Diversity and engineering of diatom metabolism for new and improved sterol products

Ana Cristina Jaramillo-Madrid

PhD by research

Submitted in fulfilment of the requirements for
The degree of Doctor of Philosophy

Climate Change Cluster
School of Life Sciences
University of Technology Sydney
2020

Certificate of original authorship

I, Ana Cristina Jaramillo Madrid declare that this thesis, is submitted in fulfilment of the requirements for the award of Doctorate of Philosophy, in the Faculty of Science at the University of Technology Sydney.

This thesis is wholly my own work unless otherwise reference or acknowledged. In addition, I certify that all information sources and literature used are indicated in the thesis.

This document has not been submitted for qualifications at any other academic institution.

This research is supported by the Australian Government Research Training Program.

Signature:

Production Note:
Signature removed prior to publication.

Ana Cristina Jaramillo Madrid
Date: 20 of December 2019

Acknowledgements

Si el Señor no construye la casa, en vano se cansan los constructores

Salmo 126

I would like to express my sincerest gratitude to my supervisors Prof. Peter Ralph and Dr. Justin Ashworth. Peter has been always a kind and supportive mentor, he believed in me from day one and encouraged me to cultivate my skills as a scientist. Thank you for providing and promoting the amazing C3 culture we enjoy today. I want to especially thank Justin for his mentorship; I barely have words to express the immense contributions he has made to the development of my scientific capabilities. He pushed me to learn and perform rigorous science. He always led by example, providing guidance and direction. His passion for science is contagious and helped me to do my job with enthusiasm. Thanks, Justin, for always keeping your door open to give me advice and encouragement. Without your expertise I would not have gotten half as far.

I would also like to thank Raffaela Abriano and Michele Fabris, the godfather of my project, for their thoughtful insights that have greatly expanded the results obtained in this thesis. A special thanks to Raffie for always having a kind and constructive attitude to guide me through the PhD journey.

Thank you to my fellow PhD students for being an amazing group of people to work with. Jestin George and Lorenzo Barolo for making the long hours in the lab more enjoyable and for the lovely dinners during game nights. To the other members of Algae Biosystems and Biotechnology group, especially Mathieu Pernice and Audrey Commault, thanks for your support and feedback, for listening to my struggles and for being there when I needed support.

Thank you to the tech staff at UTS for all the help provided. Thanks to Unnikrishnan Kuzhiumparambil, Taya Lapshina and Kun Xiao for methodological guidance in GC/MS analysis and Flow Cytometry.

I wish to acknowledge and give thanks for the funding I have received from the UTS International Research Scholarship and President's Scholarship, as well as a Scholarship from the non-profit Colfuturo Foundation of Colombia, the Climate Change Cluster (C3) postgraduate funding and the UTS conference funding schemes.

A special thank you to Angelina Hormaza, my undergrad and master's supervisor back in Colombia, who always recognised and cultivated my potential. She has been a very close mentor throughout my scientific career and encouraged me to pursue my PhD studies abroad. She is one of the great examples of women in science and an inspiration to me.

To the German, Julian Kofler, for his patience and support throughout this PhD process. You were always there and helped me to keep going during the stressful times. To my parents, Gabriel Jaramillo and Gladis Madrid, for being the people I can always count on for anything. They have constantly sustained and encouraged me to pursue my dreams. They taught me to be hard working and to stay true to myself. Thanks for believing in me and setting a great example for me. A special thanks to my dad for the amazing job editing of this thesis in LaTeX, it certainly looks better than my Word version.

I am very grateful to you all for your input in helping me deliver this thesis and supporting my love of science.

Contents

List of Figures

List of Tables

Abbreviation List

AACCO	Aceto-acetyl-CoA
AACT	Acetoacetyl-CoA thiolase
ACCOA	Acetyl-CoA
AltSQE	Alternative squalene epoxidase enzyme
CDP-ME	4-(cytidine 5′-diphospho)-2-C-methyl-D-erythritol
CDP-MEP	2-phospho-4-(cytidine 5′-diphospho)-2-C-methyl-D-erythritol
CMK	4-(cytidine 5′-diphospho)-2-Cmethyl-d-erythritol kinase
DMAPP	Dimethylallyl diphosphate
DXP	1-deoxy-D-xylulose-5-phosphate
DXR	1-deoxy-d-xylulose 5-phosphate reductoisomerase
DXS	1-deoxy-dxylulose 5-phosphate synthase
EPA	Eicosapentaenoic acid
ER	Endoplasmic reticulum
FPP	Farnesyl diphosphate
FPPS	Farnesyl diphosphate synthase
GC-MS	Gas chromatography-mass spectrometry
GPP	Geranyl diphosphate
GPPS	Geranyl pyrophosphate synthase
HDR	4-hydroxy-3-methylbut-2-en-1-yl diphosphate reductase
HDS	4-hydroxy-3-methylbut-2-en-1-yl diphosphate synthase
HMBPP	1-hydroxy-2-methyl-2butenyl-4-diphosphate
HMG-CoA	3-hydroxy-3-methylglutaryl-CoA
HMGR	3- hydroxy-3-methylglutaryl-CoA reductase
HMGS	Hydroxymethylglutaryl-CoA synthase
HMMs	Hidden Markov Models
IDI	Isopentenyl diphosphate
IDI-SQS	Isopentenyl diphosphate isomerase-squalene synthase
IPK	Isopentenyl phosphate kinase
IPP	Isopentenyl diphosphate
MCT	2-C-methyl-d-erythritol 4-phosphate cytidylyltransferase

MDS	2-C-methyl-d-erythritol 2,4-cyclodiphosphate synthase
ME-cPP	2-C-methyl-D-erythritol2,4-cyclodiphosphate
MEP	Methylerithriol phosphate pathway
MMETSP	Marine Microbial Eukaryote Transcriptome Sequencing Project
MVA	Mevalonate pathway
MVAP	Mevalonate phosphate
MVAPP	Mevalonate diphosphate
MVK	Mevalonate kinase
OSC	Oxidosqualene cyclase
SCAP	SREBP cleavage activating protein
Si	Silicon
SQE	Squalene epoxydase
SQS	Squalene synthase
SRE	Sterol Regulatory Element
SREBPs	Sterol Regulatory Element Binding Proteins
SSD	Sterol sensing domain
tHMGR	Truncated 3- hydroxy-3-methylglutaryl-CoA reductase

Thesis Abstract

Diatoms are a large group of eukaryotic microalgae that arose through secondary endosymbiosis and are renowned for their wide ecological distribution. Diatoms have genetically diversified their physiology, metabolism and natural products, while adapting to dynamic environments. Among these metabolic products are an expanded repertoire of phytosterols, a class of essential terpenoids that are involved in the regulation of membrane dynamics, signalling, and membrane-bound protein functions in higher plants, algae, fungi, and vertebrates. Phytosterols are considered a marker of eukaryotic life and have been used to identify and date evolutionary events. They are also useful natural products due to their wide range of biological applications. The principal therapeutic and nutraceutical properties of phytosterols include cholesterol-lowering, anti-inflammatory and anti-diabetic activities. The global phytosterol market by 2013 was US$ 300 million and it is growing at about 7-9% per annum. In order to meet this demand, diatom microalgae are proposed as an alternative source of natural products.

The function, distribution and biosynthesis of sterols is well characterised and conserved in model animal, plant and fungal organisms. However, the biological role and metabolism of the high diversity of sterols produced by diatoms is not well understood. To establish diatoms as a suitable platform for phytosterols production, in this PhD project we provide insight into key aspects of sterol compounds from diatoms: i) The response of sterol levels to changes in environmental conditions, ii) The reconstruction of the sterol biosynthesis pathways of multiple diatom species, and iii) Genetic investigations and engineering of diatoms to alter sterol product profiles.

In Chapter 1, I provide an updated review of the phytosterol repertoire in diatoms, including the biology and regulation of sterol biosynthesis according to the latest primary studies, and new genetic approaches by which the productive metabolisms of these organisms can be further optimised.

In the first data chapter, Chapter 2, I investigated the occurrence of different sterol types in twelve different diatom species, as well as the effect of temperature reduction and changes in salinity on the sterol contents of three model diatoms. In Chapter 3, I experimentally examined the sterol biosynthesis pathways of three divergent diatom species, using empirical biochemical profiling and comparative 'omics. This Chapter experimentally explored hypotheses with regard to what extent the sterol biosynthesis pathways

of three diatom species are conserved, and where each of these has diverged to produce different phytosterols. This study introduces in-depth multi-species analyses in order to compare and contrast the biosynthesis pathways of distantly related species. The results expand our understanding of sterol biosynthesis in diatoms, including a new model for cholesterol synthesis in diatoms.

Finally, in Chapter 4, I implemented and performed genetic engineering technologies to test the extent to which natural sterol levels can be rationally manipulated in the diatoms *Thalassiosira pseudonana* and *Phaeodactylum tricornutum*. Three different genetic targets were chosen, including i) The overexpression of a rate-limiting enzyme in sterol biosynthesis, HMGR, and ii) the expression of a N-terminal truncated HMGR and introduction of a heterologous squalene epoxidase enzyme from the microalgae *Nannochloropsis oceanica*.

This thesis is structured with one introduction chapter (Chapter 1), currently published as a review, three data Chapters (Chapters 2 to 4), each written in the form of a journal manuscript for peer-review and a conclusion Chapter (Chapter 5). At the time of thesis submission, all chapters, except conclusion chapter, have been either published, are under peer-review, or in final draft for submission.

The overarching aim of this research project was to investigate and optimise the production of bioactive sterols in diatoms for commercial applications. This project first investigated the diversity and differential production of sterols by several diatom species under different growth conditions. Inhibitors of the key enzymes in the sterol metabolic pathway will then be used to identify relevant intermediate compounds in the biosynthesis of sterols. Finally, enzymes responsible for the synthesis of sterol compounds will be genetically targeted for metabolic engineering of the selected diatom species. The specific aims of this project were:

Aim 1: Characterise the sterols produced under different environmental conditions by several diatoms strains.

Objectives:

- Identify the most abundant sterols produced by diatoms growing in enriched medium.
- Determine the sterols produced under different culture conditions.

$\mathbf{H}_0$: Sterol production does not vary according to diatom strain and culture conditions.
$\mathbf{H}_a$: Sterol production varies according to diatom strain and culture conditions.

Aim 2: Identify enzymes and genes putatively involved in the sterol biosynthesis pathway

Objectives:

- Identify target intermediate compounds in the metabolic pathway of sterols using chemical inhibitors.
- Assemble a general sterol biosynthesis pathway to identify genetic targets for the enhanced production of sterol compounds.

$\mathbf{H}_0$: Inhibition of enzymes involved in the sterol metabolic pathway of diatoms does not result in the production of phytosterol intermediate compounds

$\mathbf{H}_0$: Sterol biosynthesis inhibitors will not differently affect the sterol profiles of different diatom species

Aim 3: Genetically engineer diatoms to probe and optimise the production of sterols

Objectives:

- Genetically over-express biosynthetic enzymes to increase production of sterols.
- Genetically up-regulate and/or disrupt native enzymes and/or regulatory genes to alter or increase production of sterols.

$\mathbf{H}_0$: It is not possible to transgenically alter the sterol products or amounts of diatoms; the natural levels are strictly balanced and regulated

$\mathbf{H}_a$: Genetic modification of enzymes participating in the sterol pathway of diatoms leads to alteration of sterol profiles.

In summary, this project addressed the following research questions:

- What is the effect of different growth conditions on the sterols produced by diatoms?
- Which are the principal intermediate compounds in the sterol metabolic pathway, and which enzymes participate in their formation?
- Does genetic engineering or disruption of genes involved in sterol biosynthesis alter the production of phytosterols by diatoms?