UNIVERSITI PUTRA MALAYSIA

MOLECULAR CLONING AND FUNCTIONAL EXPRESSION OF DELTA 9-FATTY ACID DESATURASE FROM ANTARCTIC Pseudomonas sp. A3 AND Pseudomonas sp. A8

LAWAL GARBA

FBSB 2017 5

MOLECULAR CLONING AND FUNCTIONAL EXPRESSION OF DELTA 9-FATTY ACID DESATURASE FROM ANTARCTIC *Pseudomonas* sp. A3 AND *Pseudomonas* sp. A8

By

LAWAL GARBA

Thesis Submitted to the School of Graduate Studies, Universiti Putra Malaysia, in Fulfilment of the Requirements for the Degree of Doctor of Philosophy

March 2017

COPYRIGHT

Abstract of thesis presented to the Senate of Universiti Putra Malaysia in fulfillment of the requirement for the degree of Doctor of Philosophy

MOLECULAR CLONING AND FUNCTIONAL EXPRESSION OF DELTA 9-FATTY ACID DESATURASE FROM ANTARCTIC *Pseudomonas* sp. A3 AND *Pseudomonas* sp. A8

By

LAWAL GARBA

March 2017

Chairman : Professor Raja Noor Zaliha Raja Abd. Rahman, PhD
Faculty : Biotechnology and Biomolecular Sciences

Fatty acid desaturase enzymes are capable of inserting double bonds between carbon atoms of saturated fatty acyl chains to produce unsaturated fatty acids. The enzymes are used by the Antarctic microorganisms to increase the amount of cellular unsaturated fatty acids, which maintain the proper membrane fluidity at low temperatures. The Δ9-fatty acid desaturase enzyme catalyses introduction of the first double bond between C9 and C10 positions of saturated fatty acyl chains, a critical step in the biosynthesis of many polyunsaturated fatty acids. Although many Δ9-fatty acid desaturases were studied from animals, plants and bacteria, to date the number of the Δ9-fatty acid desaturase enzymes reported from Antarctic bacteria is very limited.The main objectives of this research were to clone and functionally express Δ9-fatty acid desaturases. Five isolates of Antarctic bacteria were screened for their ability to produce unsaturated fatty acids at low temperatures and identified as *Pseudomonas* sp. A3, *Pseudomonas* sp. A8, *Arthrobacter* sp. 1B, *Arthrobacter* sp. 3B and *Arthrobacter* sp. PB based on 16S rDNA identification supported by morphological characteristics and biochemical tests. The bacteria were positive palmitoleic and oleic acids producers except *Arthrobacter* sp. 1B. Presence of a double bond at C9 positions of these fatty acids suggested that the isolates would be potential Δ9-fatty acid desaturase producers. Total unsaturation was observed to be higher in *Arthrobacter* sp. 3B (47.24%), followed by *Pseudomonas* sp. A8 (45.09%), *Pseudomonas* sp. A3 (33.17%), and *Arthrobacter* sp. PB (31.92%). Although *Arthrobacter* sp. 3B had the highest unsaturation, isolation of Δ9-fatty acid desaturase gene from this bacterium was unsuccessful. Hence, *Pseudomonas* sp. A3 and *Pseudomonas* sp. A8 were selected for Δ9-fatty acid desaturase gene isolation. The genes were successfully PCR amplified from *Pseudomonas* sp. A3 and *Pseudomonas* sp. A8, cloned and heterologously expressed in *Escherichia coli* Transetta (DE3) under the control of T7 promoter. The genes isolated from *Pseudomonas* sp. A3 and *Pseudomonas sp*. A8 were designated as *PA3FAD9* and *PA8FAD9*, respectively each having an open reading frame of 1,185 bp coding for 394 amino acids with a predicted molecular weight of 45 kDa. Three dimensional structures of both the Δ9-fatty acid

desaturases were predicted using YASARA software with suitable templates and used to perform molecular docking of palmitic acid, which predicted the ability of the enzymes to use palmitic acid as a substrate during the *in vivo* studies. Functional expression of each gene was confirmed by GCMS, which showed functionally expressed Δ9-fatty acid desaturases capable of increasing the overall cellular palmitoleic acid content of the recombinant *E. coli* cells that carried *PA3FAD9* and *PA8FAD9* genes leading to two-fold increase upon expression at 15 and 20 ºC, respectively. Exogenous stearic acid was incorporated into the *E. coli* phospholipids before it was desaturated by the Δ9-fatty acid desaturases to produce oleic acids when added into the *E. coli* growth medium. The results confirmed novel Δ9-fatty acid desaturases that could be used to enhance unsaturated fatty acid production in suitable hosts. In conclusion, the two Δ9-fatty acid desaturase proteins were functionally expressed and increased the overall cellular unsaturated fatty acid contents of the recombinant *E. coli*. The enzymes could be used in polyunsaturated fatty acids production through co-expression with other genes of desaturases and elongases in a host that can produce unsaturated fatty acids using the recombinant DNA technology.

Abstrak tesis yang dikemukakan kepada Senat Universiti Putra Malaysia sebagai memenuhi keperluan untuk ijazah Doktor Falsafah

PENGKLONAN MOLEKUL DAN EKSPRESI FUNGSI DELTA 9-ASID LEMAK DESATURASE DARI ANTARKTIKA *Pseudomonas* sp. A3 DAN *Pseudomonas* sp. A8

Oleh

LAWAL GARBA

Mac 2017

Pengerusi : Profesor Raja Noor Zaliha Raja Abd. Rahman, PhD
Fakulti : Bioteknologi dan Sains Biomolekul

Enzim desaturase asid lemak mampu memasukkan ikatan antara atom karbon tepu rantaian asil lemak untuk menghasilkan asid lemak tak tepu. Enzim ini digunakan oleh mikroorganisma Antarktika untuk meningkatkan jumlah asid lemak tak tepu di dalam sel, yang mengekalkan kebendaliran membran yang betul pada suhu rendah. Enzim Δ9-asid lemak desaturase menjadi pemangkin pengenalan kepada ikatan berganda yang pertama antara C9 dan C10 pada kedudukan rantaian acyl lemak tepu, satu langkah yang kritikal dalam biosintesis pelbagai asid lemak tidak tepu. Problem. Walaupun banyak desaturases asid lemak Δ9 telah dikaji daripada haiwan, tumbuh-tumbuhan dan bakteria, setakat ini hanya sejumlah enzim yang sangat terhad telah dikemukakan daripada bakteria Antartika. Objektif utama kajian ini adalah untuk mengklon dan mengzahirkan fungsi Δ9-asid lemak desaturases. Δ9-Asid lemak enzim desaturase mejadi pemangkin kepada pengenalan ikatan kembar pertama antara C9 dan kedudukan C10 tepu rantaian lemak asil, satu langkah yang kritikal dalam biosintesis asid lemak tak tepu yang banyak. Sebahagian besar "Open Reading Frame" (ORF) pada Δ9-asid lemak desaturase enzim telah diterjemah dari genom spesies Antarktika *Pseudomonas* dan dikemukakan kepada pangkalan data awam tanpa apa-apa maklumat mengenai fungsi mereka. Objektif utama kajian ini adalah untuk mengkaji pengklonan molekul dan pengzahiran fungsi Δ9-asid lemak desaturases. Lima pencilan bakteria Antarktika telah disaring untuk keupayaan mereka menghasilkan asid lemak tak tepu pada suhu rendah dan dikenal pasti sebagai *Pseudomonas* sp. A3, *Pseudomonas* sp. A8, *Arthrobacter* sp. 1B, *Arthrobacter* sp. 3B dan *Arthrobacter* sp. PB berdasarkan pengenalan 16S rRNA disokong oleh ciri-ciri morfologi dan ujian biokimia. Bakteria-bakteria tersebut adalah penghasil asid palmitoleic positif dan asid oleik kecuali *Arthrobacter* sp. 1B. Kehadiran ikatan kembar pada kedudukan C9 asid lemak menunjukkan bahawa pencilan-pencilan tersebut berpotensi sebagai penghasil Δ9-asid lemak desaturase. Jumlah asid tak tepu yang dihasilkan di dalam *Arthrobacter* sp. adalah paling tinggi, 3B (47.24%) diikuti oleh *Pseudomonas* sp. A8 (45.09%), *Pseudomonas* sp. A3 (33.17%) dan *Arthrobacter* sp. PB (31.92%). Walaupun *Arthrobacter* sp. 3B mempunyai asid lemak tak tepu tertinggi,

pengasingan gen Δ9-asid lemak desaturase dari bakteria ini masih sukar difahami. Oleh itu, *Pseudomonas* sp. A3 dan *Pseudomonas* sp. A8 telah dipilih untuk pengasingan Δ9-asid lemak desaturase gen. Gen Δ9-asid lemak desaturase telah berjaya digandakan oleh PCR bagi *Pseudomonas* sp. A3 dan *Pseudomonas* sp. A8, kemudian gen tersebut diklon dan dizahir secara heterolog di dalam *Escherichia coli* Transetta (DE3) di bawah kawalan T7 promoter. Gen yang diasingkan daripada *Pseudomonas* sp. A3 dan *Pseudomonas* sp. A8 telah diberikan panggilan sebagai *PA3FAD9* dan *PA8FAD9*, di mana gen tersebut mempunyai bingkai bacaan terbuka sebanyak 1185 bp pengekodan untuk 394 asid amino dengan berat molekul yang dianggarkan sebanyak 45 kDa. Ketiga-tiga dimensi Δ9-asid lemak desaturases tersebut telah dianalisa dalam perisian YASARA menggunakan templat yang sesuai dan digunakan untuk melakukan dok molekul asid palmitik. Penzahiran fungsi setiap gen telah disahkan oleh GCMS yang menunjukkan penzahiran Δ9-asid lemak desaturases yang mampu berfungsi untuk meningkatkan jumlah palmitoleate di dalam sel *E. coli* rekombinan secara keseluruhan yang sekaligus membawa *PA3FAD9* dan *PA8FAD9* gen kepada peningkatan dua kali ganda penzahiran pada suhu 15 dan 20 ^{o}C. Asid stearik luaran telah dimasukkan ke dalam *E. coli* phospholipid sebelum ia dinyahtepukan oleh Δ9-asid lemak desaturases untuk menghasilkan asid oleik apabila ditambah ke dalam medium pertumbuhan *E. coli*. Hal ini telah membuktikan bahawa Δ9-asid lemak desaturases yang baru dari spesies Antarktika *Pseudomonas* boleh digunakan bagi meningkatkan pengeluaran asid lemak tak tepu dalam bakteria yang sesuai. Kesimpulannya, kedua-dua gen pengekodan bagi protein Δ9-asid lemak desaturase ini berjaya berfungsi dan dizahirkan bagi meningkatkan kesuluruhan kandungan asid lemak tak tepu di dalam sel *E. coli* rekombinan. Hasil kajian ini boleh digunakan untuk meningkatkan pengeluaran asid lemak politaktepu oleh penzahiran bersekali gen desaturases dan elongases yang lain dalam hos yang boleh menghasilkan asid lemak tak tepu melalui teknologi rekombinan DNA.

ACKNOWLEDGEMENTS

All praise is due to ALLAH, the Almighty who preserved me in good health to witness the successful completion of my PhD programme in the field of Microbial Biotechnology at the Faculty of Biotechnology and Biomolecular Sciences, Universiti Putra Malaysia. I believe without HIS unlimited blessings my goals would not have been achieved. I pray that this report and the knowledge acquired would be beneficial to fellow researchers and the humanity in general.

I am highly indebted to Professor Dr. Raja Noor Zaliha binti Raja Abdul Rahman, the chairman of my supervisory committee for taking time out of her tight schedules to ensure the smooth conduct of this research. Her supports, perseverance, tolerance and unscheduled appointments provided would never be forgotten. May ALLAH, the Almighty continue to bless and reward her in abundance. Members of my supervisory committee including Associate Professor Dr. Mohd Shukuri bin Mohamad Ali and Dr. Siti Nurbaya binti Oslan have played a crucial role throughout the conduct of this research. I wish them the best in all their endeavours.

I sincerely thank the management of Gombe State University, Gombe State, Nigeria. I am cognizant that without their cooperation, tolerance, moral and financial supports this research would not have been successful.

I am so grateful to my parents (malam Abubakar Muhammad and malama Dayyibah Muhammad) who may not be alive to witness this achievement. May ALLAH reward them with Jannatul firdous. In the same vein, I wish to express my profound gratitude to my family members including malam Hamisu Abubakar, Ridwanu Abubakar, Binta Abubakar and Hauwa'u Abubakar who had provided an extra drive at different points in time but all of them may not be alive to witness the successful completion of my PhD programme. May their gentle souls continue to rest in perfect peace. Other siblings include Mahmud Abubakar, Sadiya Abubakar and Mansur Abubakar. My late wife, Khadija Yusuf Ibrahim is still in my memory, may her gentle soul continue to rest in perfect peace. My fiancée in person of Nabilah Muhammad Dauda deserves the highest accolade for her understanding and more importantly standing by my side throughout the period of this programme despite the little time she received from me.

I sincerely thank the entire principal investigators, Enzyme and Microbial Technology Research group (EMTech), Faculty of Biotechnology and Biomolecular Sciences for their excellent guidance throughout the conduct of this research. I wish to extend my sincere gratitude to my labmates for their support, tolerance, and cooperation throughout the period of my research work at Lab 140 (Biotech 2), protein engineering lab (Biotech 3), Enzyme and Microbial Technology Laboratories at chemistry department, Faculty of Science and Institute of Bioscience (IBS), Universiti Putra Malaysia (UPM). I sincerely thank RMC, UPM for financial support to this research. Endless prayers offered by my mentors, loved ones, colleagues, personal friends and well-wishers are well appreciated.

I certify that a Thesis Examination Committee has met on 20 March 2017 to conduct the final examination of Lawal Garba on his thesis entitled "Molecular Cloning and Functional Expression of Delta 9-Fatty Acid Desaturase from Antarctic *Pseudomonas* sp. A3 and *Pseudomonas* sp. A8" in accordance with the Universities and University Colleges Act 1971 and the Constitution of the Universiti Putra Malaysia [P.U.(A) 106] 15 March 1998. The Committee recommends that the student be awarded the Doctor of Philosophy.

Members of the Thesis Examination Committee were as follows:

Umi Kalsom binti Md Shah, PhD
Associate Professor
Faculty of Biotechnology and Biomolecular Sciences
Universiti Putra Malaysia
(Chairman)

Muhajir bin Hamid, PhD
Associate Professor
Faculty of Biotechnology and Biomolecular Sciences
Universiti Putra Malaysia
(Internal Examiner)

Janna Ong binti Abdullah, PhD
Associate Professor
Faculty of Biotechnology and Biomolecular Sciences
Universiti Putra Malaysia
(Internal Examiner)

Naeem Rashid, PhD
Professor
University of the Punjab
Pakistan
(External Examiner)

NOR AINI AB. SHUKOR, PhD
Professor and Deputy Dean
School of Graduate Studies
Universiti Putra Malaysia

Date: 28 April 2017

This thesis has been presented to the Senate of Universiti Putra Malaysia and has been accepted as fulfillment of requirement for the degree of Doctor of Philosophy. Members of the supervisory committee were as follows:

Raja Noor Zaliha Raja Abdul Rahman, PhD
Professor
Faculty of Biotechnology and Biomolecular Sciences
Universiti Putra Malaysia
(Chairman)

Mohd Shukuri Mohamad Ali, PhD
Associate Professor
Faculty of Biotechnology and Biomolecular Sciences
Universiti Putra Malaysia
(Member)

Siti Nurbaya Oslan, PhD
Senior lecturer
Faculty of Biotechnology and Biomolecular Sciences
Universiti Putra Malaysia
(Member)

ROBIAH BINTI YUNUS, PhD
Professor and Dean
School of Graduate Studies
Universiti Putra Malaysia

Date:

Declaration by graduate student

I hereby confirm that:

- This thesis is my original work;
- Quotations, illustrations and citations have been duly referenced
- The thesis has not been submitted previously or concurrently for any other degree at any other institutions;
- Intellectual property from the thesis and copyright of thesis are fully-owned by the Universiti Putra Malaysia, as according to the Universiti Putra Malaysia (Research) Rules 2012;
- Written permission must be obtained from supervisor and office of the deputy Vice-Chancellor (Research and Innovation) before this thesis is published (in the form of written, printed or in electronic form) including books, journals, modules, proceedings, popular writings, seminar papers, manuscripts, posters, reports, lecture notes, learning modules or any other materials as stated in the Universiti Putra Malaysia (Research) Rules 2012;
- There is no plagiarism or data falsification/fabrication in the thesis, and scholarly integrity is upheld as according to the Universiti Putra Malaysia (Graduate Studies) Rules 2003 (Revision 2012-2013) and the Universiti Putra Malaysia (Research) Rules 2012. The thesis has undergone plagiarism detection software.

Signature:_____ Date:_____

Name and Matric No: Lawal Garba, GS37049

Declaration by Members of Supervisory Committee

This is to confirm that:

- the research conducted as well as the writing of this thesis was under our supervision;
- Supervision responsibilities as stated in the Universiti Putra Malaysia (Graduate Studies) Rules 2003 (Revision 2012-2013) are adhered to.

Signature :_____
Name of Chairman Supervisory Committee : Professor Dr. Raja Noor Zaliha binti Raja Abd Rahman

Signature :_____
Name of Member Supervisory Committee : Associate Professor Dr. Mohd Shukuri Mohamad Ali

Signature :_____
Name of Member Supervisory Committee : Dr. Siti Nurbaya Oslan

TABLE OF CONTENTS

			Page
ABSTRACT			i
ABSTRAK			iii
ACKNOWLEDGEMENTS			v
APPROVAL			vi
DECLARATION			viii
LIST OF TABLES			xiv
LIST OF FIGURES			xv
LIST OF APPENDICES			xviii
LIST OF ABBREVIATIONS			xix
CHAPTER			
1	**INTRODUCTION**		1
	1.1	Problem statement	1
	1.2	Hypothesis	1
	1.3	Objectives	2
2	**LITERATURE REVIEW**		3
	2.1	Extreme environments	3
	2.2	Psychrophiles	4
	2.3	Regulation of membrane fluidity in response to temperature changes	5
	2.4	Unsaturated fatty acids	7
		2.4.1 Synthesis of Unsaturated fatty acids in Bacteria	7
		2.4.1.1 Anaerobic synthesis	8
		2.4.1.2 Aerobic synthesis	8
	2.5	Fatty acid desaturases	10
		2.5.1 Types and sources of fatty acid desaturases	10
		2.5.1.1 Soluble fatty acid desaturases	11
		2.5.1.2 Integral membrane fatty acid desaturases	11
		2.5.1.3 Front-end and methyl-end fatty acid desaturases	12
	2.6	Cloning of fatty acid desaturase genes	13
	2.7	Functional expression of genes for fatty acid desaturase enzymes	13
		2.7.1 Functional expression of animal fatty acid desaturase genes	15
		2.7.2 Functional expression of plants fatty acid desaturase genes	15
		2.7.3 Functional expression of fungal fatty acid desaturase genes	17
		2.7.4 Functional expression of bacterial fatty acid desaturase genes	17
3	**MATERIALS AND METHODS**		19
	3.1	Bacterial isolates	19
	3.2	Preparation of glycerol stocks of the bacteria	19
	3.3	Screening of the bacteria for unsaturated fatty acids	19

3.3.1 Preparation of fatty acid methyl esters (FAMEs) 19
3.3.1.1 Saponification step 19
3.3.1.2 Methylation step 20
3.3.1.3 Extraction step 20
3.3.1.4 Base wash step 20
3.3.2 Gas chromatography-mass spectrometry analysis of FAMEs 20
3.4 16S rDNA identification of the bacteria 20
3.4.1 Extraction of genomic DNA 20
3.4.2 Polymerase chain reaction (PCR) amplification of 16S rDNA gene 21
3.4.3 Analysis of 16S rDNA gene and construction of phylogenetic tree 21
3.5 Morphology and biochemical tests 21
3.5.1 Morphological characteristics 21
3.5.1.1 Gram staining 21
3.5.1.2 Spore staining 21
3.5.1.3 Capsule staining 22
3.5.1.4 Motility test 22
3.5.2 Biochemical tests 22
3.5.2.1 Triple sugar iron (TSI) test 22
3.5.2.2 Citrate utilization test 22
3.5.2.3 Oxidase test 22
3.5.2.4 Sulfide and indole production tests 23
3.5.2.5 Urease test 23
3.5.2.6 Catalase test 23
3.5.2.7 Methyl red-Voges Proskauer test 23
3.6 Isolation of Δ9-fatty acid desaturase genes 23
3.6.1 Genomic DNA extraction 23
3.6.2 Analysis of genomic DNA 24
3.6.3 Primer design and PCR amplification of Δ9-fatty acid desaturase genes 24
3.6.4 Gel electrophoresis analysis and purification of PCR products 25
3.7 Preparation of *Escherichia coli* competent cells 25
3.8 Cloning of Δ9- fatty acid desaturase genes 25
3.8.1 Cloning of Δ9- fatty acid desaturase genes into pGEM-T Easy vector 25
3.8.1.1 Extraction of recombinant pGEM-T Easy vector 26
3.8.1.2 Confirmation of the insert 26
3.8.1.3 Analyses of Δ9-fatty acid desaturase genes and amino acid sequences 26
3.8.1.4 Structure prediction of Δ9-fatty acid desaturases 27
3.8.2 Preparation of expression constructs 27
3.9 Expression of Δ9-fatty acid desaturase genes 28
3.9.1 Expression and *in vivo* activity of Δ9-fatty acid desaturases in *E. coli* 28
3.9.1.1 Optimum IPTG concentration 28

3.9.1.2 Optimum expression temperature 28
3.9.1.3 Optimum expression time 29
3.9.1.4 SDS-PAGE of the recombinant Δ9-fatty acid desaturases 29
3.9.1.5 Western blot analysis of Δ9-fatty acid desaturase proteins 29
3.9.1.6 Functional analyses of Δ9-fatty acid desaturases in E. coli 30
3.10 Statistical analysis 31

4 RESULTS AND DISCUSSION 32
4.1 Screening and identification of the Antarctic bacteria 32
4.1.1 Fatty acids profiles of the wild-type Antarctic bacteria 32
4.1.2 Identification of the bacteria 36
4.2 Cloning and expression of Δ9-fatty acid desaturase genes 40
4.2.1 Cloning of Δ9-fatty acid desaturase genes 40
4.2.1.1 Isolation of Δ9-fatty acid desaturase genes 40
4.2.1.2 Ligation of full-length Δ9-fatty acid desaturase genes into pGEM-T Easy vector and transformation of E. coli TOP10 42
4.2.1.3 Confirmation of the insert 43
4.2.1.4 Analyses of gene sequences 46
4.2.1.5 Analyses of amino acid sequences 46
4.2.1.6 Structure prediction of the Δ9-fatty acid desaturases 49
4.2.1.7 Quality verification of Pseudomonas sp. A3 Δ9-fatty acid desaturase models 52
4.2.1.8 Docking of palmitic acid onto the modeled structures of Δ9-fatty acid desaturases 55
4.2.1.9 4.2.1.9 Membrane topology and signal peptide prediction of Δ9-fatty acid desaturases 61
4.2.2 Expression of Δ9-fatty acid desaturase genes 63
4.2.2.1 Effect of inducer concentration on the expression of Pseudomonas sp. A3 and Pseudomonas sp. A8 Δ9-fatty acid desaturase genes 66
4.2.2.2 Time course study on the expression of Pseudomonas sp. A3 and Pseudomonas sp. A8 Δ9-fatty acid desaturase genes 66
4.2.2.3 4.2.2.3 Effect of temperature on the expression of Pseudomonas sp. A3 and Pseudomonas sp. A8 Δ9-fatty acid desaturase genes 70
4.2.2.4 Western blot analysis of Pseudomonas sp. A3 and Pseudomonas sp. A8 Δ9-fatty acid desaturases 76
4.3 Functional expression of the Δ9-fatty acid desaturases in *E. coli* 77
4.3.1 Effect of incubation time on cellular palmitoleic acids of *E. coli* 77
4.3.2 4.3.2 Effect of incubation temperature on cellular palmitoleic acids of *E. coli* 77

4.3.2.1 Gas chromatograms of major fatty acids identified at lower temperatures 83
4.3.3 *In vivo* activity of the Δ9-fatty acid desaturases on exogenous stearic acid 87

5 SUMMARY, CONCLUSIONS AND RECOMMENDATIONS 95
5.1 Summary 95
5.2 Conclusions 96
5.3 Recommendations for further study 97

REFERENCES 98
APPENDICES 109
BIODATA OF STUDENT 138
LIST OF PUBLICATIONS 139

LIST OF TABLES

Table		Page
1	Different fatty acid desaturase encoding genes cloned from various organismd	14
2	Commonly used hosts for functional expression of fatty acid desaturases	14
3	Primers used to amplify Δ9-fatty acid desaturase genes	24
4	Vector and sequences of primers used for cloning of desaturase gene	28
5	Constituents of SDS-PAGE gel	30
6	Overall percentage (%) fatty acids composition of the bacteria	33
7	Morphological and biochemical characteristics of the bacteria	38

LIST OF FIGURES

Figure		Page
1	Effects of temperature changes on membrane structure and lipid bilayers	6
2	Anaerobic mechanism of unsaturated fatty acid production in *Escherichia coli*	9
3	A representative desaturation reaction catalysed by Δ9-fatty acid desaturase enzyme	10
4	Diagrammatic representation of important electron transport proteins involved in desaturation related to stearoyl-CoA	12
5	Gas chromatogram of major fatty acids identified from isolate 3B	34
6	Gas chromatogram of major fatty acids identified from isolate A8	34
7	Gas chromatogram of major fatty acids identified from isolate A3	35
8	Gas chromatogram of major fatty acids identified from isolate PB	35
9	Genomic DNAs and 16S rDNA genes of the Antarctic bacteria on 1% (w/v) agarose gel	37
10	Phylogenetic analysis of the Antarctic bacteria	39
11	Multiple sequences alignments of fatty acid desaturases	41
12	Agarose gel electrophoresis of PCR products on 1% (w/v)agarose gel	42
13	Complete sequence of *Pseudomonas* sp. A3 Δ9-fatty acid desaturase gene	44
14	Complete sequence of *Pseudomonas* sp. A8 Δ9-fatty acid Desaturasegene	45
15	Phylogenetic relationship of Δ9-fatty acid desaturase genes in *Pseudomonas* sp. A3, *Pseudomonas* sp. A8,fungi and animals	47
16	Phylogenetic analysis of Δ9-fatty acid desaturase sequences in some *Pseudomonas* species, *Pseudomonas* sp. A3 and *Pseudomonas* sp. A8	48
17	The predicted structure of *Pseudomonas* sp. A3 Δ9-fatty acid desaturase built with YASARA software	50
18	Structure of *Pseudomonas* sp. A8 Δ9-fatty acid desaturase predicted with YASARA software	51
19	Quality verification plot of *Pseudomonas* sp. A3 Δ9-fatty acid desaturase model performed using ERRAT	53
20	Quality verification plot of *Pseudomonas* sp. A8 Δ9-fatty acid desaturase model performed using ERRAT	54

21	Ramachandran plot of *Pseudomonas* sp. A3 Δ9-fatty acid desaturase model predicted with YASARA software	56
22	Ramachandran plot of *Pseudomonas* sp. A8 Δ9-fatty acid desaturase model predicted with YASARA software	57
23	Residues of *Pseudomonas* sp. A3 Δ9-fatty acid desaturase model interacting with palmitic acid	59
24	Residues of *Pseudomonas* sp. A8 Δ9-fatty acid desaturase model interacting with palmitic acid	60
25	Kyte-Doolittle hydropathy analysis of amino acids sequences of *Pseudomonas* sp. A3 (A) and *Pseudomonas* sp. A8 (B) Δ9-fatty acid desaturases	62
26	Signal Peptide analysis of the predicted amino acid sequences of *Pseudomonas* sp. A3 Δ9-fatty acid desaturase	64
27	Signal Peptide analysis of the predicted amino acid sequence of *Pseudomonas* sp. A8 Δ9-fatty acid desaturases	65
28	SDS-PAGE analyses of soluble (A) and insoluble (B) fractions of *E. coli* expressing *PA3FAD9* gene at different IPTG concentrations	67
29	SDS-PAGE analyses of soluble (A) and insoluble (B) fractions of *E. coli* expressing *PA8FAD9* gene at different IPTG concentrations	68
30	SDS-PAGE analysis of soluble and insoluble fractions of *E. coli* expressing *PA3FAD9* gene at different incubation time	69
31	SDS-PAGE analysis of soluble (A) and insoluble (B) fractions of *E. coli* expressing *PA3FAD9* gene at lower temperatures	71
32	SDS-PAGE analysis of soluble (A) and insoluble (B) Fractions of *E. coli* expressing *PA3FAD9* gene at higher temperatures	72
33	SDS-PAGE analysis of crude lysates from *E. coli* expressing *PA3FAD9* gene at 15 ºC	73
34	SDS-PAGE analysis of soluble (A) and insoluble (B) fractions of *E. coli* expressing *PA8FAD9* gene at lower temperatures	74
35	SDS-PAGE analysis of soluble (A) and insoluble (B) Fractions of *E. coli* expressing *PA8FAD9* gene at higher temperatures	75
36	SDS-PAGE analysis of crude lysates from *E. coli* expressing *PA8FAD9* gene at 20 ºC	76
37	Western-blot analyses of recombinant Δ9-fatty acid desaturases in *E.coli*	77
38	Pattern of palmitoleic acid (C16:1Δ9) production in *E. coli* at different incubation time	78
39	Fatty acids profile of *E. coli* expressing *Pseudomonas* sp. A3 Δ9-fatty acid desaturase at different temperatures	79

40	Ratio of palmitoleic to palmitic acid in *E. coli* cells expressing *PA3FAD9* gene or transformed with an empty vector	81
41	Fatty acids profile of *E. coli* expressing *Pseudomonas* sp. A8 Δ9-fatty acid desaturase at different temperatures	82
42	Ratios of palmitoleic to palmitic acid in *E. coli* cells expressing *PA8FAD9* gene or transformed with an empty vector	84
43	Gas chromatograms of fatty acids identified from *E. coli* expressing *Pseudomonas* sp. A3 Δ9-fatty acid desaturase at different temperatures	85
44	Gas chromatograms of fatty acids identified from *E. coli* expressing *Pseudomonas* sp. A8 Δ9-fatty acid desaturase at different temperatures	86
45	Fatty acids composition of *E. coli* that carried *PA3FAD9* gene grown at 15 ºC in the presence of 0.4 mM stearic acid after 12 h	88
46	Gas chromatograms of C16 and C18 fatty acids analysed From *E. coli* control cells (A) or cells expressing *PA3FAD9* gene (B)	89
47	Gas chromatogram of oleic acid (C18:1Δ9) and cis-vaccenic acid (C18:1Δ11) analysed from the *E. coli* cells expressing *PA3FAD9* gene at 15 ºC in the presence of 0.4 mM stearic acid after 12 h	90
48	Fatty acids composition of *E. coli* that carried *PA8FAD9* gene at 20 ºC in the presence of 0.4 mM stearic acid after 12 h	91
49	Gas chromatograms of C16 and C18 fatty acids analysed from *E. coli* control cells (A) and cells expressing *PA8FAD9* gene (B)	92
50	Gas chromatogram of oleic acid (C18:1Δ9) and cis-vaccenic acid (C18:1Δ11) analysed from *E. coli* cells expressing *PA8FAD9* gene at 15 ºC in the presence of 0.4 mM stearic acid after 12 h	93

LIST OF APPENDICES

Appendix		Page
A	Cultural characteristics of the bacteria	109
B	Gram staining reactions of the bacteria	110
C	Partial 16S Rrna gene sequences of th ebacteria	111
D	Motility test	118
E	Spore staining test	119
F	Capsule staining test	120
G	Catalase test	121
H	Oxidase test	122
I	Indole test	123
J	Citrate utilization test	124
K	Triple sugar ion (TSI) test	125
L	MR-VP test	126
M	Nucleotide sequences of the Δ9-fatty acid desaturases	127
N	Vectors	131
O	Statistical analyses	133

LIST OF ABBREVIATIONS

EDTA	ethylene diamine tetraacetic acid
IPTG	isopropyl β-D Thiogalactoside
TCA	trichloroacetic acid
kDa	kilo Dalton
ORF	open reading frame
PCR	polymerase chain reaction
PMSF	phenylmethylsulfonyl fluoride
SDS	sodium dodecyl sulphate
SDS-PAGE	sodium dodecyl sulphate polyacrylamide gel electrophoresis
TEMED	N, N, N, N-Tetramethyllenediamide
GC	gas chromatography
GCMS	gas chromatography- Mass spectrometry
FAMEs	fatty acid methyl esters
UFA	unsaturated fatty acid
MUFA	monounsaturated fatty acids
PUFA	polyunsaturated fatty acid
LB	Luria Bertani
DHA	docosahexaenoic acid
EPA	ecosapentaenoic acid
ALA	α-linolenic acid
LA	linoleic acid
NADPH	nicotinamide adenine dinucleotide phosphate
FabA	β-hydroxydecanoyl-ACP dehydrase
FabB	β-kitoacyl-ACP synthase
SCD	Stearoyl-CoA Desaturase
VLDL	very low density lipoprotein

CHAPTER 1

INTRODUCTION

Fatty acid desaturase enzymes create double bonds between carbon atoms of fatty acyl chains to produce mono- or polyunsaturated fatty acids through desaturation reactions. The reactions require molecular oxygen with reducing equivalents derived from the electron transport systems and usually eliminate two hydrogen atoms at position of the double bonds insertion (Shanklin, 2009). The Δ9-fatty acid desaturases are critical enzymes of lipid metabolism that introduced the first double bond between number 9 and 10 carbon atoms of stearic acid (C18:0) and palmitic acid (C16:0) to produce oleic acid (C18:1) and palmitoleic acid (C16:1), respectively. These are monounsaturated fatty acids making-up the key ingredients of of polyunsaturated fatty acids (PUFAs) synthesis such as docosahexaenoic acid (DHA, 22:6*n*-3) and eicosapentaenoic acid (EPA, 20:5*n*-3) (Lee *et al*., 2016; Xue *et al*., 2016). PUFAs play several biological roles such as preventing non-alcoholic fatty liver disease (Gormaz *et al*., 2010; Liu *et al*., 2011), autoimmune responses (Simopoulos, 2008) and other chronic diseases such as cardiovascular disease (Superko *et al*., 2014), cancers (Fasano *et al*., 2015), and diabetes (Zheng *et al*., 2012).

1.1 Problem statement

Many Δ9-fatty acid desaturases have been reported from animals, plants and bacteria. However, to date, very limited number of Δ9-fatty acid desaturases was reported from Antarctic bacteria. Moreover, several open reading frames (ORF) of Δ9-fatty acid desaturases have been sequenced in the genomes of many Antarctic *Pseudomonas* species and submitted to public database without any knowledge about their functions.

1.2 Hypothesis

The Δ9-fatty acid desaturase of *Pseudomonas* sp. A3 and *Pseudomonas* sp. A8 may increase the overall unsaturated fatty acid contents of *Escherichia coli* when they are functionally expressed.

1.3 Objectives

The main objectives of this research were to clone and functionally express Δ9-fatty acid desaturase from Antarctic bacteria through the following specific objectives:

1 To screen and identify unsaturated fatty acids producing-Antarctic bacteria.
2. To clone and express Δ9-fatty acid desaturase gene from the identified bacteria.
3 To predict three dimensional structure of the Δ9-fatty acid desaturase and to conduct molecular docking with palmitic acid.
4. To functionally express the Δ9-fatty acid desaturase in *Escherichia coli*.

REFERENCES

Aguilar, P. S., Cronan, J. E., and De Mendoza, D. (1998). A *Bacillus subtilis* gene induced by cold shock encodes a membrane phospholipid desaturase. *Journal of Bacteriology, 180*(8), 2194-2200.

Aguilar, P. S., and De Mendoza, D. (2006). Control of fatty acid desaturation: a mechanism conserved from bacteria to humans. *Molecular Microbiology, 62*(6), 1507-1514.

Ali, M. S. M., Mohd Fuzi, S. F., Ganasen, M., Abdul Rahman, R. N. Z. R., Basri, M., and Salleh, A. B. (2013). Structural adaptation of cold-active RTX lipase from *Pseudomonas* sp. strain AMS8 revealed via homology and molecular dynamics simulation approaches. *BioMed Research International, 2013*.

Altabe, S. G., Mansilla, M. C., and de Mendoza, D. (2013). Remodeling of Membrane Phospholipids by Bacterial Desaturases. *Stearoyl-CoA Desaturase Genes in Lipid Metabolism*: 209-231.

Anderson, B. M., and Ma, D. (2009). Are all n-3 polyunsaturated fatty acids created equal. *Lipids Health Dis, 8*(1), 33.

Avelange-Macherel, M. H., Macherel, D., Wada, H., and Murata, N. (1995). Site - directed mutagenesis of histidine residues in the Δ12 acyl - lipid desaturase of *Synechocystis*. *FEBS Letters, 361*(1), 111-114.

Bai, Y., McCoy, J. G., Levin, E. J., Sobrado, P., Rajashankar, K. R., Fox, B. G., and Zhou, M. (2015). X-ray structure of a mammalian stearoyl-CoA desaturase. *Nature*.

Brown, A. P., Kroon, J. T., Swarbreck, D., Febrer, M., Larson, T. R., Graham, I. A., Caccamo, M., and Slabas, A. R. (2012). Tissue-specific whole transcriptome sequencing in castor, directed at understanding triacylglycerol lipid biosynthetic pathways. *PLoS ONE, 7*(2), e30100.

Burdge, G. C., and Calder, P. C. (2005). Conversion of\ alpha-linolenic acid to longer-chain polyunsaturated fatty acids in human adults. *Reproduction Nutrition Development, 45*(5), 581-598.

Cao, S., Zhou, X. R., Wood, C. C., Green, A. G., Singh, S. P., Liu, L., and Liu, Q. (2013). A large and functionally diverse family of Fad2 genes in safflower (*Carthamus tinctorius* L.). *BMC Plant Biology, 13*(1), 5.

Cavicchioli, R. (2006). Cold-adapted archaea. *Nature Reviews Microbiology, 4*(5), 331-343.

Chajès, V., Torres-Mejía, G., Biessy, C., Ortega-Olvera, C., Angeles-Llerenas, A., Ferrari, P., Lazcano-Ponce, E., and Romieu, I. (2012). ω-3 and ω-6 polyunsaturated fatty acid intakes and the risk of breast cancer in Mexican women: impact of obesity status. *Cancer Epidemiology Biomarkers and Prevention, 21*(2), 319-326.

Chan, A. P., Crabtree, J., Zhao, Q., Lorenzi, H., Orvis, J., Puiu, D., Melake-Berhan, A., Jones, K. M., Redman, J., and Chen, G. (2010). Draft genome sequence of the oilseed species *Ricinus communis*. *Nature Biotechnology, 28*(9), 951-956.

Chang, B. E., Hsieh, S. L., and Kuo, C. M. (2001). Molecular cloning of full‐length cDNA encoding delta‐9 desaturase through PCR strategies and its genomic organization and expression in grass carp (*Ctenopharyngodon idella*). *Molecular Reproduction and Development, 58*(3), 245-254.

Cheesbrough, M. (2006). District laboratory practice in tropical countries: Cambridge university press.

Chintalapati, S., Kiran, M., and Shivaji, S. (2004). Role of membrane lipid fatty acids in cold adaptation. *Cellular and Molecular Biology (Noisy-le-Grand, France), 50*(5), 631-642.

Chen, H., Gu, Z., Zhang, H., Wang, M., Chen, W., Lowther, W. T., and Chen, Y. Q. (2013). Expression and purification of integral membrane fatty acid desaturases. *PLoS ONE, 8*(3), e58139.

Clustal, W. (1994). improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice Thompson, Julie D.; Higgins, Desmond G.; Gibson, Toby J. *Nucleic Acids Research, 22*(22), 4673-4680.

Clustal, W., and Clustal, X. (2007). version 2.0. *Bioinformatics, 23*, 2947-2948.

Colovos, C., and Yeates, T. O. (1993). Verification of protein structures: patterns of nonbonded atomic interactions. *Protein Science, 2*(9), 1511-1519.

Contreras, C., Franco, M., Place, N. J., and Nespolo, R. F. (2014). The effects of poly-unsaturated fatty acids on the physiology of hibernation in a South American marsupial, *Dromiciops gliroides*. *Comparative Biochemistry and Physiology Part A: Molecular and Integrative Physiology, 177*, 62-69.

D'Amico, S., Collins, T., Marx, J. C., Feller, G., and Gerday, C. (2006). Psychrophilic microorganisms: challenges for life. *EMBO Reports, 7*(4), 385-389.

Das, U. N. (2006). Essential fatty acids: biochemistry, physiology and pathology. *Biotechnology Journal, 1*(4), 420-439.

Davydov, R., Behrouzian, B., Smoukov, S., Stubbe, J., Hoffman, B. M., and Shanklin, J. (2005). Effect of substrate on the diiron (III) site in stearoyl acyl carrier protein Δ9-desaturase as disclosed by cryoreduction electron paramagnetic

resonance/electron nuclear double resonance spectroscopy. *Biochemistry, 44*(4), 1309-1315.

De Lorgeril, M., and Salen, P. (2012). New insights into the health effects of dietary saturated and omega-6 and omega-3 polyunsaturated fatty acids. *BMC Medicine, 10*(1), 50.

DeLano, W. L., and De Lano Scientific, L. (2006). PyMOL v. 0.99. *DeLano Scientific, LLC: San Francisco*.

Diaz, A. R., Mansilla, M. A. C., Vila, A. J., and de Mendoza, D. (2002). Membrane topology of the acyl-lipid desaturase from *Bacillus subtilis*. *Journal of Biological Chemistry, 277*(50), 48099-48106.

Dillon, J. T., Aponte, J. C., Tarozo, R., and Huang, Y. (2013). Purification of omega-3 polyunsaturated fatty acids from fish oil using silver-thiolate chromatographic material and high performance liquid chromatography. *Journal of Chromatography A,* 1312, 18-25.

Dlakić, M. (2002). A model of the replication fork blocking protein Fob1p based on the catalytic core domain of retroviral integrases. *Protein Science,* 11(5), 1274-1277.

Dobson, S., Colwell, R., McMeekin, T., and Franzmann, P. (1993). Direct sequencing of the polymerase chain reaction-amplified 16S rRNA gene of *Flavobacterium gondwanense* sp. nov. and *Flavobacterium salegens* sp. nov., two new species from a hypersaline Antarctic lake. *International Journal of Systematic Bacteriology,* 43(1), 77-83.

Fasano, E., Serini, S., Cittadini, A., and Calviello, G. (2015). Long-Chain n-3 PUFA against breast and prostate cancer: which are the appropriate doses for intervention studies in animals and humans? *Critical Reviews in Food Science and Nutrition.*

Feng, Y., and Cronan, J. E. (2011). Complex binding of the FabR repressor of bacterial unsaturated fatty acid biosynthesis to its cognate promoters. *Molecular Microbiology, 80*(1), 195-218.

Fox, B. G., Lyle, K. S., and Rogge, C. E. (2004). Reactions of the diiron enzyme stearoyl-acyl carrier protein desaturase. *Accounts of Chemical Research,* 37(7), 421-429.

Fuentes-Grünewald, C., Garcés, E., Alacid, E., Sampedro, N., Rossi, S., and Camp, J. (2012). Improvement of lipid production in the marine strains *Alexandrium minutum* and *Heterosigma akashiwo* by utilizing abiotic parameters. *Journal of Industrial Microbiology and Biotechnology, 39*(1), 207-216.

Fujii, T., Suzuki, M. G., Katsuma, S., Ito, K., Rong, Y., Matsumoto, S., Ando, T., and Ishikawa, Y. (2013). Discovery of a disused desaturase gene from the pheromone gland of the moth *Ascotis selenaria*, which secretes an

epoxyalkenyl sex pheromone. *Biochemical and Biophysical Research Communications, 441*(4), 849-855.

García-Maroto, F., Garrido-Cárdénas, J., Rodríguez-Ruiz, J., Vilches-Ferrón, M., Adam, A., Polaina, J., and López Alonso, D. (2002). Cloning and molecular characterization of the Δ6-desaturase from two Echium plant species: Production of GLA by heterologous expression in yeast and tobacco. *Lipids,* 37(4), 417-426. doi: 10.1007/s1145-002-0910-6

Goren, M. A., and Fox, B. G. (2008). Wheat germ cell-free translation, purification, and assembly of a functional human stearoyl-CoA desaturase complex. *Protein Expression and Purification, 62*(2), 171-178.

Gormaz, J. G., Rodrigo, R., Videla, L. A., and Beems, M. (2010). Biosynthesis and bioavailability of long-chain polyunsaturated fatty acids in non-alcoholic fatty liver disease. *Progress in Lipid Research, 49*(4), 407-419.

Guedes, A., Amaro, H. M., and Malcata, F. X. (2011). Microalgae as sources of high added‐value compounds—a brief review of recent work. *Biotechnology Progress,* 27(3), 597-613.

Guo, Z. H., Yang, Z. G., Cheng, Y.X., Ji, L.Y., Que, Y.Q., Liu, Z.W., and Zeng, Q.T. (2013). Molecular characterization, tissue expression of acyl-CoAΔ 9-desaturase-like gene, and effects of dietary lipid levels on its expression in the hepatopancreas of the Chinese mitten crab (*Eriocheir sinensis*). *Aquaculture, 402*, 58-65.

Gupta, A., Vongsvivut, J., Barrow, C. J., and Puri, M. (2012). Molecular identification of marine yeast and its spectroscopic analysis establishes unsaturated fatty acid accumulation. *Journal of Bioscience and Bioengineering, 114*(4), 411-417.

Heinemann, F. S., and Ozols, J. (2003). Stearoyl-CoA desaturase, a short-lived protein of endoplasmic reticulum with multiple control mechanisms. *Prostaglandins, Leukotrienes and Essential Fatty acids, 68*(2), 123-133.

Huang, C., and Ebersole, J. (2010). A novel bioactivity of omega‐3 polyunsaturated fatty acids and their ester derivatives. *Molecular Oral Microbiology, 25*(1), 75-80.

Hulbert, A. J., Turner, N., Storlien, L., and Else, P. (2005). Dietary fats and membrane function: implications for metabolism and disease. *Biological Reviews, 80*(01), 155-169.

Innis, S. M. (2007). Fatty acids and early human development. *Early Human Development, 83*(12), 761-766.

Irlinger, F., Bimet, F., Delettre, J., Lefèvre, M., and Grimont, P. A. (2005). *Arthrobacter bergerei* sp. nov. and *Arthrobacter arilaitensis* sp. nov., novel coryneform species isolated from the surfaces of cheeses. *International Journal of Systematic and Evolutionary Microbiology, 55*(1), 457-462.

Jadhav, V. V., Jamle, M. M., Pawar, P. D., Devare, M. N., and Bhadekar, R. K. (2010). Fatty acid profiles of PUFA producing Antarctic bacteria: correlation with RAPD analysis. *Annals of Microbiology, 60*(4), 693-699.

Jadhav, V. V., Yadav, A., Shouche, Y. S., and Bhadekar, R. K. (2013). Isolation and cellular fatty acid composition of psychrotrophic Halomonas strains from Antarctic sea water. *Songklanakarin Journal of Science and Technology, 35*(3), 287-292.

Kikukawa, H., Sakuradani, E., Kishino, S., Park, S.-B., Ando, A., Shima, J., Ochiai, M., Shimizu, S., and Ogawa, J. (2013). Characterization of a trifunctional fatty acid desaturase from oleaginous filamentous fungus *Mortierella alpina* 1S-4 using a yeast expression system. *Journal of Bioscience and Bioengineering, 116*(6), 672-676.

Kim, M. J., Kim, H., Shin, J. S., Chung, C. H., Ohlrogge, J. B., and Suh, M. C. (2006). Seed-specific expression of sesame microsomal oleic acid desaturase is controlled by combinatorial properties between negative cis-regulatory elements in the SeFAD2 promoter and enhancers in the 5′-UTR intron. *Molecular Genetics and Genomics, 276*(4), 351-368.

Kyte, J., and Doolittle, R. F. (1982). A simple method for displaying the hydropathic character of a protein. *Journal of Molecular Biology, 157*(1), 105-132.

Laemmli, U. K. (1970). Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature, 227*(5259), 680-685.

Laskowski, R. A., Moss, D. S., and Thornton, J. M. (1993). Main-chain bond lengths and bond angles in protein structures. *Journal of Molecular Biology, 231*(4), 1049-1067.

Lee, J. M., Lee, H., Kang, S., and Park, W. J. (2016). Fatty acid desaturases, polyunsaturated fatty acid regulation, and biotechnological advances. *Nutrients, 8*(1), 23.

Li, F., Bian, C. S., Xu, J. F., fu Pang, W., Liu, J., Duan, S. G., Lei, Z.-G., Jiwan, P., and Jin, L.-P. (2015). Cloning and functional characterization of SAD genes in potato. *PLoS ONE, 10*(3), e0122036.

Li, L., Wang, X., Gai, J., and Yu, D. (2007). Molecular cloning and characterization of a novel microsomal oleate desaturase gene from soybean. *Journal of Plant Physiology, 164*(11), 1516-1526.

Li, Q., Du, W., and Liu, D. (2008a). Perspectives of microbial oils for biodiesel production. *Applied Microbiology and Biotechnology, 80*(5), 749-756.

Li, Y., Dietrich, M., Schmid, R. D., He, B., Ouyang, P., and Urlacher, V. B. (2008b). Identification and functional expression of a Δ9-fatty acid desaturase from *Psychrobacter urativorans* in *Escherichia coli*. *Lipids, 43*(3), 207-213.

Li, Y., Xu, X., Dietrich, M., Urlacher, V. B., Schmid, R. D., Ouyang, P., and He, B. (2009). Identification and functional expression of a Δ9 fatty acid desaturase from the marine bacterium *Pseudoalteromonas* sp. MLY15. *Journal of Molecular Catalysis B: Enzymatic, 56*(2), 96-101.

Liu, X., Strable, M. S., and Ntambi, J. M. (2011). Stearoyl CoA desaturase 1: role in cellular inflammation and stress. *Advances in Nutrition: An International Review Journal, 2*(1), 15-22.

Los, D. A., Mironov, K. S., and Allakhverdiev, S. I. (2013). Regulatory role of membrane fluidity in gene expression and physiological functions. *Photosynthesis Research, 116*(2-3), 489-509.

Los, D. A., and Murata, N. (1998). Structure and expression of fatty acid desaturases. *Biochimica et Biophysica Acta (BBA)-Lipids and Lipid Metabolism, 1394*(1), 3-15.

Lozinsky, S., Yang, H., Forseille, L., Cook, G. R., Ramirez-Erosa, I., and Smith, M. A. (2014). Characterization of an oleate 12-desaturase from *Physaria fendleri* and identification of 5′ UTR introns in divergent FAD2 family genes. *Plant Physiology and Biochemistry, 75*, 114-122.

Lu, C., and Wallis, J. G. (2007). An analysis of expressed sequence tags of developing castor endosperm using a full-length cDNA library. *BMC Plant Biology, 7*(1), 42.

Maali, R., Schimschilaschvili, H., Pchelkin, V., Tsydendambaev, V., Nosov, A., Los, D., and Goldenkova-Pavlova, I. (2007). Comparative expression in *Escherichia coli* of the native and hybrid genes for acyl-lipid Δ9 desaturase. *Russian Journal of Genetics, 43*(2), 121-126.

MacKenzie, D. A., Carter, A. T., Wongwathanarat, P., Eagles, J., Salt, J., and Archer, D. B. (2002). A third fatty acid Δ9-desaturase from *Mortierella alpina* with a different substrate specificity to ole1p and ole2p. *Microbiology, 148*(6), 1725-1735.

Madduri, K. M., Schafer, B. W., Hasler, J. M., Lin, G., Foster, M. L., Embrey, S. K., Sastry-Dent, L., Song, P., Larrinua, I. M., and Gachotte, D. J. (2012). Preliminary safety assessment of a membrane-bound delta 9 desaturase candidate protein for transgenic oilseed crops. *Food and Chemical Toxicology, 50*(10), 3776-3784.

Mahgoub, E. O., and Bolad, A. (2013). Correctness and accuracy of template-based modeled single chain fragment variable (scFv) protein anti-breast cancer cell line (MCF-7). *Open Journal of Genetics, 3*(03), 183.

Man, W. C., Miyazaki, M., Chu, K., and Ntambi, J. M. (2006). Membrane topology of mouse stearoyl-CoA desaturase 1. *Journal of Biological Chemistry, 281*(2), 1251-1260.

Mansilla, M., Banchio, C., and Mendoza, D. (2008). Signalling Pathways Controlling Fatty Acid Desaturation. In P. Quinn andX. Wang (Eds.), *Lipids in Health and Disease* (Vol. 49, pp. 71-99): Springer Netherlands.

Meesapyodsuk, D., and Qiu, X. (2012). The front-end desaturase: structure, function, evolution and biotechnological use. *Lipids, 47*(3), 227-237.

Meng, X.-Y., Zhang, H.-X., Mezei, M., and Cui, M. (2011). Molecular docking: a powerful approach for structure-based drug discovery. *Current Computer-aided Drug Design, 7*(2), 146-157.

Miyazaki, M., Bruggink, S. M., and Ntambi, J. M. (2006). Identification of mouse palmitoyl-coenzyme A Δ9-desaturase. *Journal of Lipid Research, 47*(4), 700-704.

Morozkina, E., Slutskaya, E., Fedorova, T., Tugay, T., Golubeva, L., and Koroleva, O. (2010). Extremophilic microorganisms: Biochemical adaptation and biotechnological application (review). *Applied Biochemistry and Microbiology, 46*(1), 1-14.

Moyer, C. L., and Morita, R. Y. (2007). Psychrophiles and psychrotrophs. *ELS*.

Ochei, J., and Kolhatkar, A. (2000). Medical laboratory science: Theory and practice. *Copy Right*, 801-804.

Paredes, D. I., Watters, K., Pitman, D. J., Bystroff, C., and Dordick, J. S. (2011). Comparative void-volume analysis of psychrophilic and mesophilic enzymes: Structural bioinformatics of psychrophilic enzymes reveals sources of core flexibility. *BMC Structural Biology, 11*(1), 42.

Parra, G., Bradnam, K., Rose, A. B., and Korf, I. (2011). Comparative and functional analysis of intron-mediated enhancement signals reveals conserved features among plants. *Nucleic Acids Research, 39*(13), 5328-5337.

Patterson, R. E., Flatt, S. W., Newman, V. A., Natarajan, L., Rock, C. L., Thomson, C. A., Caan, B. J., Parker, B. A., and Pierce, J. P. (2011). Marine fatty acid intake is associated with breast cancer prognosis. *The Journal of Nutrition, 141*(2), 201-206.

Paulucci, N. S., Dardanelli, M. S., and de Lema, M. G. (2014). Biochemical and molecular evidence of a Δ9 fatty acid desaturase from *Ensifer meliloti* 1021. *Microbiological Research, 169*(5), 463-468.

Pereira, H., Barreira, L., Figueiredo, F., Custódio, L., Vizetto-Duarte, C., Polo, C., Rešek, E., Engelen, A., and Varela, J. (2012). Polyunsaturated fatty acids of marine macroalgae: potential for nutritional and pharmaceutical applications. *Marine Drugs, 10*(9), 1920-1935.

Pereira, S. L., Leonard, A. E., and Mukerji, P. (2003). Recent advances in the study of fatty acid desaturases from animals and lower eukaryotes. *Prostaglandins, Leukotrienes and Essential Fatty acids, 68*(2), 97-106.

Pikuta, E. V., Hoover, R. B., and Tang, J. (2007). Microbial extremophiles at the limits of life. *Critical Reviews in Microbiology, 33*(3), 183-209.

Rajwade, A. V., Kadoo, N. Y., Borikar, S. P., Harsulkar, A. M., Ghorpade, P. B., and Gupta, V. S. (2014). Differential transcriptional activity of SAD, FAD2 and FAD3 desaturase genes in developing seeds of linseed contributes to varietal variation in α-linolenic acid content. *Phytochemistry, 98*, 41-53.

Ramachandran, G. N., Ramakrishnan, C., and Sasisekharan, V. (1963). Stereochemistry of polypeptide chain configurations. *Journal of Molecular Biology, 7*(1), 95-99.

Ramesh, A. M., Kesari, V., and Rangan, L. (2014). Characterization of a stearoyl-acyl carrier protein desaturase gene from potential biofuel plant, *Pongamia pinnata* L. *Gene, 542*(2), 113-121.

Rampelotto, P. H. (2010). Resistance of microorganisms to extreme environmental conditions and its contribution to astrobiology. *Sustainability, 2*(6), 1602-1623.

Ritchie, D. W., and Kemp, G. J. (2000). Protein docking using spherical polar Fourier correlations. *Proteins: Structure, Function, and Bioinformatics, 39*(2), 178-194.

Rodrigues, D. F., da C Jesus, E., Ayala-del-Río, H. L., Pellizari, V. H., Gilichinsky, D., Sepulveda-Torres, L., and Tiedje, J. M. (2009). Biogeography of two cold-adapted genera: Psychrobacter and Exiguobacterium. *The ISME Journal, 3*(6), 658-665.

Rothschild, L. (2007). Extremophiles: defining the envelope for the search for life in the universe. *Planetary Systems and the Origins of Life, 3*, 113.

Russell, N. J. (1978). The positional specificity of a desaturase in the psychrophilic bacterium *Micrococcus cryophilus* (ATCC 15174). *Biochimica et Biophysica Acta (BBA)-Lipids and Lipid Metabolism, 531*(2), 179-186.

Ruxton, C., Reed, S. C., Simpson, M., and Millington, K. (2004). The health benefits of omega‐3 polyunsaturated fatty acids: a review of the evidence. *Journal of Human Nutrition and Dietetics, 17*(5), 449-459.

Saitou, N., and Nei, M. (1987). The neighbor-joining method: a new method for reconstructing phylogenetic trees. *Molecular Biology and Evolution, 4*(4), 406-425.

Sakaguchi, M., Matsuzaki, M., Niimiya, K., Seino, J., Sugahara, Y., and Kawakita, M. (2007). Role of proline residues in conferring thermostability on aqualysin I. *Journal of Biochemistry, 141*(2), 213-220.

Sakuradani, E., Abe, T., Iguchi, K., and Shimizu, S. (2005). A novel fungal ω3-desaturase with wide substrate specificity from arachidonic acid-producing *Mortierella alpina* 1S-4. *Applied Microbiology and Biotechnology, 66*(6), 648-654.

Sambrook, J., and Russell, D. W. (2001). Molecular cloning: a laboratory manual. third. *Cold pring Harbor Laboratory Press, New York*.

SAnchez, R., and Sali, A. (1998). Large-scale protein structure modeling of the *Saccharomyces cerevisiae* genome. *Proceedings of the National Academy of Sciences, 95*(23), 13597-13602.

Schweizer, H. P., and Choi, K. H. (2011). *Pseudomonas aeruginosa* aerobic fatty acid desaturase DesB is important for virulence factor production. *Archives of Microbiology, 193*(3), 227-234.

Shanklin, J., Guy, J. E., Mishra, G., and Lindqvist, Y. (2009). Desaturases: emerging models for understanding functional diversification of diiron-containing enzymes. *Journal of Biological Chemistry, 284*(28), 18559-18563.

Shanklin, J., Whittle, E., and Fox, B. G. (1994). Eight histidine residues are catalytically essential in a membrane-associated iron enzyme, stearoyl-CoA desaturase, and are conserved in alkane hydroxylase and xylene monooxygenase. *Biochemistry, 33*(43), 12787-12794.

Shimizu, S., and Kobayashi, M. (2002). Δ9-desaturase gene: Google Patents.

Simopoulos, A. P. (2008). The importance of the omega-6/omega-3 fatty acid ratio in cardiovascular disease and other chronic diseases. *Experimental Biology and Medicine, 233*(6), 674-688.

Sperling, P., Ternes, P., Zank, T., and Heinz, E. (2003). The evolution of desaturases. *Prostaglandins, Leukotrienes, and Essential Fatty Acids, 68*(2), 73.

Stan-Lotter, H., and Fendrihan, S. (2012). Adaption of microbial life to environmental extremes. *Vienna New York: Springer*.

Struvay, C., and Feller, G. (2012). Optimization to low temperature activity in psychrophilic enzymes. *International Journal of Molecular Sciences, 13*(9), 11643-11665.

Stryjewska, A., Kiepura, K., Librowski, T., and Lochyński, S. (2013). Biotechnology and genetic engineering in the new drug development. Part I. DNA technology and recombinant proteins. *Pharmacological Reports, 65*(5), 1075-1085.

Subrahmanyam, S., and Cronan, J. E. (1998). Overproduction of a Functional Fatty Acid Biosynthetic Enzyme Blocks Fatty Acid Synthesis in *Escherichia coli*. *Journal of Bacteriology, 180*(17), 4596-4602.

Superko, H. R., Superko, A. R., Lundberg, G. P., Margolis, B., Garrett, B. C., Nasir, K., and Agatston, A. S. (2014). Omega-3 fatty acid blood levels clinical significance update. *Current Cardiovascular Risk Reports, 8*(11), 1-8.

Tamura, K., Stecher, G., Peterson, D., Filipski, A., and Kumar, S. (2013). MEGA6: molecular evolutionary genetics analysis version 6.0. *Molecular Biology and Evolution, 30*(12), 2725-2729.

Tanomman, S., Ketudat-Cairns, M., Jangprai, A., and Boonanuntanasarn, S. (2013). Characterization of fatty acid delta-6 desaturase gene in Nile tilapia and heterogenous expression in *Saccharomyces cerevisiae*. *Comparative Biochemistry and Physiology Part B: Biochemistry and Molecular Biology, 166*(2), 148-156.

Tinberg, C. E. (2010). Exploring the reactivity of bacterial multicomponent monooxygenases. Massachusetts Institute of Technology.

Torres-Franklin, M. L., Repellin, A., Huynh, V. B., d'Arcy-Lameta, A., Zuily-Fodil, Y., and Pham-Thi, A. T. (2009). Omega-3 fatty acid desaturase FAD3, FAD7, FAD8) gene expression and linolenic acid content in cowpea leaves submitted to drought and after rehydration. *Environmental and Experimental Botany, 65*(2), 162-169.

Uğur, A., Ceylan, Ö., Aslım, B., Sheibani, S., Ghadiri, H., Eshghi, S., Safizadeh, M. R., Jamali, B., and Sarseifi, M. (2012). Characterization of *Pseudomonas* spp. from seawater of the southwest coast of Turkey. *Journalof Biolohical and Environmental Sciences, 6*(16), 15-23.

van Beilen, J. B., Penninga, D., and Witholt, B. (1992). Topology of the membrane-bound alkane hydroxylase of *Pseudomonas oleovorans*. *Journal of Biological Chemistry, 267*(13), 9194-9201.

Venegas-Calerón, M., Muro-Pastor, A., Garcés, R., and Martínez-Force, E. (2006). Functional characterization of a plastidial omega-3 desaturase from sunflower (*Helianthus annuus*) in cyanobacteria. *Plant Physiology and Biochemistry, 44*(10), 517-525.

Wan, X., Liang, Z., Gong, Y., Zhang, Y., and Jiang, M. (2013). Characterization of three Δ9-fatty acid desaturases with distinct substrate specificity from an oleaginous fungus *Cunninghamella echinulata*. *Molecular Biology Reports, 40*(7), 4483-4489.

Warude, D., Joshi, K., and Harsulkar, A. (2006). Polyunsaturated fatty acids: Biotechnology. *Critical Reviews in Biotechnology, 26*(2), 83-93.

Watanabe, K., Oura, T., Sakai, H., and Kajiwara, S. (2004). Yeast Δ12 fatty acid desaturase: gene cloning, expression, and function. *Bioscience, Biotechnology, and Biochemistry, 68*(3), 721-727.

Waters, S., Kelly, J., O'Boyle, P., Moloney, A., and Kenny, D. (2009). Effect of level and duration of dietary n-3 polyunsaturated fatty acid supplementation on the transcriptional regulation of Δ-desaturase in muscle of beef cattle. *Journal of Animal Science, 87*(1), 244-252.

Wei, D., Li, M., Zhang, X., Ren, Y., and Xing, L. (2004). Identification and characterization of a novel Δ 12-fatty acid desaturase gene from *Rhizopus arrhizus*. *FEBS Letters, 573*(1), 45-50.

Xing, H., Zhang, X., Yang, Q., Liu, R., Bao, Z., Su, B., Yang, Y., and Ren, Q. (2014). Separation of long chain fatty acids with different number of unsaturated bonds by fractional extraction: Experimental and COSMO-RS study. *Food Chemistry, 143*, 411-417.

Xue, W.-B., Liu, F., Sun, Z., and Zhou, Z.-G. (2016). A Δ-9 Fatty Acid Desaturase Gene in the Microalga Myrmecia incisa Reisigl: Cloning and Functional Analysis. *International Journal of Molecular Sciences, 17*(7), 1143.

Yi, C., Shi, J., Kramer, J., Xue, S., Jiang, Y., Zhang, M., Ma, Y., and Pohorly, J. (2009). Fatty acid composition and phenolic antioxidants of winemaking pomace powder. *Food Chemistry, 114*(2), 570-576.

Zhao, C.-H., Zhang, T., Li, M., and Chi, Z.-M. (2010). Single cell oil production from hydrolysates of inulin and extract of tubers of *Jerusalem artichoke* by *Rhodotorula mucilaginosa* TJY15a. *Process Biochemistry, 45*(7), 1121-1126.

Zheng, J.-S., Huang, T., Yang, J., Fu, Y.-Q., and Li, D. (2012). Marine N-3 polyunsaturated fatty acids are inversely associated with risk of type 2 diabetes in Asians: a systematic review and meta-analysis. *PLoS ONE, 7*(9), e44525.

Zhu, K., Choi, K. H., Schweizer, H. P., Rock, C. O., and Zhang, Y. M. (2006). Two aerobic pathways for the formation of unsaturated fatty acids in *Pseudomonas aeruginosa*. *Molecular Microbiology, 60*(2), 260-273.

Zhu, L., Cheng, J., Luo, B., Feng, S., Lin, J., Wang, S., Cronan, J. E., and Wang, H. (2009). Functions of the *Clostridium acetobutylicium* FabF and FabZ proteins in unsaturated fatty acid biosynthesis. *BMC Microbiology, 9*(1), 119.