İSTANBUL TECHNICAL UNIVERSITY ★ GRADUATE SCHOOL OF SCIENCE ENGINEERING AND TECHNOLOGY

OPTIMIZATION OF CULTIVATION CONDITIONS AND ENGINEERING BY RANDOM MUTAGENESIS FOR HIGH LIPID PRODUCTION IN *SCHIZOCHYTRIUM* SP. S31

Ph.D. THESIS

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DECEMBER 2016

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SCHIZOCHYTRIUM SP. S31'İN ÜRETİM KOŞULLARININ İYİLEŞTİRİLMESİ VE RASTLANTISAL MUTASYON İLE GELİŞTİRİLMESİYLE YÜKSEK ORANDA LİPİD ELDESİ

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To my family,

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TABLE OF CONTENTS

Page

FOREWORD ..... **ix**
TABLE OF CONTENTS ..... **xi**
ABBREVIATIONS ..... **xv**
SYMBOLS ..... **xvii**
LIST OF TABLES ..... **xix**
LIST OF FIGURES ..... **xxi**
SUMMARY ..... **xxiii**
ÖZET ..... **xxvii**
1. INTRODUCTION **1**
1.1 Classification of Algae 1
1.1.1 Macroalgae 1
1.1.2 Cyanobacteria 1
1.1.3 Microalgae 2
1.2 Microalgal Cultivation Systems 2
1.2.1 Open pond 3
1.2.2 Closed photobioreactors 3
1.2.3 Heterotrophic cultivation 3
1.3 Major Chemical Composition of Microalgal Biomass 4
1.3.1 Protein 5
1.3.2 Carbohydrate 5
1.3.3 Nutraceuticals 6
1.3.4 Lipids 6
1.3.4.1 Temperature 10
1.3.4.2 pH and salinity 10
1.3.4.3 Aeration rate 11
1.3.4.4 Nutrient limited conditions 12
1.3.4.5 Alternative raw materials 12
1.3.4.6 Sunlight or artificial light 13
1.3.4.7 Genetic manipulation 13
1.4 Long Chain Polyunsaturated Fatty Acids (PUFAs) 14
1.4.1 Biosynthesis of PUFA 16
1.4.1.1 Conventional biosynthetic pathway 16
1.4.1.2 Novel polyketide synthase biosynthetic pathway 17
1.4.2 Current sources of PUFAs 19
1.5 Thraustochytrids 20
1.5.1 Taxonomy of thraustochytrids 20
1.5.2 Production of *Schizochytrium*. 21
1.6 Factorial Designs 21
1.6.1 Plackett burman design 22
1.6.2 Central composite design. 23

1.7 Downstream Process 24
1.7.1 Harvesting microalgal culture 25
1.7.2 Drying cell biomass 26
1.7.3 Cell disruption. 27
1.7.4 Lipid extraction. 29
1.7.4.1 Organic solvent extraction 29
1.7.4.2 Supercritical liquid carbondioxide extraction 32
1.8 Mutagenesis 33
1.8.1 Flow cytometer 35
1.8.2 Screening with ImageStream 38
1.9 Market Analysis 38
1.10 Purpose of Thesis 43
2. MATERIALS AND METHODS 45
2.1 Microorganism and Medium 45
2.2 Cryoprotection of Microalgae 46
2.2.1 Evaluation of survival rate 47
2.2.2 Bacterial contamination test 48
2.3 Determination of Growth Curve 48
2.4 Optimization Experiments 51
2.4.1 Optimization of chemical composition of the medium 51
2.4.2 Optimization of the physical conditions 52
2.4.3 Statistical analysis 52
2.4.3.1 Plackett burman 52
2.4.3.2 Central composite design and response surface methodology 54
2.5 Large Scale Growth 54
2.5.1 Batch fermentation 57
2.5.2 Fed-batch fermentation. 58
2.6 Downstream Processing Steps 58
2.7 Analytical Methods 63
2.7.1 Cell dry weight measurement 63
2.7.2 Glucose quantification 63
2.7.2.1 Chemical assay 63
2.7.2.2 High pressure liquid chromatography assay 64
2.7.3 Nitrogen quantificaiton 64
2.7.3.1 Indophenol blue method 64
2.7.3.2 High pressure liquid chromatography assay 65
2.7.4 Lipid characterization 66
2.7.4.1 Total lipid analysis 66
2.7.4.2 Fatty acid analysis 66
2.7.4.3 Gas chromatography analysis 66
2.7.5 Kinetic analysis 66
2.7.5.1 Cell growth kinetics 67
2.7.5.2 Glucose utilization kinetics 67
2.7.5.3 Nitrogen uptake kinetics 67
2.7.6 Lipid qualification analysis 68
2.7.6.1 Peroxide analysis 68
2.7.6.2 Free fatty acid (FFA) analysis 68
2.8 Genome Analysis 69
2.8.1 Genomic DNA extraction 69
2.8.2 Genome size expectation 70

2.9 Random Mutagenesis 71
2.9.1 Death curve analysis 71
2.9.2 Construction of mutant library 71
2.9.3 Screening the mutant libray 72
2.9.3.1 Staining by fluorescent dye BODIPY 72
2.9.3.2 Fluorescent activated cell sorting (FACS) 73
2.9.4 Characterization of mutants 75
2.9.4.1 Image Stream analysis 75
2.9.4.2 Analysis of fatty acid profile of selected mutants 77
3. RESULTS AND DISCUSSION 79
3.1 *Schizochytrium* sp. S31 Growth Curve 79
3.2 Cryoprotection Efficiency 79
3.3 The Influence of Medium Composition on Biomass and Lipid Concentration 80
3.4 The Influence of Physical Conditions on Biomass and Lipid Concentration .. 82
3.5 Effect of The Selected Factors on CDW and DHA Production 83
3.6 Optimization of Biomass Production by Response Surface Methodology 86
3.7 Large Scale Growth Parameters 88
3.7.1 PID control 88
3.7.2 pH, temperature and antifoam controller 90
3.8 Heterotrophic Growth of *Schizochytrium* sp.S31 91
3.8.1 Cellular growth 91
3.8.2 Monitoring medium components 92
3.8.3 Lipid analysis 93
3.8.4 Analytical calculations 95
3.8.5 Kinetic calculations 97
3.9 Optimization of Cell Lysis and Lipid Extraction 101
3.10 Analysis of SFE Model 102
3.10.1 Effect of pressure-temperature 104
3.10.2 Effect of temperature-extraction time 105
3.10.3 Effect of pressure-extraction time 105
3.11 Strain improvement by Random Mutagenesis 106
3.11.1 Death curve analysis *Schizochytrium* sp. S31 106
3.11.2 Nutrient starvation 108
3.11.3 UV mutagenesis and screening 109
3.11.4 Characterization of selected mutants 113
4. CONCLUSIONS 117
REFERENCES 123
APPENDICES 137
CURRICULUM VITAE 157

ABBREVIATIONS

ALA : Alpha linolenic acid
ARA : Arachidonic acid
ATCC : American Type Culture Collection
CCD : Central Composite Design
CDW : Cell Dry Weight
CFU : Colony Forming Units
DAG : Diacylglycerol
DHA : Docosahexaenoic Acid
DMSO : Dimethyl Sulfoxide
DNS : Dinitrosalicylic Acid
DO : Dissolved Oxygen
EMS : Ethyl Methansulfonate
EPA : Eicosapentaenoic Acid
Fas : Fatty Acids
FAME : Fatty Acid Methyl Ester
FAO : Food and Agriculture Organization
FFA : Free Fatty Acid
FFD : Full Factorial Design
FSC : Forward Scatter
GC : Gas Chromatography
GCMS : Gas Chromatography Mass Spectrometry
GL : Glycolipids
GLA : Gamma linolenik asit
HPLC : High Pressure Liquid Chromatography
IS : Image Stream
MAG : Monoacylglycerol
MMS : Methanesulphonate
MSG : Monosodium Glutamate
NIH : National Institue of Health
PB : Plackett Burman
PKS : Polyketide Synthase
PL : Phospholipids
PUFA : Polyunsaturated Fatty Acids
PQ : Pure Quadratic
RSM : Response Surface Methodology
SFE : Supercritical Fluid Extraction
SSC : Side scatter
TAG : Triacylglycerol
TFA : Total Fatty Acid
TWI : Two Way Interaction
UV : Ultraviolet Radiation
WHO : World Health Organization

SYMBOLS

μ_m	: Maximum Specific Growth Rate
q	: Maintenance Coefficient
β	: Product formation Constant
$Y_{X/S}$	: Yield Factor
rn	: Monosodium Glutamate Uptade Rate
rs	: Glucose Utilization Rate
rx	: Growth Kinetics

LIST OF TABLES

Page

Table 1.1 : Lipid content of different microalgae species..... **9**
Table 1.2 : Recommended adequate intakes for omega-3 fatty acids..... **41**
Table 2.1 : Growth medium for *Schizochytrium* sp.S31 ..... **45**
Table 2.2 : Levels of factors in the Plackett Burman design **53**
Table 2.3 : Substrate profile of 5 lt fermenter culture..... **58**
Table 2.4 : Codded and uncoded levels of independent variables for CCD and responses..... **62**
Table 3.1 : Alternative medium components and their responses on biomass and lipid production..... **81**
Table 3.2 : Different physical conditions and their responses on biomass production. **82**
Table 3.3 : Plackett Burman design for screening of selected factors **83**
Table 3.4 : Effect estimates for CDW and DHA production from the results of Plackett Burman design. **85**
Table 3.5 : Analysis of varience of CCD for CDW and lipid production by *Schizochytrium* sp.S31. ..... **86**
Table 3.6 : PID values obtained by ZN method..... **88**
Table 3.7 : Fatty acid profile of *Schizochytrium* sp.S31 microalgae..... **94**
Table 3.8 : Analytical results of *Schizochytrium* sp. S31 production. **95**
Table 3.9 : Chemical characteristics of *Schizochytrium* sp.S31. ..... **96**
Table 3.10 : Elemental composition of *Schizochytrium* sp.S31..... **96**
Table 3.11 : Kinetic calculation results..... **97**
Table 3.12 : Comparison of different cell lysis and lipid extraction method..... **101**
Table 3.13 : Coded and uncoded levels of independent variables for CCD and responses..... **103**
Table 3.14 : Quadratic model constants and regression analysis for total extraction yield and DHA% **103**
Table 3.15 : ImageStream report of 30 sec. mutant and wild type **111**
Table 3.16 : Comparative results of the Mutants and Wt *Schizochytrium* sp. **114**
Table A2.1 :GCMS results of Mutant1..... **143**
Table A2.2 :GCMS results of Mutant2..... **145**
Table A2.3 :GCMS results of Mutant3..... **147**
Table A2.4 :GCMS results of Mutant4..... **149**
Table A2.5 :GCMS results of Mutant5..... **151**
Table A2.6 :GCMS results of Mutant6..... **153**
Table A2.7 :GCMS results of wild type. **155**

LIST OF FIGURES

Page

Figure 1.1 : LC PUFA production from microalgae. **2**
Figure 1.2 : Chemical composition of microalgae biomass **5**
Figure 1.3 : Triacylglycerol production reaction **6**
Figure 1.4 : Categorization of lipids and fatty acid chains **7**
Figure 1.5 : Saturated,monounsaturated and polyunsaturated fat **8**
Figure 1.6 : Classification of PUFAs **14**
Figure 1.7 : Structure of docosahexaenoic acid **15**
Figure 1.8 : Conventional biosynthetic pathway of the formation of PUFAs **17**
Figure 1.9 : PUFA biosynthesis via the polyketide synthase (PKS) in *Schizochytrium* sp. **18**
Figure 1.10 : Morphology of *Schizochytrium* sp. cell **20**
Figure 1.11 : Central composit design matrix for two factors **23**
Figure 1.12 : Downstream processing steps **25**
Figure 1.13 : Different cell disruption methods **27**
Figure 1.14 : Fatty acid methyl ester (FAME) reaction **31**
Figure 1.15 : Mutation selection cycle for improved lipid production **35**
Figure 1.16 : Different lasers and filters in flow cytometer system **37**
Figure 1.17 : Forward and side scatter **37**
Figure 1.18 : Principle of fluorescent activated cell sorting (FACS) **38**
Figure 2.1: Cell stock lot system **47**
Figure 2.2: Shematic diagram of optimization strategy **51**
Figure 2.3: The Biostat A plus bioreactor and computer based control program **55**
Figure 2.4: Ultraviolet (UV) radiation mutant library constraction and screening .. **72**
Figure 3.1: Growth curve of *Schizochytrium* sp.S31 microalgae **79**
Figure 3.2 : Survival rate of *Schizochytrium* sp.S31 with different cryoprotectant and storage conditions. **80**
Figure 3.3 : Three dimensional surface plot and two dimensional contour plot of biomass production **87**
Figure 3.4 : Three dimensional surface plot and two dimensional contour plot of lipid production. **87**
Figure 3.5 : Dissolve oxygen and stirrer speed change during the incubation of *Schizochytrium* sp.S31 ..... **89**
Figure 3.6 : pH, temperature and antifoam change during the incubation of *Schizochytrium* sp.S31 ..... **90**
Figure 3.7: Cell dry weight and O.D750 results during the incubation of *Schizochytrium* sp.S31 ..... **91**
Figure 3.8 : Residual, added and consumed glucose during the incubation **92**
Figure 3.9 : Residual, added and consumed MSG during the incubation **93**
Figure 3.10 : Lipid accumulation in *Schizochytrium* sp. during the incubation **94**
Figure 3.11 : Calculation of μmax and Ks by Lineweaver-Burke method **98**

Figure 3.12 : Calculation of μmax and Ks by Monod equation..... **99**
Figure 3.13 : Glucose utilization rate calculation **100**
Figure 3.14 : Monosodium glutamate utilization rate calculation **101**
Figure 3.15 : Effect of temperature-pressure interaction on lipid extraction **104**
Figure 3.16 : Effect of extraction time- temperature on lipid extraction **105**
Figure 3.17 : Effect of extraction time- pressure on lipid extraction..... **106**
Figure 3.18 : *Schizochytrium* sp. death curve under different U.V exposure time . **107**
Figure 3.19 : *Schizochytrium* sp. death curve under different EMS exposure time **107**
Figure 3.20 : Flow cytometer analysis of *Schizochytrium* sp. during time under normal and limited conditions **108**
Figure 3.21: Comparison of mean values of two different cultures..... **109**
Figure 3.22: Lipid accumulation of wt *Schizochytrium* sp. S31 during time by limitation..... **110**
Figure 3.23: Time course ImageStream analysis of mutants and wt *Schizochytrium* sp.S31..... **112**
Figure 3.24: Light intensity difference between mutants and wt on ImageStream analysis..... **112**
Figure 3.25: Comparative O.D.750 and CDW result of the mutant and wt *Schizochytrium* sp. **113**
Figure 3.26: Comparative lipid % result of the mutant and wt *Schizochytrium* sp. **114**
Figure A1.1 : Mutant1 time course experimental data..... **139**
Figure A1.2 : Mutant2 time course experimental data..... **139**
Figure A1.3 : Mutant3 time course experimental data..... **140**
Figure A1.4 : Mutant4 time course experimental data..... **140**
Figure A1.5 : Mutant5 time course experimental data..... **141**
Figure A1.6 : Mutant6 time course experimental data..... **141**
Figure A1.7 : Wild type time course experimental data **142**
Figure A2.1 : GCMS results of Mutant1 ..... **144**
Figure A2.2 : GCMS results of Mutant2 ..... **146**
Figure A2.3 : GCMS results of Mutant3 ..... **148**
Figure A2.4 : GCMS results of Mutant4 ..... **150**
Figure A2.5 : GCMS results of Mutant5 ..... **152**
Figure A2.6 : GCMS results of Mutant6 ..... **154**
Figure A2.7 : GCMS results of Wild type **156**

OPTIMIZATION OF CULTIVATION CONDITIONS AND ENGINEERING BY RANDOM MUTAGENESIS FOR HIGH LIPID PRODUCTION IN *SCHIZOCHYTRIUM* SP. S31

SUMMARY

Schizochytrium sp. is widely studied microalgae to obtain high content of polyunsaturated fatty acids especially docosahexaenoic acid (DHA). They contain various bioactive compounds that can be used as pharmaceutical raw material, food additive, aquaculture and animal feed. They also play a crucial role in biofuel production which can be used as an alternative energy source. *Schizochytrium* sp. which is grown rapidly and produced high amount of DHA can be used as an alternative to fish oils.

In this study, cultivation conditions were optimized to increase biomass and total lipid productivity of the *Schizochytrium* sp. S31 by using response surface methodology. After optimizing growth conditions, large scale production of *Schizochytrium* sp. S31 under favorable conditions were performed and downstream process including cell lysis, drying and lipid extraction were compared and optimized to maximize total lipid specially DHA extracted from *Schizochytrium* sp. S31. Finally, random mutation was applied by ultraviolet (UV) radiation or chemical mutagen and high yield of lipid accumulating mutants of a *Schizochytrium* sp. were selected by flow cytometric-based selection.

Many factors may affect the cultivation conditions of *Schizochytrium* sp. such as medium composition, pH, salinity and temperature. Since polyunsaturated fatty acids have gained significance due to their role in human health, industrial and commercial usage, research on enhancement of biomass and lipid production has increased. Therefore, optimization of medium composition and environmental conditions to improve biomass and total lipid content of the organism is important. Applying a statistical strategy was an effective tool for optimization of the production and extraction process, which would also reduce the production costs through maximizing the yield. Statistical methodologies such as Plackett burman and central composite design have been extensively used to optimize several factors and their interactions. Plackett burman design was used recently as the first step in optimizing different bioprocesses to identify the factors with a significant effect on desired responses. Following the selection of the most significant factors, response surface methodology with central composite design is used to determine the optimum values of these factors. Based on contour plots and canonical analysis, a maximum biomass production of 26.86g·L-1 was obtained with 2.29 $g.L^{-1}$ monosodium glutamate (MSG), pH 5.8 cultivation conditions. Maximum lipid production of 35% was obtained with 0.49 $g.L^{-1}$ MSG at 17.6 °C temperature conditions.

Schizochytrium sp. S31 can be grown heterotrophically in bioreactor by supplying with alternative raw materials such as sugars, organic acids and alcohols. Thus,

heterotrophic systems assure a cost effective way to obtain valuable microalgae-produced DHA by using cheap substrates on large scale. Large scale heterotrophic growth technologies has lower contamination risk, lower harvesting costs, eliminated light limitation, high degree of process control and reproducible. In most instances, heterotrophic culture commonly results in much higher cell densities and lipid productivity than phototrophic culture because there is no light limitation in heterotrophic culture and controllable. According to large scale production results, 65 g/l of cell dry weight with the initial growth rate of 0.312 h^{-1} was obtained. C/N ratio of the *Schizochytrium* sp. culture was calculated as 19.32. The total lipid content was 27.5 % of the cell dry weight after applying optimized conditions. DHA percentage of the *Schizochytrium* sp. culture in 5 liter was calculated as 30.18%. Biomass, lipid and DHA productivity was calculated as 0.65 g/l.h, 0.17g/l.h and 0.053g/l.h respectively.

Downstream processing steps required to obtain lipid from microalgal biomass once large scale production process is completed. Downstream process include harvesting, dewatering, cell disruption, lipid extraction. Harvesting microalgal culture includes centrifugation, filtration, and flocculation methods to concentrate microalgal culture. Drying step is known as post-dewatering step in which the cell pellet is completely dewatered. The cell pellet is exposed drying process for enhancing the efficiency of subsequent lipid extraction and lowering the cost of downstream process. Cell disruption is a key step in influencing lipid extraction yields. Sonication, high-pressure homogenizers, grinding, enzymatic reactions, chemical hydrolysis are the most known cell disruption methods applied for microalgae. Different types of solvents and extraction methods have been used in the literature to recover microalgal lipids. Hexane, methanol, ethanol, isopropanol are the typical solvents used for lipid extraction. According to the cell lysis and lipid extraction results, ultrasonication with hexane method increased the total lipid yield significantly with clear appearance. Sonication resulted in a 1.4-fold increase in lipid yield when compared with solvent alone. However, these traditional lipid extraction methods use large amounts of solvents that are mostly toxic. Supercritical liquid extraction (SFE) generally uses carbondioxide as a solvent at high pressure to extract lipid and/or nutraceutical products with higher selectivity in shorter extraction times. The effect of extraction temperature, pressure and time of SFE on the lipid yield and %DHA amount were investigated by using RSM. According to SFE results, pressure and temperature have significant effect ($p < 0.05$) on total lipid yield and DHA concentration. Based on contour plot analysis, optimum extraction conditions were found to be 425 bar pressure at 40.5°C for 97.5min. After optimization of pressure, temperature and time variables of SFE, 30.2% lipid yield was obtained.

Isolation of high yield lipid accumulating mutants of microalgae by flow cytometric-based selection can be performed by introducing mutations in the genome. There are mainly two mutation agents for random mutagenesis: UV radiation and chemical mutagenizing agent. Both methods do not require any genetic information about microalgae. Fluorescent activated cell sorting is a high throughput technique for selection of mutants with desired phenotype. This study highlighted that UV mutagenesis and high throughput selection improved lipid productivity in *Schizochytrium* sp. S31. Nutrient limitation conditions, such as nitrogen (N), phosphorus (P) starvation, and temperature limitation induced lipid accumulation in *Schizochytrium* sp. According to characterization results, mutants which were treated to UV for 30 second accumulate lipid faster than wild type. Time course experiment were conducted to understand the lipid accumulation profile difference between

mutants and wild type. Total lipid was increased by 28.4 % for Mutant1 and 10.8% for Mutant2 comparing to wild type. The results obtained from gas chromatography mass spectrometry analyses confirmed the results obtained by flow cytometry, showing an increase in DHA in UV treated cultures (30sec) compared to untreated controls. There was a 17.9% and 12.1% increase compare to wild type in terms of DHA percentage in two different mutants respectively.

SCHIZOCHYTRIUM SP. S31'İN ÜRETİM KOŞULLARININ İYİLEŞTİRİLMESİ VE RASTLANTISAL MUTASYON İLE GELİŞTİRİLMESİYLE YÜKSEK ORANDA LİPİD ELDESİ

ÖZET

Schizochytrium sp. yüksek oranda çoklu doymamış yağ asitleri özellikle dokosaheksaenoik asit (DHA) elde etmek amacıyla yaygın olarak çalışılan mikroalgdir. Farmasötik hammadde, gıda katkısı, su kültürü ve hayvan yemi olarak kullanılan çeşitli biyoaktif bileşikler içerirler. Ayrıca alternatif enerji kaynağı olarak kullanılan biyoyakıt üretiminde önemli role sahiptir. Hızlı büyüyen ve yüksek DHA oranına sahip *Schizochytrium* sp. balık yağına alternatif olarak kullanılabilir.

Bu çalışmada, *Schizochytrium* sp. S31 mikroalginin toplam biyokütle ve yağ oranını artırmak amacıyla hücre büyüme koşullarının tepki yüzey metodu ile optimizasyonu gerçekleştirilmiştir. Optimize edilen büyüme koşulları kullanılarak *Schizochytrium* sp. S31'in büyük hacimde üretimi gerçekleştirilmiş ve yüksek oranda yağ elde etmek amacıyla hücre parçalama, kurutma ve yağ ekstraksiyonu içeren akış proseslerinin karşılaştırılması ve optimizasyonu tamamlanmıştır. Son bölümde, ultraviyole (UV) ışın veya kimyasal mutajen kullanarak rastlantısal mutasyon uygulaması ve floresan sitometri yöntemiyle yüksek oranda yağ üreten *Schizochytrium* sp. mutantın seçilimi gerçekleştirilmiştir.

Schizochytrium sp. üretimini besiyeri içeriği, pH, tuzluluk ve sıcaklık gibi birçok faktör etkileyebilir. Çoklu doymamış yağ asitlerinin insan sağlığı, endüstriyel ve ticari kullanımında önemli bir rol oynamasından dolayı biyokütle ve lipid üretiminin geliştirilmesi üzerindeki çalışmalar son zamanda artmıştır. Bu sebeple, besiyeri içeriği ve çevresel koşulların optimizasyonu, organizmanın biyokütlesinin ve toplam lipid içeriğinin geliştirilmesi için önemlidir. İstatistiksel yaklaşımlar, biyokütle üretim ve yağ ekstraksiyon optimizasyonu için uygun bir yol olmakla birlikte ürün verimliliğini artırarak üretim maliyetini düşürmüştür. Plackett burman ve merkezi kompozit tasarım gibi istatistiksel metotlar, çeşitli faktörleri ve onların birbiriyle etkileşiminin optimizasyonunda yaygın olarak kullanılmıştır. Plackett Burman tasarımı, biyoproseslerin optimizasyonunda belirlenen cevaplara etki eden faktörlerin tanımlanmasında ilk adım olarak kullanılır. En önemli faktörlerin seçimini takiben merkezi kompozit tasarımlı tepki yüzey yöntemi, bu faktörlerin optimum değerlerini tespit etmek amacıyla kullanılır. İstatistiksel analiz sonuçlarına göre, maksimum biyokütle (26.86g/L) üretimi, 2.29 g/l monosodyum glutamat (MSG), pH 5.8 yetiştirme koşulları ile elde edilmiştir. Maksimum lipid (% 35) üretimi 0.49 $g.L^{-1}$ MSG, 17.6 °C sıcaklık koşullarında elde edilmiştir.

Schizochytrium sp. S31, şeker, organik asitler ve alkol gibi alternatif hammaddeler kullanılarak biyoreaktörde heterotrofik olarak üretilebilir. Bu sebeple heterotrof sistemler, alternatif substratlar kullanarak yüksek kalitede büyük ölçekli DHA üretilmesinde uygun maliyetli bir yoldur. Yüksek hacimde heterotrofik üretim teknolojileri, kirlilik riski ve üretim maliyetini düşürmüş, ışık kısıtlaması olmaksızın

yüksek oranda proses kontrolü ve yeniden üretilebilirlik sağlamıştır. Birçok durumda, heterotrof mikroalg kültürü ışık kısıtlaması ve kontrol edilebilirliği sayesinde fototrof kültüre göre daha yüksek hücre yoğunluğu ve lipid verimliliği sağlamıştır. Yüksek hacimde üretim sonuçlarına göre, başlangıç üreme hızı 0.312 h^{-1} olan 65g/l kuru hücre ağırlığı elde edilmiştir. Karbon azot oranı (C/N) 19.32 olarak hesaplanmıştır. Optimize edilen koşulların uygulanması sonucunda kuru hücre ağırlığının %27.5'i oranında lipid elde edilmiştir. 5 litre biyoreaktörde üretilen *Schizochytrium* sp. kültüründen %30.18 oranında DHA elde edilmiştir. Biyokütle, lipid ve DHA üretim verimlilikleri sırasıyla 0.65 g/l.h, 0.17g/l.h ve 0.053g/l.h olarak hesaplanmıştır.

Büyük hacimde üretim tamamlandıktan sonra istenilen ürünün hücre kütlesinden belirli aşamalarla ayrılarak saflaştırılması işlemi gerçekleştirilir. Bu saflaştırma işlemleri hasat alma, su giderme, hücre parçalanması ve lipid ekstraksiyon işlemlerini içermektedir. Mikroalg kültürünün hasadı, biyokütle konsantrasyonunu arttırmak için santrifüj, filtrasyon ve çöktürme yöntemlerini içerir. Kurutma aşaması, su giderme işlemi olarak da bilinir ve hücre peletleri tamamen sudan uzaklaştırılmış olur. Lipid ekstraksiyonun verimliliğini arttırmak ve ayrıca maliyeti düşürmek için hücre peleti kurutma işlemine maruz bırakılır. Hücre parçalanması, lipid ekstraksiyonun veriminde anahtar bir rol oynamaktadır. Sonikasyon, yüksek basınçlı homojenizatörler, öğütme, enzimatik reaksiyonlar ve kimyasal hidroliz mikroalgler için yaygın olarak kullanılan hücre parçalama yöntemleridir. Mikroalg lipid ekstraksiyonu için literatürde farklı türde solvent ve ekstraksiyon yöntemleri kullanılmıştır. Genellikle hekzan, metanol, etanol ve izopropanol lipid ekstraksiyonu için kullanılan solventlerdir. Hücre parçalama ve lipid ekstraksiyon sonuçlarına göre, hekzan ile birlikte uygulanan ultrasonikasyon metodu toplam lipid oranını önemli ölçüde artırmıştır. Sonikasyon metodu uygulanarak toplam lipid oranı 1.4 kat artırılmıştır. Bununla birlikte, geleneksel lipid ekstraksiyon yöntemleri yüksek oranda toksik solvent kullanmaktadır. Süperkritik akışkan ekstraksiyonu genellikle solvent olarak yüksek basınçta karbondioksit kullanarak yüksek seçicilikle lipid ve/veya nutrasötik ürünlerin ekstraksiyonu için kullanılır. Süperkritik akışkan ekstraksiyonun süre, sıcaklık ve basıncın yağ verimi ve %DHA üzerindeki etkisi tepki yüzey metoduyla tespit edilmiştir. Süperkritik akışkan ekstraksiyonu sonuçlarına göre, basınç ve sıcaklık toplam lipid verim ve DHA konsantrasyonu üzerinde önemli etkiye sahiptir ($p < 0.05$). Sabit sıcaklıkta, basınç arttıkça lipid ve DHA verimi artarken sabit basınçta sıcaklık arttıkça lipid ve DHA verimi azalmaktadır. İstatistiksel kontur analizlerine göre, optimum koşullar 425bar basınç, 40.5°C ve 97.5 dakika olarak tespit edilmiştir. SFE koşullarının optimizasyonu sonucu %30.2 yağ verimi elde edilmiştir.

Yüksek yağ içeriğine sahip mikroalg mutantlarının flow sitometri yöntemiyle seçilimi, genoma rastlantısal mutasyon tanıtımı ile gerçekleşebilir. Rastlantısal mutasyon için genel olarak iki mutajen uygulanmaktadır: UV radyasyon ve kimyasal mutajen. Her iki yöntemde mikroalgler ile ilgili genetik bilgiye gereksinim duyulmaz. Floresan aktif hücre ayırma, istenilen fenotipte mutant seçiminde yüksek verimlilik sağlamaktadır. Bu çalışma ile *Schizochytrium* sp. mikroalginin lipid üretiminin artırılmasında UV mutasyonu ve flow sitometre bazlı seçilimin uygulanabilir bir yaklaşım olabileceği gösterilmiştir. Azot (N), fosfor (P) gibi besin kısıtlayıcı ve sıcaklık sınırlayıcı koşullar, *Schizochytrium* sp.'nin lipid içeriğinde önemli bir artışa sebep olmuştur. Karakterizasyon sonuçlarına göre, 30 saniye UV ışınına maruz kalmış mutantlar yabanıl tipe göre daha hızlı lipid biriktirmektedir. Mutantlar ve yabanıl tip arasındaki lipid biriktirme profili farkını anlayabilmek için sürece bağlı deneyler uygulanmıştır. Sonuçta, toplam yağ oranı Mutant1'de %28.4, Mutant2'de ise %10.8 oranında

artmıştır. Gaz kromatografisi kütle spektrometresi analizlerinden elde edilen sonuçlar flow sitometriden elde edilen sonuçlarla tutarlılık göstermekte olup 30 saniye boyunca UV'ye maruz kalmış kültürlerin DHA oranında artış olduğu gözlemlenmiştir. Yabanıl tip ile diğer iki mutant karşılaştırıldığında DHA oranında sırasıyla %17.9 ve %12.1 oranında artış gözlemlenmiştir.

1. INTRODUCTION

1.1 Classification of Algae

The classification of algae into taxonomic groups is based on the ability to perform photosynthesis and live completely in an aquatic environment (both fresh and marine). Algal cells can be single or multicellular. They also can be prokaryotes or eukaryotes. Different morphological features such as organelle, cell membrane, flagella and cell divison process have been demonstrated for classification of algae. Today, algae are generally divided into three major groups: macroalgae, cyanobacteria and microalgae (Baweja and Sahoo, 2015).

1.1.1 Macroalgae

Macroalgae are macroscopic aquatic photosynthetic plants and commonly known as seaweed. They differ from microalgae in respect to cellular structure. While multicellular marine macroalgae can be visible by eye, microalgae can only be seen by microscope. The size of macroalgae ranges from a few millimetres to plants up to 3-4 m high. They can appear red (Rhodophyta), brown (Ochrophyta) or green (Chlorophyta) due to different photosynthetic pigments each group possesses (Kılınç et al, 2013). Macroalgae are either grown in the wild or cultivated in laboratory to be used generally as edible food, medicine, pharmaceutical raw material and energy production.

1.1.2 Cyanobacteria

Cyanobacteria (Cyanoprokaryota, Cyanophyceae) formerly called "blue-green algae" are a group of photosynthetic and aquatic organisms. Cyanobacteria are relatively simple, ancient phylum of bacteria that obtain their energy via photosynthesis (Oren, 2014). These prokaryotic and unicellular cells are typically much larger than bacteria and photosynthesize like algae. Cyanobacteria are considered one of the most important groups of organisms in respect to ecological change of earth's history. Also, according to endosymbiotic theory all chloroplasts in plants and eukaryotic algae

evolved from the endosymbiosis of cyanobacteria cells. Spirulina is most known cyonobacteria that contain 60-70% protein.

1.1.3 Microalgae

Microalgae are highly diverse unicellular group which present in all existing earth ecosystems mostly in ocean and freshwater. It is predicted that there are approximately more than 50.000 species exist, but only a limited number have been analyzed. *Schizochytrium sp*. S31 is a marine thraustochytrid that contains lipid rich in polyunsaturated fatty acids (PUFAs) especially docosahexaenoic acid (DHA) (Yokochi et al, 1998). They contain various bioactive and high value compounds that can be used as pharmaceutical raw material, food additive and supplement . They also play a crucial role in biofuel production which can be used as an alternative energy and aquaculture (Spolaore et al, 2006).

Microalgae are classified in groups based on different morphological variations such as round, oval, cylindrical and/or their sizes which is range from 0,20 µm to 200 µm. Microalgae can grow rapidly and be cultivated either phototrophically or heterotrophically. Microalgae offer a promising source of LC-PUFA which can be used in wide range of areas specifically in pharmaceutical industry (Figure 1.1).

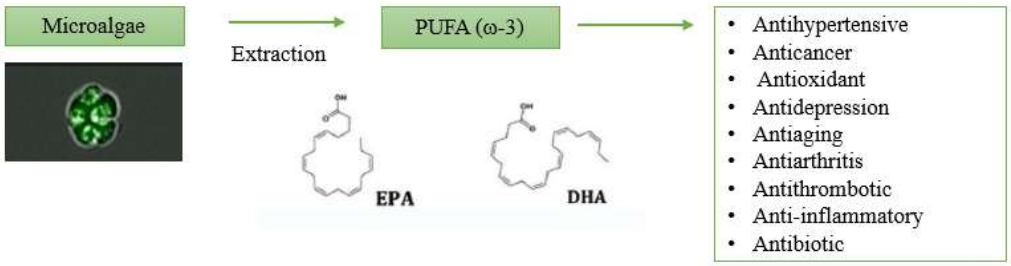


Figure 1.1: LC-PUFA production from microalgae (Martins et al, 2013).

1.2 Microalgal Cultivation Systems

Microalgal cultivation systems have been generally categorized into photoautotrophic and heterotrophic systems. While the heterotrophic systems requires organic substrates such as carbon and nitrogen, photoautotrophic cultivation system only requires sunlight. Nevertheless, heterotrophic cultivation is considered superior to photoautotrophic cultivation due to the fact that light, the growth limiting factor, is eliminated (Bumbak et al, 2011). Open pond and closed photobioreactors which are

the main types of photoautotrophic systems use artificial and/or natural (sunlight) light rather than organic substrate for cultivation (Chauton et al, 2015). Batch, continuous and fed-batch systems are the main types of heterotrophic cultivation and all requires organic substrates and controlled conditions. The development of suitable microalgal cultivation systems is important for the production of pharmaceutical and commercially important products.

1.2.1 Open pond

Open pond sysytem is considered as most prevalent and commercial system employed for microalgae cultivation (Mata et al, 2010). This system typically built in circular or raceway configuration that use free sunlight. However, low volumetric productivity and high energy costs are the disadvantages of this system (Suali and Sarbatly, 2012). Because of there is a high risk of microbial contamination on open pond system, cultivation of *Dunaliella salina* which can tolerate high salinity and *Spirulina, Arthrospira* which can grow well at high pHs can be applied to open pond system.

1.2.2 Closed photobioreactors

This type of systems such as optical, tubular and helical reactors, use the large illumination surface to volume ratio to generate higher biomass concentration (Perez-Garcia et al, 2011). Closed bioreactors are easy to control of conditions and can be used as alternative to disadvantageous open pond system. Open ponds has high contamination risk and more experience on bigger scale whereas the photobioreactors are more resistant to contamination and can be used for small scale. Close photobioreactors have been employed for DHA production using different species specially *Isochrysis galbana* (Duong et al, 2012).

1.2.3 Heterotrophic cultivation

It is alternative cultivation in fermentors for culturing microalgae under the conditions of light is eliminated and organic substrates are utilized as the source of carbon and energy. Heterotrophic microalgae culture commonly results in much higher cell densities than phototrophic culture because there is no light limitation in heterotrophic culture and controllable. The majority of microalgae are phototrophic. They use light to generate energy but some microalgae species are able to utilize organic substrates as an energy source. Microalgae that have been shown to grow heterotrophically

include *Cyclotella, Chlorella, Tetraselmis, Nitzschia, Crypthecodinium* and *Schizochytrium* species (Bumbak et al, 2011). Heterotrophic algae commonly produce omega-3 long chain polyunsaturated fatty acids (LC-PUFAs) specially DHA and eicosapentaenoic acid acid (EPA) as phospholipid and/or triacylglycerol (TAG) form depending on species. Bowles et al. (1999) reported that the high omega-3 fatty acid (DHA) yield were obtained by *Thraustochytrium* strain which is an heterotrophic microalgae in bioreactors. Fed-batch or continuous culture can be used by modifying the batch culture system to enhance the biomass yield and DHA productivity. Microalgae can also be grown in fermenter supplemented with alternative raw materials such as sugars, organic acids and alcohols. Thus, heterotrophic systems assure a cost-effective way to obtain valuable microalgae based products by using cheap substrates on large scale. (Bumbak et al, 2011). Heterotrophic cultivation have many benefits; for example, established fermentation technologies, lower contamination risk, lower harvesting costs, elimination of light limitation, high degree of process control and reproducible. In most instances, heterotrophically grown algae of one species has much higher lipid productivity than phototrophically grown algae of the same species. One study indicate that when *Chlorella* sp. cells were grown heterotrophically, they accumulated 55.2% lipids, however produce only 14.6% of lipid when phototrophically grown (Miao and Wu 2006) The main advantages of heterotrophic cultivation are the higher densities that can be achieved with controlled conditions and low contamination risk and the large scale can be obtained from single cells in fermenters.

1.3 Major Chemical Composition of Microalgal Biomass

The majority of microalgae biomass contains proteins, carbohydrates, and lipids. In general, algae biomass contains approximately 20-25% carbohydrate, 15-70% lipid, and 25-40% protein (Singh et al, 2011) . In addition to the three main components, cells can contain smaller amounts of nucleic acids, pigments such as carotenoids, omega-3 and -6 fatty acids and valuable products such as antioxidants, cratenoids, vitamins, phytosterols, lutein and bioactive peptide molecules. Figure1.2 shows the chemical composition of microalgae biomass. However, chemical composition of the microalgae can be manipulated by different methods to favor a particular end product.

For example, if nitrogen and phosphate is starved, *Schizochytrium* sp. microalgae tend to produce more lipid (Chisti, 2007).

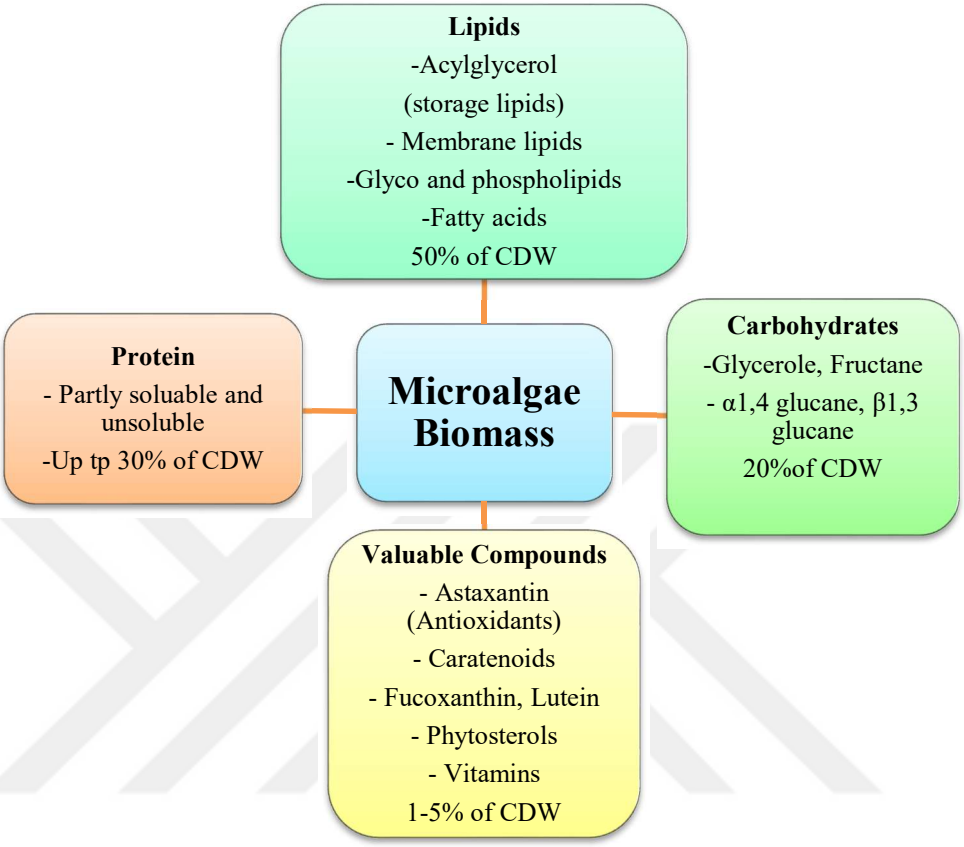


Figure 1.2: Chemical composition of microalgae biomass.

1.3.1 Protein

Protein level in the microalgae biomass generally constitute approximately 25-40% of the dry weight even though protein levels vary greatly from species to species. Spirulina is most known algae that has high protein content (65%) (Dillon et al, 1995). Because of algal protein has a good and valuable amino acid profile, World Health Organization (WHO) and Food and Agriculture Organization (FAO) defined that algae is a very promising protein source (Noack, 1974). Currently, algae protein is mostly used as animal feed and supplement for vegetarian/vegan people.

1.3.2 Carbohydrate

Microalgae have utilized different carbohydrates as energy and carbon storage molecules, and altered the location of storage of those carbohydrates in the cell, resulting in dramatic differences in storage forms and locations (Ball et al, 2011,

Hildebrand et al, 2013). Carbohydrate stores range from insoluble starch, hydrosoluble glycogen, laminaran, fucoidin mannitol and water soluble β-1,3-glucans such as chrysolaminarin. *Thalassiosira pseudonana* diatom accumulate chrysolaminarin as a carbon source (Roessler, 1987). Differences in carbohydrate type and storage could affect a variety of aspects of cellular function, including photosynthetic efficiency, carbon fixation, carbon flux and partitioning, carbohydrate storage, lipid accumulation and cellular energetics. Algal carbohydrates can be used for two main reasons. The first reason is to be used for a feed source for livestock. The second reason is to be used as a sugar source in an alcohol fermentation process.

1.3.3 Nutraceuticals

Microalgae has been investigated for various nutraceutical products. The major nutraceuticals produced by algae include: omega-3/6 fatty acids which are specially important for improving both brain and heart health; bioactive peptide molecules which has potential pharmaceutical, biotechnological properties, anti-tumour and antiviral activities; carotenoids and vitamins, which have antioxidant properties; sterols which show anti-diabetic and sulfated polysaccharides which have anticoagulant properties (De Jesus Raposo, 2013).

1.3.4 Lipids

Lipids are natural organic molecules that contain hydrocarbon in their structure. Biological functions of the lipids are cellular structure specially membrane, energy storage, signaling molecules (Lehninger, 1982). Fats (solid) and oils (liquids) which are nutritionally important lipids consist of fatty acids with 12-20 carbons and are made by reaction of hydroxyl groups on the glycerol (an alcohol with a hydroxyl group on each of its three carbons) backbone with the carboxyl groups of fatty acids by dehydration synthesis reaction (Figure 1.3).

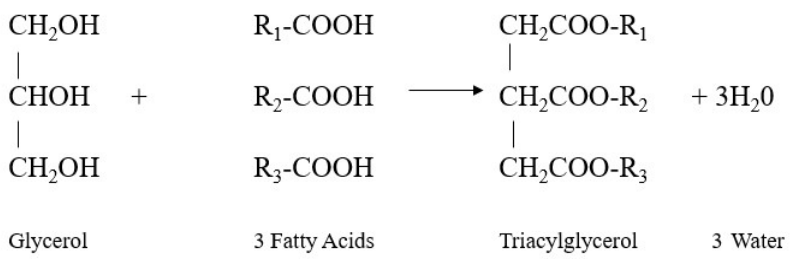


Figure 1.3: Triacylglycerol (TAG) production reaction.

This group of molecules includes fats and oils, waxes, phospholipids, steroids (like cholesterol) are most important type of lipids and they are characterized by their solubility in organic solvents (polar or neutral lipids) and general insolubility in water (Luckey, 2014). Common feature of these lipids is having a long chain fatty acids in their structure. Polar lipids are hydrophilic lipids and consist mainly of polar phosphorus or carbohydrate moiety and fatty acids (FAs) backbone. Glycolipids (GL) and phospholipids (PL) are the examples of polar lipids. Neutral lipids are hyrophobic lipids and consist of monoacylglycerol (MAG), diacylglycerol (DAG), triacylglycerol (TAG) and sterols. While polar lipids generally form bilayer cell membrane, neutral lipids are used primarily in cytoplasm as energy storage. The percentage of polar and neutral lipids in microalgae are unknown because they produce predominantly polar lipids under optimal conditions while produce significantly higher concentrations of neutral lipids (TAGs) under stressed conditions. A fatty acid (FA) molecule consists of a hydrophilic carboxylate group attached to long aliphatic hydrocarbon chain which could be saturated or unsaturated (Figure 1.4). Fatty acids are constituents of both neutral and polar lipid molecules and categorized based on either the number of double bonds or total number of carbon atoms in the chain. Saturated fatty acids have no double bond, while unsaturated fatty acids consist of at least one double bond.

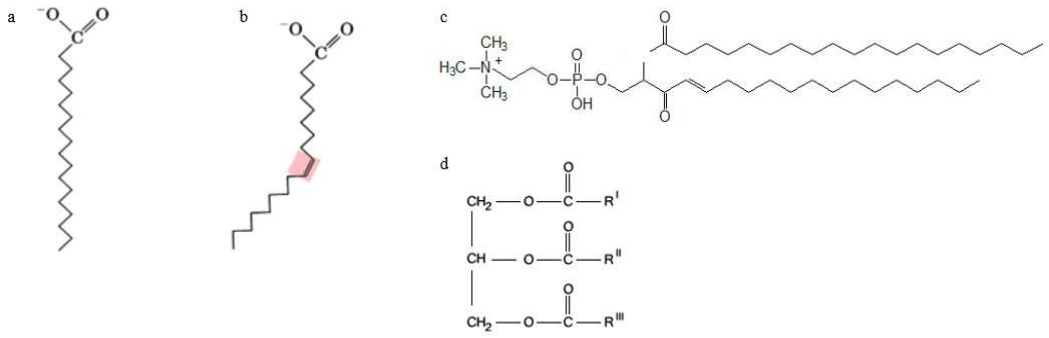


Figure 1.4: Categorization of lipids and fatty acid chains. a: Saturated fatty acid (C18:0), b: Unsaturated fatty acid (C18:1); c: Phospholipid (polar lipid), d: Triacylglycerol (neutral lipid).

Saturation degree of fatty acids (FAs) can be grouped as saturated fats, monounsaturated fats and polyunsaturated fats (Figure 1.5). Saturated FAs is obtained by the saturation of unsaturated fatty acids with H^+ atom (hydrogenation reaction), so contain no double bonds in the carbon chain. Saturated fats are generally solid at room

temperature, have a high melting point and are mainly found in animal products (butter, cheese and meat) and vegetables (coconut and palm oils) sources. Unsaturated FAs are fatty acid has at least one double bond along the fatty acid chain. Monounsaturated fatty acids contain double bond within the hydrocarbon chain and found in olive, avocados, canola oils, nuts, peanut and olives). PUFAs contain two or more double bonds and found in fish, sunflower seed oils and corn.

Microalgal fatty acids range from 12 to 22 carbons in length and the number of double bonds never exceeds six. Unsaturated fatty acids are generally in cis isomers form. The major fatty acids in most microalgae cells are palmitic acid (C16:0), oleic acid (C18:1), and linoleic acid (C18:2) or α-linolenic acid (C18:3). Some microalgal species are able to produce LC-PUFAs such as omega-3, omega-6 fatty acids. This species are important because of their nutritional benefits (Lee, 2012).

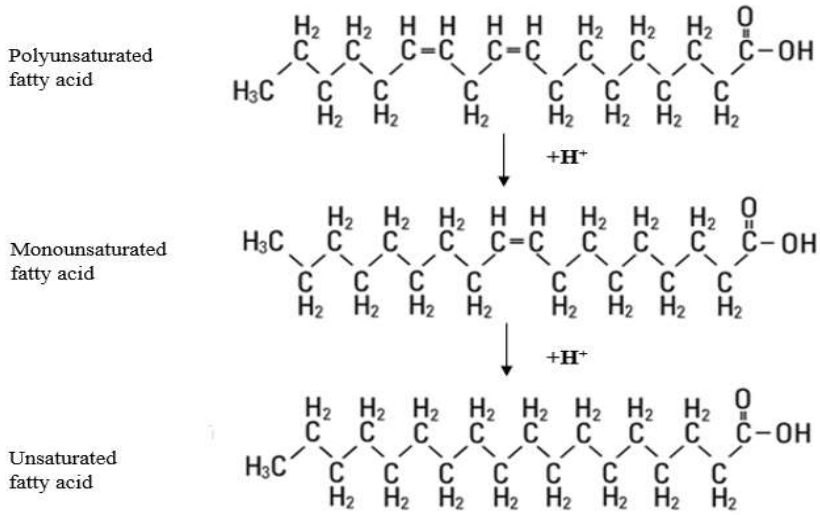


Figure 1.5: Saturated, monounsaturated and polyunsaturated fatty acids (Lehninger, 1982).

A significant amount of research has been done to characterize the lipid contents of different species of algae. Microalgal lipid content varies significantly from one species to another as 5 to 70% dry weight biomass (Table 1.1), (Mata et al, 2010; Brown et al, 1997).

Table 1.1: Lipid content (% dry weight biomass) of different microalgae species

Marine and freshwater microalgae species	Lipid content (% dry weight biomass)
Chlorella sorokiniana	20–22
Chlorella sp.	10–50
Crypthecodinium cohnii	20–50
Dunaliella salina	6–23
Dunaliella primolecta	23
Dunaliella sp.	17.5–67
Euglena gracilis	14.0–20
Haematococcus pluvialis	25.0
Isochrysis galbana	7.0–40
Isochrysis sp.	7.1–33
Monallanthus salina	20.0–22
Nannochloris sp.	20.0–56
Nannochloropsis oculata.	22.7–30
Neochloris oleoabundans	29.0–65
Pavlova salina	30,9
Pavlova lutheri	35.5
Phaeodactylum tricornutum	18.0–57
Scenedesmus obliquus	11.0–55
Skeletonema sp.	14–32
Spirulina platensis	4.0–18
Schizochytrium sp.	30-57
Thalassiosira pseudonana	21

Lv et al. (2010) indicate that lipid composition vary between microalgal species and microalgae are richer in neutral lipids than other species. The composition and fatty acid profile of lipids (saturated and polyunsaturated) affected by the microalgal life cycle as well as the physiochemical conditions, such as temperature, pH, aeration rate, medium composition, stress conditions, illumination intensity (Guzman et al, 2010; Rao et al, 2007). In addition to these parameters, microalgal cells has different polar lipid content in various growth stages. They generally have lower polar lipid contents in stationary phase than logarithmic phase (Dunstan et al, 1993). Lipid metabolism of algae affected strongly by stress or unfavorable condition such as nutrients, temperature, salinity, pH, growth phase, light, etc., and generally these conditions leads to the accumulation of more lipids (Hu et al, 2008).

1.3.4.1 Temperature

Growth rate of microalgae generally positively affected by increased temperatures and decrease with further temperature increases like many microorganisms. The key controlling factor in lipid specifically in PUFA synthesis is the temperature (Zeng et al, 2011). As it is known, long lipid chains with double bonds which is more fluid and have more surface area than saturated lipids organized in the cell membrane to keep membrane fludity and viscosity at low temperatures (Murata and Los 1997).

Bacterial PUFAs production was reached to highest point between 5-25°C whereas the production decreased dramatically at temperatures higher than 25°C (Yang et al, 2007). This factor is very important for open pond culture where the temperature control is hard and limited. In general, low temperatures don't kill the algae however, temperatures above the optimum can kill the algae. Lower temperature was found to be the most significant factor for higher DHA omega-3 fatty acid production (max. 3 g/l) by the microalgae *Schizochytrium limacinum* (Chi et al, 2007). It is explained that *Schizochytrium limacinum* could grow in temperatures between 16 to 37°C but the optimum temperature for DHA production was found to be at 28°C (Luying et al, 2008, Nakahara et al, 1996). EPA production reduced in *Shewanella olleyana* was increased from 10.2% to 23.6% of the total fatty acids (TFA) by decreasing the temperature from 24°C to 4°C while the concentration of monosaturated fatty acids was reduced. (Skerratt et al, 2002). Expression of the desaturase enzyme of the *Mucor circinelloides* was increased by shifting culture temperature from 28°C to 15°C (Michinaka et al, 2003) This led GLA to increase up to 30% of the total fatty acid. Due to the impact of temperature variation on PUFAs synthesis in algae culture, it is important to select an appropriate temperature for the microalgal growth and fatty acid production.

1.3.4.2 pH and salinity

Microalgae are sensitive to pH changes in surrounding environment. Even though the reason of lipid accumulation due to pH stress is not yet known, there are some reports about it. Lipid accumulation can be inhibited by H^+ ion toxicity in microalgae culture. In many production systems, pH is kept between 5-8. Guckert and Cooksey (1990) predicted that alkaline pH induce lipid accumulation in *Chlorella* by reducing cell release from non-flagellated spore. Wu et al. (2005) indicated that *Schizochytrium* sp. S31 could not intake the carbon from the medium at initial pH 8.0 therefore grow poor.

Yazawa et al. (1988) screened marine microorganisms for EPA production and found 1.7% of them has highest yield of EPA at pH7, 20-25°C while no productivity was observed at pH 5 and pH 10 at the same temperature.

Salt stress significantly affects the cellular growth and lipid accumulation in microalgae cells. It is known that the increase in salinity negatively affect the cellular growth but can increase the lipid content of microalgae. Therefore, the optimum salinity conditions on the growth and lipid content of microalgal species must be investigated. Microalgae can adapt to salinity and other stress conditions. The ability of cells to survive in saline environment under osmotic stress is a challenging factor. Microalgae produce polar lipids under favorable growth conditions, which enrich mostly cellular membranes. However, polar lipid accumulation switch to neutral lipids under unfavorable growth conditions which is generally located in the cytoplasm (Asulabh, 2012). The cells can adapt themselves to stress conditions by changing biochemical and physiological processes as well as in morphological structure. The increase in salinity affects the cell membrane permeability, photosynthetic activity mineral distribution, ion toxicity, rate of respiration in the cell probably due to change in membrane electron transport system (Sudhir, 2004; Zhang et al, 2010). One research showed that, high NaCl concentration cause lower in saturated fatty acid but higher in unsaturated fatty acid in microalgae (Kirrolia et al, 2011).

1.3.4.3 Aeration rate

Aeration of the culture especially large volumes plays an important role to ensure all cells in the culture receive an equal amount of nutrients and oxygen. Some researchers showed that stirrer speed was critical factor compared to temperature for *Mucor recurvus* to produce both EPA and DHA. Highest productivity obtained at 160 rpm and the biomass productivity dramatically decrease when the shaker speed above 180 rpm (Li et al, 2008). One research showed that optimizing the culture conditions specially aeration rate of the fungus *Thraustochytrium aureum* doubled the DHA production (460 mg/l) compared to previous reports (Iida et al, 1996). The main reason in previous report was found that cells showed a tendency to coagulate due to the mechanical stirrer rate in the reactor. Some microalgal species have been known to doubled their lipid contents during oxygen deprivation (Dunstan et al, 1993).

1.3.4.4 Nutrient limited conditions

Like many organisms, algae require various organic and inorganic nutrients to achieve a healthy culture with high biomass and lipid. These basic nutrients are macronutrients (carbon, nitrogen and phosphorus), vitamins and trace elements (chelated salts of iron, manganese, selenium zinc, cobalt, and nickel). In most cultures, however nutrients are often added in excess in order to minimize nutrient limitation.

Under nutrient starvation conditions, microalgal cells generally change their lipid biosynthetic pathways to accumulate neutral lipids, especially in TAG form (Minhas et al, 2016). It offers microalgae to tolerate unfavorable environmental conditions by storage. Some researchers showed that maximum amount of lipid content was obtained with temperature and nitrogen-limited culture conditions (Rios et al, 2015; Jiang and Chen 2000).

1.3.4.5 Alternative raw materials

Different carbon (glucose, glycerol, glucose syrup, molasses), nitrogen (ammonium sulphate, ammonium carbonate, diammonium tartrate, monosodium glutamate), salt (sodium chloride, sodium sulphate, mineral salt) sources have been used in the media and showed marked influences on PUFA production. Omega-3 productivity can be increased and production costs can be decreased by using alternative carbon and nitrogen sources, like molasses and ammonium hydroxide. Molasses, which is one of the cheapest carbon sources used for the production of PUFAs from *Mucor recurvus* (Li et al, 2008) for industrial scale. The authors obtained to 5.74 g/l PUFA by using sugarcane molasses as a carbon source. Optimum PUFA production conditions were found to be as 15% sugarcane molasses with using urea in a C/N: 35 ratio, pH 6.0, 28°C, for 5 days at 160 rpm resulting 0.4 g/l EPA and 0.3 g/l DHA production. Yazawa, (1996) obtained 200 mg of EPA/l by using marine industrial waste and corn steep liquor as an alternative carbon source for *Shewanella putrefaciens* to reduce the overall large scale production costs. Gema et al. 2002 increase the amount of GLA production from *Cunninghamella echinulata* up to 80mg/g biomass by cultivating on agro-industrial by-product as a carbon source. Similar research about GLA production from *Cunninghamella echinulata* CCRC 31840 was conducted by Chen and Chang, 1996 and obtained 0.9 g/l GLA by cultivating on 10% soluble starch, 0.11% NH_4NO_3

as an alternative sources but higher growth and productivity was achieved when , urea and ammonium nitrate were used in the same study.

1.3.4.6 Sunlight or artificial light

Sunlight or artificial light is the main energy source and crucial for phototrophic algal cells. Photosyhthesis efficiency depends on light intensity. The photosynthetic activity increases periodically with light intensity until reaching a threshold point (light saturation point). After this point, the photosynthetic activity start to decrease because of higher intensity can damage light receptors in the chloroplasts of the cells. Liu J. et al, 2012 found that highest biomass yield and lipid content were achieved when algae at 400 µmol photons m^{-2} s^{-1} than 250 µmol photons m^{-2} s^{-1}. Mutual shading is a general problem for photoototrophic cultivation because the cells closest to the surface receive more light and the cells below receive very low light (Totaro, 1989). Researches have been conducted to solve this problem by optimizing light distribution evenly, mixing and liquid depth.

1.3.4.7 Genetic Manipulation

In addition to optimizing the chemical and physical growth conditions, genetic manipulation of the PUFA producers could lead to significant increase in the production. Amiri-Jami et al. (2006) obtained *Shewanella baltica* mutants by inserting the Tn5 transposon. These mutants finally produced 3-5 times more EPA than the wild type. Orikasa et al. 2007 co-express the vector *vktA (catalase gene)* in a recombinant *E. coli* DH5 to enhance the productivity of EPA resulting increased EPA production for 4 times. Higher plants are normally unable to synthesize PUFAs with more than 18 carbons, due to the lack of the enzymes required for such process (Wallis et al, 2002). Transgenic plants, in which PUFA production genes were inserted into genome, were able to produce LC-PUFAs. Another important study succeed to increase both EPA and DHA by 33% in *Pavlova lutheri* by UV mutagenesis (Meireles et al, 2003). EPA content of the *Phaeodactylum tricornutum* was increased by 37% with same technique (Alonso et al, 1996). Chaturvedi and Fujita (2006) used EMS to increase the yield of eicosapentaenoic acid (EPA) in *Nannochloropsis*. They reported that mutants were more thermotolerance than wt strain.

1.4 Long Chain Polyunsaturated Fatty Acids (PUFAs)

Polyunsaturated fatty acids (PUFAs) are long chain fatty acids containing multiple carbon-carbon double bonds. PUFAs can be classified into three main groups (Omega-3, 6 and 9) depending on the position of the first double bond from the methyl end (Figure 1.6). Omega-3 has a double bond (C=C) at the third carbon atom from the methyl or omega end (ω), Omega-6 has the first double bond at the sixth carbon atom from the methyl terminus and Omega-9 which have carbon–carbon double bond in the ninth bond when counting from the methyl end of the fatty acid (Chow, C. K. 2007). Examples of chemical structures of typical PUFAs are shown in Figure 1.6.

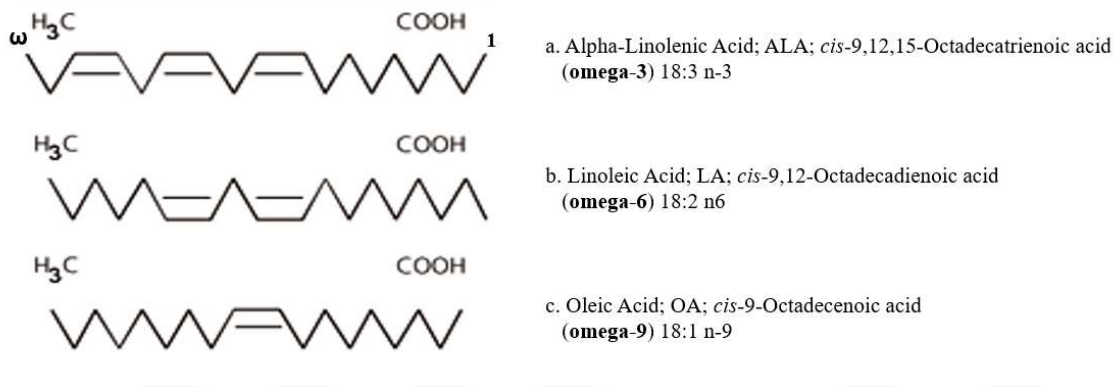


Figure 1.6: Classification of PUFAs, (a) PUFA from ω-3 family, (b) PUFA from ω-6 family, (c) PUFA from ω -9 family.

There are three types of omega-3 fatty acids involved in human diet: α-linolenic acid (ALA), eicosapentaenoic acid (EPA), and docosahexaenoic acid (DHA). While ALA is mostly found in plant oils, EPA and DHA commonly found in marine oils (Swanson et al, 2012; Ruxton et al,2004). Marine algae and microalgae are primary sources of omega-3 fatty acids. Omega-3 fatty acids especially EPA and DHA are known as components of phospholipids and glycolipids therefore they are important structural and functional components of cell and plasma membranes. They have important functions in the cell such as acting as precursors for many hormones and enzymes, storing energy, being structural component of cell membrane (Hussein, J. S., 2013). They are generally used in pharmaceutical, nutraceuticals and food supplements. Eicosapentaenoic acid (EPA) that has chemical structure of C20:5n-3 is twenty carbon length omega-3 fatty acid, which has five cis double bonds (cis-5,8,11,14,17) in their hydrocarbon chain. The first double bond is located at the third carbon when counting from the methyl end. EPA is essential fatty acids and can be obtained mostly from the

fish and algae (Gupta et al, 2012). Most important health benefits of eicosapentaenoic acid (EPA) among all are protection function against atherosclerosis, lowering of plasma cholesterol and decreasing the incidence of breast, colon and pancreatic cancers (Lopez-Huertas, 2010; Swanson et al, 2012). DHA that has chemical structure of C22:6n-3 is a type of polyunsaturated fatty acids (omega 3 fatty acids) with twenty two hydrocarbon chain length which has six cis double bond (cis-4,7,10,13,16,19) among the chain, the first double bond is located at the third carbon from the omega end (Gupta et al, 2012). (Figure1.7). DHA is the longest and the most unsaturated fatty acid among all of the PUFAs and has very important functions in the cell specially phospholipid cell membrane. Approximately, 60% of the TFA in the cellular membrane of the retina consist of DHA. Because of it is only synthesized by plant and algae, it is an essential fatty acid for human. DHA has an enormous benefit to the human body, especially in the human brain and retina therefore has a critical role in proper visual and neurological development in infants (Uauy et al, 2001). It also plays an important role in protection against several diseases such as cancer, cardiovascular and neurological diseases. It has been reported that omega-3 fatty acids especially DHA has promising antiaging, anticancer, antihypertensive, antidepression and anti-cataract effects (Siriwardhana et al, 2012). It is widely known that, fish and fish oils are the main source of DHA but contrary to popular belief, one rich source of DHA is microalgae. Animals such as fish do not have enzyme to synthesize DHA, they obtain it by consuming photosynthetic and heterotrophic microalgae. Currently, DHA is commercially manufactured from *Crypthecodinium cohnii* and *Schizochytrium* sp. microalgae species (Spolaore et al, 2006).

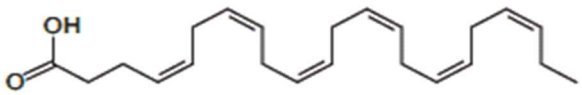


Figure 1.7: Structure of Docosahexaenoic acid, 22:6n-3 (all-cis-docosa-4,7,10,13,16,19-hexaenoic acid) (Von Schacky, 2007).

In addition to EPA and DHA, omega-6 fatty acids such as γ-linolenic acid (GLA C18:3, n-6) was reported to have an anti-tumor function specially against malignant glioma cells and breast cancer and helps to reduce eczema and acne (Fujiwara et al, 1986). Arachidonic acid (ARA 20:4, n-6) is a precursor of the biologically active prostacyclins, thromboxanes, prostaglandins, and leukotrienes, therefore Food and Agriculture Organization/World Health Organization (FAO/WHO) recommends the

addition of arachidonic acid to infant milk (World Health Organization and UNICEF, 2003). Marine algae, microalgae, fungi and bacteria are main sources of PUFAs (Dominguez et al, 2012). Bacterial EPA and DHA are produced only within the cellular membrane phospholipids not in cytoplasm. Microalgal omega-3 fatty acids are accumulated in both cellular/chloroplast membrane and cytoplasm. Large-scale production of PUFA producing microorganism outcomes good and renewable source of PUFAs. Conversion of low value-added compounds to omega-3 fatty acids by microalgae has been studied. For example, *Schizochytrium* strain has been used to convert agro-industrial raw materials into valuable lipids such as DHA (Song et al, 2015).

1.4.1 Biosynthesis of PUFAs

1.4.1.1 Conventional biosynthetic pathway

Biosynthesis of PUFAs depends on the genetic sequence responsible for their production, enzymes and proteins in the organism. Biosynthetic pathway of the fatty acid in all organisms terminates once the C16 or C18 saturated fatty acids forms, then fatty acids are modified into PUFAs via desaturase and elongase enzymes (Figure 1.8).

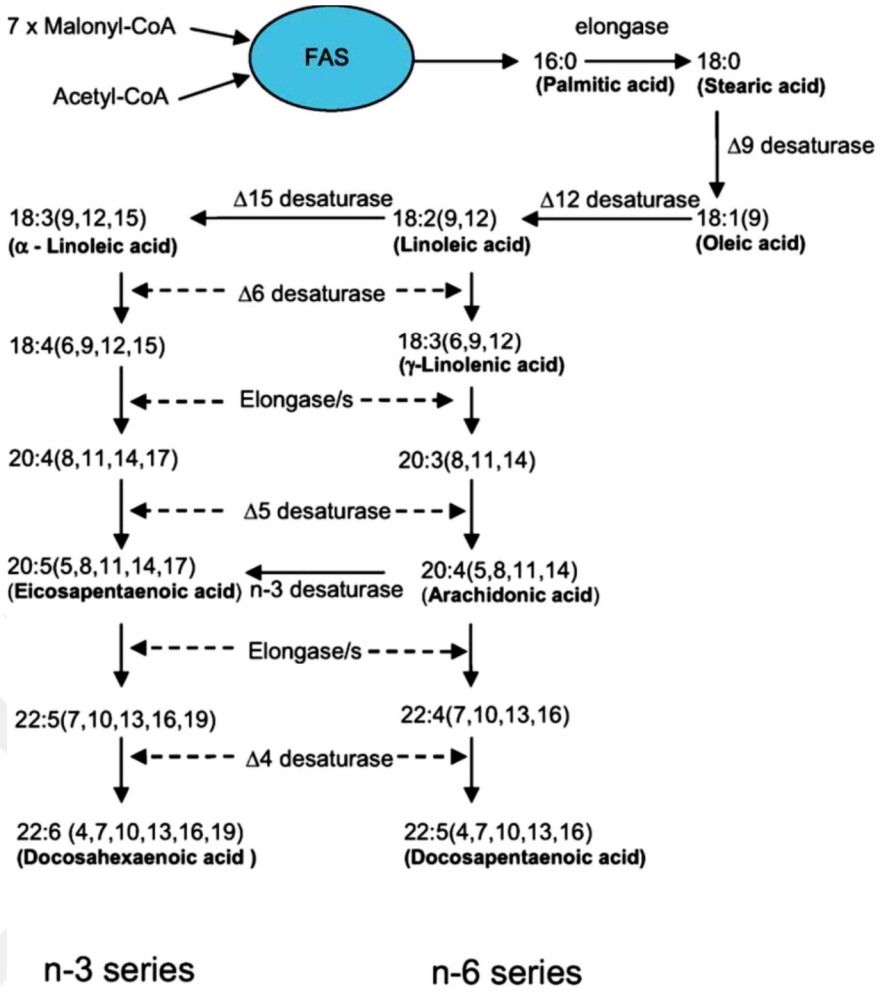


Figure 1.8: Conventional biosynthetic pathway of the formation of PUFAs (Ratledge, 2004).

Palmitic acid (C16) are synthesized from acetyl-CoA and malonyl-CoA precursors, using the FAS enzyme complex and then stearic acid (C18) is then sequentially desaturated and elongated through a succession of reactions leading to the formation of different PUFAs.

1.4.1.2 Novel polyketide synthase biosynthetic pathway

A novel polyketide synthase (PKS) biosynthetic pathway for PUFA production using multi-enzyme complexes was suggested by Metz et al. (2001). The eight PKS domains were identified which are 3-ketoacyl synthase, malonyl-CoA:ACP acyl transferase, acyl carrier protein, 3-ketoacyl-ACP reductase, acyltransferase, chain length factor, enoyl reductase, and dehydrase. This pathway involves condensation and isomerization for introducing double bonds and chain elongation while does not involve desaturation. It does not require molecular oxygen when introducing double bond to chain. In PKS system acyl-CoA and malonyl-CoA are still required as precursors but reduction of intermediates doesn't involved. This pathway involves polyketide synthase instead of

fatty acid synthase for the repeated cycle. Allen and Bartlett (2002) indicate that PKS pathway of PUFA-producing marine bacteria genes could be common in with *Shewanella* EPA gene cluster because of the gene homology. It has been demonstrated that *Schizochytrium* sp. ATCC 20888 uses PKS pathway for DHA biosynthesis.

The PKS pathway has been detected in some other microorganisms for production of PUFAs. These organisms such as *Schizochytrium* sp. contain a polyketide synthase (PKS) system for biosynthesis of DHA (Ratledge, 2004). PUFA biosynthesis via the PKS pathway in *Schizochytrium* is summarised in Figure 1.9

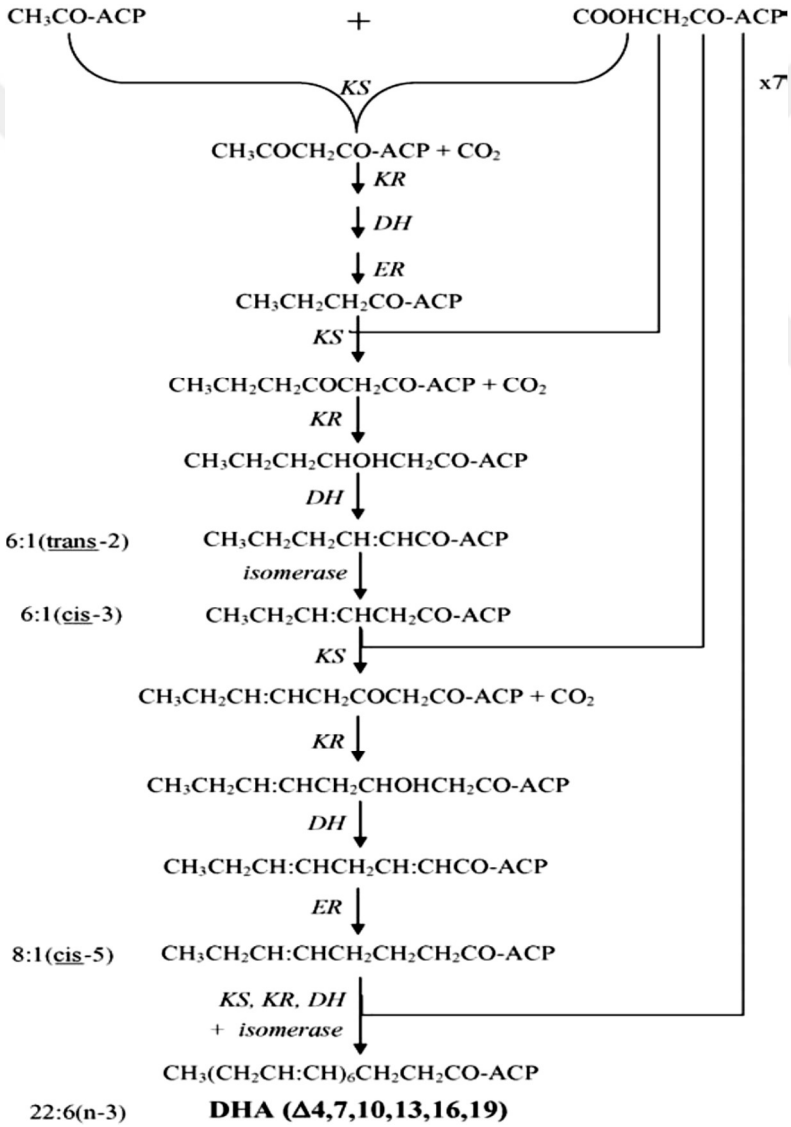


Figure 1.9: PUFA biosynthesis via the Polyketide Synthase (PKS) pathway in *Schizochytrium* (Ratledge, 2004).

While conventional biosynthetic pathway occurs in fungi and nematodes, PKS systems take place in some strains of microalgae and bacteria. (Sato et al, 2008).

1.4.2 Current sources of PUFAs

PUFAs are natural product due to the difficulty of producing double bond synthetically. PUFAs are also essential because animal cells do not have the enzyme for synthesis, only plant cells can produce PUFA. Flaxseed, linseed and its oil, walnuts, canola oil, quinoa are most known botanical source of the omega-3 fatty acid (Dubois et al, 2007). Krill oil, squid oil (calamari oil), fish (mostly salmon), sardines, tuna, herring and mackerel are basic marine animal sources for the omega-3 PUFAs including EPA and DHA (Rasmussen and Morrissey, 2007).

However, fish originated PUFAs has unfavorable charactestics such as unpleasant odour and taste, heavy metal content, toxic chemicals due to sea pollution, resource sustainability of fish supply, overfishing, expensive and unnatural purification steps for PUFAs extraction from the complex mixture of fatty acids in the fish oil (Barclay et al, 1994; Tocher, 2009; Ratledge, 2004).

The most important concern regarding the use of fish oil is heavy metal, dioxins and phenolic compounds, which are accumulated in fish because of the pollution of marine ecosystems causing a high hazard to human health (Domingo et al, 2007). In addition, species of fish, season changes and the refining conditions can negatively affect the quality of PUFAs (Shene et al, 2010).

Because of all these negative factors, scientists have tried to find alternative ways to obtain high quality PUFAs. Microbial sources PUFAs were found to be most reliable and remarkable potential sources (Ratledge, 2004). Advantages of microbial PUFAs compared to the fish oil as,

- There is no toxic chemical, heavy metal risks in microbial based PUFA products
- Microorganisms can be cultivated under controlled conditions to enhance PUFA production
- There is not a bad odour and taste in microbial based PUFA products
- Microorganisms can be grown on a wide variety of substrates including wastes and by-products.

1.5 Thraustochytrids

1.5.1 Taxonomy of thraustochytrids

Schizochytrium sp. is a heterotrophic marine thraustochytrid that contains oil rich in polyunsaturated fatty acids especially DHA (Yokochi et al, 1998). *Schizochytrium* sp. is a spherical microalgae with the size between 20-50 µm display repeated binary division to form diads, tetrads and clusters by forming biflagetta zoospore (Kamlangdee & Fan, 2003) (Figure 1.10). The taxonomy detail of *Schizochytrium* sp. is as follows (Leipe et al, 1994):

Kingdom : Chromista (Stramenopilia)
Phylum : Heterokonta
Class : Thraustochytridae
Order : Thraustochytriales
Family : Thraustochytriaceae
Genus : Schizochytrium
Species : *Schizochytrium* sp.

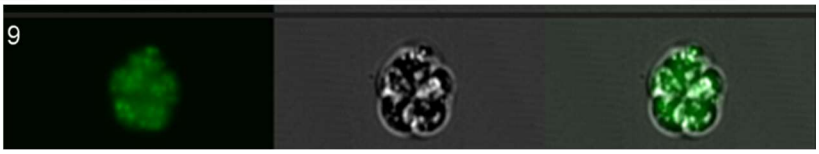


Figure 1.10: Morfology of *Schizochytrium* sp. cell.

They contain various bioactive compounds specially DHA that can be used as pharmaceutical raw material, food additive, aquaculture, animal feed, cosmetic and play a crucial role in biodisel production (Spolaore et al, 2006). *Schizochytrium* sp. which is grown rapidly and produced high amount of DHA as well as independent on light as energy source and environmental factors can be used as an alternative to replace of fish oils (Ren et al, 2010). Martek which is most known American food supplement industry, produced omega-3 oil named AlgalOil (37% of DHA and 16% of EPA) from a different strain of *Schizochytrium* sp. Lonza which is another known food supplement industry has several food ingredient and dietary supplement products from *Schizochytrium* sp. A number of safety assesment studies confirmed the safety of *Schizochytrium* sp. based DHA algal oil and it is generally recognize as safe

(GRAS) according to Food and Drug Administration and can be used as dietary supplements (FDA, 1997).

1.5.2 Production of *Schizochytrium*

Cultivation of *Schizochytrium* sp. on lab scale is found to be easier than other strains (Wu et al, 2005). Many factors may effect the production of *Schizochytrium* sp. such as medium composition, pH, salinity and temperature. Different types of carbon and nitrogen can ben used for production of high value DHA from *Schizochytrium* sp. While glucose, fructose, sugaecane, molasses and glycerol could be used as carbon sources, yeast extract, peptone, tryptone, ammonium sulphate, urea, monosodium glutamate, sodium nitrate, ammonium chloride could be used as nitrogen sources to grow *Schizochytrium* sp. (Wu et al, 2005). In addition to media components, initial pH, salinity, temperature and aeration also affects on *Schizochytrium* sp. growth. The acidity of the medium affects cellular growth, cell membrane functions, the uptake of nutrients, secondary metabolit production as well as the product synthesis (Kim et al, 2005). *Schizochytrium* sp. is generally lives in mangrove environment, therefore salinity of the medium is a critical factor. Salinity of the surrounding environment affects the cytoplasmic ion gradient and the osmatic pressure of the cell (Ho and Chou, 2001). *Schizochytrium* sp. could grow wide range of salinity from 1 ppt to 35ppt (Kamlangdee and Fan, 2003). However sodium, calcium, potassium and magnesium are essential for the growth of microalgae (Bahnweg, 1979). Temperature is another important factor for the biomass and lipid production. It has been demonstrated that *Schizochytrium limacinum* can grow wide range of temperatures between 15°C -37°C. Optimum temperature for DHA production was found as 23°C (Luying et al, 2008).

1.6 Factorial Designs

Since polyunsaturated fatty acids have gained significance due to their role in human health, industrial and commercial usage, research on enhancement of biomass and lipid production has increased. Growth rate and lipid production are affected by both environmental conditions and media composition (Sajbidor 1997). Therefore, optimization of medium composition and environmental conditions to improve biomass and total lipid content of the organism is important. Optimization of the process parameters and culture conditions with *Schizochytrium* sp. has been studied

before with different medium components (Song et al, 2007; Luthra et al, 2014a, b; Wu and Lin, 2003). However, it is necessary to study the alternative raw materials and lipid extraction methods influencing the production and quality of the lipid. Applying a statistically optimum strategy was an effective tool for optimization of the production and extraction process which would also reduce the production costs through maximizing the yield. Factorial experiments consist of two or more factors on the response variable and able to measure effect of all factors and interactions between them. Full Factorial Design (FFD) study all of the possible treatment combinations and their levels associated with the factors on the response variable. Most of FFD test the two levels factor (min/max) and are called two level Full Factorial Designs. Mathematical calculation of the number of runs required to test is $2n$, where n is the number variables. Full factorial design gives reliable data with the highest level of confidence at the en of the experiment (Montgomery, 2000). Full Factorial Design is suitable for small number of variables rather than large number of variables (Lazic, 2004). The most important feature of the Full Factorial Design is to reduce the number of trials (Lazic, 2004). A Fractional Factorial Design is derived from the carefully chosen subsets of the full factorial matrices with reduced number of runs. Factor levels are commonly coded as +1 (higher level), 0 (intermediate value) and −1 (lower level). There is an another commonly used design with a lower number of trials can be applied for screening method. This screening factorial design only tests the linear effect of each variable not the interaction between them. The most known factorial design is the Plackett-Burman (PB) design (Lazic, 2004; Montgomery, 2004). Statistical methodologies such as PB and CCD have been extensively used to optimize several factors and their interactions using a reduced number of experiments. PB design was used recently as the first step in optimizing different bioprocesses to identify the factors with a significant effect on desired responses. Following the selection of the most significant factors, response surface methodology (RSM) with CCD is used to determine the optimum values of these factors (Park et al, 2005; Wen and Chen 2001).

1.6.1 Plackett burman design

PB is a two level fractional factorial design that is often used for screening the factors with important effects on the process. (Plackett and Burman, 1946). The number of trials in a PB design must be the multiple of four and the PB design is calculated with $n+1$ formula where the n is the number of variables.

A Plackett Burman design was generally used as a first step optimization of the process to reduce number of experiments. It is also used for screening significant (confidence level >90%) factors from a list of variables including medium components, environmental factors and interactions between them (Chauhan et al, 2007). Haider and Pakshirajan (2007) found that MgSO4 and FeSO4 were the most important factors on lipase activity when screening the all variables with PB. PB was also used for determing the important factors affecting PUFA prorodyction in fungus *Mortierella alpine.* Seven significant factors which are incubation time, temperature, substrate particle size, yeast extract, glucose, glutamate supply and moisture content were screened for ARA production (Ghobadi et al, 2011). Another study were performed with nine variables on PUFA production in *Physcomitrella.* Sucrose, pH, temperature, calcium chloride and magnesium sulphate were found to be the important factors effecting PUFA production in both way (Chodok et al, 2010). Chi et al, (2007) used the glycerol as an alternative raw material for microalga *Schizochytrium limacinum* and obtained maximum DHA yield with using PB to optimize the growth conditions.

1.6.2 Central composite design (CCD)

A Central Composite Design is generally used for creating a second order (quadratic) model for the response variable and contains fractional factorial design with star points that allow the detection of curvature. Figure 1.11 illustrates the distribution of the nine star points used to create the CCD matrix for two factors. This design has been generally used with PB to optimize bioprocesses.

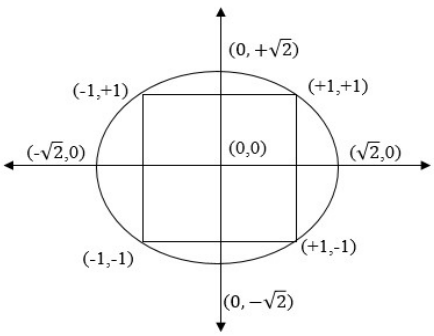


Figure 1.11: Central Composite Design matrix for two factors (Lee et al, 2007).

Once the most significant factors were chosen by PB, determination of optimum values of these factors and testing the interactions between them is required for a full

optimization. CCD and RSM are powerful tools for determining the main effects, optimum values of the significant factors and defining interactions between variables on the process. RSM is collection of mathematical and statistical techniques for exploring the relationships between input variables and response (output) variables (Khodaiyan et al, 2007). RSM has been widely used for optimization of different manufacture processes (Park et al, 2005). CCD was use to formulate quadratic polynomial models by computer simulation to optimize the variables. Maximum yield of extracellular nuclease from *Bacillus cereus* were achieved by using CCD model (Zhou et al, 2010). Most important applications of CCD is the optimization of the scaling up the cultivation process and media optimization to maximize favorable products. Optimization of nutrients concentrations and cultivation conditions of *Schizochytrium* sp. was performed to enhance DHA yield. Glucose, yeast extract, sodium chloride, pH and temperature were found to be most important factors effecting DHA production. 516 mg/L DHA were obtained at optimum conditions (Wu and Lin, 2003). Another study for optimization of culture conditions to maximize growth and the production of DHA by *Schizochytrium limacinum* BR2.1.2 were performed by Kawsakul et al. (2012). Three factors, which are temperature, glycerol concentration and peptone concentration were found to influence biomass and DHA production by the PB. Low temperature, high glycerol and high peptone concentrations positively influence DHA production by CCD. The yield of EPA production by the diatom *Nitzschia laevis* was increased after applying CCD. pH, temperature, sodium chloride and calcium chloride were optimized to enhance the yield of EPA (Wen and Chen, 2001).

1.7 Downstream Process

Once large scale growth process is completed, microalgal culture is harvested by centrifugation. After harvesting, microalgal culture concentrated in a dewatering step. Pretreatment step is then applied to the cell pellet to prepare it for lipid extraction. Lipid extraction is performed by applying extraction solvent to dried cell biomass. After several hours of incubation, the lipids will be separated from the cellular debris. Lipid-solvent-water mixture is then separated by evaporation to obtain pure lipid and finally converted to fatty acid methyl esters (FAMEs) in the transesterification step for

determining fatty acid profile with gas chromatography (GC). Figure 1.12 shows the downstream processing steps required to obtain lipid from microalgal biomass.

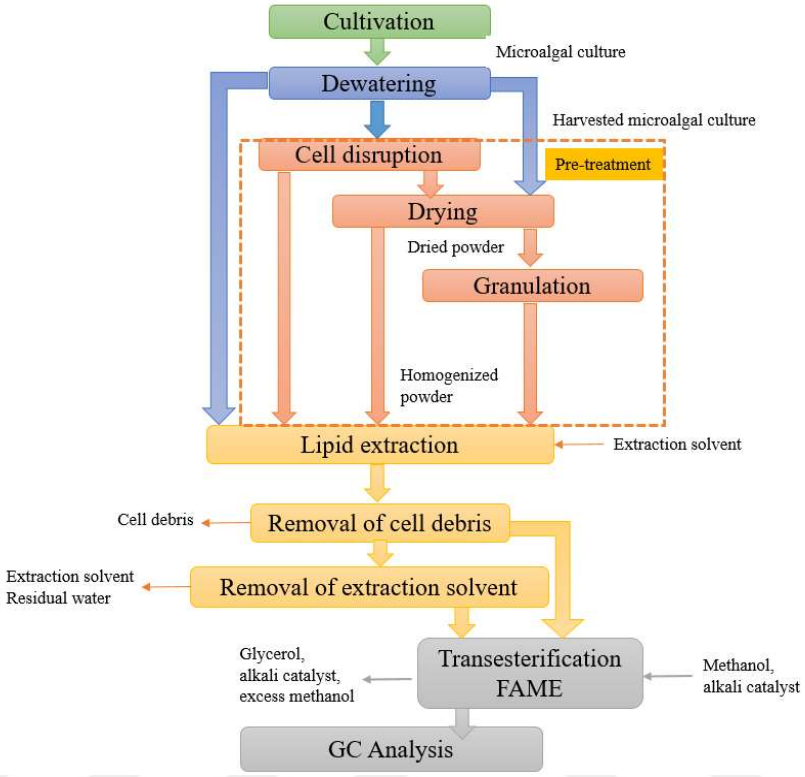


Figure 1.12: Downstream processing steps.

1.7.1 Harvesting microalgal culture

Harvesting microalgal culture includes centrifugation, filtration, and flocculation methods to concentrate microalgal culture. During flocculation, microalgal cells tend to accumulate to form aggregates. Negatively or positively charged polymers and are generally cause the flocculation microalgal cells. *Schizochytrium* sp. S31 is an example of the microalgae cell that aggregates each another. Among the harvesting technologies, flocculation requires low energy, because of that it could be the most advantageous technique (Uduman , 2010; Wijffels and Barbosa, 2010). Concentrated microalgal culture is generally referred as a cell pellet. Aqueous suspension still exists after harvesting the microalgal culture, therefore it needs to be concentrated in order to obtain high quality of lipid and reduce the cost of downstream processing (Danquah et al, 2009; Grima et al, 2003). In this stage, cell pellet is applied to heat treatment (generally 65°C for 2 hours) to inhibit cellular lipase activity of the cell. Antioxidant

such as tocopherol can be added in 1:1000 ratio to the cell pellet for prezervation against lipid oxidation.

1.7.2 Drying cell biomass

This step is known as post-dewatering step in which the cell pellet is completely dewatered. The cell pellet or paste expose drying process for enhancing the efficiency of subsequent lipid extraction and lowering the cost of downstrem process (Lee , 1998, 2010). De-watering process can be choosen according to desired biomass alterations. Lyophilization (freeze-drying), spray drying and drum drying are most known and sutable techiques for drying microalgae pellet.

Lyophilizer consist of a vacuum chamber, sample table and freezer units to freeze the sample by evaporative cooling and freezing (Abdelwahed et al, 2006). Freeze-drying works by freezing the material and then lowering the pressure to evaporate allow the frozen water. Approximately 200g/L dired biomass can be obtain by freeze-drying the cell pellet at -54°C under vacuum for 70h.

Spray dryer has a which has peristaltic pump that delivers the liquid cell culture from a container through a small diameter jet into the main chamber. While liquid cell culture is delivered into main chamber, heated air is blown through the chamber to evaporate water. The dried biomass are then separated from the exhaust air flow and collected in collection bottle. Generally spray dryer has 1000-1500ml/h evaporation rate (Berk, 2008).

Drum drying minimize the moisture content of the material by contacting it with a heated gas. The rotary drum dryer is made up of a rotating cylindrical tube where the liquid sample is heated and dried. The feeding end of the dryer is higher than the discharge end in order to transport the liquid materials through the system (Berk, 2008). When the liquid material is entered into system, rotary drum dryer starts to rotate causing samples to contact with fins lining the inner wall of the dryer. Dried cell biomass falls back down to the bottom of the dryer. While drum dryer and spray dryer need large amount of sample for drying, small amount of sample can be dried by freeze dryer (Desobry et al, 1997). Sometimes, no pre-treatment is performed and the concentrate is directly processed for lipid extraction.

1.7.3 Cell disruption

Cell disruption is a key step in influencing lipid extraction yields (McMillan et al, 2013; Araujo et al, 2013; Zheng et al, 2011). Therefore, applying an appropriate cell disruption method is essential for lipid extraction. The most efficient cell lysis method has not been determined yet for microalgae due to cell wall variations among the species (Lee et al, 2010). Sonication (ultrasound), high-pressure homogenizers, grinding, enzymatic reactions (protease), chemical hydrolysis (NaCl, CTAB) are most known cell disruption methods applied for microalgae. The cell wall properties of the microalgae play a critical role in the extraction of lipid, as it mentioned above (Brennan and Owende, 2010; Mata et al, 2010). The ideal cell disruption method should lyse the cell wall to maximize the lipid yield and can be used in large scale. It should also not cause any contamination to product and hinder further steps of the process (Goettel et al, 2013). Figure 1.13 shows the different cell disruption methods used for cell lysis.

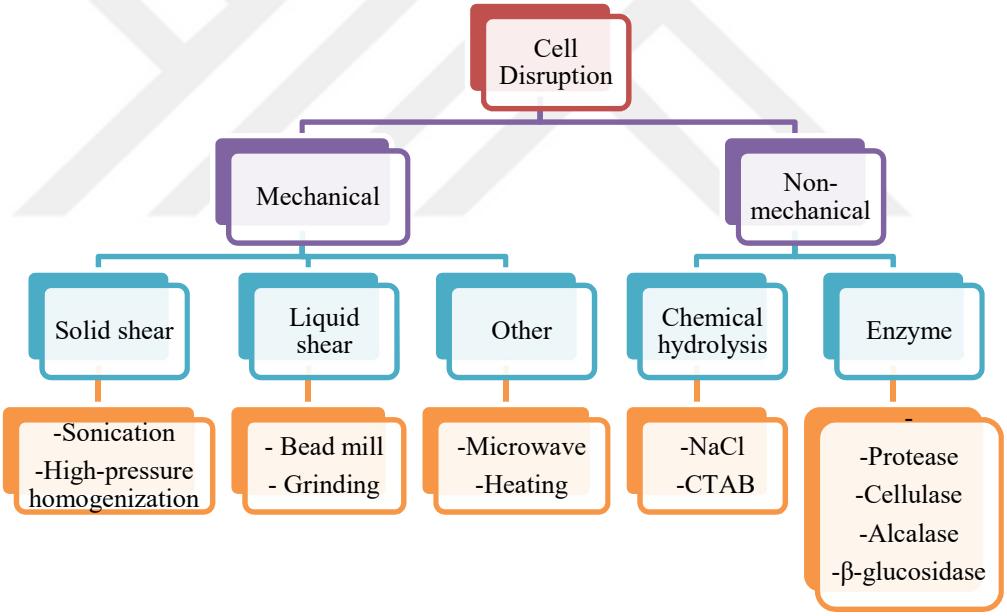


Figure 1.13: Different cell distribution methods.

Sonication is a method that uses ultrasound to agitate cells in a sample by using ultrasonic probe. Araujo et al. (2013) showed that ultrasounic distribution of *C. vulgaris* resulted in the highest lipid extraction, suggesting a favorable potential for biodiesel production. Another research with C. *Minutissima, Thalassiosira fluviatilis* and *Thalassiosira pseudonana* indicated that sonication-assisted method with n-hexane was efficient method for lipid extraction in these strains (Neto , 2013).

High-pressure homogenization pre-treatment such as french press homogenization forces the cells to pass through a narrow valve to distrupt the cell wall and release of intracellular lipids by introducing pressure to cell culture.

Grinding of the frozen sample is a mechanical distruption method that generally used for plant cell. Microalgae sample can be frozen by incubating it with liquid nitrogen or dry ice. Zheng et al. (2011) has tried several cell disruption methods such as grinding in liquid nitrogen, ultrasonication, bead milling, enzymatic lysis and microwaves for *C. vulgaris* and found that grinding in liquid nitrogen was the most effective method in terms of disruption efficiency and time.

Enzymatic distribution is more moderate method than mechanical distribution. Protease, cellulase, β-glucosidase, alcalase and combined mixture of enzyme are generally used for microalgal cell lysis for lipid extraction purposes. Wang et al, 2015 analyzed different cell lysis techniques including combined enzyme method. The combined process was found to be more efficient (92.6% lipid recovery) and economical compared with the individual process. Fu et al. (2010) used immobilized cellulase to degrade microalgae cell walls. Extraction of lipids from microalgae increased from 32% to 56% after enzyme treatment.

Chemical pre-treatment is another distribution method that generally use detergents or chemical agents to lyse the cell wall. Byreddy et al. (2015) compared the efficiency of several cell distribution methods including osmotic shock with NaCl. The research results indicated that the most efficient method was osmotic shock with NaCl (10%) leading lipid recovery 48.7% from *Schizochytrium* sp. S31 and 29.1% from *Thraustochytrium* sp.

Other pre-treatment methods such as microwave and/or heat have been employed on microalgae in order to facilitate lipid extraction. Lee et al. (2010) compared the different cell lysis method including mechanical and chemical distributions on *Botryococcus* sp., *Chlorella vulgaris*, and *Scenedesmus* sp. microalgae. The research results indicated that even though the efficiency of lipid extraction differ according to species, the microwave method was found to be the simplest and most effective method among the tested methods for these microalgae. (Lee et al, 2010). Kita et al. (2010) applied thermal pre-treatment ranging from 75-120°C in a reactor for 10 minutes. After subjecting the microalgae cell culture to heat treatment hexane

extraction was used. The results showed that the recovery of hexane-soluble materials improved around 90% when heat pre-treatment was applied at very low cell concentrations.

1.7.4 Lipid extraction

Different types of solvents and extraction methods have been used in the literature to recover microalgal lipids (Ryckebosch et al, 2012). The pretreated microalgal biomass could be in the form of disrupted concentrate or dried powder. While disrupted concentrate such as sonicated hexane-microalge mixture still contains a certain level of residual water, dried powder will be completely devoid of residual water.

Either organic solvent (hexane) or supercritical fluid is used to extract microalgal lipid. Extraction solvent extracts the cellular and membrane lipids out of the matrices during lipid extraction. Extraction temperature and time are the critical factors for lipid extraction. Either soxhlet or incubation method is used when organic solvent is applied as extraction solvent. Soxhlet lipid extraction was originally designed for continuous extraction of analytes from a solid into an organic solvent. As the flask containing the solvent is heated, vapors rise in the larger outside tube, through the thimble containing the sample, enter the water-cooled condenser, and liquefy. When the liquid level in the extractor reaches the top of the siphon arm, the extract-enriched solvent returns to the flask (De Castro , 1998). Incubation method is easier way to extract lipid from microalge.The dryed biomass-oganic solvent mixture containing erlen-mayer is incubated at room temprature in shaker for 4-6 hours. This method is also called as cold extraction because of the extraction is performed at room temperature.

1.7.4.1 Organic solvent extraction

Neutral lipids are generally known as storage lipids such as TAG and extracted with non-polar solvents such as hexane, chloroform, benzene, diethyl ether. Neutral lipids interact with their long hydrophobic fatty acid chains with van der waals attraction. Therefore, non-polar lipids in cytoplasm come together and form globules (Kates, 1986; Medina et al, 1998). Polar lipids are generally associated with cellular membrane and dissolve in polar solvents such as ethanol or methanol. The mechanism of organic solvent extraction can be divided into 5 steps (Halim et al, 2012). When a non-polar organic solvent, such as hexane, is subjected to microalgal cell culture, it penetrates into the cytoplasm (step 1). Non-polar organic solvent interacts with the neutral lipids

by van der Waals attraction (step 2). Organic solvent-lipid complex is formed (step 3). This complex diffuses out of the the cell membrane (step 4) Organic solvent-lipid complex pass through the static film surrounding the cell and come into the bulk mixture out of the cell (step 5). The reason of the organic solvent film out of the cell is interaction of organic solvent with cell wall. Briefly, the neutral lipids are dissolved in the non-polar organic solvent and extracted out of the cell. Polar lipids are generally located in cell membrane. Some neutral lipids in the cytoplasm interact with polar lipids in the membrane to form a complex. This complex generally strongly linked to membrane protein by hydrogen bond. The van der Waals interactions is not efficent to distrup this hyrogen bond. Polar organic solvents such as methanol, ethanol, isopropanol can be used to distrupt this strong bond by forming hydrogen bond with polar lipids in the protein-lipid complex (Grima , 2013). The extraction mechanism of the organic solvent-membrane lipid-protein mixture can also be divided into 5 steps (Halim et al, 2012) . The organic polar and apolar solvent mixture penetrates through the cell membrane into the cytoplasm (step 1). This mixture interacts with the lipid complex which is located in cell membrane (step 2).While apolar organic solvent forms van der Waals associations with the neutral lipids in the complex, polar organic solvent forms hydrogen bonds with the polar lipids in the complex. The hydrogen bonds between the polar lipids-polar solvent are strong enough to dislocate the lipid–protein complex from the cell membrane (step 3). This organic solvent–lipids complex diffuses out of the the cell membrane (step 4). Organic solvent-lipid complex pass through the static film surrounding the cell and come into the bulk mixture out of the cell (step 5). Therefore, addition of a polar organic solvent into a non-polar organic solvent cause the extraction of membrane-associated neutral lipid complexes. Nevertheless, using the mixture of polar-apolar solvent (such as hexane/isopropanol or chloroform/methanol) leads to the co-extraction of polar lipids.

After solvent extraction , cell debris is removed from the mixture by centrifugation or filtration. Then, biphasic separation is applied to remove both residual water and non-lipid contaminants such as carbohydrate and protein from the mixture of organic solvents and lipids. Biphasic separation is performed by adding non-polar organic solvent and water. After centrifugation, neutral and polar lipids will be separated in the organic phase, while the contaminants such as protein and carbohydrate are accumulated in the aqueous phase which consist of a water and polar organic solvent (Kates, 1986; Medina et al, 1998). The organic phase is then taken carefully and

evaporated to obtain crude lipid. When only non-polar organic solvent is used for extraction, vacuum evaporation such as rotary evaporator can be used to evaporate organic solvent and residual water from the mixture.

Briefly, gravimetric determination of neutral lipids performed gradually. First, lipid extraction from the cells is performed and then any possible contaminants from the extract are removed. Solvent mixture is evaporated to concentrate lipid. The lipid content is then determined by comparing the initial weight of the sample with the weight of the lipid fraction recovered. Each step will effect the lipid purity and percentage. Total lipids, can be gravimetrically quantified after all the steps. Total lipids obtained from microalgae contain not only acylglycerols but also polar lipids and non-acylglycerol neutral lipids (such as free fatty acids, hydrocarbons, sterols, ketones, carotenes, and chlorophylls). Due to crude lipids are often contain non-acylglycerols, it is generally subjected to a fractionation step before they are transesterified. Different purification methods, such as urea crystallizations and acid precipitation are used for lipid fractionation (Medina, 1998). During transesterification, the crude lipids containing the fatty acids are reacted with alcohol (methanol, ethanol) and converted to fatty acid alkyl esters. When methanol is used, the reaction produces FAME. Generally, an alkali (such as NaOH or KOH) is used as a catalyst to cleave the ester bonds between the fatty acids and the glycerol backbone (Figure 1.14), (Christie, 2007; Volkman et al, 1989).

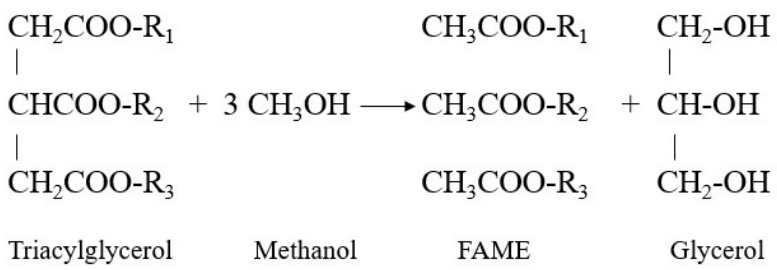


Figure 1.14: Fatty acid metyl ester (FAME) reaction.

Previous studies have indicated that abundant presence of water in the crude lipids reacts with the monoacylglycerol to form free fatty acid under alkaline conditions. When the FFA reacts with the alkali (KOH), soap and water form (saponification). However, alkaline-catalyzed transesterification has low efficiency when applied to polar lipids and free fatty acids. After transesterification, by product contaminants

such as glycerol, alkali catalyst, excess methanol, and un-transesterified lipids are removed by post-transesterification (Halim et al, 2012). Fractionated and transesterified lipid is analyzed by for gas chromatography (GC) to determine fatty acid profile.

1.7.4.2 Supercritical liquid carbondioxide extraction

Lipids have been recovered from microalgae by using different types of solvents and extraction methods (Ryckebosch et al, 2012). However, these traditional lipid extraction methods use large amounts of solvents that are mostly toxic. Unlike traditional extraction, supercritical liquid extraction (SFE) does not use toxic solvent. SFE generally uses carbondioxide as a solvent at high pressure to extract lipid and/or neutroceutical products with higher selectivity. Extraction process can be performed in shorter extraction times without leaving any solvent residue comparing to other systems (Herrero et al, 2006; Mouahid et al, 2013). Moreover the CO_2 which is used as extracting agent is non-toxic, inert, nonflammable and tasteless. Because of that, products that are extracted by SFE carbon dioxide can be used in food, aromas, essential oils and nutraceutical industries (Bhusnure et al, 2015). SFE was used for lipid extraction from *Schizochytrium* sp. S31 since it is the most effective and efficient way to extract valuable constituent. Pressure, temperature and extraction time are critical factors of SFE and vary depending on the species. Because of that, these factors should be optimized before the extraction. The components of the system are a pump for the CO_2, an extraction chamber, a recovery chamber and collection tube. Extractions can be performed in three modes: static, dynamic or recirculating mode. When CO_2 is used as extracting agent, it is pumped to a heating zone and pressurized above its critical pressure in a pump. Heated and pressurized supercritical fluid is allowed to equilibrate. This stage is called static extraction. Supercritical CO_2 passes into the extraction chamber, where the sample to be extracted is placed to contact with the material. The dissolved material is transferred from the extraction cell into a recovery chamber at lower pressure. This stage is called dynamic mode. The CO_2 can then be cooled and recycled, or discharged to atmosphere. This stage is recirculating mode (Hedrick et al, 1992). Appropriate software such as CAMO Software AS. (Version 10.3, Norway) is generally used to perform the experimental design and statistical analysis of SFE extraction conditions data. This method offers some

advantages for high value products such as high diffusion and easy separation with no extra step. There is no toxicity, oxidation or thermal degradation risk in this method.

1.8 Mutagenesis

Mutation can enhance not only the lipid content and also growth rate of microalgae. There are mainly two mutation agents for random mutagenesis: high energetic irradiation and mutagenizing agent. UV light or alkylation chemicals are most common methods for mutagenesis (Kodym and Afza, 2003). Both methods do not require any genetic information about microalgae and can be implemented on a range of microalgal species such as *Schizochytrium* sp., *Nannochloropsis oculata*, *Haematococcus pluvialis*, *Phaeodactylum tricornutum*, *Chlorella sorokiana*, *Pavlova lutheri*, *Scenedesmus obliquus*, *Isochrysis galbana*, *Dunaliella salina* to produce mutants with increased lipid content and growth rate (Lim , 2015).

UV irradiation induce the formation of pyrimidine dimer, specially thymin dimes, in the genome. This kind of change in the genome can cause different types of mutations such as deletion, transition and transversion (Rastogi et al, 2010). Pyrimidine dimers can also cause the formation of long single strand DNA (ssDNA) which activates homologous recombination (Deering and Setlow, 1963; Haruta et al, 2012).

Chemical mutagenizing agent such as ethyl methanesulfonate (EMS), methyl methanesulphonate (MMS) can cause the base substitutions (transition and transversion). MMS alkylates DNA by transferring methyl group onto bases in the genome. Alkylated bases such as O6-Methylguanine or O4-Methylthymine can not make a bond with their usual complementary base (Wyatt and Pittman, 2006). Therefore, transition and transversion (GC to TA or a TA to GC) occur in the genome. Alkylation on nitrogen atom (N3-Adenine, N1-Adenine, N7-Adenine or N3-Guanine) generally cause indirect mutations by inducing SOS response (Shrivastav et al, 2010). UV mutagenesis is used in one of the study to enhance the neutral lipid productivity in the Microalga *Isochrysis Affinis Galbana* (T-Iso) by a mutation-selection procedure (Bougaran , 2012). Shaish et al. (1991) applied UV irradiation *Dunaliella bardawil* strains to increase the induction of beta-carotene and also obtain the beta-carotene resistant mutants. Another important study succeeded to inrease both EPA and DHA by 33% in *Pavlova lutheri* by UV mutagenesis (Meireles et al, 2003). EPA content of the *Phaeodactylum tricornutum* was increased by 37% with same technique (Alonso

et al, 1996). It is known that different organisms have different response to UV exposure. Until now, it has been reported that UV mutagenesis can improve biomass or the lipid content in microalgae in most of the studies. There are some other studies about obtaining high temperature resistant *Chlorella* sp. by chemical mutagenesis (Ong et. al., 2010). Chaturvedi and Fujita (2006) used EMS to increase the yield of eicosapentaenoic acid (EPA) in *Nannochloropsis*. They reported that mutants were more thermotolerance than wt strain. Mendoza (2008) obtained the carotenoid hyperproducing strains of *Dunaliella salina* by exposing it with EMS. Combination of UV mutagenesis with fluorescence activated cell sorting (FACS) was generally used as a method for the isolation potential mutants with increased lipid content of microalgae without compromising on cellular growth. This procedure combines allows the high throughput screening the highest lipid producing cells based on Nile-red luminescence (Dempster and Sommerfeld, 1998). The use of UV mutagenesis in combination with FACS has been described several studies in which the high lipid producing *I.galbana* and carotenoid hyperproducing *D. salina* strains (Bougaran et al, 2012; Mendoza et al, 2008). *Crypthecodinium cohnii* and *Schizochytrium* sp. which are generally isolated from the ocean or mangrove have attracted by researchers because of the high amount of DHA production in their metabolism. Industrial production of DHA at large scale depends on some important parameters. Low productivity, degeneration and weak adaptability are the drawbacks of the strain for large-scale production. Random mutagenesis is widely used to solve these type of problems. Alonso et al. (1996) increased the yields of EPA production in *Phaeodactylum tricornutum* microalgae by UV mutagenesis. As it is described before, there are two types of fatty acid synthesis pathway existed in the thraustochytrids: conventional fatty acid synthesis pathway which comprise a series of desaturation and elongation steps of fatty acids existed in *Thraustochytrium* sp. and novel polyketide synthase system which is generally existed in *Schizochytrium* sp. However, no matter which kind of pathway is used, NADPH and acetyl–CoA are the important factors for achieving enhanced lipid production in microorganisms (Meng et al, 2009). Any mutation on the gene coding of these key factors directly effects the lipid production. Mutant library construction, screening and comparing with control groups steps are described in Figure 1.15. Wild type culture is exposed to mutagen (UV or chemical agent) for optimum dosage and time. After overnight incubation, mutated culture is screened and sorted by fluorescence activated cell sorting according to Nile red

luminescence. Selected mutants are grown on liquid medium containing antibiotic to grow aseptically and then nutrient deprived medium to induce lipid accumulation. After three rounds of sorting, selected mutants are screened and characterized by using image stream (IS). Fluorescence percentage, lipid accumulation rate and cell biovolume can be analyzed on IS. Characterization of the selected mutants are completed by analyzing fatty acid profile. Finally, comparison of mutants and control groups based upon biomass productivity, DHA percentage and lipid production capacity are performed.

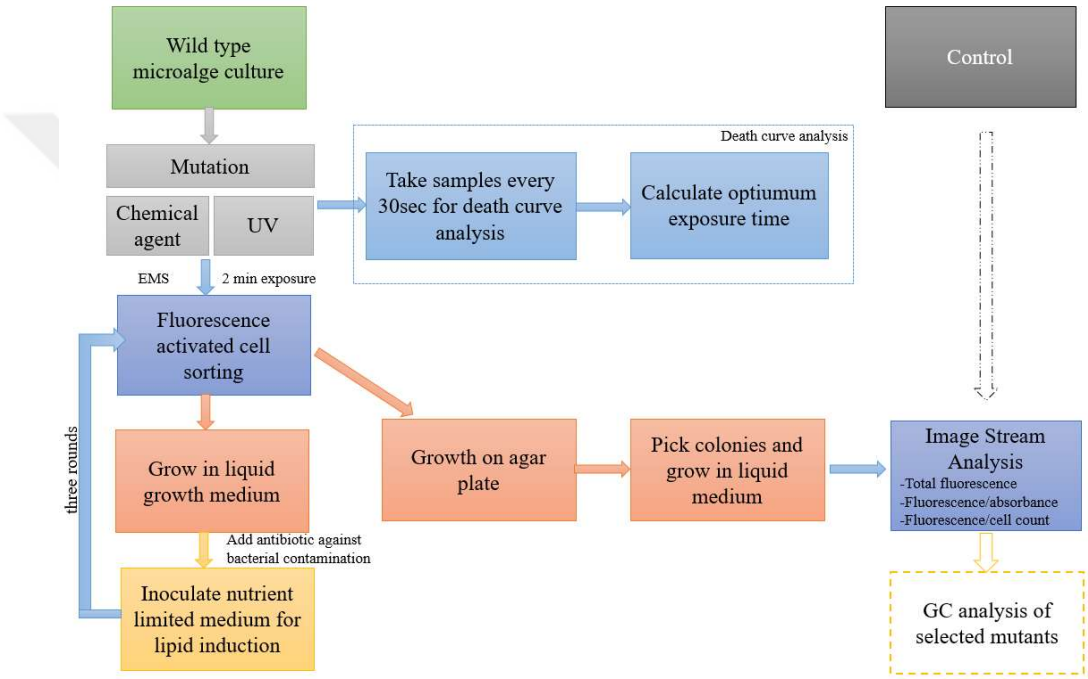


Figure 1.15: Mutation selection cycle for improved lipid production.

1.8.1 Flow cytometer

Selection of cells with particular traits can be done manually by infinite dilution or micro manipulation. However, automatic sorting techniques are much faster, resulting in a higher number of sorted cells and, hence, a faster recovery of the resulting population. The combined use of fluorochrome and flow cytometry sorting was only recently described for the selection of high-lipid content strains of *Tetraselmis suecica* (Montero et al, 2011) and for the selection of carotenoid hyper-producing strains for the green algae *Dunaliella salina* (Mendoza et al, 2008). Flow cytometry (FCM) is a laser-based technology for counting, sorting and analyzing microscopic particles, such as cells, particles and chromosomes in the fluid stream by an electronic detection

apparatus (Givan, 2013;Yentsch et al, 1983; Veal et al, 2000). It is used to analyze the physicochemical characteristics of up to thousands of particles per second. Particles such as cells can be sorted based on their physical and chemical properties. Small sub-populations in the cell culture and specific molecules can be characterized, according to fluorescence decay, robust statistics and heterogenity within a population can be obtained from the flow cytometry data (Shapiro, 2005; Dittrich and Schwille, 2003). In the flow cytometry system, there are different types of lasers and filters which enables to detect different types of particles (Shapiro, 2005), (Figure 1.16).

Polarization filter blocks or transmits light according to its polarization.

Dichroic filters transmit the undesirable wavelength to the remainder.

Monochromatic filters are able to pass narrow range of wavelengths through the system.

Bandpass filters allow only a certain wavelength to pass. 520/30 bandpass filter would transmit light between 510-530 nm.

A **longpass filter** transmits longer wavelengths.

A **shortpass filter** transmits shorter wavelengths.

Different types of lasers can be used in the system according to the research. However, five lasers in the flow cytometer systems are generally used: 488nm, 457nm, 532nm, 355nm and 640nm.

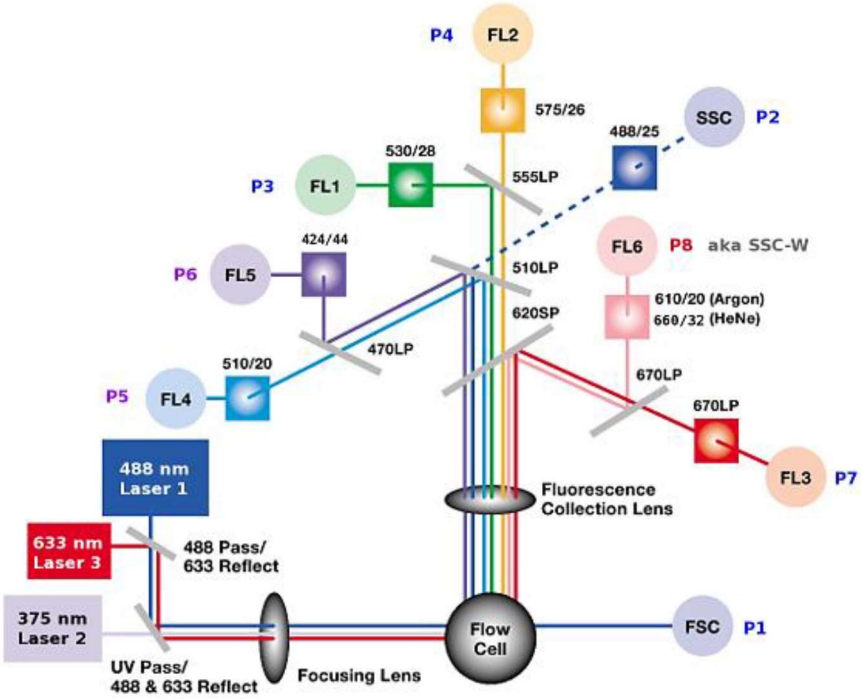


Figure 1.16: Different lasers and filters in flow cytometry system.

As a particle passes through the laser, light is scattered in all directions. The Forward Scatter (FSC) detector collect the light that is scattered up to a 20° angle from the excitation beam while side scatter (SSC) detects light at a 90° angle to the laser source point (Figure 1.17). The forward scatter gives information about the particle size. SSC gives information on granularity and internal complexity (Picot et al, 2012).

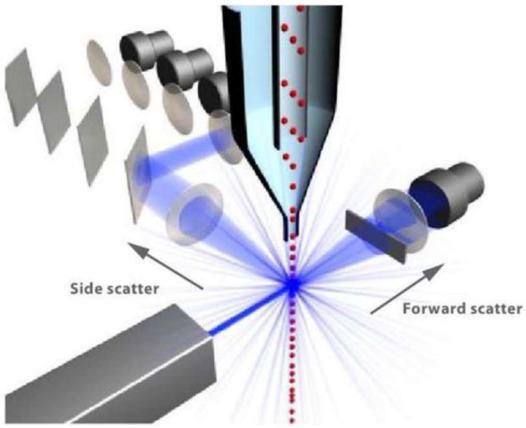


Figure 1.17: Forward and side scatter.

Fluorescence activated cell sorting is generally used to screen the cell population and sort the cell according to fluorescence intensities (Givan, 2013). The principle of the flow sorting is shown in Figure 1.18. When the piezo vibration forms droplets, it passes through the stream. The stream can be charged by pressing flash charge, which

eventually forms charged droplets. Charged droplets can be separated by deflection plates that allows isolation of cells of interests.

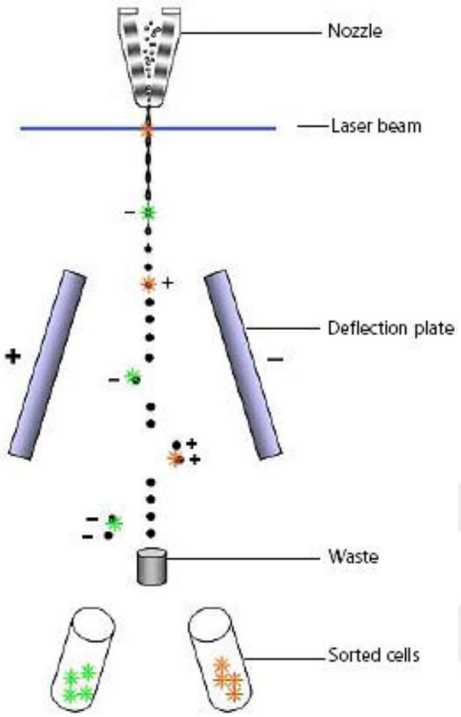


Figure 1.18: Principle of flourescent activated cell sorting.

1.8.2 Screening with ImageStream

The instrument combines a flow cytometer with a high resolution fluorescence and brightfield microscope and rapid response imaging system. Image Stream able to image each cell in the channels (1-6) as they pass through the detectors. Thousands of cells can be imaged in a few minutes (Basiji et al, 2007). Very small differences can be detected in the population with accuracy due to the enormous number of data points (20,000). Standard error in these measurements is <0.13% of the mean equivalent to 0.15 μm^2.

1.9 Market Analysis

The economic, cultural and scientific development of societies has risen the importance of our food habits and life-style. Consumers are looking for health enhancing, nutritious, great tasting, natural, safe and well-being health promoting foods. Nowadays it is generally accepted that an unbalanced diet is an important risk factor for the occurrence of certain diseases, and that a varied and balanced diet may be a protective factor. However, our daily lifestyle is at least partly responsible for us

to have left healthy eating habits which have been part of our food tradition and culture for years. According to WHO data, non-communicable or chronic diseases, primarily cardiovascular diseases (like heart attacks and stroke), cancer, chronic respiratory diseases (such as chronic obstructed pulmonary disease and asthma) and diabetes were by far the leading cause of death in the world in 2013, representing 63% of all annual deaths, which means more than 36 million people out of 57 million global deaths each year (World Health Organization, 2013). Non-communicable diseases force many people into, or entrench them in poverty due to catastrophic expenditures for treatment. If the major risk factors were eliminated regarding to non-communicable diseases, at around 75% of stroke, type 2 diabetes and heart disease and 40% of cancer would be prevented (World Health Organization, 2013). Among the main ten risk factors identified, five are closely related to diet and lifestyle: overweight, sedentary lifestyle, high blood pressure, high cholesterol and insufficient consumption of fruit and vegetables. Therefore, social impact of functional foods (those which have active components with beneficial effects in one or more body functions, which means health improvements or disease risk reductions) is undeniable. Considering that cardiovascular diseases are, together with cancer, the main cause of death in developed countries and any measure to reduce their incidence is welcomed. Globally, the rise of the middle class and their economical means and the increase of working and better educated women boost the potential of functional foods market. According to the market report "Future Directions for the global functional foods market", led by the British independent organization Leatherhead Food Research in 2014, growth within the global functional foods market has been hindered due to economic recession, that caused many consumers to prefer cheaper groceries. The situation in Europe has not been changed by health regulatory framework changes. Nevertheless, the global market in USA continues to grow mostly because of people seeking out functional foods in place of dietary supplements. The percentage of the adult people who is 20 and over years old in the world classed as either overweight or obese increased 11% from 1980 to 2008, which equates to approximately 1.46 billion people (WHO-Obesity and overweight). Recent data shows that 30% of the global population which equates to 2.1 billion people are obese/overweight. In this context, the global functional foods market was worth an estimated USD43.27bn in 2013. When it is compare with 2009, this has increased by 26.7% and continues to grow. As a result, its share of the global industry increased slightly from 37.3% to 38.1%. It is expected that the global market

for functional foods reaches USD 54 billion in 2017, equivalent to an increase of 25% compared with the latest data available (2013). During the period between 2009 and 2013, growth expanded by more than 29% in Australian and US markets. Specifically, the omega-3 ingredients market shows a continued development and growth, mainly because the application areas of omega-3 are increasing. While the demand of omega-3 ingredients was 21.9 kilo tons in 2012, it is expected to increase to over 60 kilo tons by 2020. This means the omega-3 global market is expected to reach USD 7.32 billion by 2020 (Hegde et al, 2016). Largest consumer of omega-3 ingredients is Europe with a share of over 60% in 2012, followed by North America. Because of Omega-3 products has health approvals from various agencies specially from FDA, demand for the product has increased. Three heart health claim that EPA and DHA which are the omega-3 fatty acids have been approved by member states of the EU. The Food and Drug Administration (FDA) has approved the claim of omega-3 reducing the triglyceride in the body and coronary heart disease. The American Dietetic Association has recommended a 500mg/day of EPA and DHA intake in order to have beneficial health effects. These recommendations are expected to increase the demand for omega-3 products. Omega-3 infant formula has credible attention in the recent past. Large amount of infant formulations containing EPA/DHA are designed, as they have significant health benefits. Companies like Nestle and Similac have a significant portfolio about omega-3 enriched infant formula and this product line have been progressively increased in recent years. In addition, omega-3 market is expected to boost next couple of years because of the awareness of benefits of omega-3 by customers. Because of the infant formula market is the world's fastest-growing packaged food category in specially in Asia (particularly China) and Eastern Europe, consumption of omega-3 infant formula is expected to be the fastest growing segment with an estimated annual growth rate of 15.3% from 2014 to 2020 (Hegde et al, 2016).United Nations FAO/WHO (Food and Agriculture Organization/World Health Organization) has been recognized the need for infant formula enrichment with DHA for infants and recommends that DHA should be added to all infant formula. Approximately 87% of infant formula was enriched with DHA in 2011. This favorable prospects and the constantly decreasing rate of the fish oil market caused opportunities for the development of alternate sources, such as algae, for the extraction of omega-3 ingredients. As omega-3 fatty acids are one of the most valuable products from microalgae, the production of other important algal bioproducts in large scale will be

eventually produced (Adarme-Vega et al, 2012). Fish oil as omega-3 source with different composition of DHA and EPA in mostly capsule and syrup forms has been served several nutraceutical companies in the market. Recommended Adequate Intakes (AI) for Omega-3 Fatty Acids (Food and Nutrition Board, USA, 2002) were shown in Table 1.2.

Table 1.2: Recommended adequate intakes (AI) for omega-3 fatty acids (Food and Nutrition Board, USA, 2002).

Life Stage	Age	Males (g/day)	Females (g/day)
Infants	0-6 months	0,5	0,5
Infants	7-12 months	0,5	0,5
Children	1-3 years	0,7	0,7
Children	4-8 years	0,9	0,9
Children	9-13 years	1,2	1,2
Children	14-18 years	1,6	1,1
Adults	19 years and older	1,6	1,1
Pregnancy	All ages	N/A	1,4
Breastfeeding	All ages	N/A	1,3

In addition to this, American Heart Association recommended that individuals with coronary heart disease should take at least 900 mg/day of DHA plus EPA. National Institue of Health (NIH) Workshop recommended that the 300 mg DHA/day should be taken during pregnancy and 650 mg of DHA plus EPA should be taken by normal healthy individuals. Designing of an efficient production process of high value ingredients from microalgae requires which means to do research about different aspects (microalgae culture, extraction processes, encapsulation and stabilization, etc.) and gain scientific expertise in all those technical fields. It is known that diffuculties of the optimization and industrial and semi-industrial level scale up process and also poor economic profitability caused the manufacturing process design of microalgae to be exploitable in the short-term. Production of microalgal lipid which will be economically viable is still a major challenge (Adarme-Vega et al, 2012). Therefore, the process developed for algae cultivation and lipid extraction need to be improved

for higher efficiency and lower costs. This improvement is essential for being able to face to the increasing demand for omega-3 of the functional food market and the pressing limitations of fish oil.

Overtaking those pressing limitations of the current sources and processes involves many other general benefits:

1. Environmental benefits:

1.1. Current main source of omega-3 fatty acids, fish oil, can be replaced by microalgal oil. This replacement reduce the serious environmental problems such as overfishing and threats to biodiversity.

1.2. It is possible to use the biomass residue after SFE from the microalgae process as feed supplement for fish and other feed industry.

1.3. Production of microalge in large scale is much faster and does not depend on climatic change comparing to fish.

2. Social benefits:

2.1. More than 36 million people died in the world in 2013 (63% of all annual deaths) because of chronic diseases, primarily cardiovascular diseases, cancer, chronic respiratory diseases and diabetes. Omega-3 fatty acids are known to reduce cardiac diseases such as arrhythmia, stroke and high blood pressure by increasing the HDL/LDL ratio and decrease the total cholesterol/HDL ratio (Ander , 2003). EPA is reported as beneficial not only for coronal heart diseases, but also responsible for regulation of blood pressure and cancer prevention. DHA also shows significant effect on cardiovascular health and cancer. Omega-3 fatty acids are also reported to alleviate psychological problems like anxiety or depression, which are also a sort of epidemic nowadays (Hooper et al, 2006).

2.2. Utilizing omega-3 from microalgae rather than fish oil avoids a presence of chemical contaminants in fish oil derived products, such as PCBs, dioxins and heavy metals, which have dangerous consequences for animals and human beings because of bioaccumulation.

2.3. Vegetarians may benefit from omega-3 from microalge as a functional food. Even non-vegetarian people who rejected omega-3 from fish oil because of unpleasant odour and taste will now benefit .

3. Enhancement of advanced food industry: the replacement of fish oil with microalgae oil as main omega-3 fatty acids source means being able to meet the growing demand of not only healthy products, but also ecological and bioproducts.

1.10 Purpose of Thesis

This study has three aims: firstly to determine medium components and environmental factors influencing biomass and total lipid production in *Schizochytrium* sp. S31 by central composite design with response surface methodology. Secondly, the large scale production in bioreactor and improvement of downstream processes. Finally, to obtain a mutant library of a *Schizochytrium* sp. S31 by introducing random mutation in the genome and screen for a high yield of lipid accumulating mutant by flow cytometric-based technology.

It is mainly focused for the production of DHA of total lipid from *Schizochytrium* sp. S31 using bioprocessing approaches. All chemical and physical factors effecting total lipid and PUFA specially DHA production has to be reviewed to understand the metabolic processes leading to PUFA production. Alternative raw materials were tested to create a suitable, low cost media for maximum production of cellular biomass. Statistical designs (Plackett Burman and Central Composite Design) were used to optimize the process parameters, such as media composition, pH, temperature and dissolve oxygen in order to increase accumulation of neutral lipid and DHA in *Schizochytrium* sp. S31. Scale up process and optimization of bioreactor conditions are another main step in this thesis. Batch fermentation experiments were started from a single colony to reach 1 lt of liquid cell culture which is used as an inoculum for 5 lt of fed-batch culture. Optimum parameters were applied to fed-batch system to obtain high amount of desired products. While physical parameters were controlled automatically, optical density of the culture, dry biomass, residual glucose and MSG, total lipid, DHA% were monitored during the incubation. Improvement of downstream processes was also critical step in this study. Alternative cell harvesting, drying, lysis and solvent based extraction methods including SFE of *Schizochytrium* sp. S31 microalge were investigated and compared. Random mutation was applied by ultraviolet (UV) radiation or chemical mutagen to isolate a high yield of lipid accumulating mutants of a *Schizochytrium* sp. by flow cytometric-based selection.

2. MATERIALS AND METHODS

2.1 Microorganism and Mediums

Schizochytrium sp. S31 (ATCC 20888), which was preserved in the American Type Culture Collection (ATCC) was used in the present study. Stock culture was maintained on F/2 medium. Large scale growth experiments were maitained both complex and fermenter medium. Contamination test was performed by using Luria-Bertani (LB) agar plates containing 0.001% actidione (cycloheximide) Ingrediends of all the mediums were listed in Table 2.1.

Table 2.1: Growth medium for *Schizochytrium* sp. S31.

F/2 Medium	
$NaNO_3$	0.075 g/l
$NaH_2PO_4.H_2O$	0.005 g/l
Trace Element Solution	1ml /l
Vitamin Solution	1ml/l
Complex Medium	
Glucose	20 g/l
Protease peptone	8 g/l
Yeast Extract	5 g/l
Sea salt	25 g/l
MOPS	21 g/l
Fermenter Medium	
Glucose	40 g/l
Monosodium Glutamate	4.22 g/l
Mineral Salt	12.5 g/l
$MgSO_4.7H_2O$	2.5 g/l
KH_2PO_4	2.5 g/l
KCl	0.5 g/l
$CaCl_2$	0.1 g/l
Trace Element Solution	5ml
Vitamin Solution	1ml

The trace element solution contained (g/1): Na_2EDTA (6), $Ni_2SO_4.6H_2O$ (0.052), $Co_2Cl.6H_2O$ (0.026, $Na_2MoO_4.2H_2O$ (0.005), $Fe_2SO_4.7H_2O$ (0.29), $ZnSO_4$ (0.06), $Cu_2SO_4.5H_2O$ (0.002), $Mn_2Cl.4H_2O$ (0.86). Na_2EDTA is dissolved first, followed by the addition of metals. The vitamin solution was filter-sterilized (0.2 µm) and contained (mg/1): thiamine (100) and cyanocobalamin (0.5). All chemicals were purchased from Sigma-Aldrich (St. Louis, MO, USA).

2.2 Cryoprotection of Microalgae

Cryopreservation is widely used for achieving long-term storage of biological materials. Cryopreservation protocol of *Schizochytrium* sp. for long term storage which is described before is used (Day and Harding 2008). It is preferred to use a healthy, dense, log-phase culture for cryopreservation. Storage temperature can be either -80 or -130°C. Dimethyl sulfoxide (DMSO), glycerol and methanol can be used as cryoprotectant solution.

<u>Cryoprotection procedure</u>

1. Prepare 5 ml of 10% (v/v) cryoprotectant in the F/2 medium and filter-sterilize the cryoprotectant solution.
2. Add 5 ml of dense, healthy culture and 5 ml sterilized cryoprotectant solution into a sterile falcon tube. Seal the tube and invert twice to ensure thorough mixing.
3. Decant 1 ml aliquots into cryovials in sterile conditions and incubate for 10 min at room temperature.
4. Fill the Passive cooler (Mr Frosty™) unit with isopropanol (C_3H_8O) to the 250ml lines on the freezing unit.
5. Transfer the cryovials to the cooling chamber of the Passive cooler (Mr Frosty™) unit and incubate it at –80 °C freezer for 1 hour.
6. Transfer the cryovials to a small dewar containing liquid nitrogen using long forceps. Do not warm cryovials up prior to plunging into liquid nitrogen or freezer. Cryovials could be stored at -80 °C for storage.

<u>Recovering the culture</u>

1. Cryovials containing cell culture are thawed by placing in a pre-heated water-bath (40°C) for recovering.

3. Transfer the 1 ml of thawed culture into a vessel containing 100 ml of an appropriate medium in laminar flow cabinet.
4. After an appropriate period (1-3 day), a normal culture should be obtained.

When freezing cells, it is optimal to use lot system that keeps early passage for producing new working stock. Lot system that preserve early passage material is described in Figure 2.1. The vials designated as master stocks are maintained separately from the working stocks.

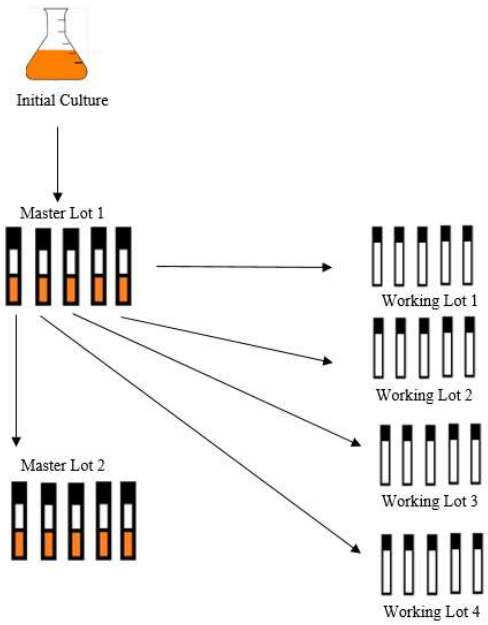


Figure 2.1: Cell stock lot system.

When the first working lot is depleted, another vial from the master lot is used to prepare a second working stock. This procedure continues until all working stocks are depleted. Last master stock vial is used to prepare a second master lot. Five cryovials are normally stored in a working bank, five vials are stored in a master/ back-up bank and three are used to check viability/ efficiency of the both cryoprotectant solution and protocol.

2.2.1 Evaluation of survival rate

Vials, which are stored for checking viability/efficiency, were diluted with distilled water and inoculated onto complex agar plates once in a month. After 2-3 days of incubation, colonies were counted. Survival rates were calculated by comparing the

colony number of the three samples, which are thawed every month for viability. Three vials were thawed for the examination of three different cryoprotectants. In addition, to investigate changes in the survival rate over months, different cryoprotectants were examined.

2.2.2 Bacterial contamination test

The protocol described in Nanotechnolgy Characterization Laboratory (NCL) is used for the quantitative determination of microbial contamination in the culture. Samples are diluted and spread on the LB agar containing 0.001% actidione (cycloheximide), and growth of bacterial colonies is monitored after 72 h incubation. Actidione at very low concentration (10ppm) permits the growth of bacteria and inhibits the growth of most yeasts, microalgae and moulds. This medium may be used for the estimation of bacterial contamination in microalgal culture.

<u>Bacterial contamination test protocol</u>

- LB plates containing 0.001% actidione (cycloheximide) were taken from refrigerator and allowed to equilibrate to room temperature.
- Totally six plates are used: two for each sample, four plates for negative and positive control. While sterile PBS or water can be used as a negative control, bacterial cultures can be used as a positive control. The negative control should contain no colony forming units (CFU). For the positive control, use the bacterial cultures dilution between 10-50 CFU/mL.
- Apply controls or sample (at each dilution) on the agar plates under sterile conditions, and distribute the sample using sterile spreaders. Incubate for 72 h at 37°C.
- Remove dishes from the incubator and count colonies (CFU/ml) as described below.

$$\boldsymbol{Bacterial\ count(CFU/ml)} = Numb \quad of\ colonies \times Dilut \quad factor \times Sampling\ factor$$

2.3 Determination of Growth Curve

Microalgal growth studies conducted via inoculation of viable cells into a sterile complex medium under optimum temperature, pH, and stirring conditions. While

microalgal cells are reproducing during the incubation period, growth curve can be charted by plotting the cell numbers and/or cell density versus time. 1ml of sample were taken at certain time points during incubation under sterile conditions. Samples were diluted and measured optical density (OD_{750}) by using spectophotometer. Cell counting of the samples were also performed by using counting chamber Hausser Scientific Co, 0,100mm deep. The stages of a typical growth curve (Lag, exponential, stationary, death phase) were determined from the growth curve.

Spectrophotometric analysis

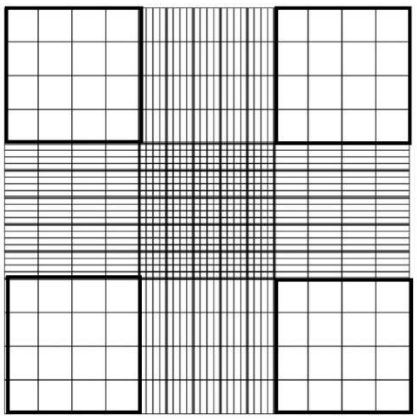
- Set and calibrate the spectrophotometer.
- Set the wavelength to 750 nm.
- Resuspend the microalgal culture and take a small amount carefully. Dilute cell culture with 1:50 ratio if the culture is too dense to measure.
- Read and record the % transmittance and the optical density (OD_{750}) of the culture.

Cell counting

- Hausser Scientific Co, 0,100mm deep Horsham,DA 19044 Cat#3200 counting chamber were used to count the cells as described below:
- 10µl of cell suspension was applied to the hemocytometer each slides. Fill both chamber underneath the coverslip
- Wait for 1-2 minutes to settle down the cells
- Focus on the grid lines with a 10X objective by using microscope
- Count the cells in the squares according to the cell sizes.

If cells are big,

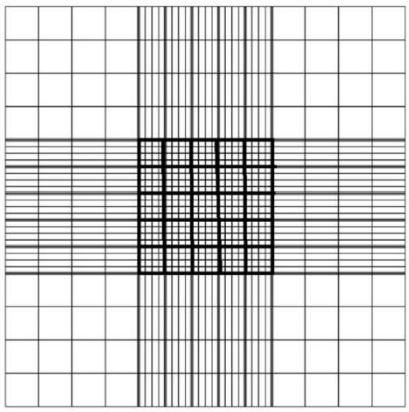
- Count the cells in the all 4 sets of 16 corners



- Take the avarage cell count
- Multiply with 10.000 (10^4) cell depth
- Multiply by dilution factor.

If cells are small,

- Count the cells in the middle of 25 squares.



- Take the avarage cell count
- Multiply with 10.000 (10^4) cell depth and dilution factor.
- Calculate cell concentration per ml and total cell

$$\boldsymbol{Cell\ Density\ (cell/ml)} = Average\ cell\ count \times Dilution\ factor \times 10^4$$

$$\boldsymbol{Total\ cell} = Cell\ density\ (cell/ml) \times Volume(ml)$$

2.4 Optimization Experiments

Optimization experiments were conducted in two steps. First step include the optimization of chemical and physical condition of culture by stepwise. Second step involves the optimization of components by statistical based methadology. The two step optimization involved testing the interaction and determining the optimum combination of the potential production media and cultivation conditions illustrated in Figure 2.2.

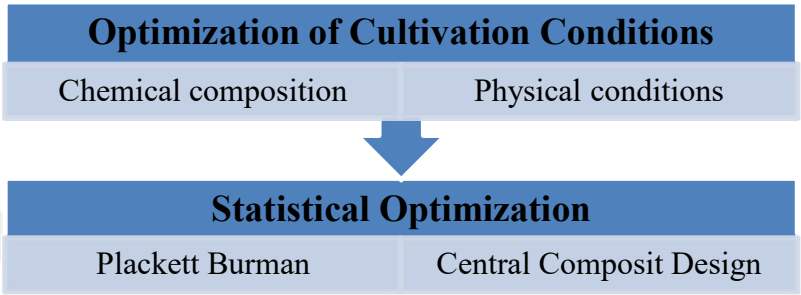


Figure 2.2: Schematic diagram of optimization strategy.

2.4.1 Optimization of chemical composition of the medium

Alternative carbon, nitrogen, salt raw materials were employed to investigate optimum cell growth and total lipid yield. Glucose, glucose syrup1 (90% glucose+10% fructose), glucose syrup2 (50% glucose+50% fructose), glycerol, molasses were studied as the initial carbon source. Fermenter medium containing different carbon sources with the same percentage of dextrose were prepared. Its incubated at 25°C, 200rpm for 48h and CDW were calculated. Alternative nitrogen raw materials experiment were conducted after determining the most suitable carbon sources. Fermenter medium containing selected carbon source and different nitrogen sources ($(NH_4)_2SO_4$, $(NH_4)_2C_4H_4O_6$, NH_4HCO_3, $C5H_8NO_4Na$) with the same percentage of nitrogen were prepared. After incubation, nitrogen source that gives best result was determined by calculation CDW. Alternative salt raw materials experiment were conducted after determining the most suitable carbon and nitrogen sources. Growth medium containing selected carbon and nitrogen sources with different salt sources (Seasalt, Na_2SO_4, NaCl, mineral salt) were prepared. After incubation, ideal salt source was determined. Three different concentrations of the raw materials were examined to determine both optimum source and concentration. The cell dry weight (g/l) and total

lipid (%) of *Schizochytrium* sp. on different carbon, nitrogen and salt sources were compared.

After determining the best composition of the growth medium, physical parameters were analyzed for optimization. Optimum temperature, pH and dissolve oxygen values were determined. Finally, chemical and physical condition of the cultivation conditions were optimized.

2.4.2 Optimization of physical conditions

Physical conditions of the culture were optimized to maximize both CDW and total lipid production. Different temperature (16, 25, 30°C), pH (5, 7, 9) and dissolve oxygen (25%, 50%) were examined. Eight different experiments were conducted stepwise with two replicate. Best condition for both biomass and lipid production were determined.

2.4.3 Statistical analysis

Statistical method was applied to screen the main effect of tested components on selected responses. The effect of selected component was calculated and compared in order to choose the most potential components and their proportion for maximum CDW and lipid production. Once the effective components were chosen, Central Composite Design (CCD) of experiments were applied to determine the optimum combination for maximum CDW and lipid production. Twelve different potential components were screened by Plackett Burman designs to investigate the most significant factors affecting Biomass, lipid and DHA% production. The screening and optimization experiments were carried out in 250 ml sterile flasks with 50 ml of given media at 25°C as a cultivation temperature for two days at 200 rpm. The factors that has the most significant effect were taken further step to compare them. The next step involved interaction between variables and determining the optimum combination of the conditions using the CCD.

2.4.3.1 Plackett Burman

The Plackett Burman design was used to identify critical factors that have a significant effects in the system. It is a two level factorial design and investigate the 'n-1' variables with at least n experiments.

The first order model of PB design is given in equation 2.1.

$$Y= A_0+A_1X_1+A_2X_2+A_3X_3+\ldots+A_nX_n \quad (2.1)$$

where Y is the response, A_0 the constant and A_1 to A_n are the coefficients of the response values. The levels of variables evaluated in this study are listed in Table 2.2. Two-level factorial design with R version 3.1.1 software was used to screen for the significant factors among Glucose, MSG, NaCl, $MgSO_4$, KCl, KH_2PO_4, $CaCl_2$, Initial pH, Dissolve Oxygen, Temperature. The selected software package was used to estimate the responses of the variables. Table 1 shows the high (+) and a low (−) level of the variables. The low levels (−) of medium components were designed as their concentrations in fermenter medium. The design matrix with 10 variables over 12 runs was developed. Preliminary experiments were carried out in 250 ml flasks, each containing growth media with all the tested variables in different concentrations in an orbital shaker incubator at 200 rpm for 48h. At 90% level of confidence, any variable with P-value < 0.1 were considered as significant. Biomass, % lipid content, %DHA of all experiments were analyzed as a response. The factors that has positive/negative effect were taken next step.

In this study, ten different factors were screened by PB designs to determine the critical factors that have significant effects on the production of biomass, total lipid and DHA%.

Table 2.2: Levels of factors in the Plackett Burman design.

	Variables	Unit	Low level (-1)	High level (+1)
A	**Glucose**	g/l	40	80
B	**MSG**	g/l	0.8	1.6
C	**Mineral salt**	g/l	6.25	12.5
D	**KH_2PO_4**	g/l	2.5	5
E	**KCl**	g/l	0.5	1
F	**$MgSO_4.7H_2O$**	g/l	2.5	5
G	**$CaCl_2$**	g/l	0.1	0.2
H	**Initial pH**	-	6	8
I	**Dissolve oxygen**	%	25	50
J	**Temperature**	°C	20	28

2.4.3.2 Central composit design and response surface methodology

Optimum values of the selected variables and interaction between them were investigated by CCD for full optimization. CCD was used to optimize the levels of the significant factors for enhancement of biomass and DHA production. In this study, two factors (MSG, initial pH) for CDW response from the PB design were tested at five levels (-1.68, -1, 0, 1, 1.68) and 12 experiments were generated. The coding of the variables was performed according to equation 2.2.

$$Y = Block.ccd + FO(x_1, x_2) + TWI(x_1, x_2) + PQ(x_1, x_2) \quad (2.2)$$

where Y is predicted response; FO the first order, TWI the two way interaction and PQ is the pure quadratic. Response was measured in the terms of CDW, total lipid and %DHA production. The software R version 3.1.1 was used for the regression analysis and also response surface graphs of the experimental data.

2.5 Large Scale Growth

Biostat A Plus bioreactor (working volume 5 liter) was used to perform both batch and fed-batch heterotrophic cultures. Figure 2.3 illustrates the experimental setup identifying the major parts of the equipment. The bioreactor was equipped with temperature, pH and dissolved oxygen probes to control important parameters. Temperature was measured with a platinum electrode Pt-100 (Sartorius BBI Systems, Type 200-4). The bioreactor temperature was kept under desired value using an heating blanket, and an external water-cooling system. An EasyFerm Plus K8 (Hamilton Bonaduz AG) electrode was used to measured the pH. Aqueous pH4, pH7 pH10 solutions were used for calibration of pH prob. The pH value of the medium was kept under control by using asid (HCl) and base (NaOH) solution connected to bioreactor by peristaltic pump. Dissolved oxygen was measured with an Oxyferm FDA (Hamilton Bonaduz AG) electrochemical sensor. Stirrer rate was adjusted cascade mode to control the dissolved oxygen at defined set-point. Air compressor was also used to control dissolve oxygen level.

Batch fermentation experiments were started from single colony to reach 1 lt liquid cell culture. 1 lt culture was then used as a inoculum for 5lt fed-batch culture. 10% of inoculum was used for large scale growth studies. Physical parameters such as pH,

temperature, dissolve oxygen were controlled in large scale growth studies. pH changes as a result of biochemical reactions were fixed at pH:7 by addition of sodium hydroxide. The amount of dissolve oxygen was fixed at 50% by changing stirrer speed and supplying air by compressor. Temperature was maintained at 25 °C by jacket. Samples were taken at 0, 6, 18, 24, 30, 42, 48 and 72h to monitor optical densitiy of the culture, dry biomass, residual glucose and MSG, total lipid, DHA% during incubation.

Figure 2.3: The Biostat A Plus (5L) bioreactor and computer based control program.

<u>The Biostat A Plus (5L) bioreactor instruction</u>

- The fermenter system is sterilized with 121°C hot steam, 3 bar for 15min in autoclave.
- The dissolved oxygen, temperature pH are controlled with PID system. Temperature is controlled by using the jacket and cooling unit. The dissolved oxygen is controlled based on air inlet pumb and speed of impeller. The pH is controlled by using strong acid and based pumbing via peristaltic pumbs.
- The temperature is generally at 37-45°C.
- The pH is generally at 5 - 7.

All fermenter system must be build on steel structure.

- The mixing speed is arrenged based on disolved oxygen ration. The RPM range is 50-200 rpm.

- The tank contain max 5 l medium and culture
- Inlets
    - Acid (HCl)
    - Base (NaOH)
    - Air (Air must be filtered)
    - Antifoam
- Outlets
    - Exit gas: Gas outlet must be top of the tank.
    - Product: Product outlet is used to empty tank after incubation
- Connections
    - Temperature
    - pH
    - Disolved oxygen
    - Level sensor (Foam sensor)
- All the connection, outlets and inlets must be good isolated aganist contamination.
- The impeller type is rushton 6 blade.

Modified large scale protocol was used to grow *Schizochytrium* sp. S31 in 5 lt bioreactor (Ganuza et al, 2008).

<u>Large Scale Growth Protocol</u>

- Thaw one of the working stock culture and plate on complex agar plate. After 72h of incubation at 25°C, single colonies is visualized.
- Pick a single colony and inoculate into 50ml of complex growth medium.
- After incubation at 25°C for 48h, inoculate 10ml of liquid culture into five flasks containing 250ml compex medium and incubated at 25°C, 225rpm for 48h.
- Turn on the fermenter and computer. Open Master Panel program from PC-Panel.
- Prepare fermenter medium and adjust to pH7 with KOH. Prepare Glucose, $MgSO_4.7H_20$, HCl, KOH and antifoam solution separately.

- Calibrate pH prob by using pH solutions (4,7,10). Check the Electrolit solution in pO2 and add if it is needed.
- Add fermenter medium into fermenter tank and close the lid of the device carefully. Cover the all outputs of the device with aluminum foil.
- Attach PTFE filters on pO2 pipe, cooling tube, sampling apparatus, acid / base bottle. The filter input and output locations must be checked carefully.
- Autoclave the fermenter tank, glucose and $MgSO_4$ separately at 121°C for 30min.
- After autoclave, make all the connections of fermented device and wait for two hours to settle down all the parameters.
- Add additionally prepared glucose, MgSO4, trace elements solution and vitamin into fermenter tank by peristaltic pump.
- Adjust the temperature, pH, pO2 and stirer speed by using computer program. Regulate substrat profile from control panel.
- Apply 10% cell inoculation and start the batch culture by pressing 'start batch' in Operator Service program.
- Take samples with regular intervals by sampling apparatus and monitore all the parameters regularly.
- Stop batch recording by a pressing 'stop batch' in Operator Service program after it is complete. Turn off thepPrograms and devices.
- Perform downstream process.

2.5.1 Batch fermentation

For the batch cultures, all nutrients, minerals and vitamin are loaded in the reactor in the beginning of the incubation and cells are harvested when the nutrients are exhausted. All materials were cleaned and sterilized before use. Sterilization was conducted in a steam autoclave, holding the temperature at 121°C for 20 minutes. Single colony were picked and inoculated into 100 ml medium in 250 ml baffled flask. Fermentation media were used for batch cultures as reported in Table 2.1. Shake-flask cultures (200 rpm, grown for 48 h) were used as the respective inocula (10% v/v) for the batch fermenter. The temperature, pH and DO were controlled by the station and monitored via Bioexpert software. Cellular growth is generally limited in batch fermentation system.

2.5.2 Fed-batch fermentation

For the fed-batch cultures, nutrients are continuously added to the culture with predefined substrate profile (Table 2.3). *Schizochytrium* sp. S31 was finally cultivated in a 5 liter working capacity bioreactor (Biostat A Plus) in which the pH was controlled by the automatic addition of substrat. Dissolved oxygen (DO) was maintained over 50% by manually increasing the stirring speed (Rushton blade impellers) from 200 to a maximum of 850 rpm. The aeration rate was always 0.5 volume air. Cells are harvested when the culture reaches stationary phase. Fed-batch system is usually used to achieve high cell density.

Table 2.3: Substrate profile of 5 liter fermenter culture.

Substrate Profile

Time	Pump rate (ml/sec)	Total amount (unit)	Added Glucose (g)
0-5h	0	0	0
5-12h	2	14	266
12-24h	2,5	30	570
24-48h	2	48	912
48-56h	1	8	152
56-70h	0,4	14	106,4
70-96h	0	0	0
Total	-	114	2.006,4

2.6 Downstream Processing Steps

In bioreactor cultivation, samples were collected at intervals, centrifuged and the pellet were freeze dried. Supernatant were used to quantified the remaining amount of each element during the process. The temperature, pH and DO were controlled by the station and monitored via Bioexpert software. O.D750, cell dry weight, μmax, C/N ratio, lipid %, DHA %, biomass productivity, lipid productivity, DHA productivity, residual glucose and nitroegen were monitored during and after incubation.

<u>Harvesting biomass</u>

Schizochytrium sp. S31 microalgae culture was mostly harvested by centrifugation at 4800xg for 15min (Beckman Allegra). Supernatant were used for further analysis. Cell pellet were washed two times and centrifuged to get rid of media competents specially

glucose. Filtration technique in which the starting solution passes along the surface of the filter sheet by pressure was also used for harvesting biomass. Different filtration sheets with varied pore sizes were tried to get best result. Filter sheets with different pore sizes (0,5mm, 0,75mm and 1mm) were used to filter *Schizochytrium* sp culture. Components larger than the filter pores are retained on filter sheet. The settled biomass was recovered by scraping off from the surface of filter sheet.

Pretreatment process

Harvested cell pellet was incubated at 65°C for 1 hour to inactivate cellular lipase enzyme that catabolize complex lipid. After inactivation, cell pellet were cool down and mix with tocopherol (vitamin E) which are fat-soluble antioxidants have important effect on lipid peroxidation by 0.1% ratio.

De-watering

Cell pellet was dried at -54°C under vacuum for 70h by placing them in a lyophilizer. Teknosem TRS-2 stainless stain lyophilizer, which has -65°C condenser temperature and 2.5m 3 vacuum capacity, was used for freeze drying of the samples.
The steps of lyophilization is summarized as follows:

- Spread pretreated cell pellet as thin layers on tray and store at -80°C for an hour.
- Loading: Place trays into lyophlizer
- Freezing at atmospheric pressure: Feeze samples at -65°C. The thermal treatment phase is the most critical in the whole freeze-drying process.
- Sublimation under vacuum: Most of the water in the sample is sublimated by heating under pressure (2.5m^3).
- Desorption: The temperature is raised, typically above 0°C, to get rid of melted water molecules in the sample under pressure
- Backfill under partial vacuum: Air is flowed into chamber slowly to lower the pressure and eventually open the lid.
- Removal of Dried Product from Freeze Dryer: Scratch dried cell biomass from the tray and weight the dried biomass to calculate CDW.

Spray dryer (SD-06-Basic LabPlant) which has 1000-1500ml/h evaporation rate was also used to dry cell biomass. The steps of spray dryer is summarized as follows:

- Place 1 liter of cell culture into container. The liquid cell culture is transferred from a container to the main chamber by peristaltic pump.
- Compressed air enters the outer tube of the jet while cell culture is transferred. Heated air evaporate the liquid content of the atomised spray.
- The dried biomass are separated and collected in the collection bottle.

Cell lysis

Different phsical and chemical cell lysis methods were examined for cell lysis to increase the total lipid content. Several methods were used to physically lyse cells, including manual grinding (mortar and pestle), granulation, high frequency sound waves (sonication), french press.

Mortar and Pestle: Manual grinding is generally used for disruption of plant cell. For disruption, cell pellet was frozen in liquid nitrogen for 1h or at -80°C for overnight. Container and other apparatus (mortar and pestle, spoon) are cooled with dry ice for 1h. Frozen sample is then crushed with a cold mortar and pestle in the container.

Granulation: Cell pellet were dried by lyophilizator and grinded with using basic grinder for 2 minutes.

Sonication: The sound waves are delivered using an sonicator (Ultrasonic Homogenizer, Model 3000, 115V/60hz) with a vibrating probe. Sample were sonicated 50W for 15min in hexane with multiple short bursts (On/Off per 30sec). Biomass-hexane mixture is placed into ice while sonication process to prevent excessive heating.

French press: Cell biomass is suspended in Tris-EDTA solution. 3ml of the sample is placed into French press (French pressure cell press, TermoSpectronic) and high pressure is applied by pressing the sample with piston. When the sample is forced to pass through a tiny hole in the press, most of the cells are lysed. Two runs at 10 MPa were conducted for each sample.

For chemical lysis methods, detergent (SDS, CTAB) based method were used.

CTAB method: 50 ml of freshly prepared extraction buffer [100 mM Tris-Cl, pH 8.0, 25 mM EDTA, 1.5 M NaCl, 2.5% CTAB, 0.2% β-mercaptoethanol (v/v) and 1%

Polyvinylpyrrolidone, MW 40,000 (PVP) (w/v)] were added to 5g of cell pellet and mixed by inversion. The mixture was incubated at 60ºC in a shaking water bath (100 rpm) for 30 min.

SDS method: 50 ml of lysis buffer [10 mM Tris-Cl, pH 8.0, 10 mM EDTA, 2% SDS and 100µg/ml proteinase K were added to cell pellet and mixed. The mixture was incubated at 65ºC in a shaking water bath (100 rpm) for overnight.

Lipid extraction

Solvent based lipid extraction which is the conventional method and supercritical carbondioxide lipid extraction method were performed for lipid extraction from *Schizochytrium* sp. S31. Hexane, hexan-IPA, Ethanol, methanol-chloroform solvents were examined with both soxhet and incubation method for lipid extraction.

Soxhet extraction: Soxhlet lipid extraction was o designed for continuous extraction of lipids from a seed and/or cell into an organic solvent. Solvent containing flask is heated to exaporate haxane. Vapor rise in the larger outside tube and then diffuse into sample containing thimble. Extracted lipid return to the solvent containing flask. This circulation continues for generally 6 hours. Soxhlet apparatus was set and 5g dryed biomass subjected to cartridge. Hexane is filled into the solvent vessel. Lipid extraction was performed at 110-130 °C for 6 hours.

Incubation method: 5g dryed biomass was dissolved in 100ml solvent containing erlen-mayer and incubated at 25°C, 225rpm for 6 hours.

Supercritical carbondioxide lipid extraction: Supercritical fluid extraction was used for lipid extraction from *Schizochytrium* sp. S31 since it is the most effective and efficient way to extract valuable constituent. Optimum lipid extraction conditions were determined by using RSM which was applied to optimize the extraction conditions for the total lipid yield (g) and DHA content (%) of dried biomass using SFE. The independent variables are pressure (325 – 425 bar), temperature (40 – 60 °C), and extraction time (90–150 min). Uncoded and coded values of the independent variables together with the experimental points used according to the central composite design were shown in Table 2.4.

The experiments were conducted in a lab-scale extractor system (*Spe-ed* SFE-2, Applied Separations Inc., Allentown, PA). The system consisted of an extractor with

an internal volume of 24 ml. 5 g of freeze dried biomass was packed inside a stainless steel extraction vessel. A certain quantity of glass wool was packed into the two ends of the vessel in order to prevent the escape of the biomass particles from the extractor. All extractions were conducted under dynamic conditions. CAMO Software AS. (Version 10.3, Norway) was used to perform the experimental design and statistical analysis of SFE extraction conditions data. Contour plots obtained for extraction yield and DHA amount were superimposed by using the same software. For extraction yield quadratic model was suggested by the software program.

Table 2.4: Coded and uncoded levels of independent variables for central composite design and responses.

	Coded level			Uncoded level			Responses	
	X_1	X_2	X_3	P (bar)	T (°C)	Extraction time (min)	Lipid Yield (g)	DHA (mg/100 g)
1	-1	-1	-1	325	40	90	1.5038	15.80
2	1	-1	-1	425	40	90	1.4632	15.98
3	-1	1	-1	325	60	90	0.8939	15.57
4	1	1	-1	425	60	90	1.3654	15.25
5	-1	-1	1	325	40	150	1.3274	14.77
6	1	-1	1	425	40	150	1.5143	16.35
7	-1	1	1	325	60	150	0.7820	13.64
8	1	1	1	425	60	150	1.0681	14.79
9	-1.681	0	0	290.9104	50	120	0.9971	16.52
10	1.6818	0	0	459.0896	50	120	1.3864	17.30
11	0	-	0	375	33.1821	120	1.3798	15.19
12	0	1.6818	0	375	66.8179	120	1.1330	14.92
13	0	0	-	375	50	69.6	0.9145	13.04
14	0	0	1.6818	375	50	170.4	1.3384	17.68
15	0	0	0	375	50	120	1.1044	14.41
16	0	0	0	375	50	120	1.1125	14.62
17	0	0	0	375	50	120	1.0896	14.18

2.7 Analytical Methods

2.7.1 Cell dry weight measurement

Cell culture was centrifuged (4800xg, 10 min) at 4 °C, and then freeze-dried for 70h at -54°C. An alternative method, 1g of dried biomass was placed in oven at 95°C. The weight of the sample and container was measured periodically until there is no further decrease. Dry cell biomass was weighed and calculated g/l of the biomass as given below:

$$\boldsymbol{Cell\ dry\ weight\ (g/L)} = \frac{weigh\ \ of\ dried\ biomas}{culture\ volume} \times 1000$$

2.7.2 Glucose quantification

2.7.2.1 Chemical assay

Dinitrosalicylic colorimetric method was used to measure residual glucose in the fermentation medium. This method tests for the presence of free carbonyl group (C=O) in the solvent. Chemical reaction shown below involves the oxidation of the aldehyde functional group to carboxyl group. 3,5-dinitrosalicylic acid (DNS) is reduced to 3-amino,5-nitrosalicylic acid at the same time under alkaline conditions.

$$\textit{aldehyde group} \xrightarrow{\textit{oxidation}} \textit{carboxyl group}$$

$$\textit{3,5-dinitrosalicylic acid} \xrightarrow{\textit{reduction}} \textit{3-amino,5-nitrosalicylic acid}$$

It is necessary to create calibration curve with the sugar used in the medium.

<u>Dinitrosalicylic colorimetric method</u>

1. Add 3 ml of DNS reagent into 3 ml of glucose sample in a test tube.
2. Heat the mixture at 90º C for 5-15 minutes to develop the red-brown color.
3. Add 1 ml of a 40% potassium sodium tartrate solution to stabilize the color.
4. After cooling to room temperature in a cold water bath, record the absorbance with a spectrophotometer at 575 nm.

Concentration of the glucose is calculated by comparing the absorbance results with standard curve.

2.7.2.2 High pressure liquid chromatography assay

HPLC analysis of residual glucose was carried out using an Aminex HPX-87H column at 60°C with a flow rate of 0.6 mL min^{-1} and 20 µL injections from 0,45 µm filtered samples in vials. 5 mM H_2SO_4 solution was used as mobile phase/eluent for HPLC measurements. Glucose standart solutions were prepared and run to obtain standard curve. Concentrations of glucose of unknown samples were identified automatically by HPLC program (Shimadzu).

2.7.3 Nitrogen quantification

2.7.3.1 Indophenol blue method

This method based on the reaction of ammonia in alkaline solution with phenol to produce a blue color. Reaction is performed with strong oxidizing agent, such as hypochlorite and can be sped up by heat or metal containing catalyst. The detection limit of this assay is reported to be about 750 µg NH4/l in the sample.

<u>Stock Reagents</u>

1) Phenol-alcohol reagent: 1g of phenol dissolved in 10ml of 95% ethyl alcohol
2) Sodium nitroprusside (nitroferricyanide): 0.1 g nitroferricyanide is dissolved in 20ml of distillated water. Wrap the tube with aluminum foil to avoid light.
3) Alkaline complexing reagent: 10 g of trisodium citrate and 0,5 g of sodium hydroxide is dissolved in DI water to a final volume of 50 ml.
4) Sodium hypochlorite: commercial bleach
5) Oxidizing solution: Mix 10 ml alkaline solution (3) with 2,5 ml sodium hypochlorite (4)

<u>Calibrants</u>

Stock Solution A: 1000 ppm ammonium (NH_4) stock. Dissolve 4.7168 g/l of $(NH_4)_2SO_4$ solution with distilled water

Stock Solution B: 100 ppm ammonium (NH_4) stock. Transfer 10 ml of Stock solution A to a 100 ml volummetric flask.

Indophenol blue method

1. Add 1 ml of standard or sample into test tubes.
2. Add 40 µl of phenol solution (1), 40 µl nitroferricyanide (2) and 100 µl ml oxidizing reagent (5) into all tubes.
3. Incubate the tubes for 1-3 h in darkness. Mix samples periodically.
4. Read absorbance of standards and unknown samples with spectrophotometer at 630 nm.
5. Use absorbance values of standards to generate a standard curve. Calculate concentrations in unknowns from standard curve.

2.7.3.2 High pressure liquid chromatography assay

For the measurement of glutamate, samples were run on HPLC (Shimadzu) equipped with a manual injector and UV/visible detector. Both the MSG standards and samples containing residual MSG were filtered with 0,45um filter and applied to DNFB derivatization reaction described as Lateef et al. (2012). All procedure should be performed under chemical hood.

DNFB derivatization and HPLC analysis

- Adjust pH of the standards and the samples to 7,8.
- Transfer 500 µl of standard solutions and samples to a test tube and add 10µL of DNFB under chemical hood.
- Shake the mixtures for 3 hours at 40°C in the dark.
- Acidify the remaining aqueous solution with 50 µL hydrochloric acid (6M)
- Extract the excess of DNFB with 0.5-1 mL diethyl ether until the ether no longer gave color
- Evaporate the traces of ether with rotary evaporator and collect the leftover residue with 500 µL methanol
- Apply 20 µl of the derivatized sample to a reverse phase column (C_{18}, 5 µM, 25 x 0.46, Kromasil),
- Mobile phase consist of methanol: water (1:1) with a flow rate of 1.2 mL / min at ambient temperature 25°C and peak was detected at 254 nm.

2.7.4 Lipid characterization

2.7.4.1 Total lipid analysis

The cell biomass, solvent and extracted lipid mixture was centrifuged to obtain a clear supernatant. Supernatant containing solvent and lipid mixture was applied to rotary evaporator (Heidolph Hei-VAP Value Rotary Evaporator) to evaporate solvent. Solvent was removed from the extract at 95°C for 30 min under reduced pressure. Amount of lipid were calculated by subtraction of the flask weight before and after the evaporation. Amount of lipid recovered and its percentage in the original sample were calculated as given below:

$$\boldsymbol{Mass\ of\ lipid} = (weight\ of\ the\ flask + extracted\ oil) - (weight\ of\ the\ flask)$$

$$\boldsymbol{Lipid\ content}\ (\%) = \frac{mass\ of\ lipid\ extracted(g)}{sample\ weight(g)} \times 100$$

2.7.4.2 Fatty acid analysis

FAME analysis was performed as described before to determine DHA content of the samples that cultivated under different conditions for statistical analysis (Schlechtriem et al, 2008). Total lipid were suspended in 5 ml 0.5 N methanolic NaOH for 10 min at 100°C, and 5 ml BF3-methanol is added for methylation. After extraction with 5 ml n-heptane, followed by evaporation and NaCl addition to the solvent, the FAMEs were collected from the upper phase.

2.7.4.3 Gas chromatography analysis

FAMEs were tranffered to bottle containing Na_2SO_4/heptane to remove any residual moisture and analyzed using an Agilent 6850 GC with Agilent HP 88 (0,25 mm, 100 m, 0,2 um) column. FAMEs were identified by chromatographic comparison with standards (Sigma Chemical Co., USA).

2.7.5 Kinetic Analysis

Kinetic analyses of specific growth rate, substrate consumption yield, metabolite productivity were calculated.

2.7.5.1 Cell growth kinetics

There are several mathematical expressions proved to be used for characterizing the growth kinetics. The kinetic model cell growth can be calculated as seen in equation 2.3.

$$\frac{dx}{dt} = \mu x = \frac{\mu_m S}{Ks + S} x$$

$$\frac{1}{\mu} = \frac{Ks}{\mu_m} \times \frac{1}{s} + \frac{1}{\mu_m} \tag{2.3}$$

where μ_m is the maximum specific growth rate (h^{-1}), Ks is the substrate concentration at which the reaction rate is half its maximal value and S is the substrate concentration. (gl^{-1}).

2.7.5.2 Glucose utilization kinetics

The substrate utilization kinetics is given by the equation 2.4, which considers substrate conversion to cell mass and maintenance.

$$\frac{dS}{dt} = r_s = \frac{r_x}{Y_{x/s}} + mX$$

$$\frac{r_s}{X} = \frac{1}{Y_{x/s}} \times \frac{r_x}{X} + m \tag{2.4}$$

Where the $Y_{X/S}$ a is yield factor, m is the maintanence coefficient, r_x/X is the specific growth rate (h^{-1}) .

2.7.5.3 Nitrogen uptake kinetics

The nitrogen uptake kinetics is given by the equation 2.5.

$$\frac{dN}{dt} = r_n = \frac{\mu_x}{Y_{X/N}} \tag{2.5}$$

Where the r_n is the nitrogen utilization rate, μ_x is the growth rate and $Y_{X/N}$ is the yield factor.

2.7.6 Lipid qualification analysis

2.7.6.1 Peroxide analysis

PUFAs are prone to autoxidation (oxidative rancidity) because of double bonds in their structure. Generally, autoxidation is determined by the peroxides which are intermediates in the autoxidation reaction. Free radical is formed in the autoxidation reaction that leads to form off-flavours. Modified peroxide value determination protocol was used (Armstrong and Browne, 1994).

- Weigh 1 g of sample into a 250 ml glass Erlenmeyer flask.
- Add 25 ml of the acetic acid - chloroform solution (15ml Acetic Acid and 10ml Chloroform)
- Swirl the flask until the sample is completely dissolved
- Add 1 ml of saturated potassium iodide solution.
- Swirl the mixture for exactly 1min.
- Add 30 ml of distilled water and shake vigorously to form chloroform layer.
- Fill the burette with 0.1N sodium thiosulfate.
- Titrate slowly with mixing until the color lightens.
- Add 1 ml of starch solution as indicator.
- Titrate until the blue gray color disappears in the aqueous (upper layer).
- Accurately record the titrant used in ml to two decimal places.

<u>Calculations:</u>

$$\boldsymbol{Peroxide\ value} = \frac{(S - B) \times N\ thiosulphate}{weight\ of\ sample} \times 1000$$

$$\boldsymbol{Peroxide\ value} = (S - B) \times\ N\ Thiosulphate\ \times\ 200$$

S = titration of sample

B= titration of blank

2.7.6.2 Free fatty acid (FFA) analysis

The free fatty acids (FFAs) and the non-polar components are separately recovered and measured in the following procedure:

- Weigh 0.1-10 g of lipid (according to the expected acid value) in glass vial and dissolve in at least 50 ml of the solvent mixture (95% ethanol/diethyl ether, v/v).
- Add a 5 drops of indicator (1 % phenolphthalein in 95% ethanol)
- Titrate the mixture with the 0,1M KOH and shake the solution until the pink color persisting for at least 10s.

Calculation:

$$\boldsymbol{Free\ Fatty\ Acid} = \frac{(V \times C \times M)}{10 \times m}$$

V= KOH consumption volume (ml)

C= KOH concentration (N)

M= Molecular weight of Oleic Acid (282 g/mol)

m= Sample weight (g)

2.8 Genome Analysis

2.8.1 Genomic DNA extraction

Phenol chloroform isoamyl alchol method described before was used to extract genomic DNA from the *Schizochytrium* sp.S31 (Chakraborti et al, 2006).

1. 400mg of cell pellet mixed with extraction buffer [100 mM Tris-Cl, pH 8.0, 10mM mM EDTA, 2% SDS, 100ug/ml proteinase K].
2. The mixture was incubated at 60ºC in a shaking water bath (100 rpm) for 30 min.
3. Equal volume of phenol : chloroform (1:1) was added and mixed by gentle inversion for about 10 min.
4. The mixture was spun at 7200 g for 5 min at 25–30ºC.
5. Upper clear aqueous layer was carefully transferred to another tube.
6. Equal volume of chloroform: Isoamyl alcohol (24:1)or chloroform was added and mixed by gentle inversion for about 5 min and spun at 7200 g for 5 min at 25–30ºC.
7. The upper clear aqueous layer was transferred to another tube.

8. One-tenth volume of 3 M Sodium acetate, pH 5.2 and double volumes of 100% chilled ethanol was added and allowed the mixture to stand at room temperature for 30 min.
9. Fibrous nucleic acid was scooped and transferred to a 1.5 ml microfuge tube. Alternatively, after mixing with sodium acetate and ethanol, the samples can be centrifuged at 11200 g for 10 min.
10. The supernatant was discarded and the pellet washed with 70% ethanol.
11. The resultant pellet was dried in a vacuum for 15 min or by keeping the tube for 1-2 h inside the laminar airflow and dissolved in 400 µl of TE (10:1) buffer
12. 8 µl RNase A (10 mg/ml) for large scale and small scale, respectively, were added and incubated at 37ºC for 30 min.
13. The mixture was extracted with equal volume of phenol: chloroform (1:1).
14. The aqueous layer was transferred to a fresh 1.5 ml microfuge tube and double volumes of 100% chilled ethanol was added, mixed and kept at -20º C for 20 min and spun at 11200 g for10 min at 25–30ºC.
15. The pellet was washed with 70% ethanol.
16. The pellet was vacuum dried for 15 min or the tube may be kept for 1-2h inside the laminar airflow for drying the pellet and dissolved in 100 µl of TE buffer for large scale and small scale preparation

2.8.2 Genome size expectation

- Growth the cells to exponential phase
- Take 1-1,5 ml of cell culture and harvest
- Mix cell pellet with 1ml of cold methanol and incubate at -20°C for O/N to get rid of pigments
- Centrifuge at 6000xg for 5min and aspirate residual methanol
- Wash the pellet for two times ans resuspend it with 500µl PBS
- Add 5µl of SyBr Green. Stain at room temperature for 20min and then placed on ice.
- Use Influx to analyze G1 and G2 phases (FSC(X)- 530(y)-[488])
- Run the known samples (E-coli, yeast, Tp diatom) to create standart curve
- Run the unknown sample. Get mean value of each peaks
- Estimate genome size.

2.9 Random Mutagenesis

2.9.1 Death curve analysis

Death curve analysis is performed before constraction of mutant library. The first stage involved mutation of the wild type *Schizochytrium* sp. culture by ultraviolet radiation (UV) using a 250nm wavelength lamp placed 7 cm over the culture. Samples consisted of 5 mL cultures at a concentration of 10^6 cell/ml. Cell culture is divided into five petri dish and exposed to UV light for 5 sec, 15sec, 30sec, 45sec, 60sec respectively for death curve analysis. Samples were then stored in darkness for 1 day to avoid photoreactivation. The mutated cultures were plated after appropriate dilution, and the resulting colonies were recovered and counted. UV irradiance time that cause fifty percent survival rate is considered to be optimal for mutant generation.

Cell culture is also mutagenized with ethyl methansulfonate (EMS, Sigma). Cell cultures at a concentration of 10^6 cells mL^{-1} were treated with 0.28 M EMS in phosphate buffer, pH 7 in the dark in the shaker. 5 % sodium thiosulfate were added at 5, 15, 30, 45, 60 and 90 min to stop the reaction. Mutated cells were washed and plated onto agar plate to determine fifty percent survival rate.

Untreated cells were used as a control.

2.9.2 Construction of mutant library

Construction of the mutant library was carried out according to protocol described by Manandhar-Shrestha and Hildebrand, (2013).

- *Schizochytrium* sp. S31 cell culture is grown to early-exponentially phase and then harvested
- Cell pellet were washed and resuspend with F/2 medium at 3×10^6 cells mL^{-1}
- Resuspended cells were placed in a sterile 60-mm petri dish covered with two layers of a Kimwipe
- Cells were exposed to UV light (UVP CX-2000) at the maximum intensity (1 joule cm^{-2}) at a distance of 7 cm for 30 sec.
- After exposure, samples were then stored in dark for overnight to avoid photoreactivation.
- For chemical mutagenesis, washed and resuspended cells were incubated with 0.28M EMS for 40 min of mutagenesis, which resulted in 50 % survival.

- The mutated cultures were inoculated into both liquid and agar growth medium and incubated at 25 °C for 2-3 days.
- Mutant culture harvested and washed with nitrogen and potassium free (NP-) fermenter medium.
- Washed cell pellet was dissolved and inoculated into NP- medium, and incubated for 24 hours at 20-25 °C for lipid induction.
- Constructed and induced library is screened by fluorescent activated cell sorting (FACS).
- Mutant library construction and screening procedure is illustrated in Fig2.4.

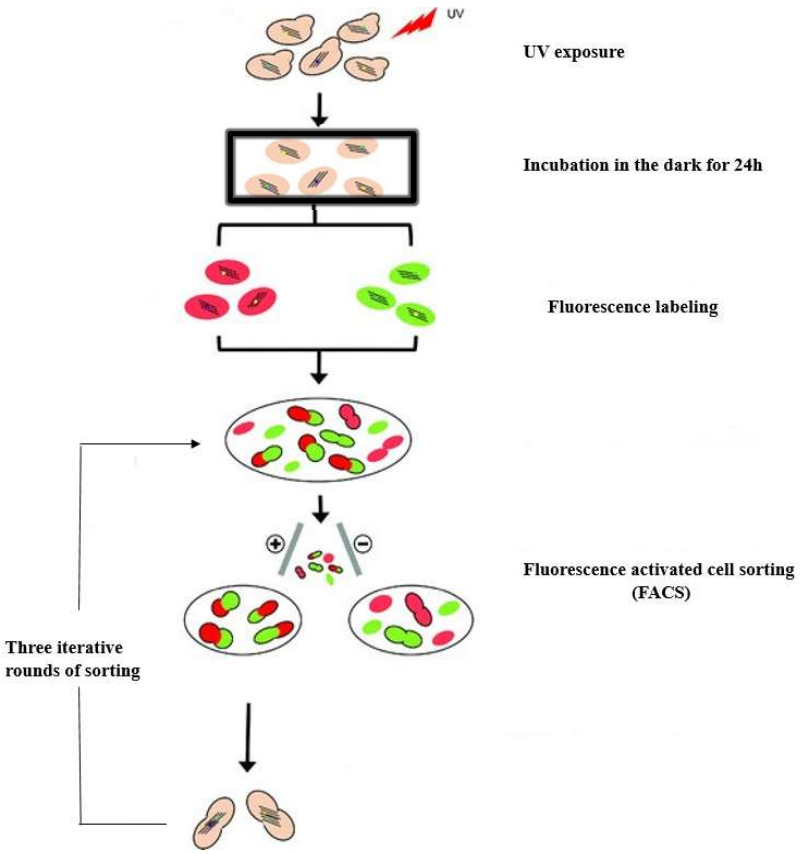


Figure 2.4: Ultraviolet (UV) radiation mutant library construction and screening.

2.9.3 Screening the mutant libray

2.9.3.1 Staining by fluorescent dye BODIPY

BODIPY (4,4-difluoro-3a,4adiaza-s-indacene) (Invitrogen), a lipophilic bright green fluorescent dye was used for detection of intracellular lipids in *Shizochytrium* sp. For analysis, cell culture were washed and resuspended in 0,1 M potassium phosphate

buffer (pH 7), and stained with 2.6 µg mL^{-1} BODIPY by incubating for 20 min at room temperature and then placed on ice.

2.9.3.2 Fluorescent activated cell sorting (FACS)

High throughput screening of the mutant library was performed by Fluorescence Activated Cell Sorting (FACS). Modified cell sorting protocol was performed as described before (Manandhar-Shrestha and Hildebrand, 2013). Cells (approximately 10^6 cells mL^{-1}) were harvested and stained with BODIPY. 488-nm laser was used for excitation on a Becton Dickinson Influx sorting flow cytometer. Cells containing high lipid content were collected from the top of cells in terms of BODIPY fluorescence. 100,000 cells were sorted for growth in the liquid medium containing antibiotic. grown cell culture were used for further resorting.

<u>Fluorescence Activated Cell Sorting (FACS) Protocol</u>

Powering up the system

- Turn on the main power.
- Turn on the electronics and cytometer computer.
- Turn the key on each laser.
- Open the BD FACS Sortware sorter software to start the software.

Preparing the fluidics tanks

- Fill the sheath tank with up to 2 L of sheath fluid (1X PBS). Close the tank lid carefully.
- Attach the sheath line to a 0.2-µm filter. Make connections of air line and tank line.
- Empty the waste tank and place it next to the sheath tank, and then attach the waste line input.
- Pressurize the both sheath and waste tank by pressing vacuum on.
- The pressure should be 33PSI for small nozzle, 17,1 PSI for large nozzle.
- Adjust the pressure level by using sheath pressure regulator knob

Flushing the system

- Remove the nozzle from the sort head and place the bucket under the nozzle

- Press RINSE and run for at least 30 seconds, checking that the lines are full of fluid.
- Stop the flow by pressing RINSE again.

Cleaning the nozzle tip

- Flush the nozzle in the opposite direction by using syringe with 0.2-µm filtered water
- After ensuring that the nozzle tip is clean, connect it to the system.

Removing bubbles from the sample line

- Fill the glass spoon with sheath fluid and place it on top of the flush bucket.
- Press PURGE to pull fluid up through the nozzle tip and remove air from the system.
- Press PULSE to free additional bubbles that might be trapped in the nozzle.
- Press RUN to start a stream with the glass spoon still in place.
- Verify that the stream is flowing straight out of the nozzle tip

Backflushing the sample line

- Press RUN to start the stream.
- Press BACKFLUSH to backflush the sample line.
- Turn backflushing off by pressing BACKFLUSH again

Introducing a sample into the system

- Use only BD Falcon 5-mL polypropylene sample tubes. Filter all samples to 40 µm to prevent nozzle clogs.
- Fill sample tube with up to 3 mL of sample
- Place the filtered sample into the sample tube holder and pull the sample tubelock lever
- Press SAMPLE to begin running the sample.
- If it is needed, press BOOST for a few seconds to temporarily boost the sample higher than the sheath pressure to introduce the sample into the sample line quickly.
- Create an Forward Scatter (FSC) vs Side scatter (SSC) dot plot to view the event scatter.
- Monitor the event scatter in the plot.

- Increase the sample pressure with the SAMPLE pressure regulator knob
- Monitor the sample flow in the pinhole monitor.
    - If the flow rate is too high, the sample core appears very large.
    - If no beads are observed flashing in the stream, then the flow rate is too low
- Create an Forward Scatter (FSC) vs BODIPY [488] plot for analyzing and sorting the cells
- Press SAMPLE to stop the sample run.
- Remove the tube and then press BACKFLUSH to allow the residual sample to backflush.
- Empty the waste tank.
- Three rounds of sorting is performed to obtain desired mutant profile.

2.9.4 Characterization of mutants

2.9.4.1 Image Stream analysis

TAG accumulation was monitored using BODIPY fluorescence by using an ImageStreamX (Amnis Corp., Seattle, WA) imaging flow cytometer. Modified cell sorting protocol was performed as described before (Manandhar-Shrestha and Hildebrand, 2013). Approximately 1×10^7 cells were taken from a culture at different times during the cultivation and stained with BODIPY for lipid analysis. For the collection of the desired data on debris, cell classifier parameters were set to capture images with a minimum and maximum area of 50 μm^2 and 300 μm^2 respectively. The bright field image was collected in channel 4, the side scatter image in channel 6 (Ex 785 nm, Em 745–800 nm), BODIPY fluorescence in channel 2 (Ex 488 nm, Em 470–560 nm) and chlorophyll autofluorescence in channel 5 (Ex 488 nm, Em 660–720 nm). The 488 nm laser was set at an intensity of 15 mW and the bright field 785 nm laser to 2 mW. Magnification is set to 40X. Core velocity value is set to 66 for the analysis. Core tracking shoud be adjusted for the bead fluids. Neutral filters can be used if the intensity is too high to measure. For analysis, cells were stained with 2.6 μgmL^{-1} BODIPY (4,4-difluoro-3a,4a- diaza-s-indacene) (Invitrogen,USA) by incubating for 20min at room temperature. After 20min, samples were run through the ImageStream by using two neutral filters. Data were collected on 5,000-10,000 cells.

ImageStream Operation Guide

- Power up ImageStream and start up the INSPIRE™
- Check the botlles. Fill the Rinse bottle with rinse fluid and Sheath bottle with shealt fluid. Empty waste bottle and fill with 50ml of 10% bleach.
- Start Initialize Fluidics to run Speedbeads through the brightfield.
- Load default template (Critical settings: BF in Ch4@ 800 counts; 785 Ex laser @2mW; Ch4 stage setting488(BODIPY) :15, Magnification: 40X; Collection filter: open; Core tracking: 5 (relative); Focus tracking: 1(relative); Autotracking: offset=0; Bining: None; Filters: Neutral density filters (2); Cell classification: upper limit= 300, lower limit= 50 for channel4; Percent Beads:100, Diameter = 10 microns, Velocity = 66 mm/sec).
- In the Image Gallery view menu, select ALL to see both beads and cells
- Click Run/Setup and choose Beads view to see only beads
- Press ASSIST tab and run calibrations and tests by clicking Start All after the Flow Speed CV is consistently less than 0.2%
- Click Flush, Lock, Load (FLL) and load the sample in the experiment. It's critical to adjust the intensity. For adjusting 466nm intesity, check column2. If the intesity of the light increased, red dots on the cell surface indicating saturated intensity can be appear and cause the misinterpret the results. Use two neutral filter to reduce the saturated intesity.
- Turn on the 488 laser and set the Laser Power to 15 Mw. Turn on the 785 laser for the brightfield.
- Set cell classification criteria (upper limit= 300, lower limit= 50) to eliminate collection of unwanted objects.
- Enter the number of cell to acquire (5000-1000), file name, destination and events to acquire.
- Click Run/Acquire to collect and save the data file
- Once the run finishes, Click Flush, Lock, Load to load the next sample.
- After analyzing, sterilyze the system by pressing Sterilize System.

Image Stream data were analyzed using IDEAS™ software and plotted as total lipid vs. [488] BODIPY fluorescence. A compensation matrix was created to eliminate crossover fluorescence. Raw data were analyzed by selecting single, focused cells in

IDEAS. After elimination of clogs and cell debris, 5000 cells were analyzed per sample.

ImageStream Data Analyzing Protocol

- Open Tools-Batch data files-Add batch-Select .rif files- Submit batch
- Select template-BODIPY.ast- convert .rif files to .daf files
- Open Report-Define statistical report-Select Parameters (Count, Intensity McCh02, Mean, St deviation of single focused cells)
- Open Report- Generate Statistical Report- Add .daf files- insert report name- press OK

2.9.4.2 Analysis of fatty acid profile of selected mutants

Each mutant and wild type strain were analyzed by gas chromatography mass spectrometry (GCMS) to determine the fatty acid profile and strains with the highest DHA content were selected.

Quantitative analysis

- Grow cell culture (both mutants and wild type) to mid exponential phase. Harvest cell culture and resuspend with NP- fermenter medium. Incubate at 25°C for 4h to induce lipid production.
- Harvest cell culture and rinse with 0.4M ammonium formate for 2 times.
- Store cell pellet at -80°C for further usage.
- Freeze-dry samples for 5h to determine weight of pellet for quantitative analysis.

Fatty Acid Methyl Ester (FAME) preparation and analysis by GCMS

- Resuspend the dry biomass in 1ml methanolic acid
- Add 50µl nonadecanoic acid methylester standard (C19:0 5mg/ml hexanes)
- Vortex tubes for 1 min.
- Incubate at 65 °C for 30 min, vortex, incubate 15 more minutes
- Cool down
- Add exactly 1 ml of hexanes by glass syringe and vortex for 30sec
- Spin tubes for 5 min to separate layers. Top: hexanes, bottom: methanol/water
- Pipet more than 0.5 of hexanes (top layer) carefully into GCMS vial.
- Store the vials at 4 °C

Standard Curve preparation

- Add 5mg of Nonadecanoic acid which is used as standard into 1ml methanolic acid in glass tube and vortex for 1 min to resuspend
- Incubate at 65°C for 15 min, vortex, incubate 15 more minutes
- Add exactly 1 ml of hexanes by glass syringe and vortex for 30sec
- Spin tubes for 5 min to separate layers. Top: hexanes, bottom: methanol/water
- Pipet more than 0.5 of hexanes (top layer) into GCMS vial.
- Add the following amounts to 1ml (total) hexane in GC-MS vials: 0µl, 1µl, 5µl, 10µl, 50µl, 100µl to otain Nonadecanoic acid standards

Running a GCMS sample

GCMS was run on an Agilent 7890A GC system connected to a 5975C VL MSD quandrupole MS (EI). Samples were separated on a 60m DB23 Agilent GCMS column using helium as carrier gas gradient of 110°C to 200°C at 15°C/min, followed by 20minutes at 200°C.

Edit the sequence (queue)

- Create a new folder by clicking datapath browse
- Fill the 'Name', 'Type', 'Vial Number', 'Method' and 'Datafile' to determine each sample name and method used in the GCMS
- Press 'Sequence >Save sequence as' to save the sample in folder
- Press 'Sequence>Run sequence' to run anaylsis.

Analyzing Data

- Launch 'GCMS data analysis' program on the computer.
- Browse the saved folder and double click on the each samples
- Chromatogram and MS spectra is shown on the screen
- For identifying peaks, right click the peak and select MS spectra of peak. Go to 'spectra', 'NIST library' to access NIST library and matches.
- For integratin peaks, Open chromatogram and click on 'integrate' symbol. Peaks will be recognized (retention time shown on chromatogram) and peaks integrated.
- For Exporting data, Export the data to an excel file by pressing to 'export', export to XLS

3. RESULTS AND DISCUSSION

3.1 *Schizochytrium* sp. S31 Growth Curve

The optical density of the *Schizochytrium* sp. S31 (ATCC20888) culture was measured by Spectronic 20D+ spectrophotometer, at the wavelength of 750 nm (OD_{750}). Figure 3.1 shows the algal growth in 50ml complex medium in erlen flask over the time.

Samples were diluted and optical density was measured by using spectophotometer. Cell counting of the samples by using counting chamber Hausser Scientific Co, 0,100mm deep and cell dry weight measurement were also performed. The stages of a typical growth curve were determined from the Figure 3.1.

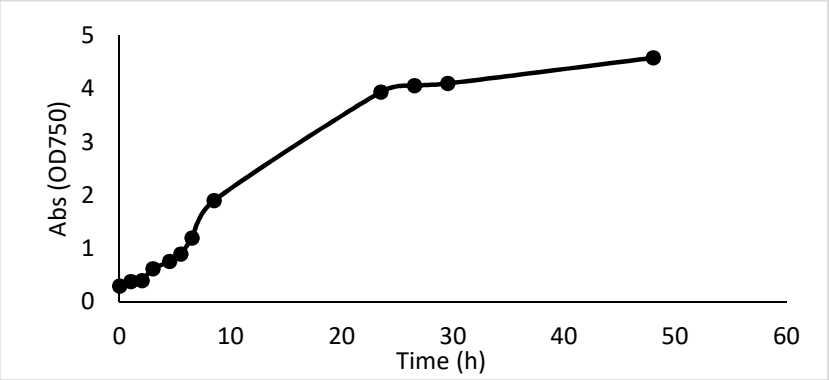


Figure 3.1: Growth curve of *Schizochytrium* sp. S31 microalgae.

According to Figure 3.1, *Schizochytrium* sp. S31 cells were in lag phase between 0-4 hours, exponential growth (log) phase between 4-30 hours and stationary phase between 30-52 hours when it was cultivated in 50ml complex medium at 25°C, 225rpm. Cellular growth were slowed down and cells were entered to stationary phase earlier than expected (at 30h) due to lack of oxygen and nutrient.

3.2 Cryoprotection Efficiency

Cryopreservation was endorsed as the best method for long term storage as the result of many other studies and reviews. *Schizochytrium* sp single colony was picked and grown in liquid medium at 25°C, 220rpm for 72h. Grown cultures were harvested and

washed with F/2 medium. Three different cryoprotectants (DMSO, glycerol and methanol) and two different storage cabinets (liquid nitrogen and -80 °C freezer) were compared (Figure 3.2). Methanol was found to be ineffective cryoprotectant for the *Schizochytrium* sp. S31 strain because of the viability was not persisted during storage period. Cell number of the stock cultures were counted during time to understand the effectiveness of cryoprotectant solution and storage method.

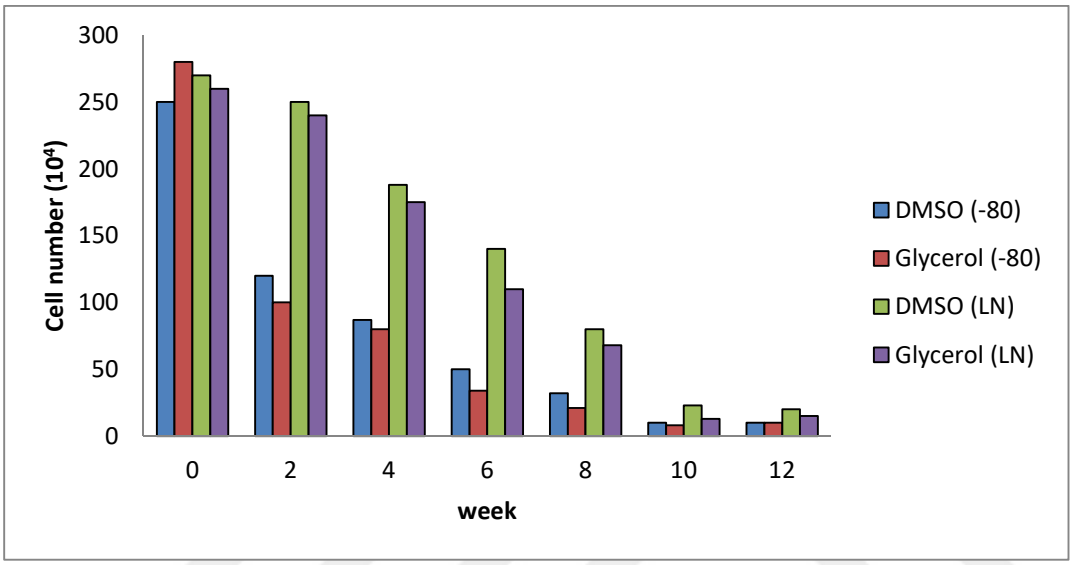


Figure 3.2: Survival rate of *Schizochytrium* sp. S31 with different cryoprotectant and storage conditions.

According to Figure 3.2, cell number of *Schizochytrium* sp. S31 decreased dramatically during time. Because of this reason, passage technique in which master stock is renewed every month is used for this study. Storing at liquid nitrogen tank was found to be more effective than storage at -80 °C freezer. Combination of the cryoprotectant solutions can also be used to increase viability. Nakanishi et al. (2011) found that approximately 50% survival rates were observed using a cryoprotectant mixture of 5% DMSO, 5% EG, and 5% proline. As a result, *Schizochytrium* sp. S31 was stored at liquid nitrogen with cryoprotectant mixture of 5% DMSO and 5% glycerol for long term storage.

3.3 The Influence of Medium Composition on Biomass and Lipid Concentration

The influence of alternative carbon, nitrogen and salt sources, and their different concentrations in the medium was illustrated in Table 3.1. In this section, an experiment was conducted to compare the effect on growth when different carbon,

nitrogen and salt sources are replaced with alternative components as a substrate. For this experiment, fermenter medium was supplemented with different carbon sources at a concentration of 40 g/L, 80 g/l, 120 g/l and different nitrogen sources were added at a concentration of 0.165 g/l, 0.33 g/l, 0.66 g/l and different salt sources were added at a concentration of 12.5 g/l, 25 g/l. As seen in table, *Schizochytrium* sp. S31 cell growth and lipid production are influenced by medium composition. Glucose, glucose syrup and glycerol supported both cell growth and lipid production. In contrast, molasses led to a poor cell growth. At the same mass concentration of the carbon substrate, the algae growing on glucose or glucose syrup exhibit a much higher growth rate and yield. Glucose provided the most effective carbon source for biomass and lipid productions. Among the four complex nitrogen sources, MSG, yeast extract was the most suitable nitrogen sources for biomass and lipid production. It is known that microalgae are able to assimilate nitrogen from several different substrates, including NH_4^+, NO_3 , urea, amino acids, and purine and pyrimidines bases (Collos and Berges, 2003). The bioavailability of each nitrogen substrate differs markedly and affects in turn the growth rate of algae. Previously, it has been reported that yeast extract and pepton are excellent nitrogen sources for the heterotrophic growth of *Schizochytrium* sp. species (Chen et al, 2010).

Table 3.1: Alternative medium components and their response on biomass and lipid production**.**

Conc. (g/l)	**Biomass (g/l)**				**Total Lipid (%)**			
	C1	**C2**	**C3**	**C4**	**C1**	**C2**	**C3**	**C4**
40	4.99	4.5	4.1	2.1	13.5	11	10.5	4
80	5.84	5.01	4.8	2.5	14	11.2	11	3.8
120	6.92	6.1	5.8	2.7	14.8	12.5	11.7	4.1
	N1	**N2**	**N3**	**N4**	**N1**	**N2**	**N3**	**N4**
0.165	2.99	5.1	8.6	9.1	5.5	13	12.5	14
0.33	2.67	6.01	9.2	11.5	6	14.1	13	13.8
0.66	2.93	7.2	10.2	11.8	6.1	14.5	11.9	14.1
	S1	**S2**	**S3**	**S4**	**S1**	**S2**	**S3**	**S4**
12.5	12	10.5	10.5	11.9	14	13.2	13	14.2
25	11.5	10.8	11	12	14.5	13.8	13.9	14.7

*C1: Glucose; C2: Glucose syrup; C3: Glycerol; C4: Molasses
*N1: Ammonium sulphate; N2:Diammonium tartrate; N3: Ammonium bicarbonate; N4: MSG
*S1: Sea salt; S2: Sodium sulphate; S3: Sodium chloride; S4: Mineral Salt

Similar research were conducted by Zhou et. al., (2007) and they found that glucose and fructose were most effective for both cell growth and DHA production. As a nitrogen sources, they observed that mixture of yeast extract, corn steep liquor, soy peptone led to more DHA yield than the single nitrogen sources (Zhou et al, 2007). In this thesis, most experiments were conducted using monosodium glutamate as a nitrogen source. Both sea salt and mineral salt can be used as salt sources for *Schizochytrium* sp. S31. Increasing the initial glucose and nitrogen concentration in the medium increased the biomass and lipid production.

3.4 The Influence of Physical Conditions on Biomass and Lipid Concentration

Physical conditions of the culture were optimized to maximize both CDW and total lipid production. Different temperature (16, 25, 30°C), pH (5, 7, 9) and dissolved oxygen (25%, 50%) were examined. Eight different experiments were conducted stepwise with two replicates. Best conditions for both biomass and lipid production were determined. Table 3.2 shows the dry biomass (g/l) result of the *Schizochytrium* sp.S31 under different physical conditions.

Table 3.2: Different physical conditions and their response on biomass production.

	Value	Biomass (g/l)
pH	5	6.01
	7	6.49
	9	4.01
Stirrer Speed (rpm)	200	6.3
	300	6.5
	400	6.9
Dissolved Oxygen (%)	25	6.5
	50	6.8
	75	7.2
Temp. (°C)	16	6.01
	25	6.47
	30	6.33

It has been demonstrated that algal growth optimum temperature was determined to be between 24-28 °C. It was also indicated that neither very low nor very high pH was suitable for algal growth (Muni , 2015). Optimum pH value for both biomass and lipid production was found to be between 7-7.5 Amount of dissolved oxygen in the culture was increased by higher stirrer speed. It is known that nutrient and dissolved oxygen in the culture distributed efficiently at higher stirer rate (Garcia-Ochoa et al, 2010). As it is seen in the Table 3.2, higher biomass was obtained at higher stirrer speed.

3.5 Effect of The Selected Factors on CDW and DHA Production

Ten variables (glucose, MSG, mineral salt, potassium dihydrogen phosphate, potassium chloride, magnesium sulphate, calcium chloride, pH, dissolve oxygen, temperature) were selected in order to study their effects on biomass and DHA production by *Schizochytrium* sp. S31 using a PB design. The experimental data corresponding CDW (g/l), total lipid (%) and DHA(%) yields are shown in Table 3.3. Twelve experiments were performed using different combinations of ten variables. Three responses (CDW, total lipid and DHA) were calculated in each combination.

Table 3.3: Plackett Burman design for screening of selected factors for CDW and DHA.

A	B	C	D	E	F	G	H	I	J	CDW (g/l)	Total Lipid (%g/g_{CDW}	DHA (%g/g_{lipid})
-1	-1	-1	-1	-1	-1	-1	-1	-1	-1	5.4	32	30.1
-1	-1	-1	1	-1	1	1	-1	1	1	5.3	23	24.9
1	1	-1	-1	-1	1	-1	1	1	-1	5.7	10.1	12.5
-1	1	1	-1	1	1	1	-1	-1	-1	8.8	10.7	13.1
1	1	-1	1	1	1	-1	-1	-1	1	9.4	2.4	10.4
-1	1	1	1	-1	-1	-1	1	-1	1	4.3	1.9	12.2
-1	1	-1	1	1	-1	1	1	1	-1	4.6	13	13.7
1	-1	1	1	1	-1	-1	-1	1	-1	4.5	31	27
1	1	1	-1	-1	-1	1	-1	1	1	9.1	3.5	12.5
1	-1	1	1	-1	1	1	1	-1	-1	1.2	29.1	32.1
1	-1	-1	-1	1	-1	1	1	-1	1	1.1	22.2	16.9
-1	-1	1	-1	1	1	-1	1	1	1	1.3	21	18.2

Highest percent lipid (32%) were obtained with 40g.L^{-1} glucose, 0.8 g.L^{-1} MSG, 6.25 g.L^{-1} mineral salt, 2.5 g.L^{-1} KH_2PO_4, 0.5 g.L^{-1} KCl, 2.5 g.L^{-1} $MgSO_4.7H_2O$, 0.1 g.L^{-1} CaCl with initial pH 6, DO 25% at 20°C. Highest percent DHA (32.1%g/gCDW) were obtained 80g.L^{-1} glucose, 0.8 g.L^{-1} MSG, 12.5 g.L^{-1} mineral salt, 5g.L^{-1} KH_2PO_4, 0.5

$g.L^{-1}$ KCl, 5 $g.L^{-1}$ $MgSO_4.7H_2O$, 0.2 $g.L^{-1}$ CaCl with initial pH 8, DO 25% at 20°C. The effect of each variable was determined with equation 3.1.

$$E = \frac{vi_{+} - vi_{-}}{N} \tag{3.1}$$

Where E is the concentration effect of the tested variable, vi_{+} and vi_ are the CDW and lipid yields from the trials at high and low concentrations, respectively, and N is the number of experiments. By applying multiple regression analysis on the experimental data, Table 3.4 was obtained automatically by programme R version 3.1.1.

For CDW production, the p value of variables MSG and initial pH are 0.0236, 0.0255 respectively (Table 3.4). These variables had confidence levels above 95% and were considered to influence CDW production by *Schizochytrium* sp. S31 significantly; at the same time, the p value of variables nitrogen source and temperature are 0.01079, 0.02357 respectively, and were considered to influence lipid production significantly. The remaining variables had confidence levels below 95% and hence, were considered insignificant. The results presented in this study showed that the cellular growth can be enhanced by adjusting MSG concentration and initial pH, while total lipid content can be increased by temperature and nitrogen limitation conditions. As it is known nitrogen is critical for protein synthesis and eventually for cell growth. Wu et al. (2005) indicated that *Schizochytrium* sp. S31 couldn't grow well and intake the carbon sources from the medium at initial pH 8.0. Under environmental stress conditions, many algae modify their lipid biosynthetic pathways to produce and accumulate neutral lipids, mainly in the form of triacylglycerol. It offers carbon and energy storage function in the cell that allows microalgae to tolerate unfavorable environmental conditions. Some researchers showed that maximum amount of lipid content was obtained with temperature and nitrogen-limited culture conditions (Rios et al, 2015; Jiang and Chen 2000).

Table 3.4: Effect estimates for DCW and DHA production from the result of Plackett Burman design**.**

	CDW Production			**Lipid Production**		
	Estimate Std.	t value	Pr(>|t|)	Estimate Std.	t value	Pr(>|t|)
(Intercept)	5.25000	63	0.0101*	1.650	99	0.00643* *
Glucose	0.25000	3	0.2048	-3.333	-2	0.29517
MSG1	2.25000	27	**0.0236***	-9.833	-59	**0.01079***
NaCl	-0.08333	-1	0.5120	-5.000	-3	0.20483
KH2PO4	-0.08333	-1	0.5230	-5.368	0	1.00000
KCl	-0.08333	-1	0.5110	1.667	1	0.50000
MgSO47H2O	0.08333	1	0.5070	-5.000	-3	0.20483
CaCl	-0.08333	-1	0.5340	3.333	2	0.29517
pH	-2.08333	-25	**0.0255***	-5.000	-3	0.20483
DO	0.08333	1	0.5000	3.333	2	0.29517
TEMP	0.08333	1	0.5510	-4.500	-27	**0.02357** *
Signif. codes: 0 '***' 0.001 '**' 0.01 '*' 0.05 '.' 0.1 ' ' 1						

The results presented in Table 3.4 showed that the cellular growth can be enhanced by adjusting MSG concentration and initial pH, while total lipid content can be increased by temperature and nitrogen limitation conditions. As it is known nitrogen is critical for protein synthesis and eventually for cell growth. At initial pH 8.0, *Schizochytrium* sp. S31 could not grow well and intake the carbon sources from the medium (Wu et al, 2005). Temperature and nitrogen limitation on several microalgae cultivations showed to be efficient in influencing both cell growth and lipid accumulation (Dean et al, 2010; Jiang and Chen, 2000). Its known that, many algae change their lipid biosynthetic pathways to accumulate neutral lipids, especially TAG under environmental stress conditions. It offers that microalgae can tolerate unfavorable environmental conditions by lipid storage function in the cell (Hu et al, 2008). Some researchers showed that maximum amount of lipid content was obtained by total nitrogen deprivation with considerable growth inhibition (Rios et al, 2015). Further experiments were conducted to determine optimum value of the selected variables to obtain highest amount of biomass and lipid production.

3.6 Optimization of Biomass Production by Response Surface Methodology

Twelve experiments were performed using different combinations of two variables. MSG (X_1) and initial pH (X_2) were analyzed to obtain maximum biomass while MSG (X_1) and temperature (X_2) were analyzed for highest lipid production. Analysis of variance (ANOVA) of the central composite design of biomass production by *Schizochytrium* sp. S31 is summarized in Table 3.5.

Table 3.5: Analysis of variance (ANOVA) of CCD for CDW and lipid production by *Schizochytrium* sp. S31.

	CDW Production				**Lipid Production**			
	Est Std.	Error	t value	Pr(>|t|)	Est Std.	Error	t value	Pr(>|t|)
Intercept	25.317	0.4563	55.47	$3.6e^{-08}$ ***	29.4121	2.097	14.0250	3.315e-05***
Block.ccd	0.1914	0.5135	0.372	0.724559	4.4217	2.360	1.8736	0.1198531
X_1	3.3763	0.2786	12.11	$6.7e^{-05}$ ***	-8.9250	1.280	-6.9699	0.000935***
X_2	-0.469	0.2786	-1.686	0.152556	-3.3593	1.280	-2.6234	0.0469033*
X_1:X_2	0.0250	0.4327	0.057	0.956165	-1.0000	1.988	-0.5029	0.6363837
X_1^2	-0.970	0.2654	-3.655	0.0146*	-5.4035	1.219	-4.4303	0.0068259**
X_2^2	-1.430	0.2654	-5.390	0.0029**	-2.2147	1.219	-1.8158	0.1290980
Signif. codes: 0 '*' 0.001 '**' 0.01 '*' 0.05 '.' 0.1 ' ' 1**								

By applying multiple regression analysis on the experimental data, the second-order polynomial was found to describe CDW production as seen in equation 3.2.

$$Y_{CDW} = 25.31720 + X_1 3.37633 - X_1^2 0.97010 - X_2^2 1.43070 \quad (3.2)$$

Where Y_{CDW} is the predicted cell dried weight, X_1 is MSG concentration and X_2 is initial pH of the medium. Total lipid production second-order polynomial was found to describe lipid production as seen in equation 3.3.

$$Y_{Lipid} = 29.4121 - X_1 8.9250 - X_2 3.3593 - X_1^2 5.4035 \quad (3.3)$$

Where Y_{Lipid} is the predicted total lipid, X_1 is MSG concentration and X_2 is temperature of the medium. The estimated standards, t-value and p-value was calculated and shown in Table 3.5. According to response surface methadology, p-value lower than $p<0.05$ is considered as significant. Significant factors on both biomass and lipid production were analyzed to obtain optimum value. The 3D surface plots and contour plots between MSG and initial pH is shown in Figure 3.3. The 3D surface plots and contour plots between MSG and temperature is shown in Figure 3.4. It explained the interaction of the variables and the optimum concentration of each variable for biomass and total lipid production.

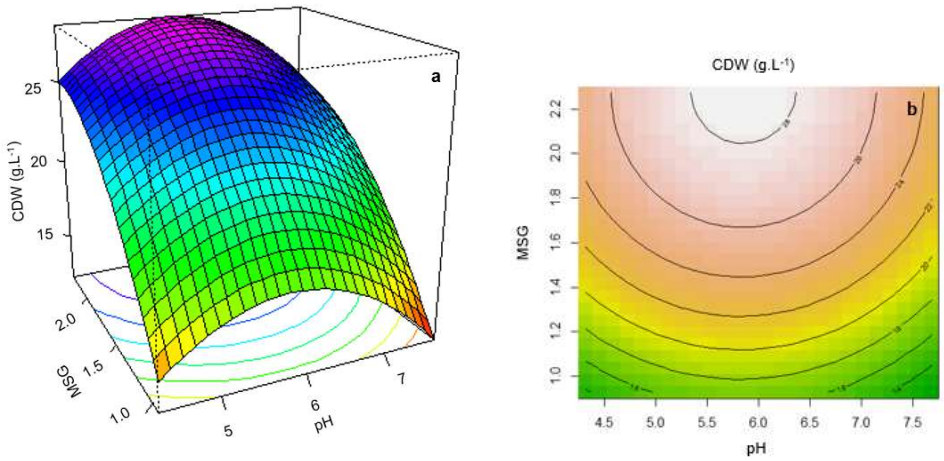


Figure 3.3: Three-dimensional response surface plot (a) and two-dimensional contour plot (b) of biomass production (CDW) showing variable interactions for MSG and initial pH.

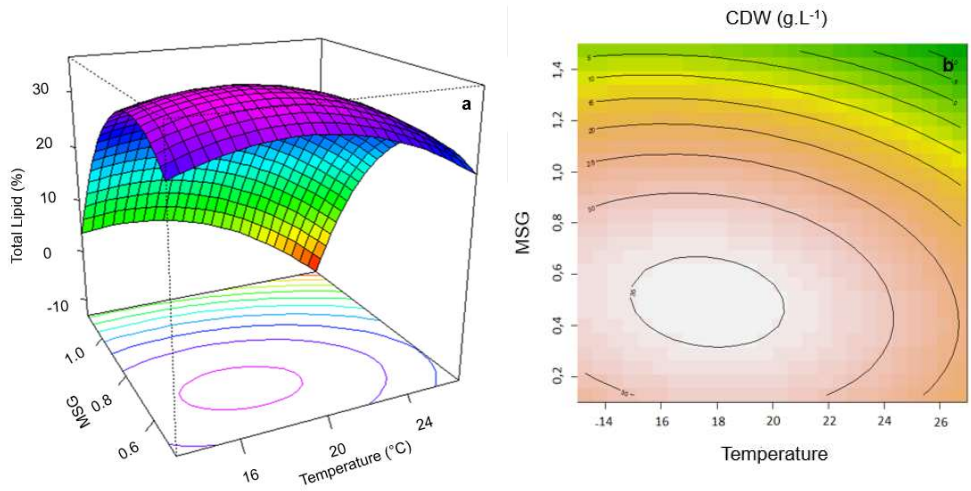


Figure 3.4: Three-dimensional response surface plot (a) and two-dimensional contour plot (b) of total lipid production (%) showing variable interactions for MSG and temperature (°C).

As shown in Figure 3.3, the optimum levels of each variable were 2.29 $g.L^{-1}$MSG, pH 5.8. Using these conditions, a maximum biomass production of 26.86$g{\cdot}L^{-1}$ was obtained. It also shows in Figure 3.4 that 0.49 $g.L^{-1}$ MSG and 17.6 °C temperature conditions are optimum for lipid production. Using these conditions, 35% lipid production was obtained. Based on contour plots and canonical analysis, 520.1 $mg.L^{-1}$ DHA have obtained with using 40 $g.L^{-1}$ glucose, 0,8$g.L^{-1}$ MSG, 6,25 $g.L^{-1}$ mineral salt, pH 6 and incubation for 48h at 20°C. Wu and Lin (2003) have also applied response surface methodology to optimize docosahexaenoic acid production by

Schizochytrium sp. S31. Based on contour plots and canonical analysis, they have obtained maximizing DHA production of 516 $mg.L^{-1}$ with using 27.98$g.L^{-1}$ glucose, 4.53 $g.L^{-1}$ yeast extraction, 24.82 $g.L^{-1}$ sodium chloride, pH 6.96 and incubation for 4 days at 30°C.

3.7 Large Scale Growth Parameters

Schizochytrium sp. is one of the heterotrophic organisms used to produce LC-PUFA. Both batch and fed-batch fermentation system is applied to produce *Schizochytrium* sp. microalgae culture under optimized conditions. OD750, Biomass, total lipid, residual glucose and nitrogen were monitored during incubation. The large scale growth of *Schizochytrium* sp. was successfully scaled up from single colony to bench-scale stirred fermentors.

Prior to using the automated control of the process variables, it was necessary to ensure that the controllers were correctly tuned. Several controls were performed before starting up.

3.7.1 PID control

An appropriate control was required to reduce the oscillation and to make reliable test. Dissolved oxygen is controlled by the stirrer rate and the aeration. Controlling the dissolved oxygen and stirrer rate is one of the most difficult challenges in scaling up a bioprocess (Rehm and Reed, 1993). Optimization of the PID controllers can be performed by using the XP, TI and TD parameters. The controller unit can be regulated by setting individual PID parameters. ZN method (best-guess-approach) was applied to test different PID values to find optimum values. The best control was obtained at the PID values shown in Table 3.6.

Table 3.6: PID values obtained by ZN method (best-guess-approach).

Field	Unit	Function	Value
MIN	%	Minimum output limitation, limit value for switch to upstream slave controller	0
MAX	%	Maximum output limitation, limit value for switch to downstream slave controller	100
DEADB	%	Dead zone in the unit of the process value	0.5

Table 3.6 (continued): PID values obtained by ZN method (best-guess-approach).

Field	Unit	Function	Value
XP	%	P share (proportional range); signal amplification of the control response proportional to the input signal	50
TI	sec	Integral portion; time function. With a higher I portion control will react more slowly (and vice versa)	50
TD	sec	Differential portion: Damping, greater D portion, damps the controller response (and vice versa)	12

Different strain generally require an appropriate PID control due to their different behavior and oxygen demand in a bioreactor. Stirring also plays a role in bioreactor allowing cells to better uptake nutrients and oxygen. The DO ("pO2") system can be controlled by the speed controller as a DO cascade control loop. When these PID values with 50 % DO as a set point applied and cascade mood is turned on, stirrer speed and dissolve oxygen change during the incubation of *Schizochytrium* sp. in 5 lt fermenter were obtained as it is shown in Figure 3.5.

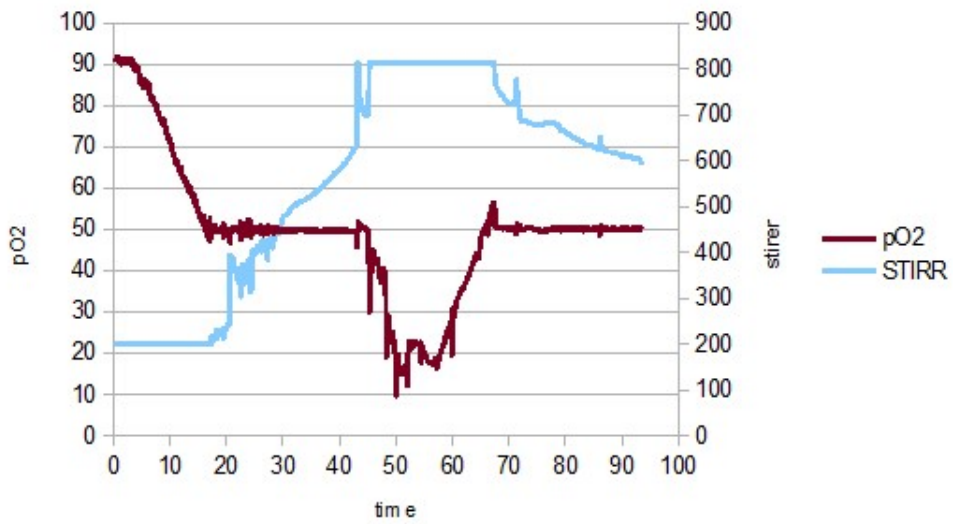


Figure 3.5: Dissolved oxygen and stirrer speed change during incubation of *Schizochytirum* sp.

In the beginning of the incubation, dissolved oxygen content was the highest level in the bioreactor. As the cellular density increased, dissolved oxygen decreased during time. Conversely, stirer speed increased to keep 50 %DO set point during the incubation. Although stirrer reaches to maximum speed between time point 45-70h, enough oxygen could not supplied to the system. Because of that, dissolved oxygen

level decrased to 10% at time point 50h. When the cellular growth reached to stationary phase, oxygen value and the stirrer speed returned to normal level.

3.7.2 pH, temperature and antifoam controller

The temperature control works like a cascaded regulation. The TEMP controller uses the temperature prob to measure the temperature in the culture vessel. The temperature of the culture was set to 25°C.When the temperature was decreased under the desired value, heat jacket was activated to heat the system. When the temperature reaches the level above the desired value, cooling chamber turned on automatically to decrease the temperature. The temperature cascade controller is operated from the master controller. The pH level of the culture was measured by pH prob and was set to pH:7 in the beginning of the fermentaion. When the pH level of the culture increased, acid pomp was activated and added several drops of HCl to the system. Conversely, when the pH level of the system decreased, base pomp was activated and added NaOH to the system until the pH level reaches to 7. Due to the tight conrol unit, both temperature and pH values were not oscillated during the incubation as it is seen in Figure 3.6. Foam occurs in bioprocesses due to the introduction of gases into the culture medium, and is further stabilised by adding antifoam (10%) to the system. Foam is detected by the probe attached to the top of fermenter lid. Antifoam solution was subjected to the culture during the incubation by peristaltic pomp to hinder overflow of the culture.

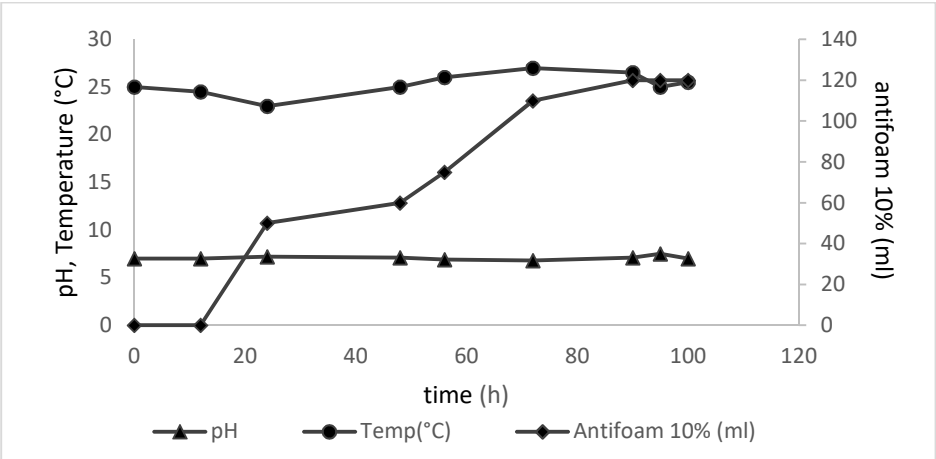


Figure 3.6: pH, temperature and antifoam change during the incubation of *Schizochytrium* sp.

Figure 3.6 shows the temperature, pH and antifoam level of the *Schizochytrium* sp. S31 culture in 5 lt bioreactor during the incubation. pH and temperature remained constant while antifoam level increased due to foaming during the incubation.

3.8 Heterotrophic Growth of *Schizochytrium* sp. S31

The large scale growth process by *Schizochytrium* sp. was successfully scaled up from single colony to bench-scale stirred fermentors. *Schizochytrium* sp. was initially cultured on agar for 72 h and a single colony was picked and grown in flasks (250 ml) containing 50 ml complex medium. Microalge culture was then inoculated into 1liter fermenter medium with 10% inoculum ratio and cultivated as batch fermentation for 48h under controlled conditions. 1 liter of culture was then used as inoculum for 5 liter fermenter medium (20% inoculum). All cultures were grown at 25°C with an initial pH of 7. *Schizochytrium* sp. was cultivated in a 5 lt working capacity bioreactor (Sartorius) in which the pH was controlled by the automatic addition of KOH (2M) and dissolve oxygen was correlated to stirrer. Fed-batch fermentation system was set to produce *Schizochytrium* sp. microalgae culture under convenient conditions. O.D750, cell dry weight, µmax, C/N ratio, %lipid, biomass productivity, DHA productivity, residual glucose and nitrogen were monitored during and after incubation.

3.8.1 Cellular growth

Cellular growth curve and growth rate were determined by measuring O.D750 and cell dry weight were measured (Figure 3.7).

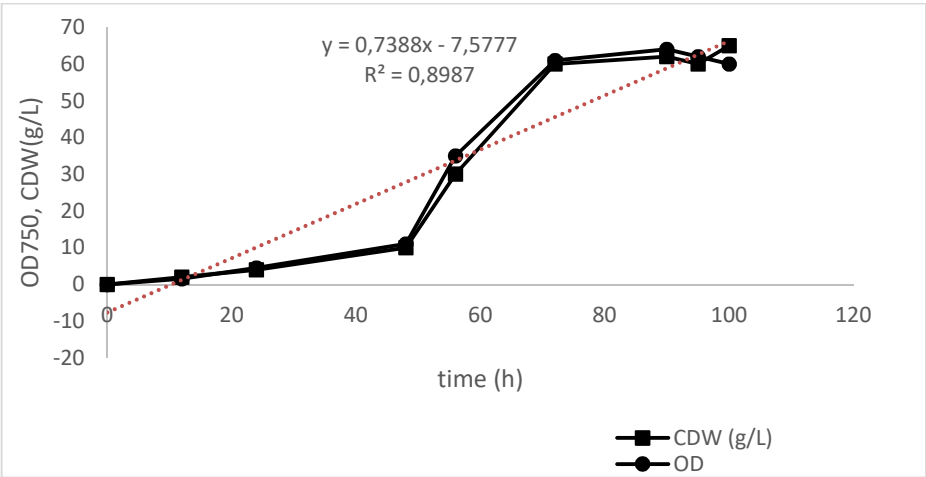


Figure 3.7: Cell dry weight and OD750 results during the incubation.

Cell dry weight results of the culture went parallel with the O.D750 results. According large scale growth curve, 0-48h is considered to be lag phase, exponential growth was occured between the 48-72h and cells were reached to stationary phase between the

72-100h. At the end of the incubation, OD_{750} was measured as 60 and CDW was calculated as 65 g/l. Higher amount of *Schizochytrium* sp. S31 cell dry weight was obtained when it was cultivated in controlled large volume bioreactor conditions due to sufficient substrate and oxygen supply during incubation.

3.8.2 Monitoring medium components

Residual glucose and monosodium glutamate in the fermenter medium were measured by HPLC. HPLC analysis of residual glucose was carried out using an Aminex HPX-87H column at 60°C with a flow rate of 0.6 mL min^{-1} and 20 µL injections from 0,45 µm filtered samples in vials. 5 mM H_2SO_4 solution was used as mobile phase/eluent for HPLC measurements. Glucose standart solutions were prepared and run to obtain standard curve. Concentrations of glucose of unknown samples were identified automatically by HPLC program (Shimadzu). Added and consumed glucose were automatically calculated by checking substrate profile on computer. Figure 3.8 shows the added and consumed glucose (g) during fed-batch fermentation and residual glucose in the medium.

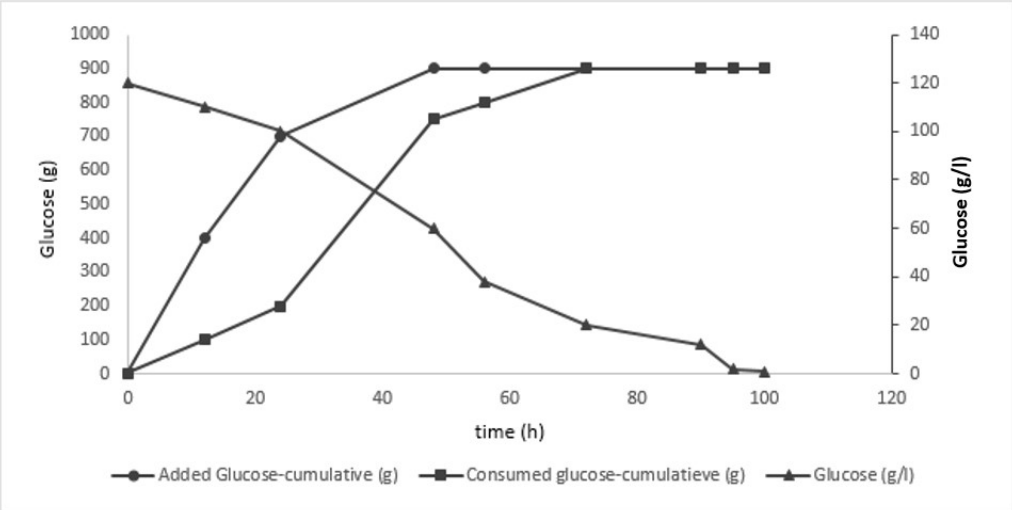


Figure 3.8: Residual, added and consumed glucose during the incubation.

HPLC analysis of resiual MSG was carried out using an Cromasil C18 reverse phase column at 25°C with a flow rate of 1 mL min^{-1} 25% acetonitrile+75% glacial acetic acid (1%) mixture were used as mobile phase. Samples were first filtered through 0,45µm filter and derivatized by DNFB. Derivatized sample were injected into HPLC

to analyze residual MSG in the medium. Figure 3.9 shows the residual MSG in the fermenter medium during incubation.

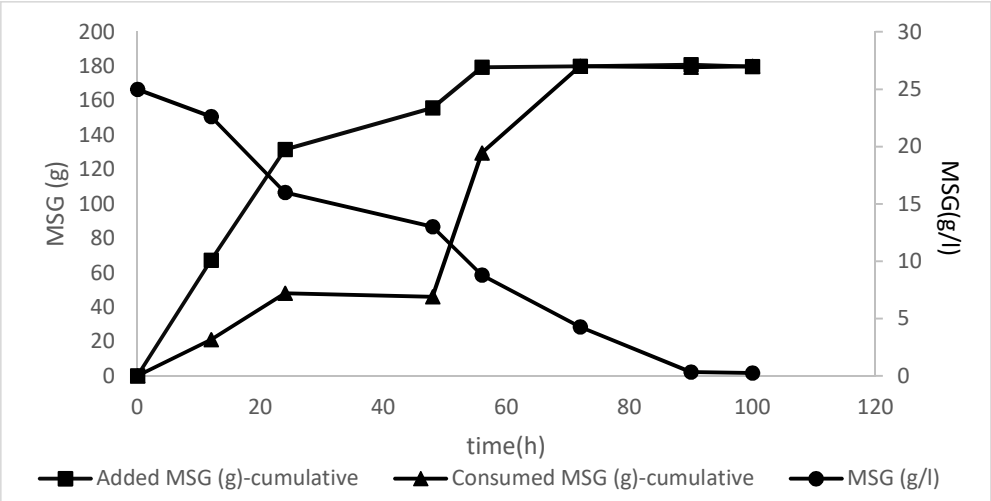


Figure 3.9: Residual, added and consumed MSG during the incubation.

Analysis of the culture media by HPLC showed an decrease in carbon and nitrogen content as cultures progressed, as illustrated in Figure 3.8 and Figure 3.9. Totally 813.8 g glucose and 180g MSG correspond to 57.6 g/l carbon and 2.98 g/l nitrogen were consumed during incubation in 5 lt fermenter. CDW/C ratio was calculated as 0.39 that could be interpreted as 39% of carbon was converted to biomass while rest of them (61%) was used as both energy and maintanence. C/N ratio of the 5lt *Schizochytrium* sp. culture was calculates as 19.32. The effect of total nutrient concentration and C/N ratio in algal growth was studied in the previous studies. The carbon to nitrogen ratio (C/N) plays a critical role in the physiology of algal cultures as shown in the previous section. According to results that Chatdumrong et al. (2007) obtained , when C/N ratio was measured between 10-20 the maximum DHA yield was obtained in microalgae.

3.8.3 Lipid analysis

Total lipid was extracted by incubation with hexane for 4 hours at 25°C, 220rpm. Solvent (hexane) based lipid extraction was performed for calculation of % lipid amount and total lipid change during large scale growth process. The cell biomass, solvent and extracted lipid mixture was centrifuged to obtain a clear supernatant. Supernatant containing solvent and lipid mixture was applied to rotary evaporator to evaporate solvent. Solvent was removed from the extract at 95°C for 30 min under reduced pressure. Amount of lipid was calculated by subtraction of the flask weight

before and after the evaporation. Amount of lipid recovered and its percentage in the original sample were calculated (Figure 3.10).

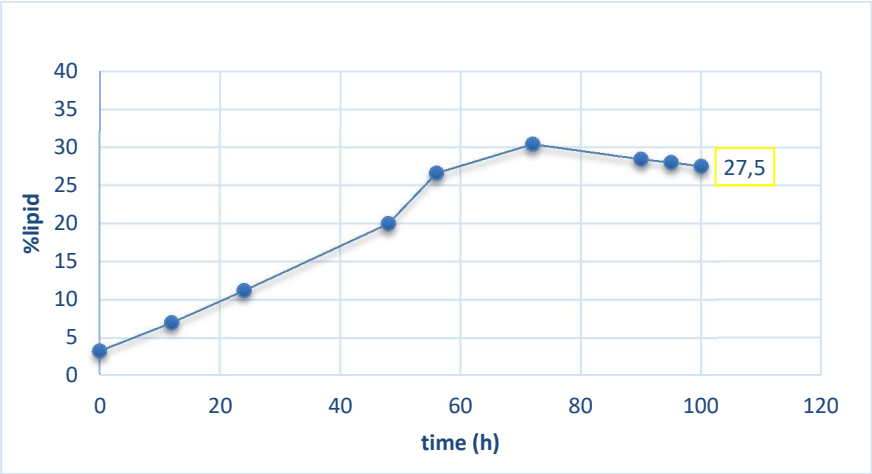


Figure 3.10: Lipid accumulation in *Schizochytrium* sp. during the incubation.

According to lipid extraction result, total lipid content was calculated as 27.5 % of the cell dry weight. The fatty acid profile of *Schizochytrium* sp. is shown in Table 3.7. As shown in the table, fatty acid compositions obtained from the GC analysis, the two fatty acids, C16:0 (Palmitic acid) and C22:6 (DHA) accounted for about 90% of total fatty acid content. These two fatty acids were found to be the major fatty acids in the algal lipid in previous studies (Pyle, 2008).

Table 3.7: Fatty acid profile of *Schizochytrium* sp. S31 microalgae.

Fatty acid	Formula	Unit	Content
Lauric acid	C12:0	%TFA	0.35
Tridecanoic acid	C13:0	%TFA	0.08
Myristic acid	C14:0	%TFA	8.69
Pentadecanoic acid	C15:0	%TFA	0.49
Palmitic acid	C16:0	%TFA	38.14
Palmitoloic acid	C16:1	%TFA	7.01
Margaric acid	C17:0	%TFA	0.21
Stearic acid	C18:0	%TFA	1.59
Oleic acid	C18:1n-9	%TFA	6.54
ɣ-Linolenic acid	C18:3n-6	%TFA	3.38
Arachidic acid	C20:0	%TFA	0.59
cis-5,8,11,14,17-Eicosapentaenoic acid	C20:5n-3	%TFA	0.75
Heneicosanoic acid	C21:0	%TFA	0.81
Behenic acid	C22:0	%TFA	0.63

Table3.7 (continued): Fatty acid profile of *Schizochytrium* sp. S31 microalgae.

Fatty acid	Formula	Unit	Content
cis-4,7,10,13,16,19-Docosahexaenoic acid	C22:6n-3	%TFA	30.18
Tricosanoic acid	C23:0	%TFA	0.55

3.8.4 Analytical calculations

All the parameter after large-scale growth were calculated and shown in Table 3.8. The initial growth rate was calculated as 0.312 h^{-1} by using growth curve. Cell dry biomass was calculated as 65 g/L after incubation. Totally 813.8 g glucose and 180g MSG correspond to 57.6 g/l carbon and 2.98 g/l nitrogen were consumed during incubation in 5l fermenter. According lipid etraction and GCMS results 27.5% of total lipid and 30.18% of DHA was obtained. Biomass, lipid and DHA productivity were calculated as 15.62 g/L.day, 0.17 g/L.hour and 0.053 g/L.hour respectively. Bacterial contamination was not observed in large scale culture.

Table 3.8: Analytical results of *Schizochytrium* sp. S31 production.

Factor	Result	value
O.D750	60	-
CDW	65	g/L
Consumed glucose	813.8	g
Consumed MSG	180	g
CDW/C	0.39	-
Consumed carbon	57.6	g/L
Consumed nitrogen	2.98	g/L
C/N	19.32	-
total lipid	27.5	%
DHA	30.18	%
Biomass productivity	15.62	g/L.day
Lipid productivity	0.17	g/L.hour
DHA productivity	0.053	g/L.hour
µmax	0.312	h^{-1}
Total bacteria	0	CFU

Qualification of extracted lipid of *Schizochytrium* sp. was tested by analyzing chemical and elemental characteristics. Chemical characteristics of *Shizochytrium* sp. S31 were reported in Table 3.9.

Table 3.9: Chemical characteristics of *Schizochytrium* sp. S31.

Chemical Characteristics			Max
FFA	ppm		0.1
Litre weight	NEN-ISO 6883	kg/l	0.9
Moisture and volatile matter	ISO 662	%	0.1
Anisidine value	ISO 6685	mg KOH/gr	20
Peroxide value	ISO 3960	meg/kg	5
Unsaponifiable matter	ISO 3596	%	4.5

Fish can accumulate toxins such as mercury, dioxins, and polychlorinated biphenyls (PCBs), and spoiled fish oil may produce peroxides. Free fatty acid, anisidin, peroxide and unsaponifiable matter values were determined below the limit when it is compared to fish oil. Because of LC-PUFAs are more prone to oxidation, it was suggested to add antioxidant solution to algal culture.

In this case, algae was grown on F/2 medium, samples were withdrawn at different times for the first 100 hours of the culture, and elemental composition was determined using a Carlo Erba EA 1108 elemental analyzer (Figure 3.10).

Table 3.10: Elemental composition of *Schizochytrium* sp.S31.

Elemental Composition		Max
Arsenic	ppm	0.1
Cadmium	ppm	0.1
Copper	ppm	0.05
Iron	ppm	0.2
Lead	ppm	0.1
Mercury	ppm	0.04

According to results, Arcenic, Cadmium, Copper, Iron, Lead and Mercury level were below the limit when it was compared to fish oil. It has been demonstrated that, no heavy metals were detected in algal biomass (Pyle, 2008). It is known that the major commercial source of omega-3 fatty acids is fish oil, which faces challenges such as odor/taste problems, heavy metal specially mercury contamination, and limited supply (Barclay et al, 1994). These heavy materials have been ingested by the fish and then

concentrated in their liver, which is then the principal organ used for obtaining oils. All these factors argue against the continued use of fish oils as a supply of EPA (and of DHA) and encourage the use of alternative sources. Heterotrophic cultivation of *Schizochytrium* sp. is an excellent opportunity to provide alternative omega-3 sources since it contain high amount of DHA with very low heavy metal content. The fatty acids can be extracted from algae and used in foods, or the biomass can be used directly as a feed additive in various animal industries such as aquaculture.

3.8.5 Kinetic calculations

Microbial processes are complex, and it is the critical step in practical applications, such as understanding, controlling, and optimizing fermentation process. Fermentation models can provide useful information for the analysis, design and operation of a fermenter without complexity (Jian , 2002; Othmane et al, 2007). Therefore, developing mathematical models of fermentation kinetics became the primary objective for fermentation behavior. In this study, experimental data from batch fermentations by *Schizochytrium* sp. S31 were examined in order to form the basis of kinetic model of the process. Table 3.11 shows the kinetic calculation results of *Schizochytrium* sp. S31 during incubation.

Table 3.11: Kinetic calculation results of *Schizochytrium* sp. S31 during incubation.

Time (h)	Biomass (g/l)	Substrate-Glucose (g/l)	Substrate-MSG (g/l)	rx (g/l.h)	rs (g/l.h)	rn (g/l.h)
0	0.36	100	16	-	-	-
1	0.4824	99.825	16	0.144	0.2125	0.02
2	0.648	99.575	15.96	0.1944	0.2875	0.004
3	0.8676	99.25	15.92	0.2592	0.4125	0.004
4	1.1628	98.75	15.88	0.3456	0.5	0.004
5	1.5588	98.25	15.84	0.4644	0.6625	0.006
6	2.0916	97.425	15.76	0.6228	1	0.008
7	2.8008	96.25	15.68	0.828	1.2125	0.012
8	3.744	95	15.52	1.116	1.625	0.016
9	5.04	93	15.36	1.494	2.25	0.02
10	6.732	90.5	15.12	1.98	3	0.028
11	9	87	14.8	2.664	4.125	0.038
12	12.06	82.25	14.36	3.582	5.375	0.52
13	16.164	76.25	13.76	4.77	7.125	0.7

Table 3.11 (continued): Kinetic calculation results of *Schizochytrium* sp. S31 during incubation.

Time (h)	Biomass (g/l)	Substrate-Glucose (g/l)	Substrate-MSG (g/l)	rx (g/l.h)	rs (g/l.h)	rn (g/l.h)
14	21.6	68	12.96	6.318	9.625	0.94
15	28.8	57	11.88	8.46	12.625	1.34
16	38.52	42.75	10.28	10.98	16.5	1.76
17	50.76	24	8.36	12.96	19.9875	2.14
18	64.44	2.775	6	7.56	12	1.2
19	65.88	-	5.96	-	-	-
20	65.88	-	5.92	-	-	-

The model appeared to provide a reasonable description for each parameter during the growth phase. Growth kinetics (rx), glucose utilization rate (rs) and monosodium glutamate uptade rate (rn) were calculated as it is shown below.

Specific growth rate calculation

Specific growth rate was calculated by using mathematical formulas showed above. Calculation of µmax and Ks by Lineweaver-Burke method were shown in Figure 3.11 and by Monod equation in Figure 3.12.

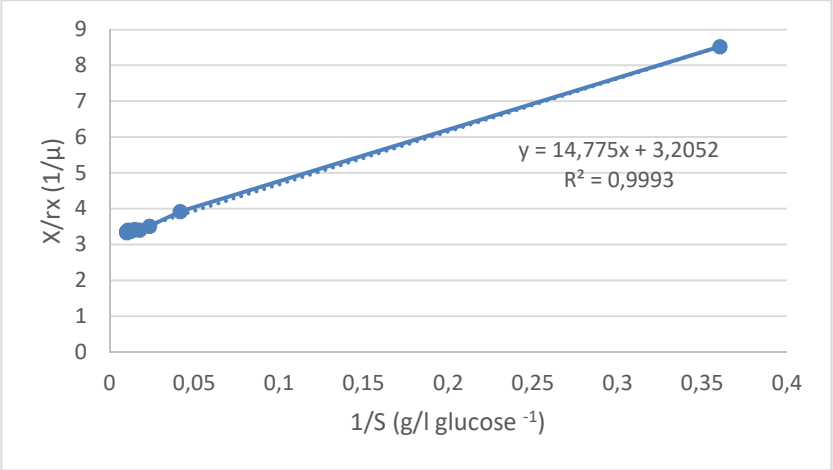


Figure 3.11: Calculation of µmax and Ks by Lineweaver-Burke method.

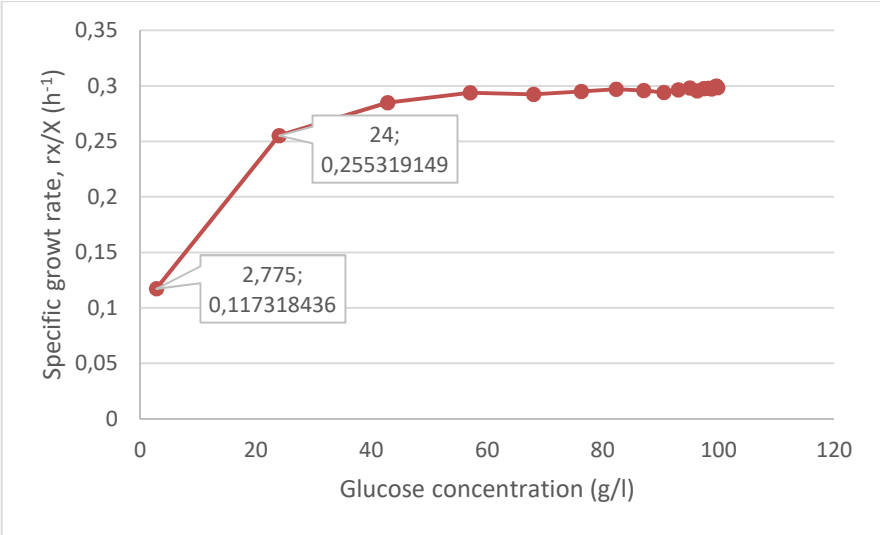


Figure 3.12: Calculation of µmax and Ks by Monod equation**.**

$$y = 0.14775x + 3.2052$$

$$\mu\boldsymbol{max} = \frac{1}{3.2052} = 0.312h^{-1}$$

$$\boldsymbol{Ks} = 0.14775 \times 0.312 = 4.6\ g\ glucose/l$$

Specific growth rate was calculated as $0.312h^{-1}$ and Ks which is the substrate concentration at half maximum rate was calculated as 4.6 g glucose/l. Finally, growth rate (rx) equation was obtained as described below.

$$\boldsymbol{rx(growt\ rate)} = \frac{0.312S}{4.6 + S}$$

<u>Rate calculation for glucose utilization</u>

Glucose utilization rate was calculated by using mathematical formulas which is given above. The substrate utilization kinetics considers substrate conversion to cell mass and maintenance. Calculation of $Y_{X/S}$ (yield factor) and m (maintenance coefficient) were shown in Figure 3.13.

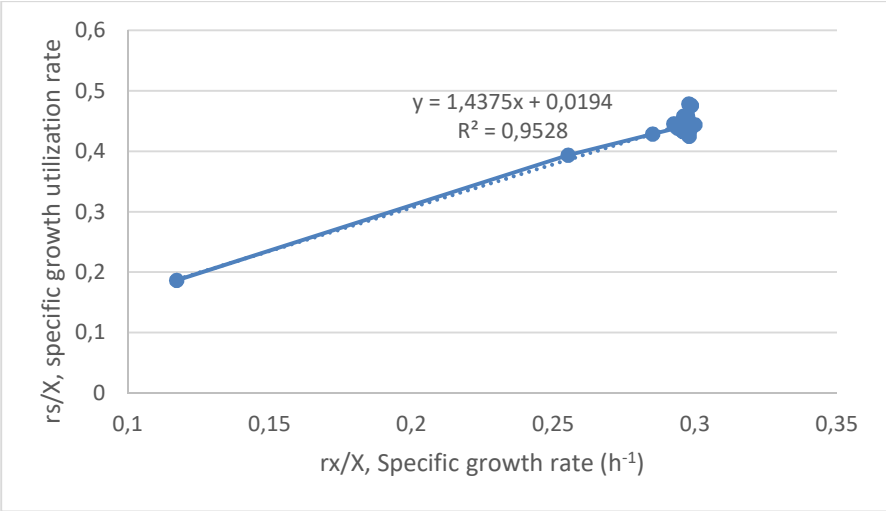


Figure 3.13: Glucose utilization rate calculation.

$$y = 1.4375x + 0.0194$$

$$\boldsymbol{m(maintenance\ coefficient)} = 0.0194$$

$$\boldsymbol{Y_{X/S}(yield\ factor)} = \frac{1}{1.4374} = 0.695\ g\ cell/g\ glucose$$

Maintenance coefficient and yield factor were calculated as 0.0194 and 0.695 g cell/g glucose respectively. Finally, glucose utilization rate (rs) equation was obtained as described below.

$$\boldsymbol{rs\ (glucose\ utilization\ rate)} = \frac{rx}{0.695} + 0.0194X$$

<u>Rate calculation for monosodium glutamate uptake</u>

The uptake rate of monosodium glutamate was calculated by using mathematical formulas which is given above. Calculation of $Y_{X/N}$ (yield factor) were shown in Figure 3.14.

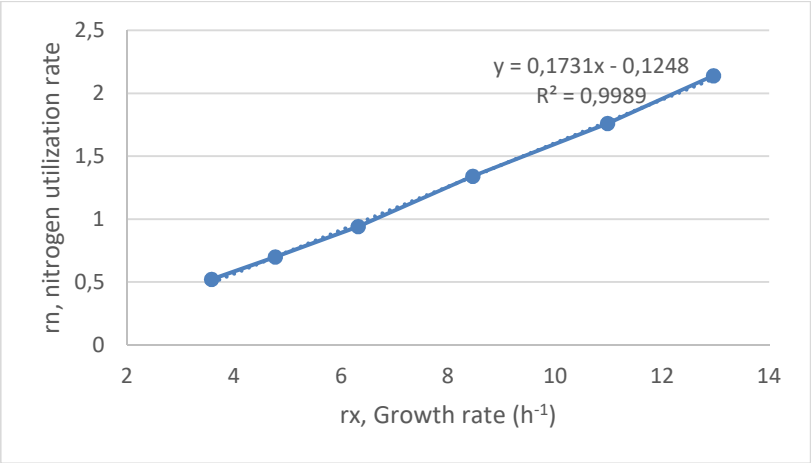


Figure 3.14: Monosodium glutamate utilization rate calculation**.**

$$y = 0.1731x - 0.1248$$

$$\boldsymbol{Y_{X/N}} = \frac{1}{0.1731} = 5.77\ g\ cell/g\ nitrogen$$

Monosodium glutamate yield factor was calculated as 5.77 g cell/ g nitrogen. Nitrogen utilization rate was obtained as described below.

$$\boldsymbol{rn\ (nitrogen\ utilization\ rate)} = \frac{rx}{5.77}$$

3.9 Optimization of Cell Lysis and Lipid Extraction

Mortar and pestle, french press, ultrasonication methods were examined for cell lysis. Comparative results of different cell lysis and lipid extraction methods were shown in Table 3.12. Mortar and pestle method is not effective for cell lysis as the 26% of total oil was extracted. The highest lipid extraction yields were obtained using sonication and french press cell disruption together with hexane extraction from *Schizochytrium* sp. S31 with 34.5% and 32% lipid yield, respectively. After optimization of pressure, temperature and time variables of SFE, 30.2% lipid yield was obtained.

Table 3.12: Comparison of different cell lysis and lipid extraction methods.

	No lysis	**Mortar and Pestle**	**French Press**	**Ultrasonication**
Hexane	24	26	32	34.5
Hexane/IPA(3:2)	19.5	20.7	28	29
Hexane/Ethanol (2:1)	17	19.2	24.4	26.5
Ethanol	18.8	20.4	24	23.5

Table 3.12 (continued): Comparison of different cell lysis and lipid extraction methods.

	No lysis	Mortar and Pestle	French Press	Ultrasonication
Chloroform/Methanol (2:1)	24.8	27	33.2	35
SFE	30.2	-	-	-

Different cell disruption techniques showed different efficiency and effects on lipid yield (Byreddy et al, 2015). Ultrasonic pretreatment exhibited the best results as indicated by Chen and Oswald (1998) in previous studies, where lipid yield was improved by up to 33%. Both sonication and high pressure homogenization (French press) could be useful methods for disruption of *Schizochytrium* sp. Solvent based extraction has been used in numerous studies; however, heat treatment can be detrimental to the sensitive bioactive components. The Soxhlet type of lipid extraction apparatus is not suitable for the extraction of thermolabile biological products, as the extracted matter is subjected to the boiling temperature. Therefore incubation method which is also known as cold extraction was used for lipid extraction. Although chloroform/methanol extraction gave the best results for lipid extraction, appearance of the lipid was not clear suggesting that unwanted molecules were co-extracted. Therefore, hexane was used for the further analysis.

3.10 Analysis of the SFE Model

Supercritical fluid extraction was also used for lipid extraction from *Schizochytrium* sp. S31. Optimum lipid extraction conditions were determined by using RSM. Response surface methodology was applied to optimize the extraction conditions for the total oil yield (g) and DHA content (%) of dried biomass using SFE. Three factor, three level CCD was used. The independent variables were pressure (325 – 425 bar), temperature (40 – 60 ^{0}C), and extraction time (90 – 150 min). Uncoded and coded values of the independent variables together with the experimental points used according to the CCD were shown in Table 3.13.

Table 3.13: Coded and uncoded levels of independent variables for CCD and responses.

	Coded level			Uncoded level			Responses	
	X1	**X2**	**X3**	**P (bar)**	**T (°C)**	**Extraction time (min)**	**Lipid Yield (g)**	**DHA (mg/100 g)**
1	-1	-1	-1	325	40	90	1.5038	15.80
2	1	-1	-1	425	40	90	1.4632	15.98
3	-1	1	-1	325	60	90	0.8939	15.57
4	1	1	-1	425	60	90	1.3654	15.25
5	-1	-1	1	325	40	150	1.3274	14.77
6	1	-1	1	425	40	150	1.5143	16.35
7	-1	1	1	325	60	150	0.7820	13.64
8	1	1	1	425	60	150	1.0681	14.79
9	-1.681	0	0	290.9104	50	120	0.9971	16.52
1	1.6818	0	0	459.0896	50	120	1.3864	17.30
1	0	-	0	375	33.18	120	1.3798	15.19
1	0	1.68	0	375	66.81	120	1.1330	14.92
1	0	0	-	375	50	69.6	0.9145	13.04
1	0	0	1.681	375	50	170.4	1.3384	17.68
1	0	0	0	375	50	120	1.1044	14.41
1	0	0	0	375	50	120	1.1125	14.62
1	0	0	0	375	50	120	1.0896	14.18

For both total extraction yield and %DHA amount of the dried biomass after SFE, quadratic models were suggested by the software The Unscrambler X 10.3. The R^2 values for total yield and %DHA were 99.3 and 94.6%, respectively indicate that only about 0.7 and 5.4% of the total variations were not satisfactorily explained by the model. The models were found to be significant according to the F-values of 31.1923 and 4.0281 for total yield and %DHA, respectively. Table 3.14 provides the regression coefficients obtained by fitting experimental data to the quadratic models for total extraction yields and %DHA amount in each extraction points, respectively.

Table 3.14: Quadratic model constants and regression analysis for total extraction yield and %DHA.

Term	Coefficient[a]	Total Yield	%DHA
Intercept	a_0	1.099408	14.44132
X_1 (pressure)	a_1	0.111482	0.3739870
X_2(temperature)	a_2	-0.288455	-0.661880
X_3 (extraction time)	a_3	-0.172280	-1.344225
$X_1.X_2$	a_{12}	0.076413	-0.116250
$X_1.X_3$	a_{13}	0.005263	0.358750
X2.X3	a_{23}	-0.035487	-0.216250

Table 3.14 (continued): Quadratic model constants and regression analysis for total extraction yield and %DHA.

Term	Coefficient[a]	Total Yield	%DHA
$X_1.X_2.X_3$	a_{123}	-0.051613	0.008750
X_1^2	a_{11}	0.041176	0.755378
X_1^3	a_{111}	0.001505	-0.050237
X_2^2	a_{22}	0.064033	0.099537
X_2^3	a_{222}	0.076043	0.205630
X_3^2	a_{33}	0.018089	0.207371
X_3^3	a_{333}	0.105467	0.962975
	R^2	0.9927	0.9458
	F	31.1923	4.0281
	Sig F	<0.05	<0.05

[a] $Y = a_0 + a_1X_1 + a_2X_2 + a_3X_3 + a_{12}X_1X_2 + a_{13}X_1X_3 + a_{23}X_2X_3 + a_{123}X_1X_2X_3 + a_{11}X_1X_1 + a_{111}X_1X_1X_1 + a_{22}X_2X_2 + a_{222}X_2X_2X_2 + a_{33}X_3X_3 + a_{333}X_3X_3X_3$

3.10.1 Effect of pressure-temperature

The pressure values between 325 and 425 bars and temperature between 40 – 60 °C were studied in order to observe the effect of pressure parameter on total lipid yield and % DHA amount in extracts. Figure 3.15 shows the effect of pressure and temperature on lipid extraction and % DHA content at 2 l/min CO_2 flow rate and 120 min extraction time, respectively.

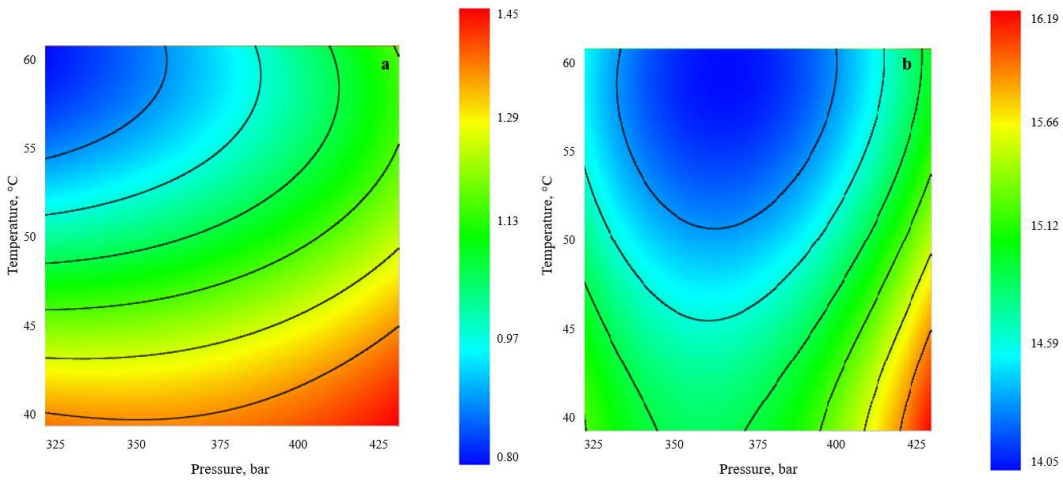


Figure 3.15: Effect of temperature-pressure interaction on lipid extraction (a); DHA % content (b).

At constant temperature, lipid yield and DHA concentration increases with pressure increase. Inversely, at constant pressure, the lipid yield and % DHA content decreases with increasing temperature. According to results of data analysis, pressure and temperature has significant effect ($p < 0.05$) on total lipid yield and DHA concentration.

This effect was expected as the solubility increases with pressure at constant temperature due to the increase in solvent density, therefore, increase in salvation power (Taher et.al., 2014).

3.10.2 Effect of temperature-extraction time

At constant pressure (375 bar); total lipid extraction yield decreased with increased extraction time and also increased temperature. The same negative effect was also observed for %DHA amount. According to data analysis interaction between temperature-extraction time had no significant effect (p>0.05) on total lipid yield and %DHA amount (Figure 3.16).

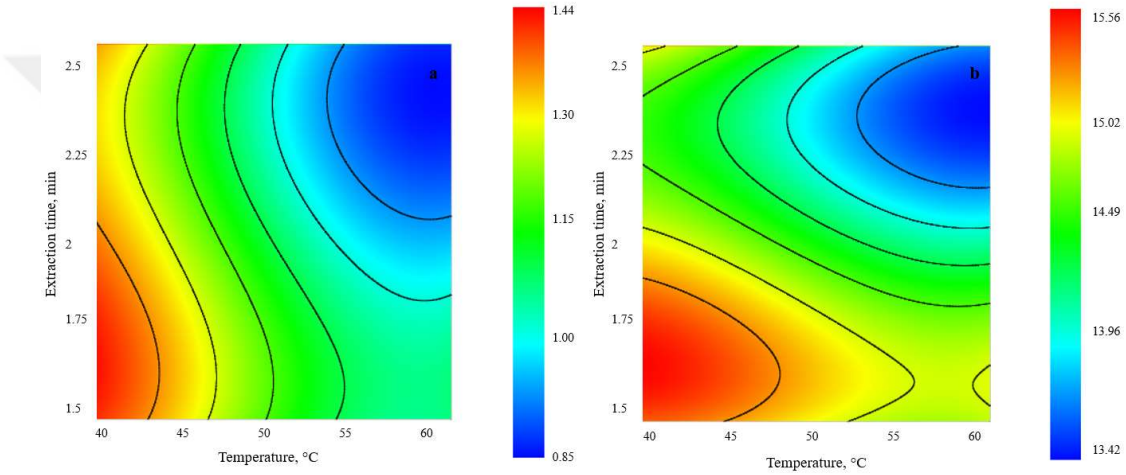


Figure 3.16: Effect of extraction time- temperature on lipid extraction (a); DHA % content (b).

3.10.3 Effect of pressure-extraction time

At constant temperature (50 ^{0}C) and extraction time (90 min); total lipid yield increased with increased pressure. In contrast to at constant pressure, lipid yield decreased with increased extraction time. (Figure 3.17). The studies about SFE extraction of lipids from microalgae were mainly focused on the effect of temperature and pressure on lipid (Herrero et al, 2006).

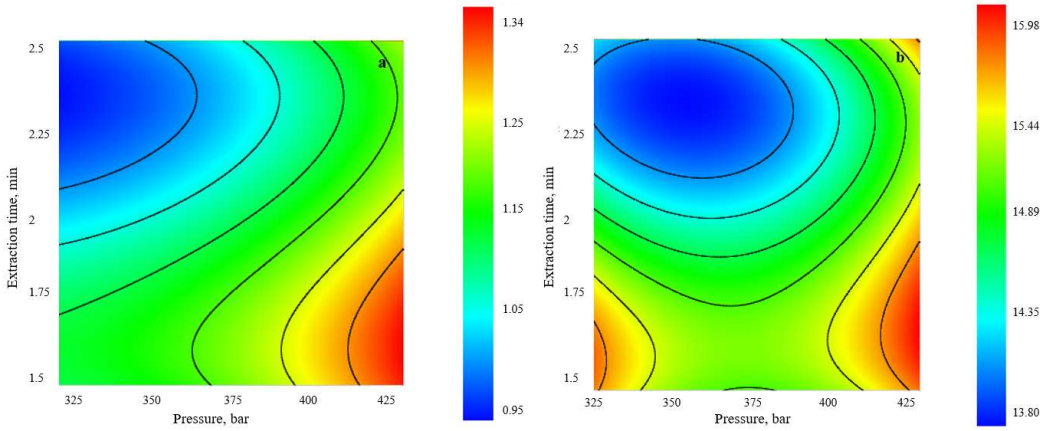


Figure 3.17: Effect of extraction time- pressure on lipid extraction (a); DHA % content (b)

Extraction time was not studied as a primer factor on the extraction yield. Mostly 90-120 minutes extraction periods were studied. Solana et al. (2014) reported that omega-3 content was negatively affected long extraction times. According to contour plot data, optimum extraction conditions were found to be 425 bar pressure at 40.5°C for 97.5min. After optimizing SFE conditions, 30.2% lipid yield was obtained.

3.11 Strain Improvement by Random Mutagenesis

High yield of lipid accumulating mutants of a *Schizochytrium* sp. S31 were isolated using flow cytometric-based selection. Characterization of the mutants were performed by using ImageStream. The first step of the mutation program was determination of the appropriate times for UV and chemical agent exposure of the parental culture. After obtaining death curves, optimum exposure time was determined for a successful strain improvement methodology.

3.11.1 Death curve analysis of *Schizochytrium* sp. S31

The mutation program used ultraviolet radiation (UV) as mutagenic agent. The UV source was a 254-nm wavelength lamp (UVP CX-2000) placed 7 cm above the culture. Volumes of 50 mL of culture were exposed to UV light for 5sec, 15sec, 30sec, 45sec and 60sec respectively. Exposed cultures were then plated after appropriate dilution, and incubated at 25°C for 72h. Cell numbers in each plate were counted to obtain death curve (Figure 3.18).

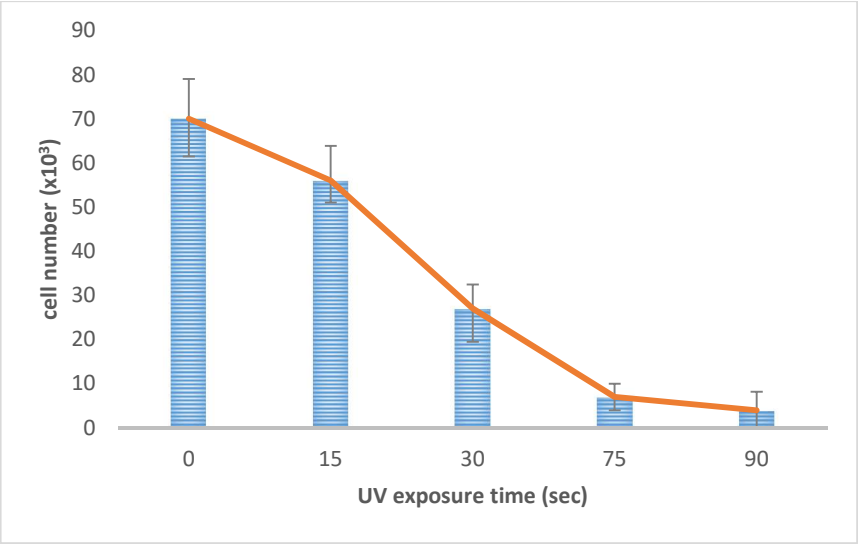


Figure 3.18: *Schizochytrium* sp. death curve under different UV exposure time.

Survival rate was found to be dependent with the exposure time. When the cell numbers were counted on agar plates, 50 %survival rate was found to be at 1 joule for 30sec (Figure 3.18). Experiments using UV radiation on *Schizochytrium* sp. showed that this external stress can stimulate lipid accumulation.

Cell culture is also mutagenized with EMS. Cell cultures at a concentration of 10^6 cells mL^{-1} were treated with 0.28 M EMS in phosphate buffer, pH 7 in the dark in the shaker. 5 % sodium thiosulfate were added at 5, 15, 30, 45, 60 and 90 min to stop the reaction. Mutated cells were washed and plated onto agar plate to determine fifty percent survival rate. Figure 3.19 shows the *Schizochytrium* sp. cell number under different EMS exposure time.

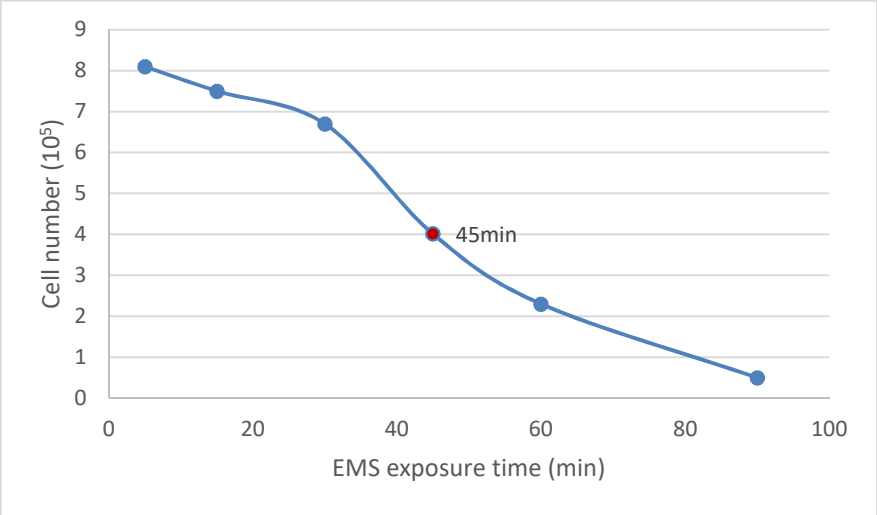


Figure 3.19: *Schizochytrium* sp. death curve under different EMS exposure time.

According to result, 45 min of mutagenesis, which resulted in 50 % survival. It has been described before that fifty percent survival was considered as optimal exposure for mutant generation (Manandhar-Shrestha, 2013)

3.11.2 Nutrient starvation

Nutrient starvation is one of the most widely used technique for lipid induction in many species including microalgae (Sharma et al, 2012). Nitrogen and phosphorus are the most important nutrient that affects the lipid metabolism. Nitrogen and phosphorus starvation on several thraustochytrid resulted in increased TAG contant in the cell. Exponentially grown cell culture were harvested and washed with nitrogen and phosphorus limited medium (NPlim). Washed cells were inoculated into NPlim medium and samples were taken at t0, t12, t24, t48. Samples were stained with BODIPY as described before and run through the FACS. 100,000 cells were screened to compare lipid accumulation difference. Figure 3.20 shows the intensity (530/40) change during time.

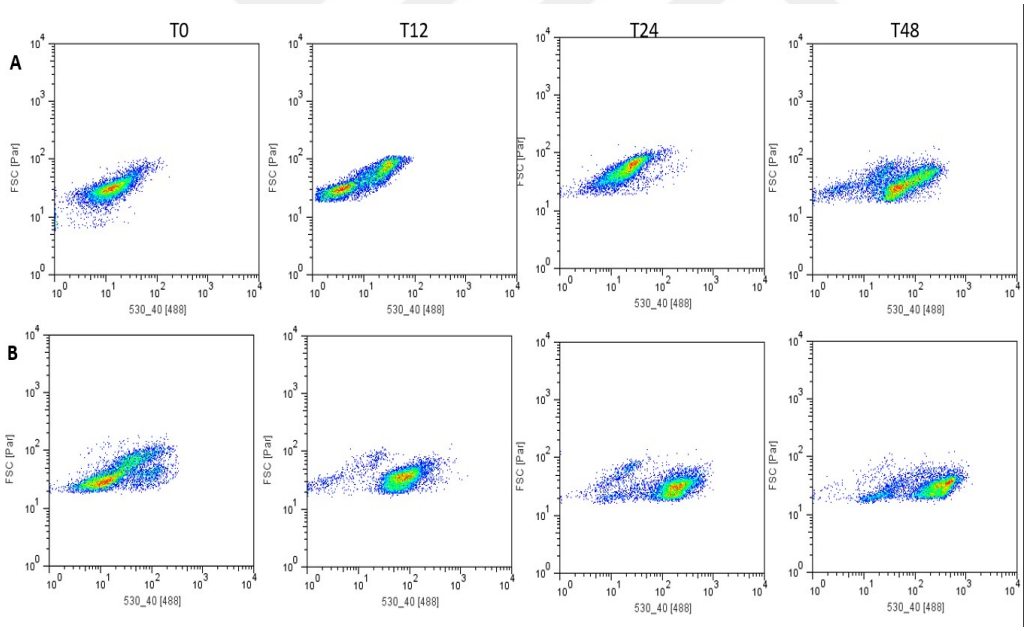


Figure 3.20: Flow cytometry analysis of the *Schizochytrium* sp. cell during time under normal and limited conditions. A: Growth medium; B: NPlim medium.

Phosphorus and nitrogen limitation was found to increase the mean value (530/40) [488] from 78 up to 290. Figure 3.21 shows the comparative results of the control and the Nitrogen+phosphorus limited *Schizochytrium* sp. S31 culture.

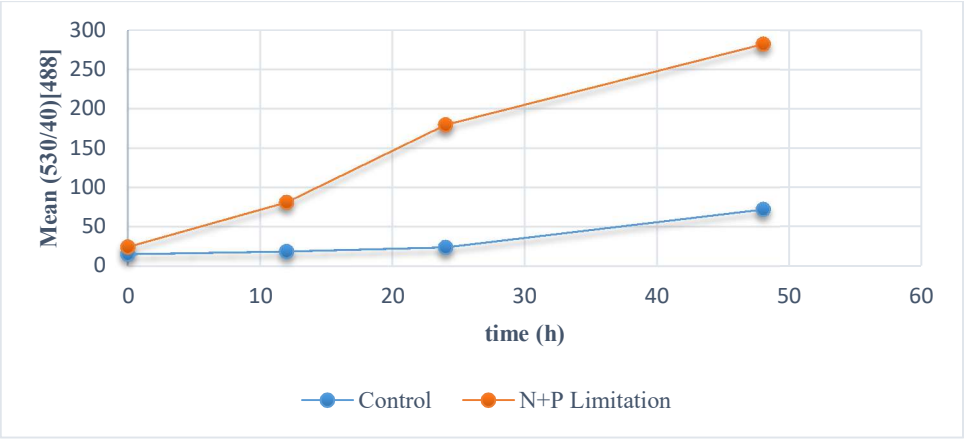


Figure 3.21: Comparision of mean values of two different cultures. Blue line: Control, Red line: Nplim culture.

According to Figure 3.21, mean value (530/40) [488] of BODIPY was increased 4-fold when the microalgal culture was introduced N+P limited medium.

3.11.3 UV mutagenesis and screening

Exponentially grown *Schizochytrium* sp. S31 cells were harvested and washed with F2 medium. Cells were exposed to UV light for 30sec minutes and allowed to recover in the dark for 24h. Cells were then inoculated into complex medium and incubated for 48h. Grown cell culture inoculated into nitrogen+phosphate free medium and incubated for 24h to induce lipid accumulation. After inducing, cell were stained with BODIPY and sorted by fluorescent activated cell sorting (FACS) device. Cells were immadiately inoculated into complex medium. Three rounds of sorting have been performed. After 3 rounds of sorting cell were plated into agar medium and incubated at 25°C for 3 days. 6 mutant colonies and 1 wild type colony were picked for IS analysis. Because of *Schizochytrium* sp algal cells tend to accumulate, they were divided into different populations. Single cells with high lipid content were collected. Three different exposure times (30sec, 40ec, 45sec) were tested for mutant library construction. Quantification based on average fluorescence intensities showed that 30sec UV exposure time always had much higher fluorescence intensities than the 40 and 45sec exposure time. 50.000cells were sorted from each treatments. Substantial improvements in lipid content were obtained, especially after 4 rounds of sorting. After sorting, cells were plated on agar and incubated at 25°C for 48h. 100 of colonies were picked and screened for lipid accumulation. According to IS results, mutants which were treated to UV for 30sec accumulate more lipid than wild type. It has been known that, UV mutagenesis is an effective method to increase lipid productivity in

microalgae. Bougaran et al. (2012) combined UV mutagenesis and FACS in an *I. galbana* mutation-selection procedure that increased lipid productivity by 80 %.

Time course experiments were conducted to understand the lipid accumulation profile difference between mutants and wt. Exponentially grown mutant and wt culture were harvested and inoculated into NPlim medium. Optimum time for lipid induction in wild type were determined by time course experiment (Figure 3.22).

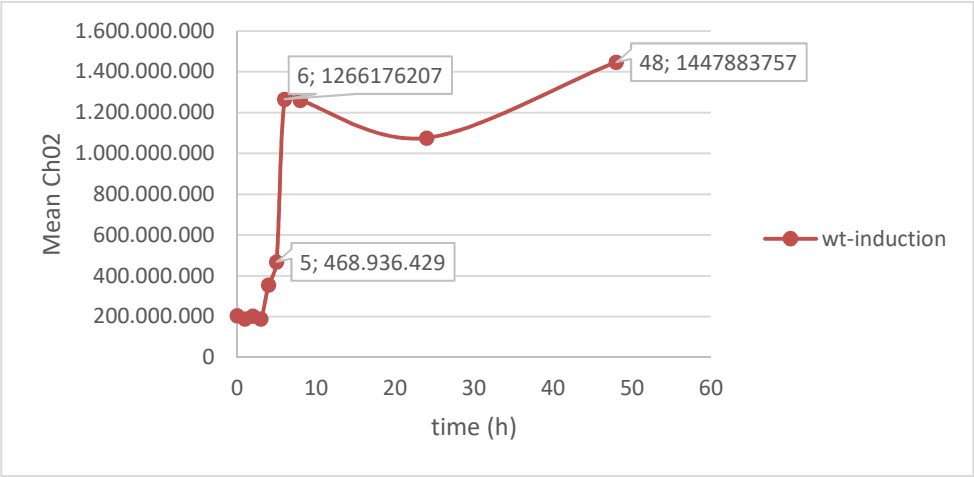


Figure 3.22: Lipid accumulation of wt *Schizochytrium* sp. S31 during time by limitation.

Figure 3.22 showed that lipid accumulation in the *Schizochytrium* sp. S31 cell occcured in the first 6 hours after induction. After that, lipid content was achieved to saturated phase. Because of that, it was better to analyse mutant and wild type in first 6 hours for correct comparsion. Timecourse ImageStream analysis of selected six different mutants and wt with two replicate were performed to differentiate the mean fluorescence level at time 0h, 2h, 4h, 6h, 8h (Table 3.15). According to IS report of 30sec mutants and wild type, Mutant1 and Mutant2 had the highest fluorescent intensity which were 2.575.849.523 and 2.372.463.769 respectively at time 6h indicating that these to mutants had the highest lipid content (Table 3.15). Therefore, these mutants were selected for further analysis.

Table 3.15: Image Stream report of 30sec mutants and wild type.

File	%Count, All	Intensity_MC_Ch02, Mean, All
M1_T0.daf	5.000	323.442.612
M1_t2.daf	5.000	880.907.594
M1_t4.daf	5.000	2.500.628.100
M1_t6.daf	5.000	2.575.849.523
M1_T8.daf	5.000	1.659.282.053
M2_t0.daf	5.000	385.978.304
M2_t2.daf	5.000	1.646.739.976
M2_t4.daf	5.000	1.944.759.633
M2_t6.daf	5.000	2.372.463.769
M2_t8.daf	5.000	2.129.672.950
M3_t0.daf	5.000	308.108.471
M3_t2.daf	5.000	717.419.427
M3_t4.daf	5.000	1.076.135.802
M3_t6.daf	5.000	1.527.346.376
M3_t8.daf	5.000	1.055.802.785
M4_t0.daf	5.000	276.462.358
M4_t2.daf	5.000	1.024.740.446
M4_t4.daf	5.000	1.124.002.963
M4_t6.daf	5.000	1.278.597.473
M4_t8.daf	5.000	958.012.921
M5_t0.daf	5.000	206.817.350
M5_t2.daf	5.000	824.411.289
M5_t4.daf	5.000	1.668.217.170
M5_t6.daf	5.000	1.978.123.931
M5_t8.daf	5.000	1.500.422.670
M6_t0.daf	5.000	260.237.387
M6_t2.daf	5.000	1.402.338.637
M6_t4.daf	5.000	1.494.223.208
M6_t6.daf	5.000	1.559.181.943
M6_t8.daf	5.000	1.071.641.374
WT_t0.daf	5.000	175.449.058
WT_t2.daf	5.000	1.080.098.985
WT_t4.daf	5.000	1.111.549.973
WT_t6.daf	5.000	1.216.466.963
WT_t8.daf	5.000	1.015.323.431

*M1: Mutant1; M2: Mutant2; M3: Mutant3; M4: Mutant4; M5: Mutant5; M6: Mutant6; WT: Wild type

Timecourse ImageStream (IS) analysis of two mutants and wild type with two replicate were performed to differenciate fluorescent intensity between them during time (Figure 3.23).

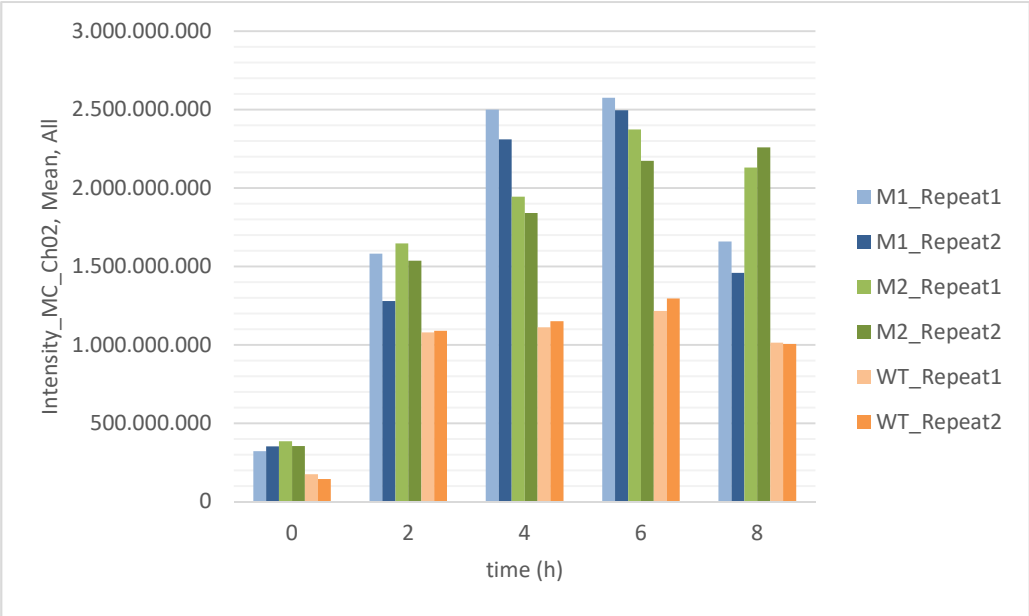


Figure 3.23: Timecourse ImageStream analysis of Mutant1, Mutant2 and wild type.

According to Table 3.15 and Figure 3.23 results, mutants and wild type strain displayed different levels of fluorescent intesity. Because of the mean fluorescence level of Mutant1 and Mutant2 was higher than all other mutants and wild type, they are selected for detailed IS analysis. 5000 cells were sorted to collect image data for comparison of mutants and wild type cell (Figure 3.24).

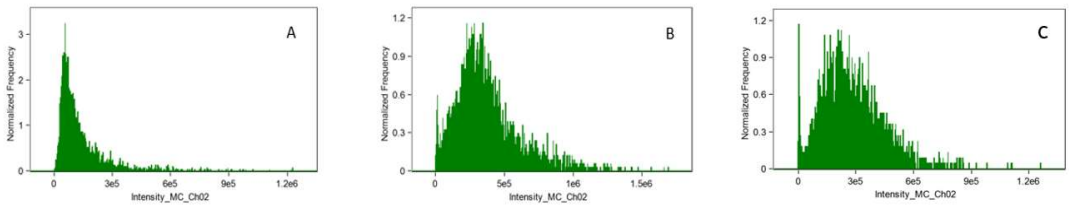


Figure 3.24: Light intensity difference between mutants and wild type on IS analysis A: Control; B: Mutant1; C: Mutant2 (30sec UV exposure).

IS data showed the light intensity difference between mutants and wild type (Figure3.24). According to data, fluorescent intensity of the mutants were increased significantly. Detailed IS graphic result of the wt and UV treated mutants were shown in Appendix A1.

3.11.4 Characterization of selected mutants

Characterization of selected mutants was performed by comparing the CDW, total lipid content, %DHA, biomas productivity and lipid productivity with wild type under the same conditions. All characterization experiments were conducted in 1 lt baffled erlen flask. Therefore, analytical results of wild type were not consistent with fermenter results. All experiments were conducted under same conditions and repeated for two times. Figure 3.25 shows the comparative O.D750 and CDW results of Mutant1, Mutant2 and wild type *Schizochytrium* sp.

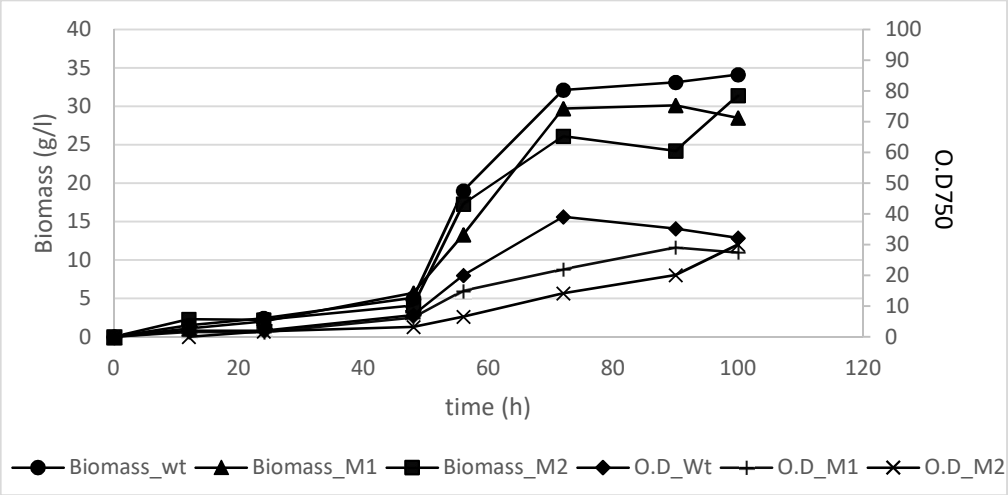


Figure 3.25: Comparative OD750 and CDW results of the Mutant1 and Mutant2 with Wt *Schizochytrium* sp.

According to results, CDW and O.D750 results of mutants were lower than wild type during the incubation. CDW result was 34.1g/l for wild type while 28.5 g/l and 31.4 g/l for Mutant1 and Mutant2 respectively. Similarly, O.D750 result was 32.2, 27.4 and 28 for for wild type, Mutant1 and Mutant2 respectively (Figure 3.25). Figure 3.26 shows the total lipid % results of the mutants and wild type.

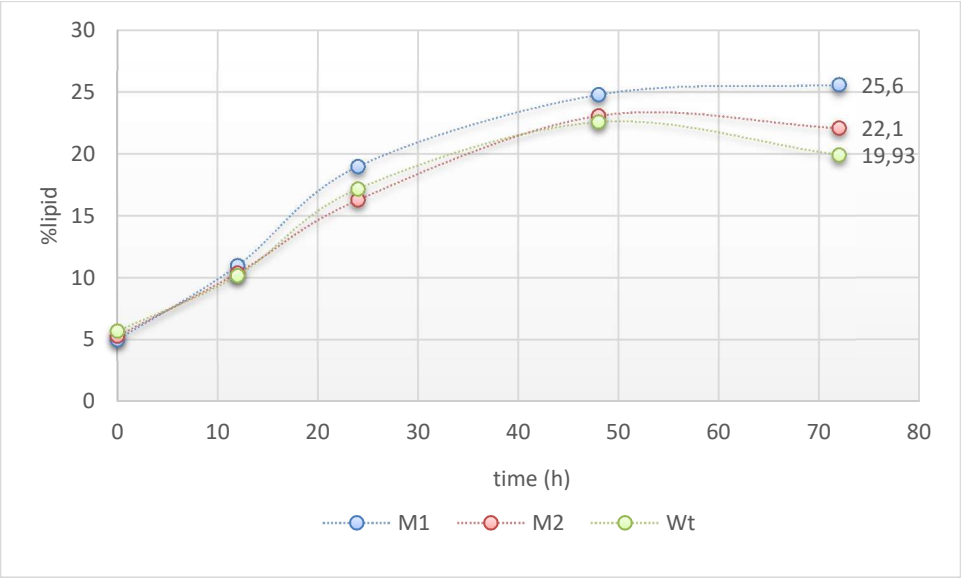


Figure 3.26: Comparative lipid % results of the Mutant1, Mutant2 and Wt *Schizochytrium* sp.

According to Figure 3.26, total lipid content of mutants were higher than wild type during the incubation. Total lipid result was 19.93, 25.6 and 22.1 for for wild type, Mutant1 and Mutant2 respectively. Total lipid was increased by 28.4 % for Mutant1 and 10.8% for Mutant2 comparing to wild type. To further quantify fatty acid contents and profile individual fatty acids, GCMS was carried out on both wt and mutant algal cultures. The area of DHA peak are $4.85x10^8$, $6.17x10^8$, $5.57x10^8$ for wild type, Mutant1 and Mutant2 respectively. GCMS graphic of the wild type and selected mutant were shown in Appendix A2. DHA percentage of the samples were shown in Table 3.16. According to results, 156mg/l, 198mg/l and 178mg/l DHA was obtained from Wt, Mutant1 and Mutant2 samples respectively.

Table 3.16: Comparative results of the Mutant1, Mutant2 and Wt *Schizochytrium* sp.

	Mutant1	**Mutant2**	**Wild type**
%lipid	25,6	22,1	19,93
lipid productivity (g/l.h)	0,0729	0,0693	0,0679
%DHA	27,13	25,79	23
DHA productivity (g/l.h)	0,0198	0,0179	0,0156

This study highlights that UV mutagenesis and high throughput selection for cell growth can be a viable combined approach to improve lipid productivity in *Schizochytrium* sp. microalgae. Nutrient limitation conditions, such as nitrogen (N), phosphorus (P) starvation, and temperature limitation can induce significant increase

in lipid content in *Schizochytrium* sp. According to characterization results, mutants which were treated with UV for 30sec accumulate lipid faster than wild type. Conversely, there was not any mutants that accumulate higher lipid than wild type on chemically mutated lines. Time course experiment were conducted to understand the lipid accumulation profile difference between mutants and wt. The results obtained from GCMS analyses confirmed the results obtained by flow cytometry, showing an increase in docosahexaenoic acid in UV treated cultures (30 sec) compared to untreated controls. There is an 17.9% and 12.1% increase compare to wild type in terms of DHA percentage in Mutant1 and Mutant2 respectively.

4. CONCLUSIONS

In this study, medium components and environmental factors influencing biomass and total lipid productivity of the *Schizochytrium* sp. S31 were optimized by statistical methodology. After optimizing growth conditions, large scale production of *Schizochytrium* sp. S31 under favorable conditions were performed. Downstream process including cell lysis, drying and lipid extraction were compared and optimized to maximize total lipid specially DHA extracted from *Schizochytrium* sp. S31. Finally, random mutation was applied and high yield of lipid accumulating mutants of *Schizochytrium* sp. were selected by flow cytometric-based technology.

It has been known that, many factors may effect the cultivation conditions of *Schizochytrium* sp. such as medium composition, pH, salinity and temperature (Guzman et al, 2010; Rao et al, 2007; Zeng et al, 2011; Wu et al, 2005, Kirrolia et al, 2011). Since PUFAs have gained significance due to their role in human health, industrial and commercial usage, research on enhancement of biomass and lipid production has increased (Uauy et al, 2001; Siriwardhana et al, 2012; Gupta et al, 2012). Therefore, optimization of medium composition and environmental conditions to improve biomass and total lipid content of the organism are important. All chemical and physical factors effecting total lipid and PUFA specially DHA production had to be reviewed to understand the metabolic processes leading to PUFA production. Omega-3 productivity can be increased and production costs can be decreased by using alternative carbon and nitrogen sources. Yazawa (1996) obtained higher amount of omega-3 fatty acid by using marine industrial waste and corn steep liquor as an alternative carbon source for *Shewanella putrefaciens* to reduce the overall large scale production costs. Therefore, alternative carbon, nitrogen, salt raw materials were employed to investigate optimum cell growth and total lipid yield of *Schizochytrium* sp. S31. According to results, glucose syrup and glycerol which are significant and cheap sources could be used by *Schizochytrium* sp. S31 for cell growth and lipid production, but molasses had negative effect on biomass and lipid production.

It was aimed to establish suitable industrial bioprocesses by testing the effect of medium components and physical factors including pH, temperature and dissolved oxygen level. The results presented in this study showed that the cellular growth can be enhanced by adjusting MSG concentration and initial pH, while total lipid content can be increased by temperature and nitrogen limitation conditions. The alternative raw materials and optimization results suggested that applying a statistically optimum strategy was a valuable tool for optimization of the production process which would eventually reduce the production costs through maximizing the yield (Yazawa, 1996). Because of this reason, it is recommended that this optimization strategy would be useful for developing microbial technology in the related industries. Statistical designs (PB and CCD) were used to optimize the process parameters, such as media composition, pH, temperature and dissolve oxygen in order to increase accumulation of neutral lipid and DHA in *Schizochytrium* sp. S31 (Lazic, 2004; Montgomery, 2004; Park et al, 2005, Wen and Chen 2001). The optimization of cultivation and environmental conditions of *Schizochytrium* sp. S31 in baffled erlen flask was accomplished by statistical design. After optimization, the maximum CDW and lipid production were 26.86 g/L and 35%, respectively. The optimum levels of CDW production were 2.29 $g.L^{-1}$MSG, pH 5.8. Using these conditions, a maximum biomass production of 26.86$g{\cdot}L^{-1}$ was obtained. On the other part, 0.49 $g.L^{-1}$ MSG and 17.6 °C temperature conditions are optimum for lipid production. Using these conditions, 35% lipid production was obtained. Based on contour plots and canonical analysis, 520.1 $mg.L^{-1}$ DHA have obtained with using 40 $g.L^{-1}$ glucose, 0,8$g.L^{-1}$ MSG, 6,25 $g.L^{-1}$ mineral salt, pH 6 and incubation for 48h at 20°C. Wu and Lin (2003) have also applied RSM to optimize docosahexaenoic acid production by *Schizochytrium* sp. They have obtained 516 $mg.L^{-1}$ DHA with using 27.98$g.L^{-1}$ glucose, 4.53 $g.L^{-1}$ yeast extraction, 24.82 $g.L^{-1}$ sodium chloride, pH 6.96 and incubation for 4 days at 30°C. When compared the results with this study, it was concluded that medium components specially nitrogen sources significantly effect the DHA production of *Schizochytrium* sp.

Scale up process and optimization of bioreactor conditions are another main step for this study. Batch fermentation experiments were started from a single colony to reach 1 lt of liquid cell culture which was used as an inoculum for 5 lt of fed-batch culture. Optimum parameters were applied to fed-batch system to obtain high amount of

desired products. While physical parameters were controlled automatically, optical densitiy of the culture, dry biomass, residual glucose and MSG, total lipid, DHA% were monitored during the incubation. According to fermenter results, 65 g/l of cell dry weight with the initial growth rate of 0.312 h^{-1} was obtained. The carbon nitrogen ratio (C/N) plays a critical role in the physiology of algal cultures as shown in the previous section. According to results that Chatdumrong et al. (2007) obtained , when C/N ratio was measured between 10-20 the maximum DHA yield was obtained in microalgae. There are, however, other nutrients and factors affecting growth and lipid production. C/N ratio of the 5 lt *Schizochytrium* sp. culture was calculated as 19.32. The total fatty acid content was about 27.5 % of the cell dry weight. Fatty acid compositions obtained from the GC analysis, the two fatty acids, C16:0 and C22:6 (DHA) accounted for about 90% of TFA content. Li et al. (2016) found that the major phospholipid fatty acids in *Schizochytrium* sp. S31 were C16:0 and C22:6. The results we obtained in this study are consistent with recent researches. DHA percentage of the fermeted *Schizochytrium* sp. culture in 5 liter was calculated as 30.18%. Biomass, lipid and DHA productivity was calculated as 0.65 g/l.h, 0.17g/l.h and 0.053g/l.h respectively.

Improvement of downstream processes was another critical step in this study. All steps has to be performed to obtain high quality of lipid and reduce the cost of downstream processing (Danquah et al, 2009; Grima et al, 2003). Alternative cell harvesting, drying, lysis and solvent based extraction methods including SFE of *Schizochytrium* sp. S31 microalge were investigated and compared. Extraction of microbial lipid is mainly conducted with solvents such as hexane, coupled with mechanical disruption techniques (Ryckebosch et al, 2012). According to the cell lysis and lipid extraction results, the highest lipid extraction yields were obtained using sonication and french press cell disruption together with hexane extraction from *Schizochytrium* sp. S31 with 34.5% and 32% lipid yield, respectively. Ultrasonication with hexane method increase the total lipid yield significantly with clear appearance. Sonication resulted in a 1.4-fold increase in lipid yield when compared with solvent alone. Araujo et al. (2013) showed that ultrasounic distribution of *C. vulgaris* resulted in the highest lipid extraction, suggesting a favorable potential for biodiesel production. Another research with C. *Minutissima, Thalassiosira fluviatilis* and *Thalassiosira pseudonana* indicated

that sonication-assisted method with n-hexane was efficient method for lipid extraction in these strains (Neto et al, 2013).

Solvent based soxhlet extraction has been used in numerous studies. However, heat treatment can be detrimental to the sensitive bioactive components (Tang et al, 2011). SFE is an alternative to liquid extraction using solvents such as hexane or chloroform. There will always be some residual solvent left in the extract and matrix, and there is always some level of environmental contamination from their use. In contrast, carbon dioxide is easy to remove simply by reducing the pressure, leaving almost no trace, and it does not require heat treatment. SFE extraction is a promising green technology that can potentially be used for food and nutraceutical application (Herrero et al, 2006; Bhusnure et al, 2015). The effect of extraction temperature, pressure and time of SFE on the lipid yield and %DHA amount were investigated by using RSM. According to SFE results, pressure and temperature has significant effect ($p < 0.05$) on total lipid yield and DHA concentration. It is shown that at constant temperature, lipid yield and DHA concentration increases with higher pressure whereas decreases with higher temperature. Based on contour plot analysis, optimum extraction conditions were found to be 425 bar pressure at 40.5°C for 97.5min. After optimizing SFE conditions, 30.2% lipid yield was obtained. Compare to traditional extraction, SFE has advantages to get solvent free value-added extracts.

As a third part of the the thesis, high yield of lipid accumulating mutants of a *Schizochytrium* sp. S31 were isolated using flow cytometric-based selection. Random mutation was applied to isolate a high yield of lipid accumulating mutants of a *Schizochytrium* sp. by flow cytometric-based selection. It is known that UV mutagenesis can induce lipid accumulation in several microalgae species. Bougaran et al. (2012) combined UV mutagenesis and FACS in an *I. galbana* mutation-selection procedure that increased lipid productivity by 80 %. Characterization of the mutants were performed by using ImageStream. This study highlights that UV mutagenesis and high throughput selection to improve lipid productivity in *Schizochytrium* sp. microalgae. Under nutrient starvation conditions, microalgal cells generally change their lipid biosynthetic pathways to accumulate neutral lipids, specially in TAG form (Minhas et al, 2016). It offers microalgae to tolerate unfavorable environmental conditions by storage. Some researchers showed that maximum amount of lipid content was obtained with temperature and nitrogen-limited culture conditions (Rios

et al, 2015, Jiang and Chen 2000). Nitrogen (N) and phosphorus (P) starvation was found to increase the mean value (530/40 [488]) from 78 up to 290.

According to characterization results, mutants which were treated to UV for 30sec accumulate lipid faster than wild type. Time course experiment were conducted to understand the lipid accumulation profile difference between mutants and wt. Total lipid content of mutants were higher than wild type during the incubation. Total lipid result was 19.93, 25.6 and 22.1 for for wild type, Mutant1 and Mutant2 respectively. Total lipid was increased by 28.4 % for Mutant1 and 10.8% for Mutant2 comparing to wild type. GCMS analysis was performed to obtain fatty acid profile of both wt and mutants. The results obtained from GCMS analyses confirmed the results obtained by flow cytometry, showing an increase in docosahexaenoic acid in UV treated cultures (30 sec) compared to untreated controls. There is an 17.9% and 12.1% increases compared to wild type in terms of DHA percentage in Mutant1 and Mutant2 respectively. Meireles et al, (2003) succeeded to increase the content of both EPA and DHA by 33% in *Pavlova lutheri* by UV mutagenesis. EPA content of the *Phaeodactylum tricornutum* was increased by 37% with same technique (Alonso et al, 1996). As a conclusion, stress conditions, such as nutrient deprivation and UV light exposure lead to higher total amounts of lipid most likely to repair cellular damage. In contrast, there was not any mutants that accumulate higher lipid than wild type on chemically mutated lines suggesting that EMS had insignificant effect on lipid accumulation in *Schizochytrium* sp.

For further study, large scale production of wild type and the mutants in bioreactor under controlled conditions can be performed to obtain growth rate and kinetic data from the large scale experiment. Second generation mutation can be performed to try to further increase biomass and DHA productivity in *Schizochytrium* sp. Since pilot-scale SFE extraction studies are lacking SFE method can be tested at larger scales beyond the laboratory for further lipid extraction strategies.

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APPENDICES

APPENDIX A.1: Image Stream Data
APPENDIX A.2: GCMS Data

APPENDIX A.1: Image Stream Data

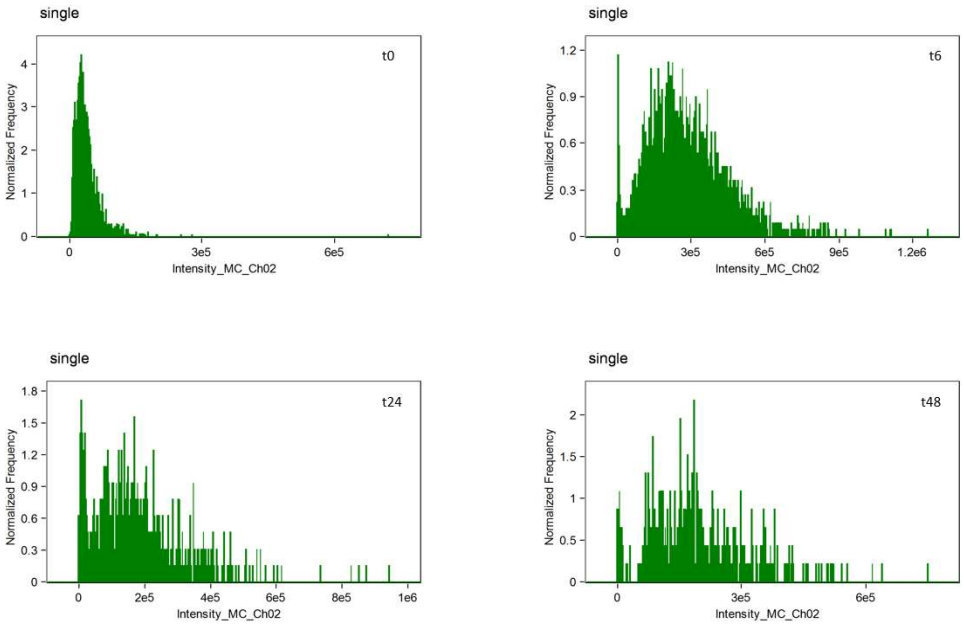

Figure A1.1: Mutant1 time course experimental data.

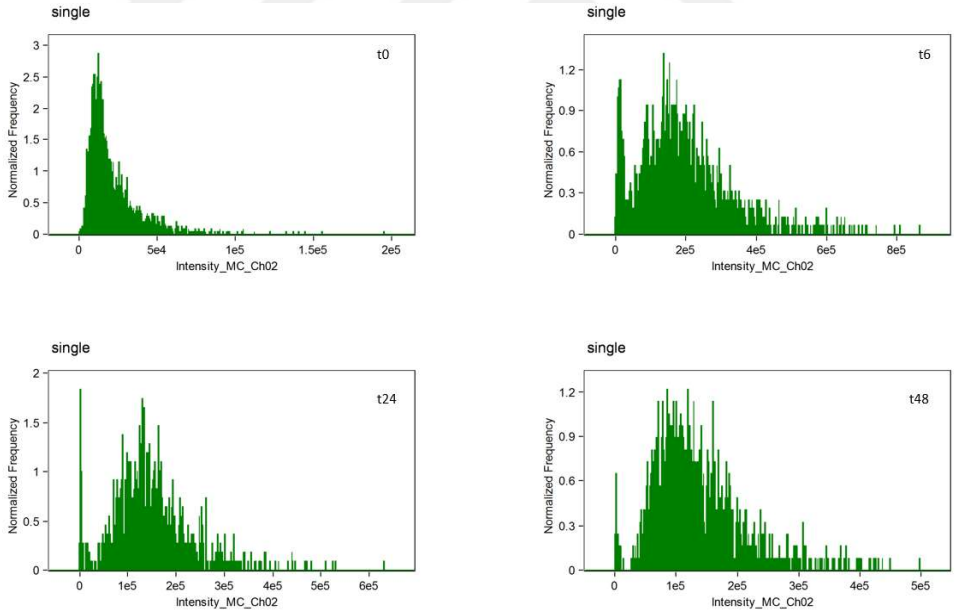

Figure A1.2: Mutant2 time course experimental data.

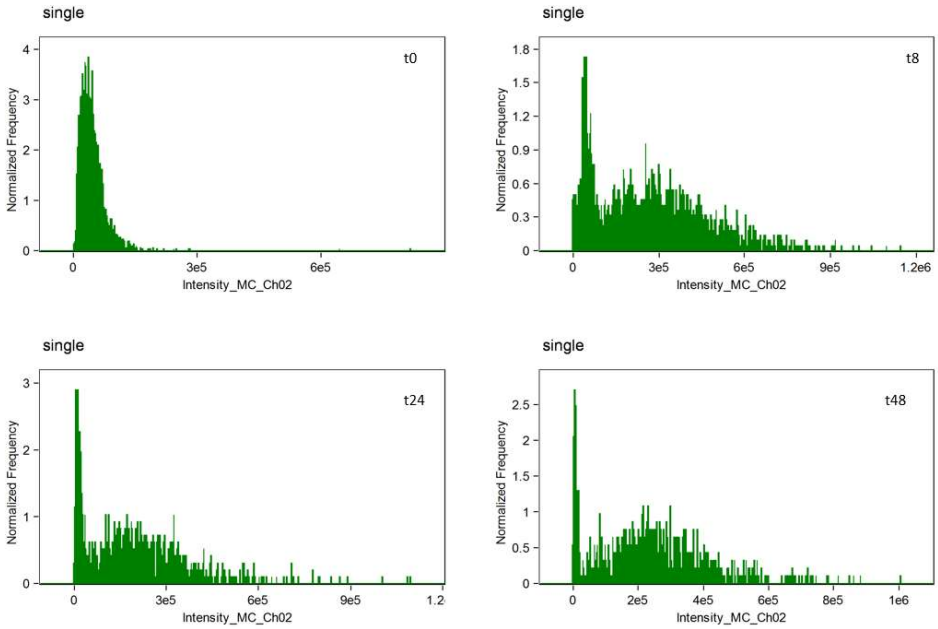

Figure A1.3: Mutant3 time course experimental data.

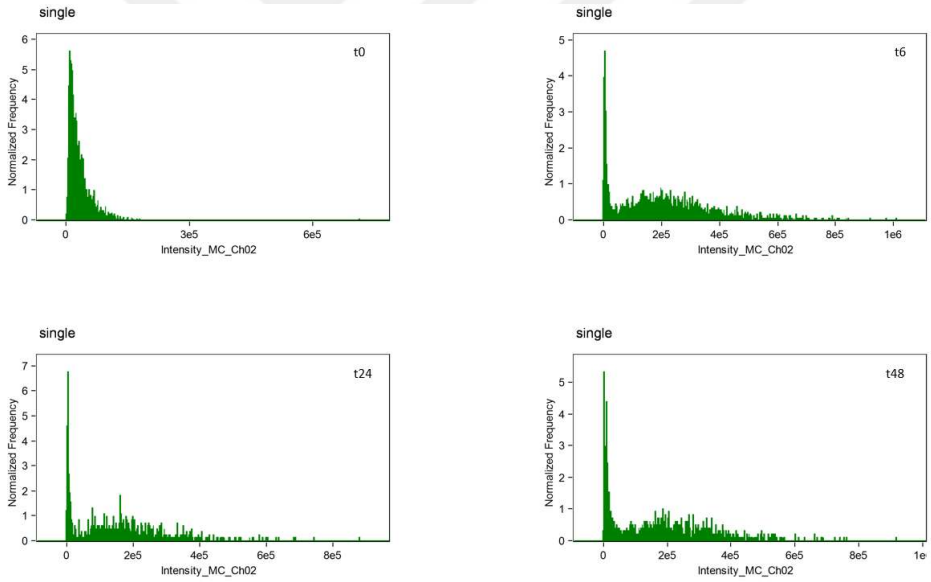

Figure A1.4: Mutant4 time course experimental data.

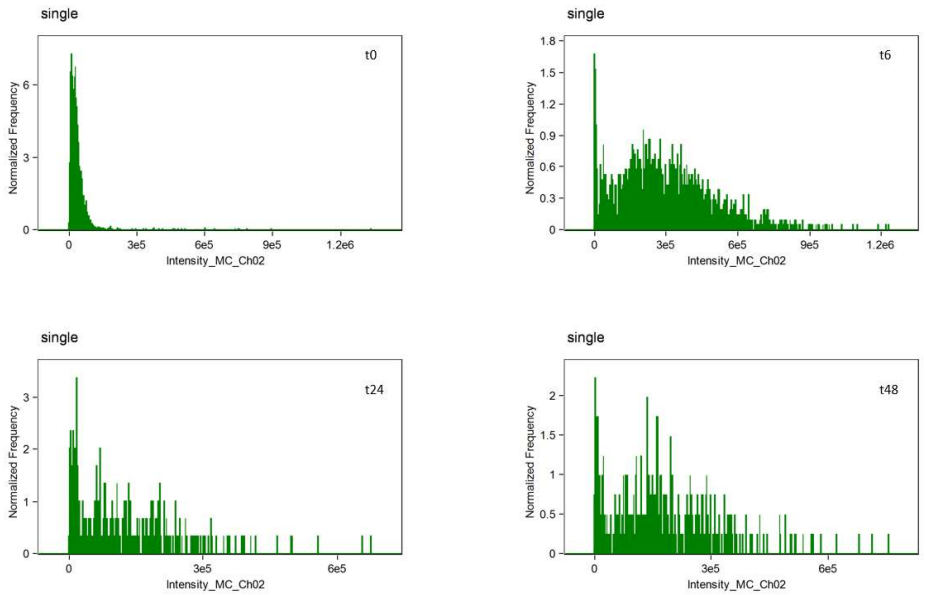

Figure A1.5: Mutant5 timecourse experimental data.

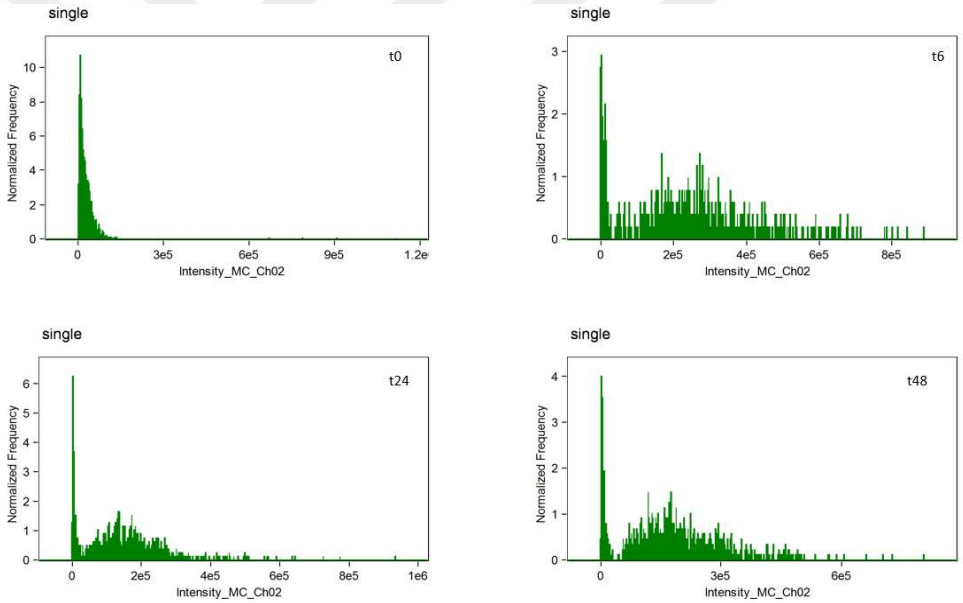

Figure A1.6: Mutant6 timecourse experimental data.

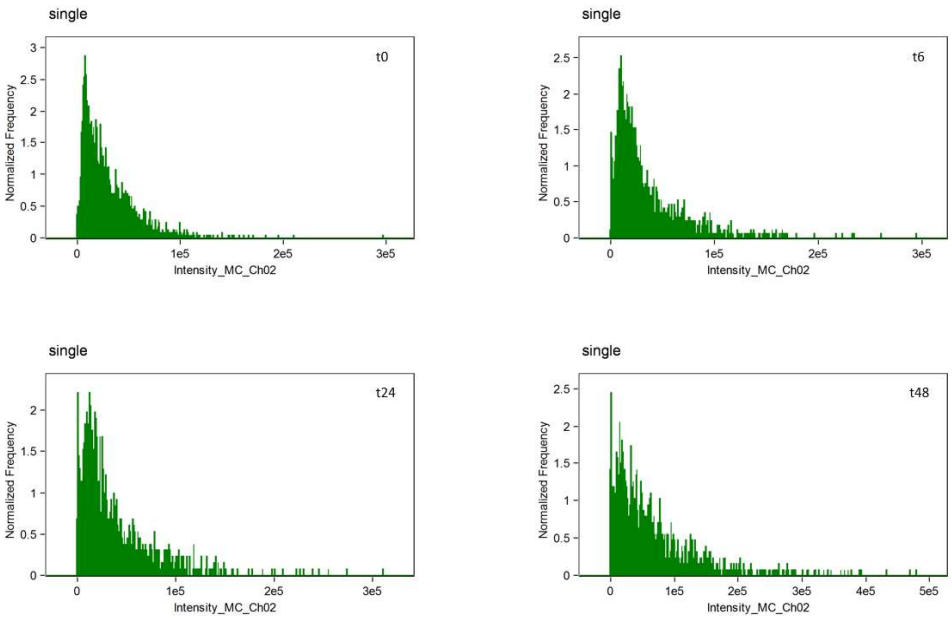

Figure A1.7: Wild type timecourse experimental data.

APPENDIX A.2: GCMS Data

Table A2.1: GCMS results of Mutant1.

	Compound name (#)	Formula	RT (min)	Area (Abs)	Peak Width 50% (min)	Start Time (min)	End Time (min)
1	Undefined	-	7,814	4532928	0,144	7,702	8,118
2	Undefined	-	9,18	876838	0,076	9,038	9,301
3	Methyl tetradecanoate	$C_{15}H_{30}O_2$	10,989	47442528	0,058	10,902	11,173
4	Pentadecanoic acid, methyl ester	$C_{16}H_{32}O_2$	12,147	17456058	0,056	11,989	12,302
5	Hexadecanoic acid, methyl ester	$C_{17}H_{34}O_2$	13,592	489302831	0,048	13,33	13,825
6	9-Hexadecenoic acid, methyl ester, (Z)-	$C_{17}H_{32}O_2$	14,178	15558376	0,061	14,006	14,329
7	Heptadecanoic acid, methyl ester	$C_{18}H_{36}O_2$	15,339	4624010	0,065	15,196	15,469
8	Undefined	-	16,259	1103511	0,08	15,893	16,413
9	Methyl 9,10-methylene-octadecanoate	$C_{20}H_{38}O_2$	17,793	5240586	0,077	17,695	17,984
10	9-Octadecenoic acid (Z)-, methyl ester	$C_{19}H_{36}O_2$	18,633	25573526	0,08	18,427	18,848
11	Androstan-17-one, 3-ethyl-3-hydroxy-, (5α)-	$C_{21}H_{34}O_2$	32,642	12511989	0,093	32,457	32,84
12	Methyl 4,7,10,13,16-docosapentaenoate	$C_{23}H_{36}O_2$	39,482	111000747	0,11	39,161	39,733
13	4,7,10,13,16,19-Docosahexaenoic acid, methyl ester	$C_{23}H_{34}O_2$	43,136	**616903033**	0,136	42,688	43,334

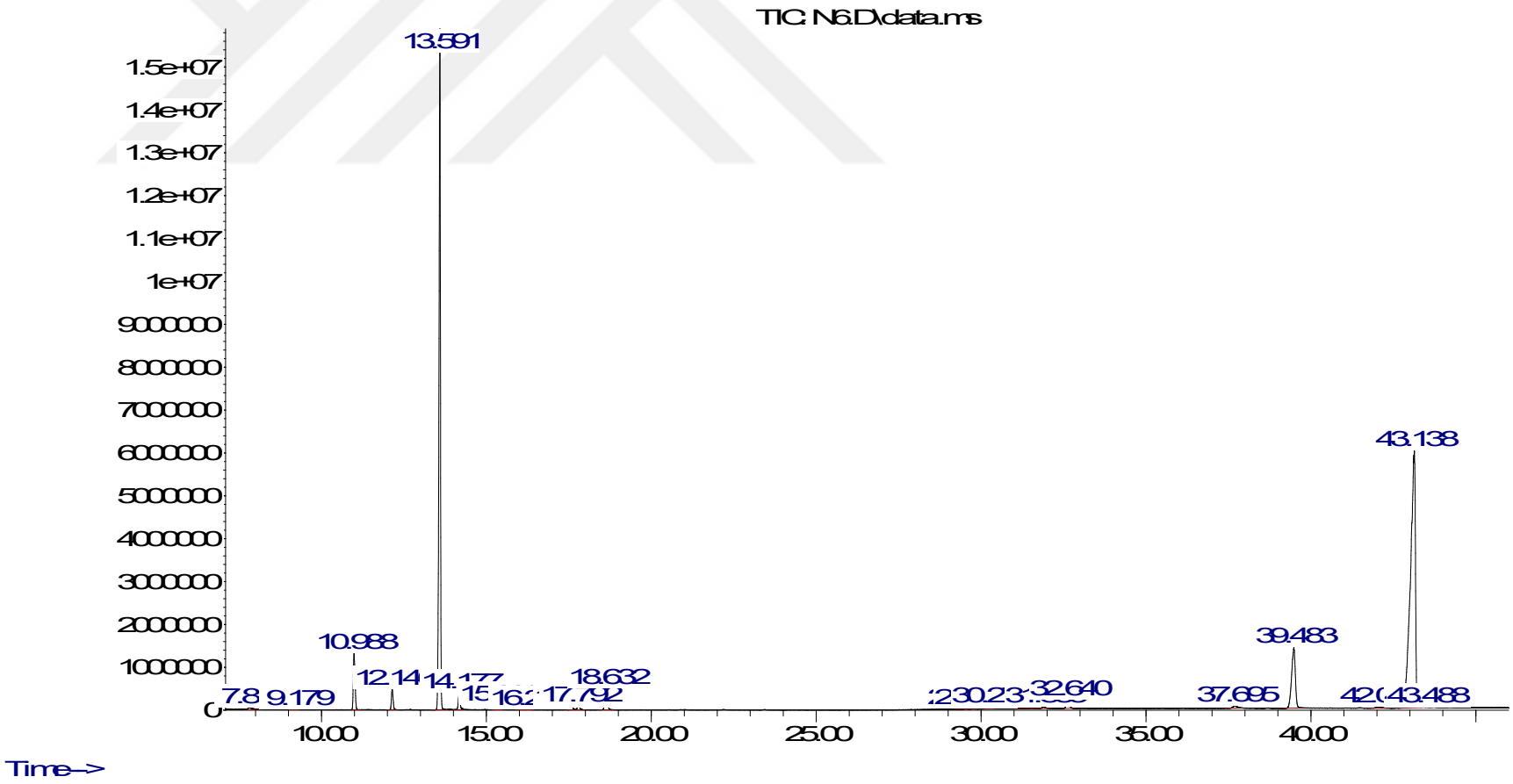

Figure A2.1: GCMS results of Mutant1.

Table A2.2: GCMS results of Mutant2.

	Compound name (#)	Formula	RT (min)	Area (Abs)	Peak Width 50% (min)	Start Time (min)	End Time (min)
1	Undefined	-	9,174	1384904	0,072	9,092	9,316
2	Methyl tetradecanoate	$C_{15}H_{30}O_2$	10,985	60270579	0,055	10,856	11,213
3	Pentadecanoic acid, methyl ester	$C_{16}H_{32}O_2$	12,14	13096620	0,056	11,978	12,353
4	Hexadecanoic acid, methyl ester	$C_{17}H_{34}O_2$	13,582	438396416	0,048	13,406	13,791
5	9-Hexadecenoic acid, methyl ester, (Z)-	$C_{17}H_{32}O_2$	14,172	12456447	0,06	13,99	14,328
6	Undefined	-	15,333	3460467	0,067	15,214	15,456
7	Heptadecanoic acid, methyl ester	$C_{18}H_{36}O_2$	16,241	874849	0,063	16,125	16,354
8	Undefined	-	17,6	1974887	0,069	17,468	17,713
9	Methyl 9,10-methylene-octadecanoate	$C_{20}H_{38}O_2$	17,784	1754247	0,073	17,713	17,933
10	9-Octadecenoic acid (Z)-, methyl ester	$C_{19}H_{36}O_2$	18,63	19482177	0,079	18,45	18,868
14	Androstan-17-one, 3-ethyl-3-hydroxy-, (5α)-	$C_{21}H_{34}O_2$	32,641	17582174	0,091	32,437	32,804
15	Methyl 4,7,10,13,16-docosapentaenoate	$C_{23}H_{36}O_2$	39,482	121100555	0,107	39,161	39,814
16	4,7,10,13,16,19-Docosahexaenoic acid, methyl ester	$C_{23}H_{34}O_2$	43,118	**557902033**	0,132	42,668	43,364

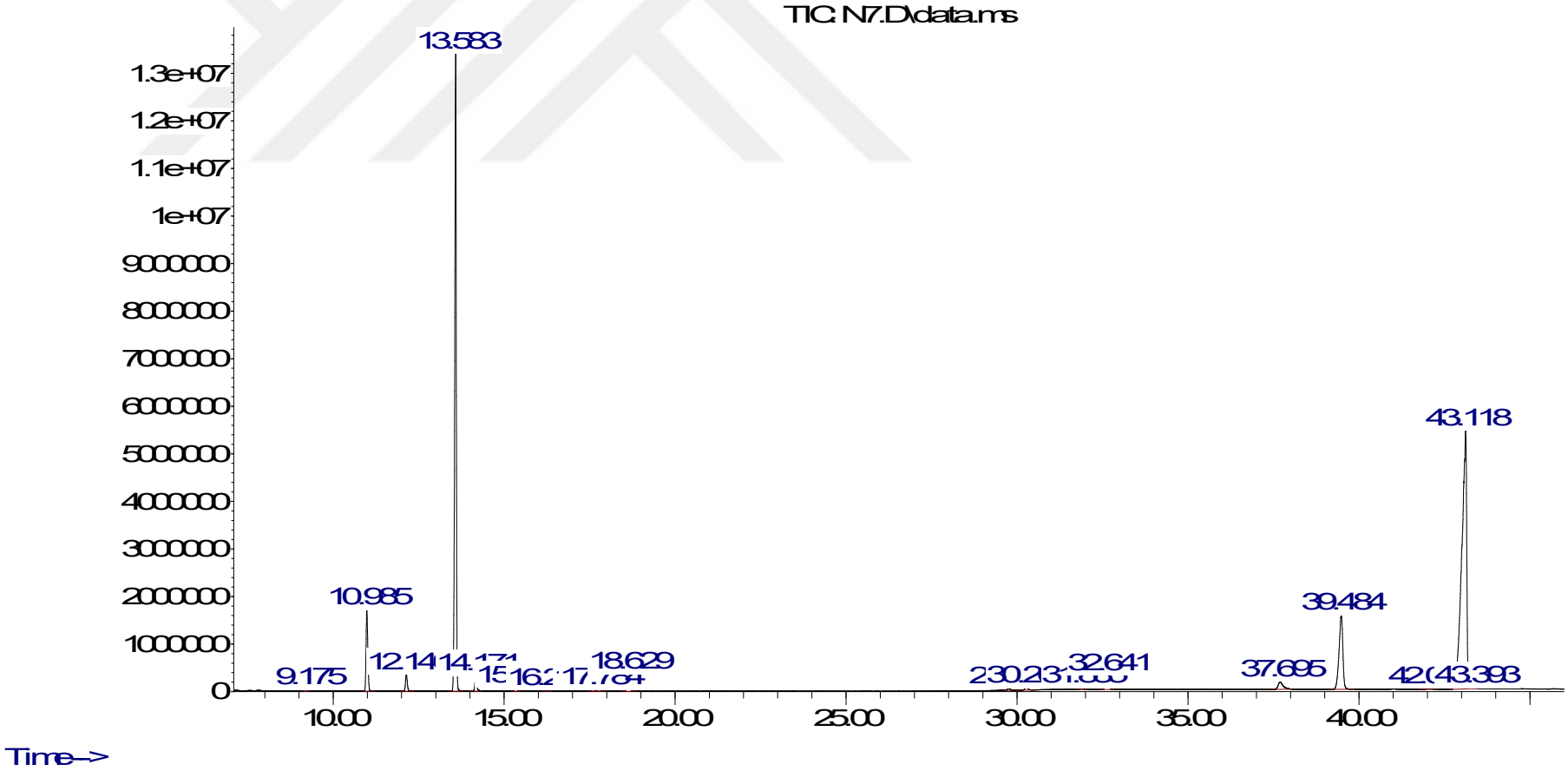

Figure A2.2: GCMS results of Mutant2.

Table A2.3: GCMS results of Mutant3.

	Compound name (#)	Formula	RT (min)	Area (Abs)	Peak Width 50% (min)	Start Time (min)	End Time (min)
1	Undefined	-	9,174	1278996	0,065	9,069	9,334
2	Methyl tetradecanoate	$C_{15}H_{30}O_2$	10,982	52884549	0,055	10,882	11,137
3	Pentadecanoic acid, methyl ester	$C_{16}H_{32}O_2$	12,137	7304745	0,056	12,016	12,358
4	Hexadecanoic acid, methyl ester	$C_{17}H_{34}O_2$	13,573	270194026	0,049	13,396	13,817
5	9-Hexadecenoic acid, methyl ester, (Z)-	$C_{17}H_{32}O_2$	14,169	14782944	0,061	14,028	14,342
6	Undefined	-	15,333	1317322	0,06	15,207	15,459
7	Heptadecanoic acid, methyl ester	$C_{18}H_{36}O_2$	17,597	1513956	0,073	17,318	17,695
8	Methyl 9,10-methylene-octadecanoate	$C_{20}H_{38}O_2$	17,787	4942085	0,079	17,695	17,981
9	9-Octadecenoic acid (Z)-, methyl ester	$C_{19}H_{36}O_2$	18,624	22656515	0,079	18,465	18,907
10	Undefined	-	29,757	1450812	0,1	29,339	29,856
11	Undefined	-	30,267	2037822	0,09	29,856	30,384
12	Undefined	-	31,896	1404349	0,079	31,731	32,014
13	Androstan-17-one, 3-ethyl-3-hydroxy-, (5α)-	$C_{21}H_{34}O_2$	32,632	9269756	0,093	32,411	32,819
14	Undefined	-	37,698	2772939	0,113	37,567	37,904
15	Methyl 4,7,10,13,16-docosapentaenoate	$C_{23}H_{36}O_2$	39,461	82404137	0,107	38,988	39,758
16	4,7,10,13,16,19-Docosahexaenoic acid, methyl ester	$C_{23}H_{34}O_2$	43,084	**478004313**	0,132	42,701	43,351

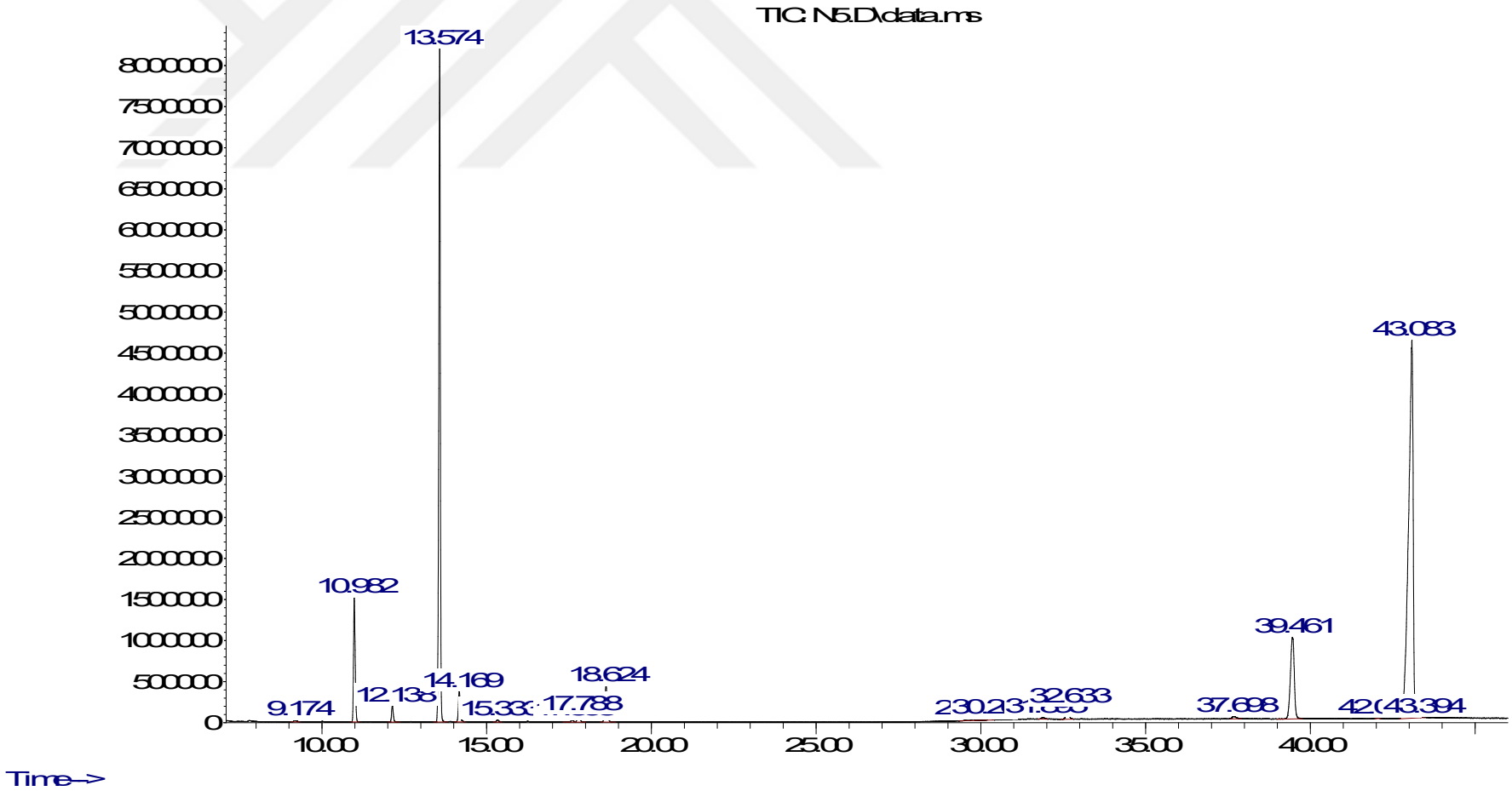

Figure A2.3: GCMS results of Mutant3.

Table A2.4: GCMS results of Mutant4.

	Compound name (#)	Formula	RT (min)	Area (Abs)	Peak Width 50% (min)	Start Time (min)	End Time (min)
1	Undefined	-	7,799	1763126	0,118	7,534	8,018
2	Undefined	-	9,192	919331	0,076	9,064	9,319
3	Methyl tetradecanoate	$C_{15}H_{30}O_2$	10,991	25504418	0,056	10,879	11,165
4	Undefined	-	11,798	702435	0,052	11,685	11,866
5	Pentadecanoic acid, methyl ester	$C_{16}H_{32}O_2$	12,143	4773003	0,06	11,894	12,317
6	Hexadecanoic acid, methyl ester	$C_{17}H_{34}O_2$	13,57	133940017	0,054	13,444	13,824
7	9-Hexadecenoic acid, methyl ester, (Z)	$C_{17}H_{32}O_2$	14,172	5426939	0,06	13,962	14,339
8	Undefined	-	14,792	888369	0,058	14,676	14,918
9	Heptadecanoic acid, methyl ester	$C_{18}H_{36}O_2$	15,336	794404	0,063	15,115	15,441
10	Methyl 9,10-methylene-octadecanoate	$C_{20}H_{38}O_2$	17,79	3079225	0,074	17,7	17,983
11	9-Octadecenoic acid (Z)-, methyl ester	$C_{19}H_{36}O_2$	18,63	7976589	0,081	18,465	18,817
12	Undefined	-	29,763	692706	0,088	29,629	29,843
13	Undefined	-	30,273	1473815	0,105	30,096	30,41
14	Androstan-17-one, 3-ethyl-3-hydroxy-, (5α)-	$C_{21}H_{34}O_2$	31,887	934581	0,081	31,759	32,034
15	Undefined	-	32,629	5052384	0,082	32,462	32,796
16	Methyl 4,7,10,13,16-docosapentaenoate	$C_{23}H_{36}O_2$	39,446	48842100	0,119	39,118	39,735
17	4,7,10,13,16,19-Docosahexaenoic acid, methyl ester	$C_{23}H_{34}O_2$	43,011	**275287054**	0,123	42,499	43,351

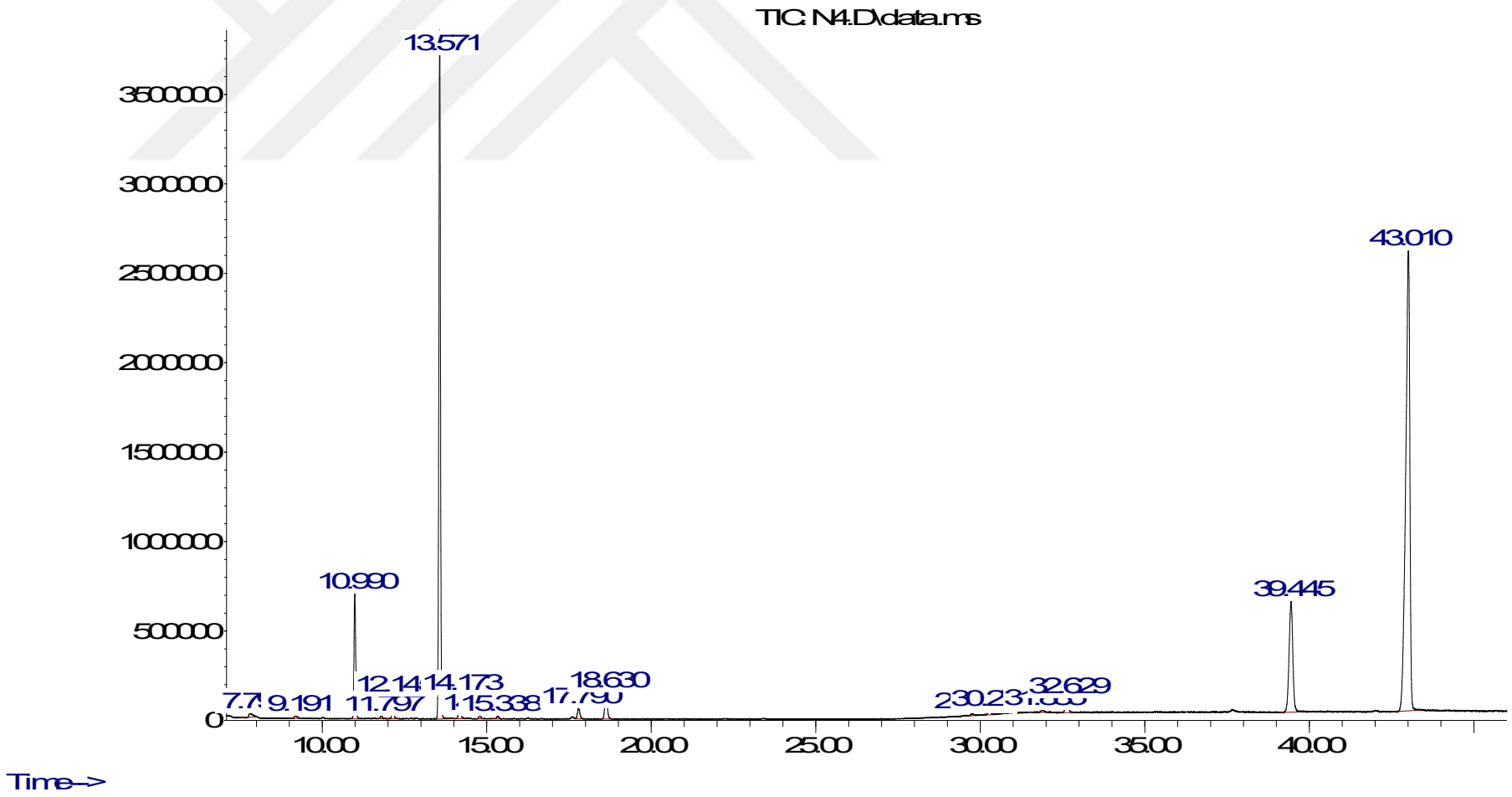

Figure A2.4: GCMS results of Mutant4.

Table A2.5: GCMS results of Mutant5.

	Compound number (#)	Formula	RT (min)	Area (Abs)	Peak Width 50% (min)	Start Time (min)	End Time (min)
1	Undefined	-	7,851	4421760	0,148	7,735	8,148
2	Methyl tetradecanoate	$C_{15}H_{30}O_2$	10,995	13535131	0,061	10,877	11,203
3	Pentadecanoic acid, methyl ester	$C_{16}H_{32}O_2$	12,149	11918434	0,056	12,009	12,315
4	Hexadecanoic acid, methyl ester	$C_{17}H_{34}O_2$	13,582	280008663	0,051	13,424	13,812
5	9-Hexadecenoic acid, methyl ester, (Z)-	$C_{17}H_{32}O_2$	14,178	3919189	0,068	13,993	14,345
6	Heptadecanoic acid, methyl ester	$C_{18}H_{36}O_2$	15,339	4817171	0,066	15,171	15,434
7	9-Octadecenoic acid (Z)-, methyl ester	$C_{19}H_{36}O_2$	17,6	2154742	0,069	17,468	17,695
8	Methyl 9,10-methylene-octadecanoate	$C_{20}H_{38}O_2$	17,796	4743799	0,077	17,695	17,968
9	Undefined	-	18,627	12371598	0,081	18,427	18,902
10	Undefined	-	30,27	1755535	0,084	30,083	30,405
11	Androstan-17-one, 3-ethyl-3-hydroxy-, (5α)-	$C_{21}H_{34}O_2$	31,893	1691222	0,077	31,621	32,047
12	Undefined	-	32,632	8706911	0,09	32,457	32,835
13	Undefined	-	37,683	3311525	0,12	37,514	37,891
14	Methyl 4,7,10,13,16-docosapentaenoate	$C_{23}H_{36}O_2$	39,458	73115150	0,107	39,208	39,694
15	Undefined	-	42,027	2716870	0,127	41,63	42,254
16	4,7,10,13,16,19-Docosahexaenoic acid, methyl ester	C23H36O2	43,063	**425839742**	0,133	42,405	43,333
17	Undefined	-	43,35	237372	0,048	43,333	43,412

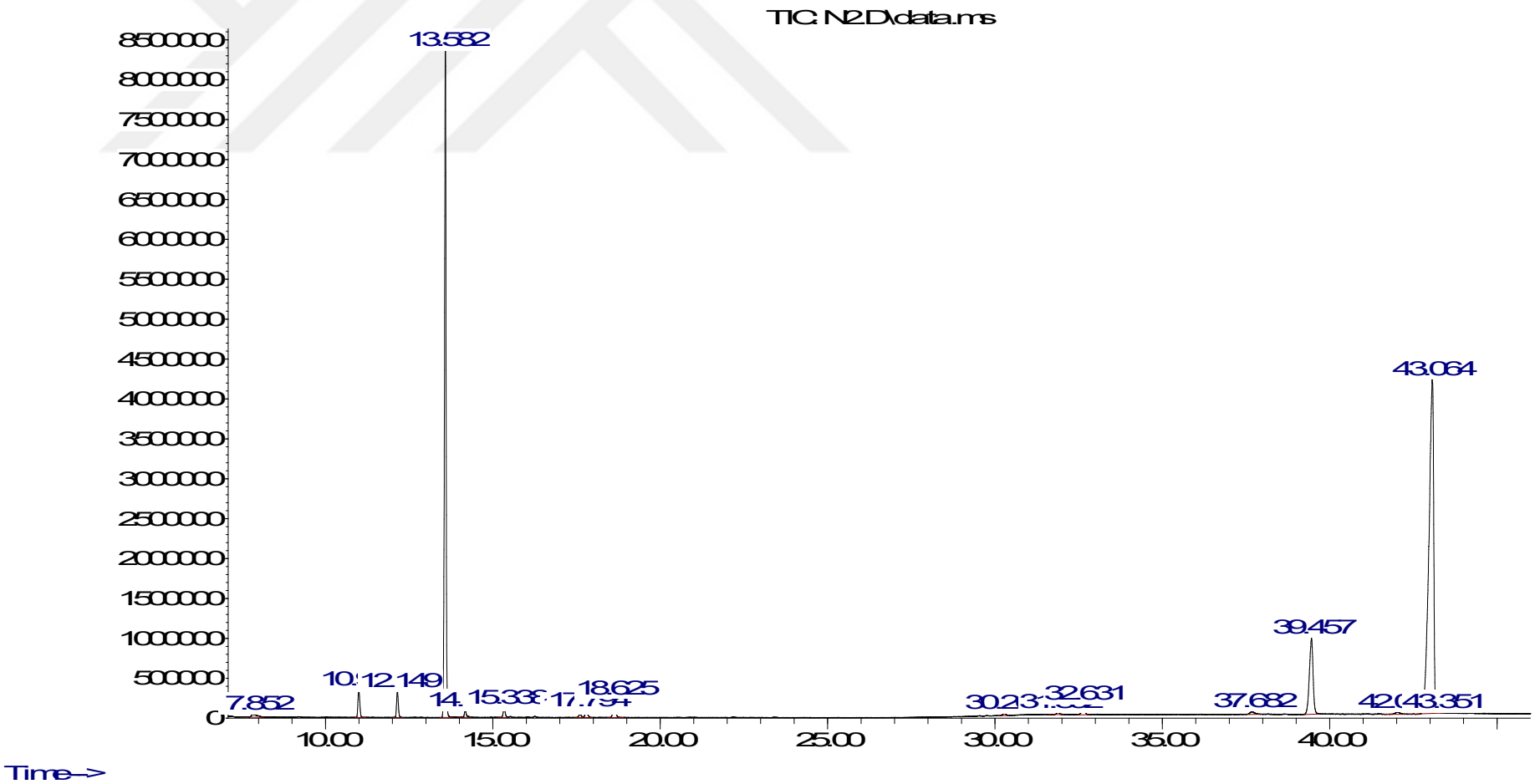

Figure A2.5: GCMS results of Mutant5.

Table A2.6: GCMS results of Mutant6.

	Compound name (#)	Formula	RT (min)	Area (Abs)	Peak Width 50% (min)	Start Time (min)	End Time (min)
1	Undefined	-	9,192	981540	0,073	9,127	9,308
2	Methyl tetradecanoate	$C_{15}H_{30}O_2$	10,998	25519890	0,059	10,902	11,198
3	Pentadecanoic acid, methyl ester	$C_{16}H_{32}O_2$	12,149	2101935	0,058	12,037	12,315
4	Hexadecanoic acid, methyl ester	$C_{17}H_{34}O_2$	13,582	175916835	0,051	13,424	13,801
5	9-Hexadecenoic acid, methyl ester, (Z)-	$C_{17}H_{32}O_2$	14,184	9650485	0,062	14,018	14,457
6	Methyl 9,10-methylene-octadecanoate	$C_{20}H_{38}O_2$	17,603	787631	0,074	17,412	17,734
7	9-Octadecenoic acid (Z)-, methyl ester	$C_{19}H_{36}O_2$	18,639	18892519	0,077	18,478	18,815
8	Undefined	-	29,751	1249283	0,093	29,634	29,92
9	Undefined	-	30,283	4344227	0,088	29,958	30,458
10	Androstan-17-one, 3-ethyl-3-hydroxy-, (5α)-	$C_{21}H_{34}O_2$	32,647	28879905	0,082	32,47	32,783
11	Undefined	-	37,695	715666	0,085	37,483	37,769
12	Methyl 4,7,10,13,16-docosapentaenoate	$C_{23}H_{36}O_2$	39,455	52507086	0,113	39,184	39,717
13	4,7,10,13,16,19-Docosahexaenoic acid, methyl ester	$C_{23}H_{34}O_2$	43,029	**306903033**	0,11	42,639	43,262
14	Undefined	-	43,286	206540	0,045	43,262	43,353

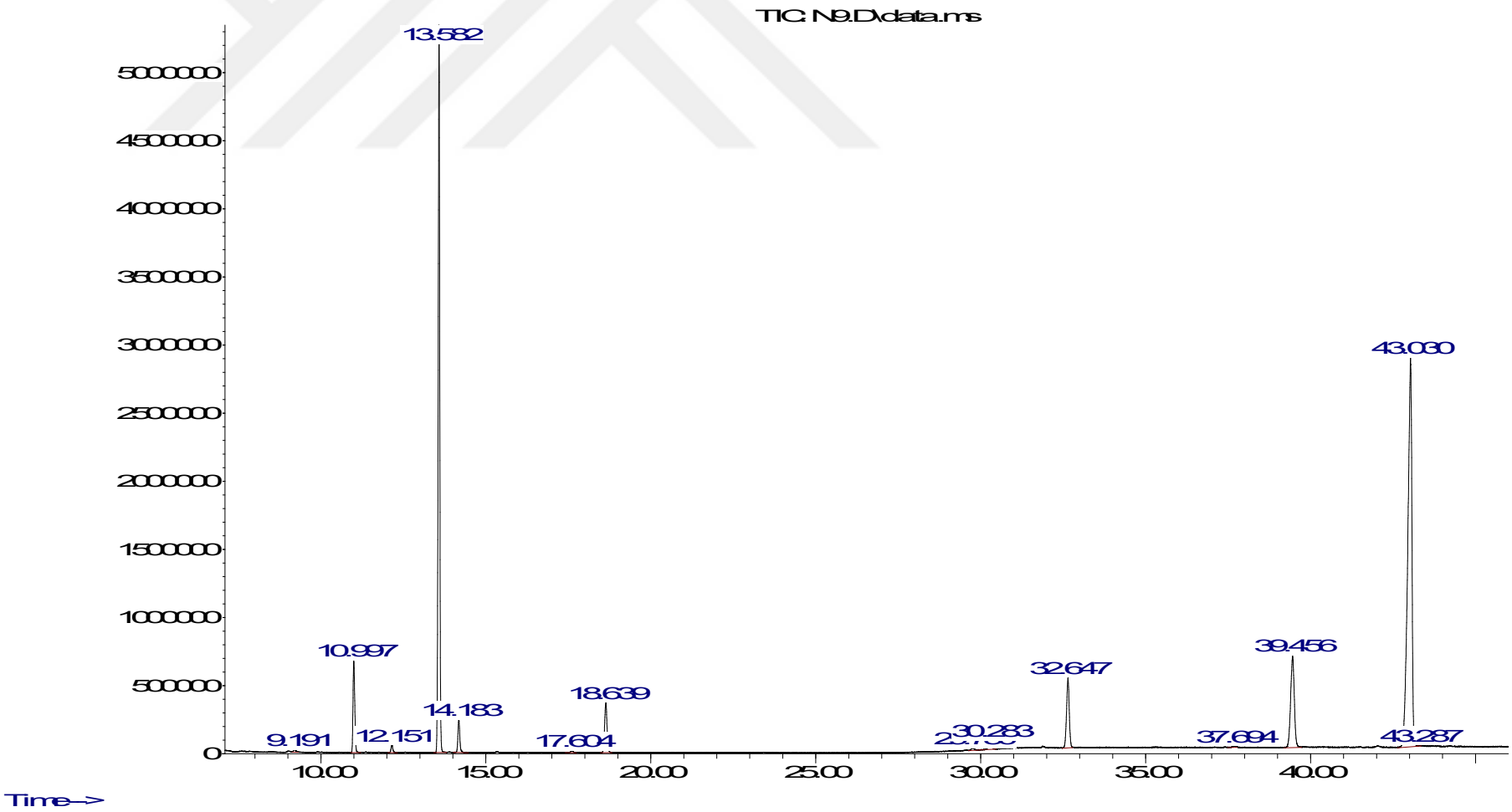

Figure A2.6: GCMS results of Mutant6.

Table A2.7: GCMS results of wild type.

	Compound number (#)	Formula	RT (min)	Area (Ab*s)	Peak Width 50% (min)	Start Time (min)	End Time (min)
1	Undefined	-	7,826	3944932	0,149	7,692	8,128
2	Undefined	-	9,183	981359	0,081	9,074	9,362
3	Methyl tetradecanoate	$C_{15}H_{30}O_2$	10,988	35232685	0,057	10,777	11,195
4	Pentadecanoic acid, methyl ester	$C_{16}H_{32}O_2$	12,143	12415969	0,061	11,767	12,358
5	Hexadecanoic acid, methyl ester	$C_{17}H_{34}O_2$	13,579	303764987	0,051	13,401	13,835
6	9-Hexadecenoic acid, methyl ester, (Z)-	$C_{17}H_{32}O_2$	14,172	9727163	0,064	13,96	14,35
7	Undefined	-	15,336	3251981	0,066	15,184	15,446
8	Heptadecanoic acid, methyl ester	$C_{18}H_{36}O_2$	17,603	1906407	0,068	17,377	17,69
9	Methyl 9,10-methylene-octadecanoate	$C_{20}H_{38}O_2$	17,79	4126939	0,079	17,69	18,014
10	9-Octadecenoic acid (Z)-, methyl ester	$C_{19}H_{36}O_2$	18,624	16049694	0,079	18,455	18,886
11	Undefined	-	29,742	720597	0,096	29,645	29,833
12	Undefined	-	30,27	2448892	0,089	29,849	30,397
13	Undefined	-	31,89	1603853	0,08	31,636	32,006
14	Androstan-17-one, 3-ethyl-3-hydroxy-, (5α)-	$C_{21}H_{34}O_2$	32,629	10528676	0,085	32,416	32,784
15	Undefined	-	37,673	2420544	0,111	37,524	37,833
16	Methyl 4,7,10,13,16-docosapentaenoate	$C_{23}H_{36}O_2$	39,464	85446674	0,107	39,23	39,893
17	4,7,10,13,16,19-Docosahexaenoic acid, methyl ester	$C_{23}H_{34}O_2$	43,075	**485502660**	0,127	42,673	43,277

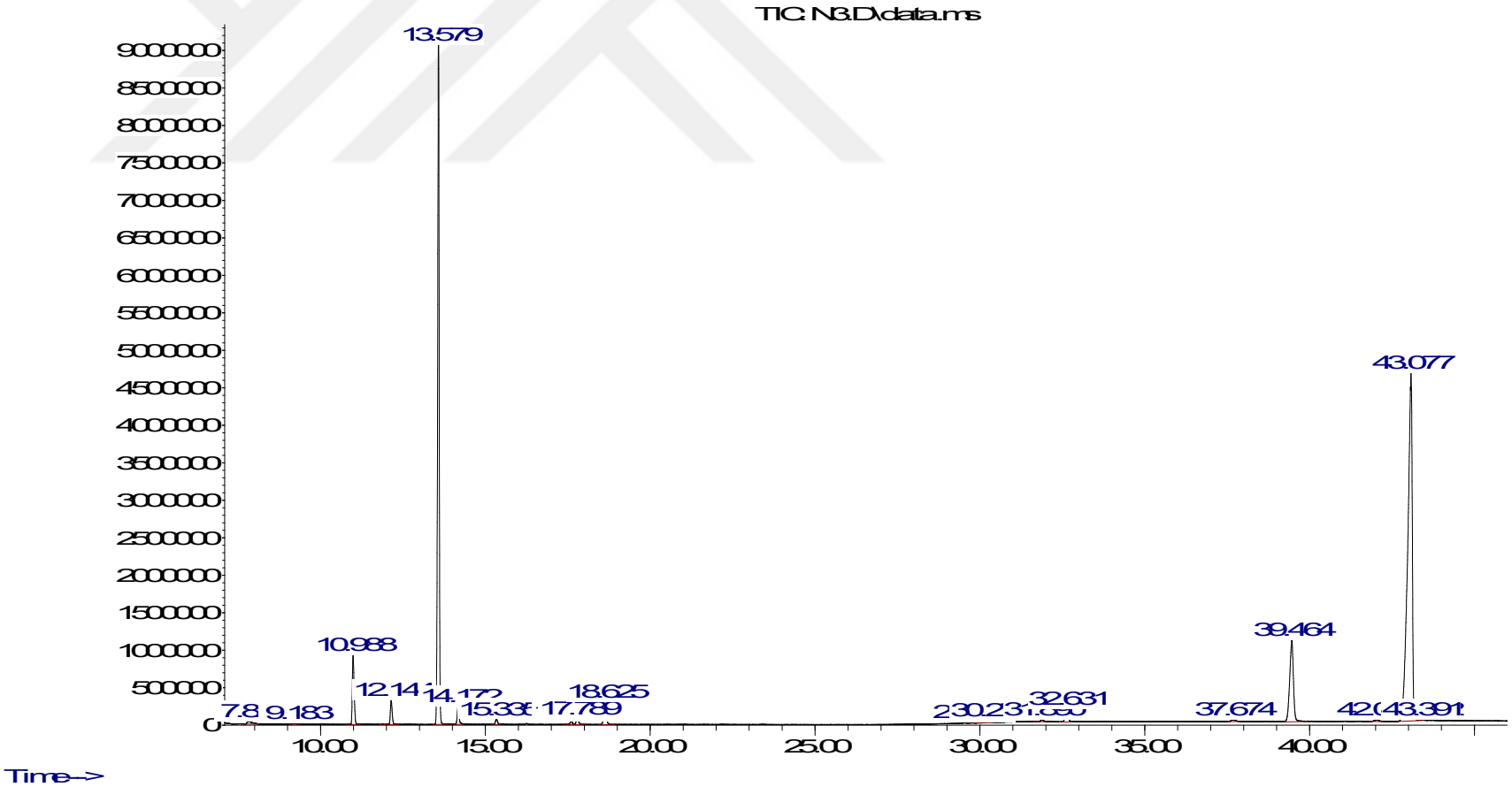

Figure A2.7: GCMS results of Wild type

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PUBLICATIONS, PRESENTATIONS AND PATENTS ON THE THESIS:

- **Nurcan Vardar,** Yiğit Ayhan, Emrah Yelboğa, Banu Bahar, Melek Tüter, Nevin Gül Karagüler, "Optimization of Cultivation and Supercritical Fluid Lipid Extraction Conditions of *Schizochytrium* sp.S31" IJSES 2016; 2(4): 13-24
- **Nurcan Vardar**, Emrah Yelboğa, Mark Hildebrand, Nevin Gül Karagüler, Melek Tüter "Improving Lipid Accumulation in the *Schizochytrium* sp. by UV mutagenesis " (In prep.)

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- Nurgül Balcı, **Nurcan Vardar**, Emrah Yelboğa, Nevin Gül Karagüler, "Bacterial community composition of sediments from Artificial Lake Maslak, Istanbul, Turkey", Environ Monit Assess 2012; 184(9): 5641-50
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