Temperature, dissolved oxygen and pH proved to have a significant effect on EPA production, either as a linear effect, two way interactions or quadratic effects.

The main challenge was reducing the oscillation occurred when the default PID values were applied to the process. The oscillation lead to a non-robust process and make the process less reproducible and controllable, as both the agitation and aeration affect the nutrient distribution and relative fluid velocities in the fermentation broth.

Enhancing EPA production by lowering the temperature supports the theory that EPA plays a vital role in sustaining the membrane fluidity under extreme cold conditions (Skerratt *et al.*, 2002b). Although the low temperature was considered as a disadvantage due to the expected high cooling cost at large scale production level, it could be considered advantageous due to reducing the contamination risks (Botao *et al.*, 2007).

In addition, another protective role of EPA is the antioxidant effect as it was reported to protect the bacterial cells against the harmful oxygen molecules (Nishida *et al.*, 2006a; Nishida *et al.*, 2007). This theory is supported by the increased production of EPA, especially as a fraction of the total fatty acids, under increased dissolved oxygen during growth.

Continuous culture was used as a tool permitting the investigation of the effects of different growth rates and the effect of temperature under constant growth rate. The data showed a non significant effect of the different tested growth rates on the EPA productivity with slight preference toward the low feed rate. The significant effect of temperature was confirmed even when the cultivation was performed at constant growth rate.

The information obtained from the chemostat may lead to a suitable feed batch strategy for maximum growth and productivity for isolate 717. Chemostat cultivations with previously developed chemically defined media may also enable the growth and production limiting media components to be explored in more detail in the future.

Although high oxygen levels increased the amount of EPA, the ability of isolate to produce EPA in the complete absence of oxygen suggests that its biosynthesis does not require oxygen, and that oxygen may be enhancing the process as an inducer. The fact that the anaerobic cultivation was carried out at low temperature (10 ^{0}C) suggests that, the amount of EPA produced (11.64 mg/g representing 8% of the total fatty acids) may be the amount of EPA required by isolate to sustain the fluidity of plasma membrane under extreme 0cold conditions, while the high amount of EPA produced during the aerobic cultivation processes may be due to the interaction between DO and temperature.

The lack of unpleasant odour, the stability, the absence of heavy metal contamination and the absence of (12-oxo-5, 8, 10-heptadecatrienoic acid and hexadeca-4, 7, 10, 13-tetraenoic acid fatty acids to interfere with chemotherapy (Roodhart *et al.*, 2011) of the bacterial oil further increase the commercial attractiveness of PUFAs produced from bacterial sources.

Microbial EPA could be extracted and supplied directly in food and pharmaceutical industry or the microbial biomass could be introduced as poultry feed and as a fish supplement in the aquaculture manufacturing (Harel *et al.*, 2002) or by incorporating microbial PUFAs into higher plants, as cereals via solid state fermentation (Certik and Adamechova, 2009) reducing the unsustainable exploitation of non-food fish species for fishmeal and oil supplements.

Chapter 8- Conclusions and future work

The screening experiment confirmed that the PUFA producers are more abundant in the cold environment than the temperate environment (Chapter 3). TTC colourimetric method for EPA screening experiment reduced the overall screening time, but further work is required to develop this method into a reliable and robust screening approach.

Isolates 717 and 66 were found to be the most promising EPA producers and with all the optimisation studies carried out at small scale, they can be clearly taken further towards the scale-up to manufacture and large scale production process. These two isolates are likely to be new strains (Chapter 3) and thus should not pose any infringement issues in process development.

The common statistically significant effects upon the EPA production in both isolates, whether negative (e.g. glucose, citric acid, palmitic acids, $Fe_2(SO_4)_3$, linseed oil, propionic acid and acetate) or positive (e.g. casein, glycerol, whey, Na_2HPO_4, L-proline, L-histidine and peptone) indicate that these compounds may be involved or interfere either directly or indirectly with the metabolic pathways of EPA biosynthesis.

Applying statistical design of experiment for screening different media components and developing potential production media for each isolate led to a significant increase in the productivity of all tested isolates. This confirms that statistical design of experiment is a vital tool for improving the performance of a manufacturing process and improvement of new processes especially PUFA production.

Because succeeding in bioreactor cultivation for a particular bio-product could be considered as a first step in scaling up from the laboratory scale to industrial scale, the bioreactor cultivation conditions for the highest producer (717) were optimised via Response Surface Methodology.

The first step in optimising the bioreactor cultivation conditions for maximising the growth and productivity of the tested isolates was to obtain an accurate control of the operating conditions will reduce the influence of the bioreactor dynamics ensuring that the culture parameters were studied precisely and any response to imposed variations in the environment was therefore an outcome of the microbial behaviour only. This was achieved by developing the optimum PID values for the process.

Central Composite Design was used to optimise the bioreactor cultivation conditions for maximising the growth and EPA productivity of isolate 717. In CCD the exact optimal values

of the tested variables were interpolated. With 45 mg/g dry weight, 350 mg/l production media and 25% of the total fatty acids, isolate 717 could be considered as one of the highest reported bacterial EPA producers. It can produce more than twice the amount of EPA reported for *Phaeodactylum tricornutum,* which produced 133 mg/l of culture media (Yongmanitchai and Ward, 1991). In addition in total fatty acids produced by isolate 717 the EPA percentage is at least twice that of reported for *Shewanella* sp. KMG427 and *Photobacterium* sp. SAMA2 which can produce 10% and 13.9% EPA, respectively (Lee *et al.*, 2008b; Freese *et al.*, 2009). This result has been achieved via the optimisation process, and without any genetic manipulation, thus avoiding any ethical concerns about the use of genetically modified organisms in the production of nutracereals for human consumption.

The obtained preliminary study on element uptake by isolate 717 (Chapter 7) suggests that EPA production may be a phosphate limited process. This conclusion needs further confirmation upon which operational strategies for bioreactor scale cultivation can be developed to maximise the EPA productivity further by controlling the phosphate supply in addition to the cultivation conditions.

The preliminary data on the quality of the produced oil indicates that the bacterial oil represents a potentially competitive source of PUFAs compared to the current commercially used fish liver oil. However further research is required to achieve this, in particular in the following aspects:

- The presence of PUFA producers in the Red sea water samples encourages further screening of that temperate region to find a bacterial isolate with the capability to produce a reasonable amount of PUFAs at a relatively high temperature to reduce the overall manufacturing cost.
- The method of colourimetric screening method requires further investigation and the native gel or chromatographic techniques, as HPLC and AKTA, can be used to identify the protein responsible for the colourimetric reaction.
- The new secondary metabolites identified in isolate 560 extract require further investigation importanceto establish their composition and useful properties (e.g. anti-microbial activity).
- Isolates 717 and 66 are initially assumed to be new strains. To confirm this hypothesis the 16S rRNA gene should be cloned and sequencing full sequence (1500 bp) analysed. Also, the biochemical and physiological properties need to be studied and compared to the closest type strain.

- The developed chemically defined media for the tested isolates (66 and 717) can be used to determine rate limiting substrate for the growth and EPA production.
- The initial hypothesis of phosphate limitation influencing the EPA production requires further exploration and new feeding/bioreactor operating strategies can be developed to further optimise the EPA production.
- The quality of the produced oil need to expose to more investigation, especially the biological activity and the oxidative stability.
- NIR approaches can be used for online monitoring for the EPA production during the fermentation process. To enhance the performance and prediction, more samples need to be screened and an optic fibre probes with a higher sensitivity can be used for better performance.
- Applying modern molecular genetic tools may help in explaining the behaviour of the organism and exploring the metabolic pathways of PUFA production which will help in fundamental understanding of the process and thus more effective control, this can be achieved via:
  - Real Time PCR technique: The *pfa* gene cluster can be analysed by this technique by monitoring the gene expression over the production time leading to the identification of the EPA overexpression mechanism. The gene sequences have already been collected from the data base and a suggested PCR primers were designed for this purpose during this project, however time constraints did not allow this investigation to be completed
  - DNA sequencing: A 2^{nd} or a 3^{rd} generation sequencer can be used to sequence the whole bacterial genome. Analysis of the genome can explore the potential for the metabolic biosynthesis of EPA. The required DNA concentration for this experiment was prepared, >300 ng/µl see Appendix E, and the samples are waiting to be sequenced.

References

Abbadi, A.D.F., Bauer, J., Napier, J.A., Welti, R., Za hringer, U., Cirpus, P. and Heinz, E. (2004) 'Biosynthesis of verylong chain polyunsaturated fatty acids in transgenic oilseeds: constraints on their accumulation', *The Plant Cell*, 16, pp. 2734–2748.

Ahuja, S.K., Ferreira, G.M. and Moreira, A.R. (2004) 'Application of Plackett-Burman design and response surface methodology to achieve exponential growth for aggregated shipworm bacterium', *Biotechnology and Bioengineering*, 85(6), pp. 666-675.

Akimoto, M., Ishii, T., Yamagaki, K., Ohtaguchi, K., Koide, K. and Yazawa, K. (1991) 'Metal salts requisite for the production of eicosapentaenoic acid by a marine bacterium isolated from mackerel intestines', *Journal of the American Oil Chemists' Society*, 68(7), pp. 504-508.

Allen, E.E. and Bartlett, D.H. (2002) 'Structure and regulation of the omega-3 polyunsaturated fatty acid synthase genes from the deep-sea bacterium *Photobacterium profundum* strain SS9', *Microbiology*, 148(6), pp. 1903-1913.

Allen, E.E., Facciotti, D. and Bartlett, D.H. (1999) 'Monounsaturated but not polyunsaturated fatty acids are required for growth of the deep-sea bacterium *Photobacterium profundum* SS9 at high pressure and low temperature', *Applied and Environmental Microbiology*, 65(4), pp. 1710-1720.

Amiri-Jami, M., Wang, H., Kakuda, Y. and Griffiths, M. (2006) 'Enhancement of polyunsaturated fatty acid production by Tn5 transposon in *Shewanella baltica*', *Biotechnology Letters*, 28(15), pp. 1187-1192.

Anderson, M.J. and Whitcomb, P.J. (2000) 'Design of Experiments', in *Kirk-Othmer Encyclopedia of Chemical Technology*. John Wiley & Sons, Inc.

Anderson, M.J. and Whitcomb, P.J. (2001) 'How to Save Runs, yet reveal breakthrough interactions, by doing only a semifoldover on medium-resolution screening designs', *Annual Quality Congress*. Charlotte,North Carolina american society for quality, pp. 433-443.

Angelis, S., Novak, A., Sydney, E., Soccol, V., Carvalho, J., Pandey, A., Noseda, M., Tholozan, J., Lorquin, J. and Soccol, C. (2012) 'Co-Culture of microalgae, cyanobacteria, and macromycetes for exopolysaccharides production: process preliminary optimization and partial characterization', *Applied Biochemistry and Biotechnology*, pp. 1-15.

Antunes, A., Ngugi, D.K. and Stingl, U. (2011) 'Microbiology of the Red Sea (and other) deep-sea anoxic brine lakes', *Environmental Microbiology Reports*, 3(4), pp. 416-433.

Bag, J. (1974) 'Diauxic growth of Vibrio cholerae: Effect of glucose on the transport & phosphoenolpyravate dependent phosphorylation of galactose & fructose', *Indian Journal of Biochemistry and Biophysics*, 11(2), pp. 148-151.

Bajpai, P. and Bajpai, P.K. (1993) 'Eicosapentaenoic acid (EPA) production from microorganisms: a review', *Journal of Biotechnology*, 30(2), pp. 161-183.

Barclay, W., Meager, K. and Abril, J. (1994) 'Heterotrophic production of long chain omega-3 fatty acids utilizing algae and algae-like microorganisms', *Journal of Applied Phycology*, 6(2), pp. 123-129.

Berzaghi, P., Dalle Zotte, A., Jansson, L.M. and Andrighetto, I. (2005) 'Near-infrared reflectance spectroscopy as a method to predict chemical composition of breast meat and discriminate between different n-3 feeding sources', *Poultry Science*, 84(1), pp. 128-136.

Blair, J.M.A. and Piddock, L.J.V. (2009) 'Structure, function and inhibition of RND efflux pumps in Gram-negative bacteria: an update', *Current Opinion in Microbiology*, 12(5), pp. 512-519.

Bligh, E.G. and Dyer, W.J. (1959) 'A rapid method of total lipid extraction and purification', *Canadian Journal of Biochemistry and Physiology*, 37(8), pp. 911-917.

Bosma, R., Van Spronsen, W., Tramper, J. and Wijffels, R. (2003) 'Ultrasound, a new separation technique to harvest microalgae', *Journal of Applied Phycology*, 15, pp. 143-153.

Botao, Z., Jinlai, M., Zhou, Z., Guodong, W., Quanfu, W., Guangyou, L. and Wanshun, L. (2007) 'Screening and optimization of EPA-producing antarctic psychrophilic bacterium *Shewanella* sp. NJ136', *High technology letters* 13, pp. 95-102

Bowman, J.P., McCammon, S.A., Nichols, D.S., Skerratt, J.H., Rea, S.M., Nichols, P.D. and McMeekin, T.A. (1997) '*Shewanella gelidimarina* sp. nov. and *Shewanella frigidimarina* sp. nov., novel antarctic species with the ability to produce eicosapentaenoic acid (20:5{omega}3) and grow anaerobically by dissimilatory Fe(III) reduction', *International Journal of Systematic Bacteriology*, 47(4), pp. 1040-1047.

Cao, X., Li, S., Wang, C. and Lu, M. (2008) 'Effects of nutritional factors on the growth and heterotrophic eicosapentaenoic acid production of diatom *Nitzschia laevis*', *Journal of Ocean University of China (English Edition)*, 7(3), pp. 333-338.

Certik, M. and Adamechova, Z. (2009) 'Cereal-based bioproducts containing polyunsaturated fatty acids', *Lipid Technology*, 21(11-12), pp. 250-253.

Certík, M., Baltészov, L. and Šajbidor, J. (1997) 'Lipid formation and γ-linolenic acid production by Mucorales fungi grown on sunflower oil', *Letters in Applied Microbiology*, 25(2), pp. 101-105.

Certik, M., Sakuradani, E. and Shimizu, S. (1998) 'Desaturase-defective fungal mutants: useful tools for the regulation and overproduction of polyunsaturated fatty acids', *Trends in Biotechnology*, 16(12), pp. 500-505.

Certik, M. and Shimizu, S. (1999) 'Biosynthesis and regulation of microbial polyunsaturated fatty acid production', *Journal of Bioscience and Bioengineering*, 87(1), pp. 1-14.

Chaturvedi, R. and Fujita, Y. (2006) 'Isolation of enhanced eicosapentaenoic acid producing mutants of *Nannochloropsis oculata* ST-6 using ethyl methane sulfonate induced mutagenesis techniques and their characterization at mRNA transcript level', *Phycological Research*, 54(3), pp. 208-219.

Chauhan, K., Trivedi, U. and Patel, K.C. (2007) 'Statistical screening of medium components by Plackett–Burman design for lactic acid production by *Lactobacillus* sp. KCP01 using date juice', *Bioresource Technology*, 98(1), pp. 98-103.

Chen, H.C. and Chang, C.C. (1996) 'Production of γ-Linolenic Acid by the Fungus *Cunninghamella echinulata* CCRC 31840', *Biotechnology Progress*, 12(3), pp. 338-341.

Chi, Z., Pyle, D., Wen, Z., Frear, C. and Chen, S. (2007) 'A laboratory study of producing docosahexaenoic acid from biodiesel-waste glycerol by microalgal fermentation', *Process Biochemistry*, 42(11), pp. 1537-1545.

Chiou, S.Y., Su, W.W. and Su, Y.C. (2001) 'Optimizing production of polyunsaturated fatty acids in *Marchantia polymorpha* cell suspension culture', *Journal of Biotechnology*, 85(3), pp. 247-257.

Chodok, P., Kanjana-Opas, A. and Kaewsuwan, S. (2010) 'The Plackett–Burman design for evaluating the production of polyunsaturated fatty acids by *Physcomitrella patens*', *Journal of the American Oil Chemists' Society*, 87(5), pp. 521-529.

Correia, M., Michel, V., Matos, A.A., Carvalho, P., Oliveira, M.J., Ferreira, R.M., Dillies, M.A., Huerre, M., Seruca, R., Figueiredo, C., Machado, J.C. and Touati, E. (2012) 'Docosahexaenoic acid inhibits *Helicobacter pylori* growth *In Vitro* and mice gastric mucosa colonization', *PLoS ONE*, 7(4), pp. 1-9.

Cozzolino, D., Murray, I., Chree, A. and Scaife, J.R. (2005) 'Multivariate determination of free fatty acids and moisture in fish oils by partial least-squares regression and near-infrared spectroscopy', *Food Science and Technology*, 38(8), pp. 821-828.

Cromwick, A.M., Birrer, G.A. and Gross, R.A. (1996) 'Effects of pH and aeration on γ-poly(glutamic acid) formation by *Bacillus licheniformis* in controlled batch fermentor cultures', *Biotechnology and Bioengineering*, 50(2), pp. 222-227.

Dai, C., Yuan, S., Li, X., Liu, J., Xing, D. and Lu, L. (2001) 'The effect of culture conditions on the fatty acid composition in the mycelium of the *Cephalosporium* sp', *Acta microbiologica Sinica*, 41(1), pp. 87-93.

Das, U.N. (2003) 'Long-chain polyunsaturated fatty acids in the growth and development of the brain and memory', *Nutrition*, 19(1), pp. 62-65.

Das, U.N. (2004) 'From bench to the clinic: γ-linolenic acid therapy of human gliomas', *Prostaglandins, Leukotrienes and Essential Fatty Acids*, 70(6), pp. 539-552.

De Gieter, M., Leermakers, M., Van Ryssen, R., Noyen, J., Goeyens, L. and Baeyens, W. (2002) 'Total and toxic arsenic levels in north sea fish', *Archives of Environmental Contamination and Toxicology*, 43(4), pp. 406-417.

De Leon, S.P., Norio I. and S.H. (1993) 'Effect of acetic and citric acids on the growth and activity of *Pseudomonas* sp.', *Bulletin of the Faculty of Fisheries. Nagasaki University*, 44(2), pp. 80-85.

Delong, E.F. and Yayanos, A.A. (1986) 'Biochemical function and ecological significance of novel bacterial lipids in deep-sea procaryotes', *Applied amd Environmental Microbiology*, 51(4), pp. 730-737.

Dettmer, A., Coelho Cavalheiro, J., Cavalli, É., Misturini Rossi, D., de Souza Gusatti, C., Záchia Ayub, M.A. and Gutterres, M. (2012) 'Optimization of the biotechnological process for hide unhairing in substitution of toxic sulfides', *Chemical Engineering & Technology*, 35(5), pp. 803-810.

Deven, J.M. and Manocha, M.S. (1975) 'Effect of glutamic acid on the fatty acid and lipid composition of *Choanephora cucurbitarum*', *Canadian Journal of Microbiology*, 21(11), pp. 1827-1833.

Doan, T.T.Y. and Obbard, J.P. (2012) 'Enhanced intracellular lipid in *Nannochloropsis* sp. via random mutagenesis and flow cytometric cell sorting', *Algal Research*, 1(1), pp. 17-21.

Domingo, J.L., Bocio, A., Falcó, G. and Llobet, J.M. (2007) 'Benefits and risks of fish consumption: Part I. A quantitative analysis of the intake of omega-3 fatty acids and chemical contaminants', *Toxicology*, 230(2–3), pp. 219-226.

Dyal, S.D., Bouzidi, L. and Narine, S.S. (2005) 'Maximizing the production of γ-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(7), pp. 815-829.

El-Sikaily, A., Khaled, A. and Nemr, A.E. (2004) 'Heavy metals monitoring using bivalves from Mediterranean Sea and Red Sea', *Environmental Monitoring and Assessment*, 98(1), pp. 41-58.

Fahy, E., Subramaniam, S., Brown, H.A., Glass, C.K., Merrill, A.H., Murphy, R.C., Raetz, C.R.H., Russell, D.W., Seyama, Y., Shaw, W., Shimizu, T., Spener, F., van Meer, G., Van Nieuwenhze, M.S., White, S.H., Witztum, J.L. and Dennis, E.A. (2005) 'A comprehensive classification system for lipids', *Journal Lipid Research*, 46(5), pp. 839-862.

Fang, J., Chiaki, K., Takako, S., Olivia, C. and David, M. (2004) 'Biosynthesis and dietary uptake of polyunsaturated fatty acids by piezophilic bacteria', *Comparative Biochemistry and Physiology Part A: Physiology*, 137(4), pp. 455-461

Fernandez-Lopez, R., Machón, C., Longshaw, C.M., Martin, S., Molin, S., Zechner, E.L., Espinosa, M., Lanka, E. and de la Cruz, F. (2005) 'Unsaturated fatty acids are inhibitors of bacterial conjugation', *Microbiology*, 151(11), pp. 3517-3526.

Freese, E., Rütters, H., Köster, J., Rullkötter, J. and Sass, H. (2009) 'Gammaproteobacteria as a possible source of eicosapentaenoic acid in anoxic intertidal sediments', *Microbial Ecology*, 57(3), pp. 444-454.

Frolova, G., Pavel, K., Shparteeva, A., Nedashkovskaya, O., Gorshkova, N., Ivanova, E. and Mikhailov, V. (2005) 'Lipid composition of novel *Shewanella* species isolated from far eastern seas', *Microbiology*, 74(6), pp. 664-669.

Funtikova, N.S., Mysyakina, I.S. and Konova, I.V. (2002) 'Synthesis of biologically active lipids by the fungus *Mucor lusitanicus* 306D grown on media with various composition', *Applied Biochemistry and Microbiology*, 38(6), pp. 553-557.

Gayathri, S., Prabhu, S., Mitra, A., Kumar, V., Sheela, J., Rajani, G., Sundar, N. and Sivvaswamy, S.N. (2010) 'Soil microorganisms produce omega-3 fatty acids', *Indian Journal of Science and Technology*, 3(5), pp. 499-503.

Gema, H., Kavadia, A., Dimou, V.D., Komaitis, T.M. and Aggelis, G. (2002) 'Production of γ-linolenic acid by *Cunninghamella echinulata* cultivated on glucose and orange peel', *Applied Microbiology and Biotechnology*, 58(3), pp. 303-307.

Geng, M. and Duan, Z. (2010) 'Prediction of oxygen solubility in pure water and brines up to high temperatures and pressures', *Geochimica et Cosmochimica Acta*, 74(19), pp. 5631-5640.

Gentile, G., Bonasera, V., Amico, C., Giuliano, L. and Yakimov, M.M. (2003) '*Shewanella* sp. GA-22, a psychrophilic hydrocarbonoclastic antarctic bacterium producing polyunsaturated fatty acids', *Journal of Applied Microbiology*, 95(5), pp. 1124-1133.

Ghobadi, Z., Hamidi-Esfahani, Z. and Azizi, M.H. (2011) 'Determination of effective variables on arachidonic acid production by *Mortierella alpina* CBS 754.68 in solid-state fermentation using Plackett-Burman screening design', *World Academy of Science, Engineering and Technology*, 81, pp. 678-680.

Gill, I. and Valivety, R. (1997) 'Polyunsaturated fatty acids, part 1: Occurrence, biological activities and applications', *Trends in Biotechnology*, 15(10), pp. 401-409.

Giusto, N.M., Pasquaré, S.J., Salvador, G.A., Castagnet, P.I., Roque, M.E. and Ilincheta de Boschero, M.G. (2000) 'Lipid metabolism in vertebrate retinal rod outer segments', *Progress in Lipid Research*, 39(4), pp. 315-391.

Gollnick, P., Babitzke, P., Antson, A. and Yanofsky, C. (2005) 'complexity in regulation of tryptophan biosynthesis in *Bacillus subtilis*', *Annual Review of Genetics*, 39(1), pp. 47-68.

Gonzalez-Baro, M. and Pollero, R.J. (1998) 'Fatty acid metabolism of *Macrobrachium borellii*: dietary origin of arachidonic and eicosapentaenoic acids', *Comparative Biochemistry and Physiology Part A: Physiology*, 119(3), pp. 747–752.

Graham, A.F. and Lund, B.M. (1986) 'The effect of citric acid on growth of proteolytic strains of *Clostridium botulinum*', *Journal of Applied Microbiology*, 61(1), pp. 39-49.

Guchhait, R.B., Polakis, S.E., Dimroth, P., Stoll, E., Moss, J. and Lane, M.D. (1974) 'Acetyl coenzyme A carboxylase system of *Escherichia coli*', *Journal of Biological Chemistry*, 249(20), pp. 6633-6645.

Gunstone, F., D, (1996) *Fatty Acid And Lipid Chemistry*. New york: Chapman and Hall.

Gurr, M.I.H., J.L. (1991) *Lipid Biochemistry*. 4th edn. London: Chapman and Hall.

Haider, M. and Pakshirajan, K. (2007) 'Screening and optimization of media constituents for enhancing lipolytic activity by a soil microorganism using statistically designed experiments', *Applied Biochemistry and Biotechnology*, 141(2), pp. 377-390.

Hajra, A. (1974) 'On extraction of acyl and alkyl dihydroxyacetone phosphate from incubation mixtures', *Lipids*, 9(8), pp. 502-505.

Hamamoto, T., Takata, N., Kudo, T. and Horikoshi, K. (1995) 'Characteristic presence of polyunsaturated fatty acids in marine psychrophilic *Vibrios*', *FEMS microbiology letters*, 129(1), pp. 51-56.

Hansson, L. and Dostálek, M. (1988) 'Effect of culture conditions on mycelial growth and production of γ-linolenic acid by the fungus *Mortierella ramanniana*', *Applied Microbiology and Biotechnology*, 28(3), pp. 240-246.

Harel, M., Koven, W., Lein, I., Bar, Y., Behrens, P., Stubblefield, J., Zohar, Y. and Place, A.R. (2002) 'Advanced DHA, EPA and ArA enrichment materials for marine aquaculture using single cell heterotrophs', *Aquaculture*, 213(1–4), pp. 347-362.

Hidetoshi, O., Yoshitake, O., Takanori, N., Kazuo, W. and Naoki, M. (2007) 'Bacterial genes responsible for the biosynthesis of eicosapentaenoic and docosahexaenoic acids and their heterologous expression', *Applied and environmental microbiology,*, 73(3), pp. 665–670.

Higashiyama, K., Murakami, K., Tsujimura, H., Matsumoto, N. and Fujikawa, S. (1999) 'Effects of dissolved oxygen on the morphology of an arachidonic acid production by *Mortierella alpina* 1S-4', *Biotechnology and Bioengineering*, 63(4), pp. 442-448.

Hirota, K., Nodasaka, Y., Orikasa, Y., Okuyama, H. and Yumoto, I. (2005) '*Shewanella pneumatophori* sp. nov., an eicosapentaenoic acid-producing marine bacterium isolated from the intestines of pacific mackerel (*Pneumatophorus japonicus*)', *International Journal of Systematic and Evolutionary Microbiology*, 55(6), pp. 2355-2359.

Iida, I., Nakahara, T., Yokochi, T., Kamisaka, Y., Yagi, H., Yamaoka, M. and Suzuki, O. (1996) 'Improvement of docosahexaenoic acid production in a culture of Thraustochytrium aureum by medium optimization', *Journal of Fermentation and Bioengineering*, 81(1), pp. 76-78.

Ivanova, E.P., Sawabe, T., Hayashi, K., Gorshkova, N.M., Zhukova, N.V., Nedashkovskaya, O.I., Mikhailov, V.V., Nicolau, D.V. and Christen, R. (2003) '*Shewanella fidelis* sp. nov., isolated from sediments and sea water', *International Journal of Systematic and Evolutionary Microbiology*, 53(2), pp. 577-582.

Ivanova, V., Oriol, M., Montes, M.J., GarcÃ-a, A. and Guinea, J. (2001) 'Secondary metabolites from a *Streptomyces* strain isolated from Livingston island, Antarctica', *Z Naturforsch C*, 56(1-2), pp. 1-5.

Janda, J.M. and Abbott, S.L. (2007) '16S rRNA gene sequencing for bacterial identification in the diagnostic laboratory: pluses, perils, and pitfalls', *Journal of Clinical Microbiology*, 45(9), pp. 2761-2764.

Jang, H.D., Lin, Y.Y. and Yang, S.S. (2005) 'Effect of culture media and conditions on polyunsaturated fatty acids production by *Mortierella alpina*', *Bioresource Technology*, 96(15), pp. 1633-1644.

Jareonkitmongkol, S., Shimizu, S. and Yamada, H. (1993) 'Production of an eicosapentaenoic acid-containing oil by a delta(12) desaturase-defective mutant of *Mortierella alpina* 1S-4', *Journal of the American Oil Chemists' Society*, 70(2), pp. 119-123.

Jiang, Y. and Chen, F. (1999) 'Effects of salinity on cell growth and docosahexaenoic acid content of the heterotrophic marine microalga *Crypthecodinium cohnii*', *Journal of Industrial Microbiology & Biotechnology*, 23(6), pp. 508-513.

Jostensen, J.P. and Landfald, B. (1996) 'Influence of growth conditions on fatty acid composition of a polyunsaturated-fatty-acid-producing Vibrio species', *Archives of Microbiology*, 165(5), pp. 306-310.

Jostensen, J.P. and Landfald, B. (1997) 'High prevalence of polyunsaturated-fatty-acid producing bacteria in arctic invertebrates', *FEMS Microbiology Letters*, 151(1), pp. 95-101.

Kavitha, A., Prabhakar, P., Vijayalakshmi, M. and Venkateswarlu, Y. (2009) 'Production of bioactive metabolites by *Nocardia levis* MK-VL_113', *Letters in Applied Microbiology*, 49(4), pp. 484-490.

Kawamoto, J., Kurihara, T., Yamamoto, K., Nagayasu, M., Tani, Y., Mihara, H., Hosokawa, M., Baba, T., Sato, S.B. and 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(2), pp. 632-640.

Kawamoto, J., Sato, T., Nakasone, K., Kato, C., Mihara, H., Esaki, N. and 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(8), pp. 2293-2298.

Kelly, F.J. (1991) 'The metabolic role of n-3 polyunsaturated fatty acids: Relationship to human disease', *Comparative Biochemistry and Physiology Part A: Physiology*, 98(3-4), pp. 581-585.

Kenny, F.S., Pinder, S.E., Ellis, I.O., Gee, J.M.W., Nicholson, R.I., Bryce, R.P. and Robertson, J.F.R. (2000) 'Gamma linolenic acid with tamoxifen as primary therapy in breast cancer', *International Journal of Cancer*, 85(5), pp. 643-648.

Khan, M.W., Priyamvada, S., Khan, S.A., Khan, S., Naqshbandi, A. and Yusufi, A.N.K. (2012) 'Protective effect of ω-3 polyunsaturated fatty acids (PUFAs) on sodium nitroprusside–induced nephrotoxicity and oxidative damage in rat kidney', *Human & Experimental Toxicology*. "in-press article"

Khodaiyan, F., Razavi, S., Emam-Djomeh, Z. and Mousavi, S. (2007) 'Optimization of canthaxanthin production by *Dietzia natronolimnaea* HS-1 using response surface methodology.', *Pakistanian Journal of Biological Sciences*, 10(15), pp. 2544-2552.

Khongto, B., Laoteng, K. and Tongta, A. (2011) 'Enhancing the production of gamma-linolenic acid in *Hansenula polymorpha* by fed-batch fermentation using response surface methodology', *Chemical Papers*, 65(2), pp. 124-131.

Khozin-Goldberg, I. and Cohen, Z. (2006) 'The effect of phosphate starvation on the lipid and fatty acid composition of the fresh water eustigmatophyte *Monodus subterraneus*', *Phytochemistry*, 67(7), pp. 696-701.

Kowluru, A., Chen, H.-Q., Modrick, L.M. and Stefanelli, C. (2001) 'Activation of Acetyl-CoA carboxylase by a glutamate- and magnesium-sensitive protein phosphatase in the islet β-Cell', *Diabetes*, 50(7), pp. 1580-1587.

Kwon, E., Kim, D.Y., Ngo, T.D., Gross, C.A., Gross, J.D. and Kim, K.K. (2012) 'The crystal structure of the periplasmic domain of *Vibrio parahaemolyticus* CpxA', *Protein Science*, 21(9), pp.1334-13343.

Lagarde, M., Burtin, M., Sprecher, H., Dechavanne, M. and Renaud, S. (1983) 'Potentiating effect of 5,8,11-eicosatrienoic acid on human platelet aggregation', *Lipids*, 18(4), pp. 291-294.

Lan, W.Z., Qin, W.M. and Yu, L.J. (2002) 'Effect of glutamate on arachidonic acid production from *Mortierella alpina*', *Letters in Applied Microbiology*, 35(4), pp. 357-360.

Lang, S., Hüners, M. and Lurtz, V. (2005) 'Bioprocess engineering data on the cultivation of marine prokaryotes and fungi marine biotechnology II', in Ulber, R. and Le Gal, Y. (eds.) Springer Berlin / Heidelberg, pp. 585-585.

Lauritzen, L., Hansen, H.S., Jorgensen, M.H. and Michaelsen, K.F. (2001) 'The essentiality of long chain n-3 fatty acids in relation to development and function of the brain and retina', *Progress in Lipid Research*, 40, pp. 1-94.

Lazic, Z., R. (2004) *Design of Experiments in Chemical Engineering*. Weinheim: WILEY-VCH Verlag GmbH & Co.

Lee, C.C., Lee, C.C., Ku, H.T., Chang, S.M. and Chiang, K.N. (2007) 'Solder joints layout design and reliability enhancements of wafer level packaging using response surface methodology', *Microelectronics Reliability*, 47(2–3), pp. 196-204.

Lee, S.J., Jeong, Y.S., Kim, D.U., Seo, J.W. and Hur, B.K. (2006) 'Eicosapentaenoic acid (EPA) biosynthetic gene cluster of *Shewanella oneidensis* MR-1: Cloning, heterologous expression, and effects of temperature and glucose on the production of EPA in *Escherichia coli*', *Biotechnology and Bioprocess Engineering*, 11(6), pp. 510-515.

Lee, S.J., Kim, C., Seo, P.S., Kwon, O., Hur, B.K. and Seo, J.W. (2008a) 'Enhancement of heterologous production of eicosapentaenoic acid in *Escherichia coli* by substitution of

promoter sequences within the biosynthesis gene cluster', *Biotechnology Letters*, 30(12), pp. 2139-2142.

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., Lee, S.H. and Kim, J.G. (2008b) 'Identification of psychrophile *Shewanella* sp KMG427 as an eicosapentaenoic acid producer', *Journal of Microbiology and Biotechnology*, 18(12), pp. 1869-1873.

Lees, A.M. and Korn, E.D. (1966) 'Metabolism of unsaturated fatty acids in protozoa', *Biochemistry*, 5(5), pp. 1475-1481.

Lewis, T.E., Nichols, P.D. and McMeekin, T.A. (1999) 'The biotechnological potential of thraustochytrids', *Marine Biotechnology*, 1(6), pp. 580-587.

Li, N., Deng, Z., Qin, Y., Chen, C. and Liang, Z. (2008a) 'Production of polyunsaturated fatty acids by *Mucor recurvus* sp. with sugarcane molasses as the carbon source', *Food Technology and Biotechnology*, 46(1), pp. 73-79.

Li, N., Deng, Z., Qin, Y., Chen, C. and Liang, Z. (2008b) 'Production of polyunsaturated fatty acids by *Mucor recurvus* sp with sugarcane molasses as the carbon source', *Food Technology and Biotechnology*, 46(1), pp. 73-79.

Liu, J.Z., Weng, L.P., Zhang, Q.L., Xu, H. and Ji, L.N. (2003) 'Optimization of glucose oxidase production by *Aspergillus niger* in a benchtop bioreactor using response surface methodology', *World Journal of Microbiology and Biotechnology*, 19(3), pp. 317-323.

Lopanik, N.B., Targett, N.M. and Lindquist, N. (2006) 'Isolation of two polyketide synthase gene fragments from the uncultured microbial symbiont of the marine bryozoan *Bugula neritina'*, *Applied and Environmental Microbiology*, pp. AEM.01277-06.

Lucas, E.M., Abreu, L.M., Marriel, I.E., Pfenning, L.H. and Takahashi, J.A. (2008) 'Phthalates production from Curvularia senegalensis (Speg.) Subram, a fungal species associated to crops of commercial value', *Microbiological Research*, 163(5), pp. 495-502.

Magnuson, K., Jackowski, S., Rock, C.O. and Cronan, J.E. (1993) 'Regulation of fatty acid biosynthesis in *Escherichia coli*', *Microbiological Reviews*, 57(3), pp. 522-542.

Mascher, T., Helmann, J.D. and Unden, G. (2006) 'Stimulus perception in bacterial signal-transducing histidine kinases', *Microbiology and Molecular Biology Reviews*, 70(4), pp. 910-938.

Metherel, A.H., Armstrong, J.M., Patterson, A.C. and Stark, K.D. (2009) 'Assessment of blood measures of n-3 polyunsaturated fatty acids with acute fish oil supplementation and washout in men and women', *Prostaglandins, Leukotrienes and Essential Fatty Acids*, 81(1), pp. 23-29.

Metz, J.G., Kuner, J., Rosenzweig, B., Lippmeier, J.C., Roessler, P. and 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(6), pp. 472-478.

Metz, J.G., Roessler, P., Facciotti, D., Levering, C., Dittrich, F., Lassner, M., Valentine, R., Lardizabal, K., Domergue, F., Yamada, A., Yazawa, K., Knauf, V. and Browse, J. (2001) 'Production of polyunsaturated fatty acids by polyketide synthases in both prokaryotes and eukaryotes', *Science*, 293(5528), pp. 290-293.

Meyer, B.J., Tsivis, E., Howe, P.R.C., Tapsell, L. and Calvert, G.D. (1999) 'Polyunsaturated fatty acid content of foods : differentiating between long and short chain omega-3 fatty acids', *Food Australia* 51, pp. 81-95

Michinaka, Y., Aki, T., Shimauchi, T., Nakajima, T., Kawamoto, S., Shigeta, S., Suzuki, O. and Ono, K. (2003) 'Differential response to low temperature of two fatty acid desaturases from *Mucor circinelloides*', *Applied Microbiology and Biotechnology*, 62(4), pp. 362-368.

Milovanovic, L., Popovic, I., Skala, D. and Saicic, S. (2006) 'Thermogravimetric analysis of the total lipids extracted from the fatty tissue of fallow deer ', *Journal of the Serbian Chemical Society*, 71(12), pp. 1281-1288.

Montgomery, D., C. (2004) *Design and Analysis of Experiments* 6th edn. New York: John Wiley & Sons.

Montgomery, D.C. (2000) *Design and Analysis of Experiments*. Arizona: John Wiley & Sons.

Montgomery, D.C. (2012) *Design and Analysis of Experiments*. 8th edn. Arizona: John Wiley & Sons.

Morita, N., Nishida, T., Tanaka, M., Yano, Y. and Okuyama, H. (2005) 'Enhancement of polyunsaturated fatty acid production by cerulenin treatment in polyunsaturated fatty acid-producing bacteria', *Biotechnology letters*, 27(6), pp. 389-393.

Mostertz, J. and Hecker, M. (2003) 'Patterns of protein carbonylation following oxidative stress in wild-type and *Bacillus subtilis* cells', *Molecular Genetics and Genomics*, 269(5), pp. 640-648.

Muhid, F., Nazatul, W., Wan, N., Abdul, N., Abdul Kader, J., Abdul Hamid, A., Mohtar, W. and Yusoff, W. (2008) 'Effects of metal ion concentrations on lipid and gamma linolenic acid production by *Cunninghamella* sp. 2A1', *Online Journal of Biological Science* 8(3), pp. 62-67.

Napier, J.A. (2002) 'Plumbing the depths of PUFA biosynthesis: a novel polyketide synthase-like pathway from marine organisms', *Trends in Plant Science* 7(2), pp. 51-54.

Napier, J.A. (2007) 'The Production of Unusual Fatty Acids in Transgenic Plants', *Annual Review of Plant Biology*, 58(1), pp. 295-319.

Narayan, M.S., Manoj, G.P., Vatchravelu, K., Bhagyalakshmi, N. and Mahadevaswamy, M. (2005) 'Utilization of glycerol as carbon source on the growth, pigment and lipid production in *Spirulina platensis*', *International Journal of Food Sciences and Nutrition*, 56(7), pp. 521-528.

Nichols, D., Bowman, J., Sanderson, K., Nichols, C.M., Lewis, T., McMeekin, T. and Nichols, P.D. (1999) 'Developments with Antarctic microorganisms: culture collections, bioactivity screening, taxonomy, PUFA production and cold-adapted enzymes', *Current Opinion in Biotechnology*, 10(3), pp. 240-246.

Nichols, D.S. and McMeekin, T.A. (2002) 'Biomarker techniques to screen for bacteria that produce polyunsaturated fatty acids', *Journal of Microbiological Methods*, 48(2-3), pp. 161-170.

Nichols, D.S., Nichols, P.D. and McMeekin, T.A. (1993) 'Polyunsaturated fatty acids in Antarctic bacteria', *Antarctic Science*, 5(02), pp. 149-160.

Nichols, D.S., Nichols, P.D., Russell, N.J., Davies, N.W. and McMeekin, T.A. (1997) 'Polyunsaturated fatty acids in the psychrophilic bacterium *Shewanella gelidimarina* ACAM 456T: molecular species analysis of major phospholipids and biosynthesis of eicosapentaenoic acid', *Biochimica et Biophysica Acta (BBA) - Lipids and Lipid Metabolism*, 1347(2–3), pp. 164-176.

Nishida, T., Hori, R., Morita, N. and 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(2), pp. 91-96.

Nishida, T., Morita, N., Yano, Y., Orikasa, Y. and Okuyama, H. (2007) 'The antioxidative function of eicosapentaenoic acid in a marine bacterium, *Shewanella marinintestina* IK-1', *FEBS Letters*, 581(22), pp. 4212-4216.

Nishida, T., Orikasa, Y., Watanabe, K. and Okuyama, H. (2006a) 'The cell membrane-shielding function of eicosapentaenoic acid for *Escherichia coli* against exogenously added hydrogen peroxide', *FEBS Letters*, 580(28-29), pp. 6690-6694.

Nishida, T., Orikasa, Y., Yu, R., Yamada, A., Watanabe, K. and Okuyama, H. (2006b) '*Escherichia coli* engineered to produce eicosapentaenoic acid becomes resistant against oxidative damages', *FEBS Letters*, 580, pp. 2731-2735.

Nogi, Y., Hosoya, S., Kato, C. and Horikoshi, K. (2007) '*Psychromonas hadalis* sp. nov., a novel piezophilic bacterium isolated from the bottom of the Japan Trench', *International Journal of Systematic and Evolutionary Microbiology*, 57(6), pp. 1360-1364.

Nogi, Y., Masui, N. and Kato, C. (1998) '*Photobacterium profundum* sp. nov., a new, moderately barophilic bacterial species isolated from a deep-sea sediment', *Extremophiles*, 2(1), pp. 1-8.

Nordoy, A., Marchioli, R., Arnesen, H. and Videbæk, J. (2001) 'n−3 Polyunsaturated fatty acids and cardiovascular diseases: To whom, how much, preparations', *Lipids*, 36(0), pp. S127-S129.

Okuyama, H., Orikasa, Y. and Nishida, T. (2007a) 'In vivo conversion of triacylglycerol to docosahexaenoic acid-containing phospholipids in a thraustochytrid-like microorganism, strain 12B', *Biotechnology letters*, 29(12), pp. 1977-1981.

Okuyama, H., Orikasa, Y., Nishida, T., Watanabe, K. and Morita, N. (2007b) 'Bacterial genes responsible for the biosynthesis of eicosapentaenoic and docosahexaenoic acids and their heterologous expression', *Applied and Environmental Microbiology*, 73(3), pp. 665-670.

Orikasa, Y., Ito, Y., Nishida, T., Watanabe, K., Morita, N., Ohwada, T., Yumoto, I. and Okuyama, H. (2007) 'Enhanced heterologous production of eicosapentaenoic acid in *Escherichia coli* cells that co-express eicosapentaenoic acid biosynthesis pfa genes and foreign DNA fragments including a high-performance catalase gene, vktA', *Biotechnology Letters*, 29, pp. 803-809.

Orikasa, Y., Tanaka, M., Sugihara, S., Hori, R., Nishida, T., Ueno, A., Morita, N., Yano, Y., Yamamoto, K., Shibahara, A., Hayashi, H., Yamada, Y., Yamada, A., Yu, R., Watanabe, K. and Okuyama, H. (2009) 'pfaB products determine the molecular species produced in bacterial polyunsaturated fatty acid biosynthesis', *FEMS Microbiology Letters*, 295(2), pp. 170-176.

Orikasa, Y., Yamada, A., Yu, R., Ito, Y., Nishida, T., Yumoto, I., Watanabe, K. and Okuyama, H. (2004) 'Characterization of the eicosapentaenoic acid biosynthesis gene cluster from *Shewanella* sp strain SCRC-2738', *Cellular and Molecular Biology*, 50(5), pp. 625-630.

Papanikolaou, S. and Aggelis, G. (2002) 'Lipid production by *Yarrowia lipolytica* growing on industrial glycerol in a single-stage continuous culture', *Bioresource Technology*, 82(1), pp. 43-49.

Park, P.K., Cho, D.H., Kim, E.Y. and Chu, K.H. (2005) 'Optimization of carotenoid production by *Rhodotorula glutinis* using statistical experimental design', *World Journal of Microbiology and Biotechnology*, 21(4), pp. 429-434.

Perveen, Z., Ando, H., Ueno, A., Ito, Y., Yamamoto, Y., Yamada, Y., Takagi, T., Kaneko, T., Kogame, K. and Okuyama, H. (2006) 'Isolation and characterization of a novel thraustochytrid-like microorganism that efficiently produces docosahexaenoic acid', *Biotechnology letters*, 28(3), pp. 197-202.

Peters, D. (2007) 'Raw materials white biotechnology', in Ulber, R. and Sell, D. (eds.) Springer Berlin / Heidelberg, pp. 1-30.

Phang, M., Garg, M.L. and Sinclair, A.J. (2009) 'Inhibition of platelet aggregation by omega-3 polyunsaturated fatty acids is gender specific--Redefining platelet response to fish oils', *Prostaglandins, Leukotrienes and Essential Fatty Acids*, 81 (1), pp. 35–40.

Plackett, R.L. and Burman, J.P. (1946) 'The design of optimum multifactorial experiments', *Biometrika*, 33(4), pp. 305-325.

Queiroga, A.C., Pintado, M.E. and Malcata, F.X. (2012) 'Use of response surface methodology to optimize protease synthesis by a novel strain of *Bacillus* sp. isolated from Portuguese sheep wool', *Journal of Applied Microbiology*, "article in press"

Ratledge, C. (2004) 'Fatty acid biosynthesis in microorganisms being used for Single Cell Oil production', *Biochimie*, 86(11), pp. 807-815.

Reddy, L.V.A., Wee, Y.J., Yun, J.S. and Ryu, H.W. (2008) 'Optimization of alkaline protease production by batch culture of *Bacillus* sp. RKY3 through Plackett–Burman and response surface methodological approaches', *Bioresource Technology*, 99(7), pp. 2242-2249.

Rehm, H.J. and Reed, G. (1993) *Biotechnology: Bioprocessing*. second edn. Weinheim, Vch.

Reis, A., Gouveia, L., Veloso, V., Fernandes, H.L., Empis, J. and Novais, J.M. (1996) 'Eicosapentaenoic acid-rich biomass production by the microalga *Phaeodactylum tricornutum* in a continuous-flow reactor', *Bioresource Technology*, 55(1), pp. 83-88.

Roach, N., Reddy, K.R. and Al-Hamdan, A.Z. (2009) 'Particle morphology and mineral structure of heavy metal-contaminated kaolin soil before and after electrokinetic remediation', *Journal of Hazardous Materials*, 165(1–3), pp. 548-557.

Rocky-Salimi, K., Hamidi-Esfahani, Z. and Abbasi, S. (2011) 'Statistical optimization of arachidonic acid production by *Mortierella alpina* CBS 754.68 in submerged fermentation', *Iranian Journal of Biotechnology*, 9, pp. 87-93.

Roodhart, Jeanine M.L., Daenen, Laura G.M., Stigter, Edwin C.A., Prins, H.-J., Gerrits, J., Houthuijzen, Julia M., Gerritsen, Marije G., Schipper, Henk S., Backer, Marieke J.G., van Amersfoort, M., Vermaat, Joost S.P., Moerer, P., Ishihara, K., Kalkhoven, E., Beijnen, Jos H., Derksen, Patrick W.B., Medema, Rene H., Martens, Anton C., Brenkman, Arjan B.

and Voest, Emile E. (2011) 'Mesenchymal stem cells induce resistance to chemotherapy through the release of platinum-induced fatty acids', *Cancer cell*, 20(3), pp. 370-383.

Rosa, S.M., Soria, M.A., Vélez, C.G. and Galvagno, M.A. (2010) 'Improvement of a two-stage fermentation process for docosahexaenoic acid production by *Aurantiochytrium limacinum* SR21 applying statistical experimental designs and data analysis', *Bioresource Technology*, 101(7), pp. 2367-2374.

Ruiz-López, N., Sayanova, O., Napier, J.A. and Haslam, R.P. (2012) 'Metabolic engineering of the omega-3 long chain polyunsaturated fatty acid biosynthetic pathway into transgenic plants', *Journal of Experimental Botany*, 63(7), pp. 2397-2410.

Russell, N.J. and Nichols, D.S. (1999a) 'Polyunsaturated fatty acids in marine bacteria -- a dogma rewritten', *Microbiology*, 145(4), pp. 767-779.

Russell, N.J. and Nichols, D.S. (1999b) 'Polyunsaturated fatty acids in marine bacteria - a dogma rewritten', *Microbiology-Sgm*, 145, pp. 767-779.

Ryan, J., Farr, H., Visnovsky, S., Vyssotski, M. and Visnovsky, G. (2010) 'A rapid method for the isolation of eicosapentaenoic acid-producing marine bacteria', *Journal of Microbiological Methods*, 82(1), pp. 49-53.

Sado-Kamdem, S.L., Vannini, L. and Guerzoni, M.E. (2009) 'Effect of [alpha]-linolenic, capric and lauric acid on the fatty acid biosynthesis in *Staphylococcus aureus*', *International Journal of Food Microbiology*, 129(3), pp. 288-294.

Saelao, S., Kanjana-Opas, A. and Kaewsuwan, S. (2011) 'Optimization of biomass and arachidonic acid production by *Aureispira maritima* using response surface methodology', *Journal of the American Oil Chemists' Society*, 88(5), pp. 619-629.

Sakuradani, E., Ando, A., Ogawa, J. and Shimizu, S. (2009) 'Improved production of various polyunsaturated fatty acids through filamentous fungus *Mortierella alpina* breeding', *Applied Microbiology and Biotechnology*, 84(1), pp. 1-10.

Sato, S., Kurihara, T., Kawamoto, J., Hosokawa, M., Sato, S. and Esaki, N. (2008) 'Cold adaptation of eicosapentaenoic acid-less mutant of *Shewanella livingstonensis* Ac10 involving uptake and remodeling of synthetic phospholipids containing various polyunsaturated fatty acids', *Extremophiles*, 12(6), pp. 753-761.

Sauer, L.A., Dauchy, R.T. and Blask, D.E. (2001) 'Polyunsaturated fatty acids, melatonin, and cancer prevention', *Biochemical Pharmacology*, 61(12), pp. 1455-1462.

Schluenzen, F., Tocilj, A., Zarivach, R., Harms, J., Gluehmann, M., Janell, D., Bashan, A., Bartels, H., Agmon, I., Franceschi, F. and Yonath, A. (2000) 'Structure of functionally activated small ribosomal subunit at 3.3 Å resolution', *Cell*, 102(5), pp. 615-623.

Schrader, K.K. and Blevins, W.T. (2001) 'Effects of carbon source, phosphorus concentration, and several micronutrients on biomass and geosmin production by *Streptomyces halstedii*', *Journal of Industrial Microbiology & Biotechnology*, 26(4), pp. 241-247.

Shene, C., Leyton, A., Esparza, Y., Flores, L., Quilodrán, B., Hinzpeter, I. and Rubilar1.M. (2010) 'Microbial oils and fatty acids: effect of carbon source on docosahexaenoic acid (c22:6 n-3, DHA) production by *Thraustochytrid* strains', *Journal of soil science and plant nutrition*, 10(3), pp. 207 - 216.

Shiba, T., Hill, R.T., Straube, W.L. and Colwell, R.R. (1995) 'Decrease in culturability of *Vibrio cholerae* caused by glucose', *Applied and Environmental Microbiology*, 61(7), pp. 2583-8.

Shimiziu, S., Kawashima, H., Shinmen, Y., Akimoto, K. and Yamada, H. (1988) 'Production of eicosapentaenoic acid by *Mortierella* fungi', *Journal of the American Oil Chemists' Society*, 65(9), pp. 1455-1459.

Shimizu, S., Kawashima, H., Akimoto, K., Shinmen, Y. and Yamada, H. (1989) 'Conversion of linseed oil to an eicosapentaenoic acid-containing oil by *Mortierella alpina* 1S-4 at low temperature', *Applied Microbiology and Biotechnology*, 32(1), pp. 1-4.

Shin, S.Y., Bajpai, V.K., Kim, H.R. and Kang, S.C. (2007) 'Antibacterial activity of eicosapentaenoic acid (EPA) against foodborne and food spoilage microorganisms', *LWT - Food Science and Technology*, 40(9), pp. 1515-1519.

Sijtsma, L. and Swaaf, M.E. (2004) 'Biotechnological production and applications of the omega-3 polyunsaturated fatty acid docosahexaenoic acid', *Applied Microbiology and Biotechnology*, 64(2), pp. 146-153.

Skerratt, J.H., Bowman, J.P. and Nichols, P.D. (2002a) '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(6), pp. 2101-6.

Skerratt, J.H., Bowman, J.P. and Nichols, P.D. (2002b) 'Shewanella olleyana sp. nov., a marine species isolated from a temperate estuary which produces high levels of polyunsaturated fatty acids', *Int J Syst Evol Microbiol*, 52(6), pp. 2101-2106.

Steinberg, D. (2007) 'Hypercholesterolemia and Atherosclerosis in humans: Causally related?', *The Cholesterol Wars*. Oxford: Academic Press, pp. 27-61.

Stülke, J. and Hillen, W. (1999) 'Carbon catabolite repression in bacteria', *Current Opinion in Microbiology*, 2(2), pp. 195-201.

Tholakalabavi, A., Zwiazek, J. and Thorpe, T. (1994) 'Effect of mannitol and glucose-induced osmotic stress on growth, water relations, and solute composition of cell suspension cultures of poplar (*Populus deltoides var. Occidentalis*) in relation to anthocyanin accumulation', *In Vitro Cellular & Developmental Biology - Plant*, 30(3), pp. 164-170.

Tocher, D.R. (2009) 'Issues surrounding fish as a source of omega-3 long-chain polyunsaturated fatty acids', *Lipid Technology*, 21 pp. 13–16.

Usui, K., Hiraki, T., Kawamoto, J., Kurihara, T., Nogi, Y., Kato, C. and 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(3), pp. 574-583.

Vamvakaki, A.-N., Kandarakis, I., Kaminarides, S., Komaitis, M. and Papanikolaou, S. (2010) 'Cheese whey as a renewable substrate for microbial lipid and biomass production by Zygomycetes', *Engineering in Life Sciences*, 10(4), pp. 348-360.

Von Wettstein-Knowles, P., Olsen, J.G., McGuire, K.A. and Henriksen, A. (2006) 'Fatty acid synthesis', *FEBS Journal*, 273(4), pp. 695-710.

Wallis, J.G., Watts, J.L. and Browse, J. (2002) 'Polyunsaturated fatty acid synthesis: what will they think of next?', *Trends in Biochemical Sciences*, 27(9), pp. 467-473.

Wang, F., Xiao, X., Ou, H.-Y., Gai, Y. and Wang, F. (2009) 'Role and regulation of fatty acid biosynthesis in the response of *Shewanella piezotolerans* WP3 to different temperatures and pressures', *Journal of Bacteriology*, 191(8), pp. 2574-2584.

Wang, Y. and Blatt, M.R. (2011) 'Anion channel sensitivity to cytosolic organic acids implicates a central role for oxaloacetate in integrating ion flux with metabolism in stomatal guard cells', *Biochemical Journal*, 439(1), pp. 161-170.

Watanabe, K., Ishikawa, C., Yazawa, K., Kondo, K. and Kawaguchi, A. (1996) 'Fatty acid and lipid composition of an eicosapentaenoic acid-producing marine bacterium ', *Journal of Marine Biotechnology*, 4 pp. 104-112.

Watanabe, Y. and Takakuwa, M. (1987) 'Change of lipid composition of *Zygosaccharomyces rouxii* after transfer to high sodium chloride culture medium', *Journal of Fermentation Technology*, 65(4), pp. 365-369.

Wen, Z.Y. and Chen, F. (2001) 'Application of statistically-based experimental designs for the optimization of eicosapentaenoic acid production by the diatom *Nitzschia laevis*', *Biotechnology and Bioengineering*, 75(2), pp. 159-169.

Wesolowski, M. (1987) 'Quality assessment of edible fish oils by thermal analysis techniques', *Lipid / Fett*, 89(3), pp. 111-116.

Wesolowski, M. and Erecinska, J. (1998) 'Thermal analysis in quality assessment of rapeseed oils', *Thermochimica Acta*, 323(1), pp. 137-143.

'WHO and FAO Joint Consultation: Fats and Oils in Human Nutrition', (1995) *Nutrition Reviews*, 53(7), pp. 202-205.

Wootton, L. (2010) 'Bacterial physiology: *Bacillus* takes the temperature', *Nature Reviews Microbiology*, 8(10), pp. 680-680.

Wu, S.T. and Lin, L.P. (2003) 'application of response surface methodology to optimize docosahexaenoic acid production by *Schizochytrium* sp. S31', *Journal of Food Biochemistry*, 27(2), pp. 127-139.

Yang, S.H., Lee, J.H., Ryu, J.S., Kato, C. and Kim, S.J. (2007) '*Shewanella donghaensis* sp. nov., a psychrophilic, piezosensitive bacterium producing high levels of polyunsaturated fatty acid, isolated from deep-sea sediments', *International Journal of Systematic and Evolutionary Microbiology*, 57(2), pp. 208-212.

Yano, Y., Nakayama, A., Ishihara, K. and Saito, H. (1998) 'Adaptive changes in membrane lipids of barophilic bacteria in response to changes in growth pressure', *Applied and Environmental Microbiology*, 64(2), pp. 479-485.

Yano, Y., Nakayama, A. and 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(7), pp. 2572-7.

Yazawa, K. (1996) 'Production of eicosapentaenoic acid from marine bacteria', *Lipids*, 31(1), pp. S297-S300.

Yazawa, K., Araki, K., Okazaki, N., Watanabe, K., Ishikawa, C., Inoue, A., Numao, N. and Kondo, K. (1988) 'Production of eicosapentaenoic acid by marine bacteria', *The Journal of Biochemistry*, 103(1), pp. 5-7.

Yongmanitchai, W. and Ward, O.P. (1991) 'Growth of and omega-3 fatty acid production by *Phaeodactylum tricornutum* under different culture conditions', *Applied and Environmental Microbiology*, 57(2), pp. 419-425.

Yoon, J.-H., Kang, K.H., Oh, T.K. and Park, Y.H. (2004) '*Shewanella gaetbuli* sp. nov., a slight halophile isolated from a tidal flat in Korea', *International Journal of Systematic and Evolutionary Microbiology*, 54(2), pp. 487-491.

Yu, R., Yamada, A., Watanabe, K., Yazawa, K., Takeyama, H., Matsunaga, T. and Kurane, R. (2000) 'Production of eicosapentaenoic acid by a recombinant marine cyanobacterium, *Synechococcus* sp', *Lipids*, 35(10), pp. 1061-1064.

Zar, H. (2009) *Biostatistical Analysis* 5th edn. Illinois, Prentice Hall.

Zhang, Q.Y., Zhou, W.W., Zhou, Y., Wang, X.F. and Xu, J.F. (2012) 'Response surface methodology to design a selective co-enrichment broth of *Escherichia coli*, *Salmonella* spp. and *Staphylococcus aureus* for simultaneous detection by multiplex PCR', *Microbiological Research*, 167(7), pp. 405-412.

Zheng, C.J., Yoo, J.-S., Lee, T.-G., Cho, H.-Y., Kim, Y.-H. and Kim, W.-G. (2005) 'Fatty acid synthesis is a target for antibacterial activity of unsaturated fatty acids', *FEBS Letters*, 579(23), pp. 5157-5162.

Zhou, L., Lu, Y., Zhou, M. and Zhao, X. (2007) 'Enhanced production of docosahexaenoic acid using *Schizochytrium* sp. by optimization of medium components', *Journal of Chemical Engineering of Japan*, 40(12), pp. 1093-1100.

Zhou, W.W., He, Y.L., Niu, T.G. and Zhong, J.J. (2010) 'Optimization of fermentation conditions for production of anti-TMV extracellular ribonuclease by *Bacillus cereus* using response surface methodology', *Bioprocess and Biosystems Engineering*, 33(6), pp. 657-663.

Zhu, L., Zhang, X., Ren, X. and Zhu, Q. (2008) 'Effects of culture conditions on growth and docosahexaenoic acid production from *Schizochytrium limacinum*', *Journal of Ocean University of China (English Edition)*, 7(1), pp. 83-88.

Ziegler, J.G. and Nichols, N.B. (1993) 'Optimum settings for automatic controllers', *Journal of Dynamic Systems, Measurement, and Control*, 115(2B), pp. 220-222.