



EXTRACTION OF CAROTENOID PIGMENTS AS  
SHARIAH COMPLIANT FOOD COLORANTS  
THROUGH CELL CULTURE OF FRESHWATER  
GREEN MICROALGAE (CHLOROPHYTA) AND  
CYANOBACTERIA

BY

NUR HIDAYAH BINTI NOH

A thesis submitted in fulfilment of the requirement for the  
degree of Master in Science (Halal Industry Science)

International Institute for Halal Research and Training  
(INHART)  
International Islamic University Malaysia

MARCH 2017

## ABSTRACT

Nowadays, halal products are gaining wider recognition as a new benchmark for safety and quality assurance. As a consequence the commercial developments of microalgae are established due to their high value chemicals, for examples,  $\beta$ -carotene, astaxanthin, phycobilin pigments and algal extracts for cosmeceuticals products. Therefore, many researchers have gained interest to study the potential of microalgae as new valuable chemicals and other products sources. The aim of the research is to explore new sources of pigments to be used as halal food colorants. This quest is not only directed in finding natural alternatives for synthetic dyes, but also to discover new taxons for the carotenoid production. Thus, there is a solid need to investigate the potential of natural pigments particularly carotenoids in microalgae to be fully utilised and commercialised especially in halal market, health advantages, food products and dye technology. A total of 10 species were evaluated for quantitative and qualitative carotenoid composition namely *Chlorella fusca*, *Chlorella vulgaris*, *Selenastrum capricornutum*, *Pandorina morum*, *Butyricococcus sudeticus*, *Chlorococcum*, *Pseudanabaena*, *Scenedesmus*, *Ankistrodesmus*, and *Butyrococcus Brauni*. *Chlorella fusca* (chlorophyta) and *Pseudanabaena* (cyanobacteria) and found three types of carotenoid out of them which are  $\beta$ -carotene,  $\beta$ -cryptoxanthin and lutein. Species that was having all three types of carotenoid was *Pandorina morum* (Figure 4.8) with lutein ( $54.64 \pm 3.11$  mg/g DW), Beta cryptoxanthin ( $2.38 \pm 0.56$  mg/g DW) and Beta carotene ( $1.39 \pm 0.38$  mg/g DW). Two types of microalgae were chosen represent their division which are *Chlorella fusca* (Chlorophyta) and *Pseudanabaena* (Cyanobacteria) were subjected to manipulation of nutrient and carbon sources. They were cultured using Bold's Basal Medium (BBM), Bristol medium and Bold's Basal Medium (BBM) modified with vitamin B with different nutrient and carbon sources. There were three types of carotenoid had produced which are zeaxanthin ( $21248.7 \pm 240.61$  mg/g DW), violaxanthin ( $16299 \pm 423.09$  mg/g DW) and lutein ( $2335.53 \pm 69.82$  mg/g DW) with their highest number respectively. Nitrate concentration also been manipulated in all types of medium that been used and it had produced lutein. The highest lutein number was produced in cultivation of *Pseudanabaena* in Bristol medium with 0.5 fold nitrate concentration ( $1317.91 \pm 40.13$  mg/g DW). *Chlorella fusca* and *Pseudanabaena* also been cultured in BBM with the addition of different elicitors which are salicylic acid (SA), polyethylene glycol (PEG) and sodium chloride (NaCl). All of these elicitors had initiate production of lutein. Among of those three elicitors, this studies had found that the implication of 25mg/L of SA had yield highest lutein concentration ( $666.45 \pm 24.66$  mg/g DW). Cell concentration of microalgae also been recorded for all studies except for profiling. Cell count were done using haemocytometer under microscope and growth pattern of microalgae been plotted to monitor microalgae cell density

## خلاصة البحث

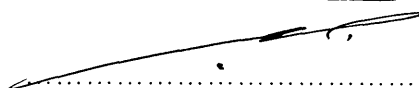
في هذه الآونة اكتسبت منتجات الحلال اعترافاً ورواجاً واسعاً، باعتبارها معياراً جديداً لضمان السلامة والجودة. وبناءً على ذلك تم تأسيس مشاريع تجارية لمنتجات الطحالب بسبب احتوائها على مواد ذات قيمة عالية مثل بيتا كاروتين، أستازانتين، وأصبغ فيكوبيلين ومستخلصات الطحالب التي تستخدم في منتجات ومستحضرات العناية بالبشرة التي تحتوي على *cosmeceuticals products* على مركبات نشطة حيويًا (بيولوجيًا)، أي لها تأثير كالأدوية على الجلد والبشرة ( لذلك، نالت دراسة الطحالب إهتمام العديد من الباحثين حول إمكانية كونها مصادر لمواد كيميائية جديدة ولمنتجات الغرض من هذا البحث هو السعي لاستكشاف مصادر جديدة للأصبغ المستخدمة كملونات غذائية أخرى ذات قيمة. حلال. هذا المسعى ليس موجهاً فقط في إيجاد بدائل طبيعية للأصبغ الاصطناعية، ولكن أيضاً لاكتشاف أصناف أو ومن ثم، هناك الحاجة الماسة لفحص إمكانات الأصبغ (carotenoid) جديدة لإنتاج كاروتينات (taxons) أنواع الطبيعية خصوصاً الكاروتينات في الطحالب لما لها من مزايا صحية كمنتجات غذائية يتم استخدامها بشكل كامل في تكنولوجيا الصبغ وتسويقها خصوصاً في سوق المنتجات الحلال. لقد تم معملياً التقويم الكمي والنوعي لعشرة أنواع من الطحالب لإنتاج وتكوين الكاروتينات وهي:

*Chlorella fusca, Chlorella vulgaris, Selenastrum capricornutum, Pandorina morum, Butyricococcus sudeticus, Chlorococcum, Pseudanabaena, Scenedesmus, Ankistrodesmus, Butyrococcus Brauni and Pseudanabaena (cyanobacteria).*

ووجد ثلاثة أنواع من كاروتينات منها ألا وهي بيتا كاروتين وكريبتوزانثين كاروتين واللوتين. والثلاث أنواع من الكاروتينات حيث كان اللوتين ( $54.64 \pm 3.11$  مليجرام/جرام "جاف")، بيتا كريبتوزانثين *Pandorina morum* وجدت في تم اختيار نوعين ( $2.38 \pm 0.56$  مليجرام/جرام "جاف") وبيتا كاروتين ( $1.39 \pm 0.38$  مليجرام/جرام "جاف"). (*Chlorella fusca* و *Cyanobacteria*) هما (*Chlorophyta*) والطحالب يمثلان قسمي ال (تم تزرع كلاهما باستخدام بولد على التوالي. وهما يتعرضان لدراسة الإجهاد وتغيير المغذيات. *Pseudanabaena*) ( المتوسطة وأيضاً المحورة وكانت هناك ثلاثة أنواع من الكاروتين قد *Bold's Basal Medium (BBM)* باصل (  $423.09 \pm 16299$  violaxanthin أنتجت وهي زياكسانثين ( $21248.7 \pm 240.61$  مليجرام/جرام "جاف")، مليجرام/جرام "جاف") واللوتين ( $2335.53 \pm 69.82$  مليجرام/جرام "جاف" مليجرام/جرام "جاف") مرتبة توالياً من الأكبر إلى الأقل. تم التلاعب بتغيير تركيز النترات في جميع أنواع الطرق المستخدمة وخلصنا إلى أن نتجت اللوتين. تم إنتاج بواسطة مغذي بريستول مع 0.5 (نصف) تركيز النترات (*Pseudanabaena* 1317.91 أكبر عدد اللوتين في تزرع مع إضافة *BBM* في *Pseudanabena*) و (*Chlorella fusca*  $40.13 \pm$  مليجرام/جرام "جاف"). وتم تزرع ( وكلوريد الصوديوم. وكلها PEG)، البولي ايثيلين جلايكول (SA المختلفة التي هي حمض الصفصاف (elicitors) SA وجدت الدراسة أن آثار من 25 مليجرام/لتر من elicitors بدأت بتنشيط إنتاج اللوتين. ومن بين تلك مختبرات أثمرت أعلى إنتاج حيث كان تركيز اللوتين ( $24.66 \pm 666.45$  مليجرام/جرام "جاف"). وتم أيضاً تسجيل تركيز وتعداد خلايا الطحالب الدقيقة لجميع الدراسات باستثناء التنميط. قد أجريت دراسة عدد الخلايا باستخدام عداد خلايا الدم تحت المجهر أما نمط نمو الطحالب قد تمت دراسته ومتابعته برصد كثافة الخلايا. (haemocytometer).

## APPROVAL PAGE

I certify that I have supervised and read this study and that in my opinion, it conforms to acceptable standards of scholarly presentation and is fully adequate, in scope and quality, as a thesis for the degree of Master of Science (Halal Industry Sciences) |

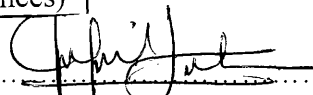


Rashidi bin Othman  
Supervisor



Mohammad Aizat bin Jamaludin  
Co-Supervisor

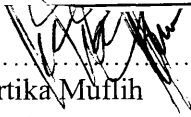
I certify that I have read this study and that in my opinion it conforms to acceptable standards of scholarly presentation and is fully adequate, in scope and quality, as a thesis for the degree of Master of Science (Halal Industry Sciences) |



Noor Faizul Hadry bin Nordin  
Internal Examiner

Zainul Amiruddin bin Zakaria  
External Examiner

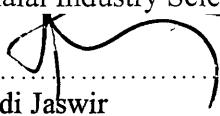
This thesis was submitted to the Department of INHART and is accepted as a fulfilment of the requirement for the degree of Master of Science (Halal Industry Sciences) |



Betania Kartika Muflih  
Deputy Director,

Academic and Training

This thesis was submitted to the Kulliyah of INHART and is accepted as a fulfilment of the requirement for the degree of Master of Science (Halal Industry Sciences) |

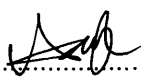


Irwandi Jaswir  
Director, INHART

## DECLARATION

I hereby declare that this dissertation is the result of my own investigations, except where otherwise stated. I also declare that it has not been previously or concurrently submitted as a whole for any other degrees at IIUM or other institutions.

Nur Hidayah Binti Noh

Signature .....  .....

Date ..... 02/2/2017 .....

**DECLARATION OF COPYRIGHT AND AFFIRMATION OF  
FAIR USE OF UNPUBLISHED RESEARCH**

**EXTRACTION OF CAROTENOID PIGMENTS AS FOOD  
COLORANTS THROUGH CELL CULTURE OF FRESHWATER  
GREEN MICROALGAE (CHLOROPHYTA) AND  
CYANOBACTERIA**

I declare that the copyright holders of this dissertation are jointly owned by the student and IIUM.

Copyright © 2016 Nur Hidayah Binti Noh and International Islamic University Malaysia. All rights reserved.

No part of this unpublished research may be reproduced, stored in a retrieval system, or transmitted, in any form or by any means, electronic, mechanical, photocopying, recording or otherwise without prior written permission of the copyright holder except as provided below

1. Any material contained in or derived from this unpublished research may be used by others in their writing with due acknowledgement.
2. IIUM or its library will have the right to make and transmit copies (print or electronic) for institutional and academic purposes.
3. The IIUM library will have the right to make, store in a retrieved system and supply copies of this unpublished research if requested by other universities and research libraries.

By signing this form, I acknowledged that I have read and understand the IIUM Intellectual Property Right and Commercialization policy.

Affirmed by Nur Hidayah Binti Noh

  
.....  
Signature

22/2/2012  
.....  
Date

## ACKNOWLEDGEMENTS

Finally.....Alhamdulillah....Thank you ALLAH , THE GREATEST, THE MOST MERCIFUL, for lending me the strength to complete my study and finish my thesis.

Firstly, special thanks to my supervisors, Assoc.Prof.Dr Rashidi bin Othman for his continuous support, encouragement and leadership, and for that, I will be forever grateful. A million thanks for the shared of knowledge, practical guidance and moral advices throughout my years of study.

A special appreciation to all members of herbarium who always giving me motivation, shared of knowledge and moral supports. A millions of thanks also dedicated to all my bestie Nur Mukmina Dasiman, Nor Ferdaus Muhammad, Siti Atika Jambari, Norbainon Baharudin, Nur Elena Zaaba, Nur Hidayah Mastor and Fatimah Azzahra Mohd Zaifuddin for standing by me through my thick and thin along the way in completing this degree. Thank you so much for shedding my tears and never left me when I need a shoulder to cry on. I want to express my gratitude to them for understanding my tantrums and my craziness when I'm in estrogen and cortisol influence and supplied me with endorphine in the time I needed the most. A very huge hug and kisses I dedicated to all of you.

It is my utmost pleasure to dedicate this work to my mother, Zaimah Jonit and my two brother Noor Ariff and Nor Ezwany, who granted me the gift of their unwavering belief in my ability to accomplish this goal: thank you for your support and patience.

I wish to express my appreciation and thanks to those who provided their time, effort and support for this project. To the members of my dissertation committee, thank you for sticking with me.

## TABLE OF CONTENTS

Abstract .....	ii
Abstract in Arabic .....	iii
Approval page .....	iv
Declaration .....	v
Copyright Page.....	vi
Acknowledgement .....	vii
Table of Contents .....	viii
List of Tables .....	xi
List of Figures .....	xivv
List of abbreviations .....	xx
<b>CHAPTER ONE : INTRODUCTION .....</b>	<b>1</b>
1.1 Background Of The Study .....	1
1.2 Statement Of The Problem.....	2
1.3 Purpose Of The Study.....	4
1.4 Research Objectives .....	5
1.5 Significance Of The Study.....	5
<b>CHAPTER TWO : LITERATURE REVIEW .....</b>	<b>7</b>
2.1 Malaysia and halal recognition .....	7
2.2 Food and nutraceuticals industries.....	7
2.2.1 Food colorant .....	7
2.2.2 Carotenoids .....	11
<b>CHAPTER THREE : MATERIALS AND METHODOLOGY.....</b>	<b>19</b>
3.1 Selection of microalgae.....	19
3.2 Mass Production of Microalgae Cell Culture .....	19
3.3 Extraction of Carotenoid Pigments.....	20
3.4 Removal of chlorophyll prior to Hplc analysis (saponification).....	21
3.5 High performance liquid chromatography (HPLC) analysis .....	21
<b>CHAPTER FOUR : CAROTENOID PROFILING ON SEVERAL SPECIES OF FRESHWATER GREEN MICROALGAE AND CYANOBACTERIA .....</b>	<b>23</b>
4.1 Introduction.....	23
4.1.1 Microalgae .....	23
4.1.2 Physical properties of microalgae .....	23
4.1.3 Division of microalgae.....	24
4.1.4 Active Pharmaceutical Ingredients (API) in Microalgae.....	25
4.1.5 Carotenoid and Algae .....	26
4.1.6 Cellular location of carotenoid in microalgae and its function.....	27
4.1.7 Carotenoid biosynthesis in microalgae .....	28

4.2 Experimental design.....	30
4.2.1 Mass production of microalgae cell culture.....	30
4.2.2 Extraction of carotenoid pigments and HPLC analysis .....	30
4.3 RESULT AND DISCUSSION.....	31
<b>CHAPTER FIVE : EFFECT OF DIFFERENT CONCENTRATIONS OF NUTRIENT AND ENERGY SOURCES TOWARDS CAROTENOID STABILITY IN MICROALGAE .....</b>	<b>44</b>
5.1 Introduction.....	44
5.1.1 Factors influencing microalgae growth .....	44
5.1.2 Stress-induced in microalage .....	46
5.2 Experimental design.....	51
5.2.1 Mass production of microalgae cell culture.....	51
5.2.2 Extraction of carotenoid pigments and HPLC analysis .....	52
5.3 Result and discussion .....	53
5.3.1 Cultivation of Pseudanabaena in Bold's Basal Medium (BBM) with different nutrient concentration and carbon sources .....	56
5.3.2 Cultivation of Chlorella fusca in Bold's Basal Medium (BBM) with different nutrient concentration and carbon sources .....	64
5.3.3 Cultivation of Pseudanabaena in Bold's Basal Medium (BBM) modified with vitamin B with different nutrient concentration and carbon sources.....	79
5.3.4 Cultivation of Chlorella fusca in Bold's Basal Medium (BBM) modified with vitamin B with different nutrient concentration and carbon source .....	86
5.3.5 Cultivation of Pseudanabaena in Bristol medium with different nutrient concentration and carbon sources .....	97
5.3.6 Cultivation of Chlorella fusca in Bristol medium with different nutrient concentration and carbon sources .....	100
5.3.1 Influence of nitrate concentration in Pseudanabeana and Chlorella fusca cultivate in Bold's Basal Medium (BBM), Bristol medium, and Bold's Basal Medium (BBM) modified with vitamin b.....	115
5.4 Discussion.....	134
5.5 Conclusion .....	137
<b>CHAPTER 6 : INFLUENCE OF ELICITORS TOWARDS THE PRODUCTION OF CAROTENOID IN MICROALGAE .....</b>	<b>138</b>
6.1 Introduction.....	138
6.1.1 Salicylic acid.....	138
6.1.2 Sodium chloride.....	142
6.1.3 Polyethylene glycol.....	144
6.2 Experimental design.....	145
6.2.1 Mass production of microalgae cell culture.....	145
6.2.2 Extraction of carotenoid pigments and HPLC analysis .....	146
6.3 Result and discussion .....	147
6.3.1 Cultivation of Pseudanabaena and Chlorella fusca in Bold's Basal Medium (BBM) with addition of several concentration of salicylic acid .....	147
6.3.2 Cultivation of Pseudanabaena and Chlorella fusca in Bold's Basal Medium (BBM) with addition of several concentration of polyethylene glycol (PEG) ..	154
6.3.3 Cultivation of Pseudanabaena and Chlorella fusca in Bold's Basal Medium (BBM) with addition of several concentration of sodium chloride (NaCl) .....	161

6.4 Discussion .....	168
6.5 Conclusion .....	170
<b>GENERAL DISCUSSION OVERALL CONCLUSION .....</b>	<b>171</b>
<b>BIBLIOGRAPHY .....</b>	<b>172</b>
APPENDIX A .....	189
APPENDIX B .....	190
APPENDIX C .....	191

## LIST OF TABLES

	<u>Page No.</u>
Table 2.1 Classification of carotenoid (Delgado Vargas & Paredes-López, 2002)	15
Table 4.1 Analysis of variance for several species of freshwater green microalgae and cyanobacteria at ( $p < 0.01$ )	32
Table 4.2 Carotenoid content on several species of freshwater green microalgae and cyanobacteria (mg/g DW)	34
Table 4.3 Microalgae and their carotenoid studies	42
Table 4.4 Microalgae and their carotenoid studies	43
Table 5.1 Impact of environmental stresses on the lipid and carotenoid production ((Minhas, Hodgson, Barrow, & Adholeya, 2016)	48
Table 5.2 Analysis of variance for <i>Psuedanabaena</i> in BBM medium with different nutrient concentration at ( $p < 0.01$ )	60
Table 5.3 Carotenoid content for <i>Psuedanabaena</i> in BBM medium with different nutrient concentration (mg/g DW)	60
Table 5.4 Analysis of variance for <i>Chlorella fusca</i> in BBM medium with different nutrient concentration at ( $p < 0.01$ )	68
Table 5.5 Carotenoid content for <i>Chlorella fusca</i> in BBM medium with different nutrient concentration (mg/g DW)	68
Table 5.6 Analysis of variance for <i>Pseudanabaena</i> in BBM modified medium with different nutrient concentration at ( $p < 0.01$ )	83
Table 5.7 Carotenoid content for <i>Pseudanabaena</i> in BBM modified medium with different nutrient concentration (mg/g DW)	83
Table 5.8 Analysis of variance for <i>Chlorella fusca</i> in BBM modified medium with different nutrient concentration at ( $p < 0.01$ )	89
Table 5.9 Carotenoid content for <i>Chlorella fusca</i> in BBM modified medium with different nutrient concentration (mg/g DW)	90
Table 5.10 Analysis of variance for <i>Pseudanabaena</i> in Bristol medium with different nutrient concentration at ( $p < 0.01$ )	98
Table 5.11 Carotenoid content for <i>Pseudanabaena</i> in Bristol medium with different nutrient concentration (mg/g DW)	99

Table 5.12 Analysis of variance for <i>Chlorella fusca</i> in Bristol medium with different nutrient concentration at ( $p < 0.01$ )	105
Table 5.13 Carotenoid content for <i>Chlorella fusca</i> in Bristol medium with different nutrient concentration (mg/g DW)	105
Table 5.14 Carotenoid content for <i>Pseudanabaena</i> in BBM with different nitrate concentration (mg/g DW)	116
Table 5.15 Carotenoid content for <i>Chlorella fusca</i> in BBM with different nitrate concentration (mg/g DW)	120
Table 5.16 Carotenoid content for <i>Chlorella fusca</i> in BBM modified with vitamin B with different nitrate concentration (mg/g DW)	122
Table 5.17 Carotenoid content for <i>Pseudanabaena</i> in BBM modified with vitamin B with different nitrate concentration (mg/g DW)	126
Table 5.18 Carotenoid content for <i>Chlorella fusca</i> in Bristol medium with different nitrate concentration (mg/g DW)	129
Table 5.19 Carotenoid content for <i>Pseudanabaena</i> in Bristol medium with different nitrate concentration (mg/g DW)	132
Table 5.20 Analysis of variance for <i>Pseudanabaena</i> and <i>Chlorella fusca</i> in several medium with different nitrate concentration at ( $p < 0.01$ )	133
Table 6.1 Carotenoid content for <i>Pseudanabaena</i> in BBM with different salicylic acid concentration (mg/g DW)	148
Table 6.2 Carotenoid content for <i>Chlorella fusca</i> in BBM with different salicylic acid concentration (mg/g DW)	151
Table 6.3 Analysis of variance for <i>Pseudanabaena</i> and <i>Chlorella fusca</i> in several medium with different salicylic acid concentration at ( $p < 0.01$ )	153
Table 6.4 Carotenoid content for <i>Chlorella fusca</i> in BBM with different PEG concentration (mg/g DW)	155
Table 6.5 Carotenoid content for <i>Pseudanabaena</i> in BBM with different PEG concentration (mg/g DW)	158
Table 6.6 Analysis of variance for <i>Pseudanabaena</i> and <i>Chlorella fusca</i> in several medium with different PEG concentration at ( $p < 0.01$ )	160
Table 6.7 Carotenoid content for <i>Chlorella fusca</i> in BBM with different NaCl concentration (mg/g DW)	162
Table 6.8 Carotenoid content for <i>Pseudanabaena</i> in BBM with different NaCl concentration (mg/g DW)	165

Table 6.9 Analysis of variance for *Pseudanabaena* and *Chlorella fusca* in several medium with different NaCl concentration at ( $p < 0.01$ )

167

## LIST OF FIGURES

	<u>Page No.</u>
Figure 2.1 Characteristic end groups of carotenoids and their names according to the semisystematic nomenclature (Delgado Vargas & Paredes-López, 2002)	13
Figure 4.1 <i>Pseudanabaena</i>	24
Figure 4.2 <i>Chlorella fusca</i>	25
Figure 4.3 Biosynthesis pathway of carotenoid in microalgae	30
Figure 4.4 Total carotenoid content on several species of microalgae and cyanobacteria	35
Figure 4.5 Lutein content on several species of microalgae and cyanobacteria	36
Figure 4.6 $\beta$ -cryptoxanthin content on several species of microalgae and cyanobacteria	37
Figure 4.7 $\beta$ -carotene content on several species of microalgae and cyanobacteria	38
Figure 4.8 <i>Pandrorina morum</i> HPLC chromatogram At Rt 15.01 = lutein (447nm wavelength) and ; Rt 21.50 = Beta cryptoxanthin (452nm wavelength) ; Rt 29.08 = Beta carotene (452nm wavelength)	39
Figure 4.9 <i>Selenestrum</i> HPLC chromatogram. At Rt 15.18 = lutein (447nm wavelength) and ; Rt 29.11 = Beta carotene (452nm wavelength)	40
Figure 5.1 Growth pattern for <i>Pseudanabaena</i> that been cultivated in Bold's Basal Media (BBM) in autotrophic condition with light exposure	57
Figure 5.2 Growth pattern for <i>Pseudanabaena</i> that been cultivated in Bold's Basal Media (BBM) in autotrophic condition with dark exposure	57
Figure 5.3 Growth pattern for <i>Pseudanabaena</i> that been cultivated in Bold's Basal Media (BBM) in heterotrophic condition	58
Figure 5.4 Growth pattern for <i>Pseudanabaena</i> that been cultivated in Bold's Basal Media (BBM) in heterotrophic conditi	58
Figure 5.5 Zeaxanthin content for <i>Psuedanabaena</i> that been cultivated in Bold's Basal Media (BBM) in autotrophic condition with light exposure (mg/g DW)	61

Figure 5.6 Zeaxanthin content for <i>Psuedanabaena</i> that been cultivated in Bold's Basal Media (BBM) in mixotrophic condition(mg/g DW)	62
Figure 5.7 Zeaxanthin content for <i>Psuedanabaena</i> that been cultivated in Bold's Basal Media (BBM) in heterotrophic condition (mg/g DW)	63
Figure 5.8 Growth pattern for <i>Chlorella fusca</i> that been cultivated in Bold's Basal Media (BBM) in autotrophic condition with light exposure	65
Figure 5.9 Growth pattern for <i>Chlorella fusca</i> that been cultivated in Bold's Basal Media (BBM) in autotrophic condition with dark exposure	65
Figure 5.10 Growth pattern for <i>Chlorella fusca</i> that been cultivated in Bold's Basal Media (BBM) in mixotrophic condition	66
Figure 5.11 Growth pattern for <i>Chlorella fusca</i> that been cultivated in Bold's Basal Media (BBM) in heterotrophic conditio	66
Figure 5.12 Total carotenoid content <i>Chlorella fusca</i> that been cultivated in Bold's Basal Media (BBM) in autotrophic condition with light exposure (mg/g DW)	69
Figure 5.13 Total carotenoid content <i>Chlorella fusca</i> that been cultivated in Bold's Basal Media (BBM) in autotrophic condition with dark exposure (mg/g DW)	69
Figure 5.14 Total carotenoid content <i>Chlorella fusca</i> that been cultivated in Bold's Basal Media (BBM) in mixotrophic condition (mg/g DW)	70
Figure 5.15 Total carotenoid content <i>Chlorella fusca</i> that been cultivated in Bold's Basal Media (BBM) in heterotrophic condition (mg/g DW)	71
Figure 5.16 Violaxanthin content for <i>Chlorella fusca</i> that been cultivated in Bold's Basal Media (BBM) in autotrophic condition with light exposure (mg/g DW)	73
Figure 5.17 Violaxanthin content for <i>Chlorella fusca</i> that been cultivated in Bold's Basal Media (BBM) in mixtotrophic (mg/g DW)	74
Figure 5.18 Lutein content for <i>Chlorella fusca</i> that been cultivated in Bold's Basal Media (BBM) in autotrophic condition with light exposure (mg/g DW)	75
Figure 5.19 Lutein content for <i>Chlorella fusca</i> that been cultivated in Bold's Basal Media (BBM) in autotrophic condition with dark exposure (mg/g DW)	76
Figure 5.20 Lutein content for <i>Chlorella fusca</i> that been cultivated in Bold's Basal Media (BBM) in mixototrophic (mg/g DW)	77

Figure 5.21 Lutein content <i>Chlorella fusca</i> that been cultivated in Bold's Basal Media (BBM) in heterotrophic (mg/g DW) for	78
Figure 5.22 Growth pattern for <i>Pseudanabaena</i> that been cultivated BBM modified with vitamin B in autotrophic condition with light exposure	80
Figure 5.23 Growth pattern for <i>Pseudanabaena</i> that been cultivated BBM modified with vitamin B in autotrophic condition with dark exposure	80
Figure 5.24 Growth pattern for <i>Pseudanabaena</i> that been cultivated BBM modified with vitamin B in mixotrophic condition	81
Figure 5.25 Growth pattern for <i>Pseudanabaena</i> that been cultivated BBM modified with vitamin B in heterotrophic condition	81
Figure 5.26 Lutein content for <i>Pseudanabaena</i> that been cultivated in BBM modified with vitamin B in autotrophic condition with light exposure (mg/g DW)	84
Figure 5.27 Lutein content for <i>Pseudanabaena</i> that been cultivated in BBM modified with vitamin B in mixotrophic condition (mg/g DW)	85
Figure 5.28 Growth pattern for <i>Chlorella fusca</i> that been cultivated BBM modified with vitamin B in autotrophic condition with light exposure	87
Figure 5.29 Growth pattern for <i>Chlorella fusca</i> that been cultivated BBM modified with vitamin B in autotrophic condition with dark exposure	87
Figure 5.30 Growth pattern for <i>Chlorella fusca</i> that been cultivated BBM modified with vitamin B in mixotrophic	88
Figure 5.31 Growth pattern for <i>Chlorella fusca</i> that been cultivated BBM modified with vitamin B in heterotrophic	88
Figure 5.32 Total carotenoid content for <i>Chlorella fusca</i> that been cultivated in BBM modified with vitamin B in autotrophic condition with light exposure (mg/g DW)	91
Figure 5.33 Total carotenoid content for <i>Chlorella fusca</i> that been cultivated in BBM modified with vitamin B in mixotrophic condition (mg/g DW)	92
Figure 5.34 Violaxanthin content for <i>Chlorella fusca</i> that been cultivated in BBM modified with vitamin B in autotrophic condition with light exposure (mg/g DW)	93
Figure 5.35 Violaxanthin content for <i>Chlorella fusca</i> that been cultivated in BBM modified with vitamin B in mixotrophic condition (mg/g DW)	94
Figure 5.36 Lutein content for <i>Chlorella fusca</i> that been cultivated in BBM modified with vitamin B in autotrophic condition with light exposure (mg/g DW)	95

Figure 5.37 Lutein content for <i>Chlorella fusca</i> that been cultivated in BBM modified with vitamin B in mixotrophic condition (mg/g DW)	96
Figure 5.38 Growth pattern for <i>Pseudanabaena</i> that been cultivated Bristol medium in autotrophic condition with light exposure	97
Figure 5.39 Lutein content for <i>Pseudanabaena</i> that been cultivated in Bristol medium in autotrophic condition with light exposure (mg/g DW)	100
Figure 5.40 Growth pattern for <i>Chlorella fusca</i> that been cultivated Bristol medium in autotrophic condition with light exposure	102
Figure 5.41 Growth pattern for <i>Chlorella fusca</i> that been cultivated Bristol medium in autotrophic condition with dark exposure	102
Figure 5.42 Growth pattern for <i>Chlorella fusca</i> that been cultivated Bristol medium in mixotrophic condition	103
Figure 5.43 Growth pattern for <i>Chlorella fusca</i> that been cultivated Bristol medium in heterotrophic condition	103
Figure 5.44 Total carotenoid content for <i>Chlorella fusca</i> that been cultivated in Bristol medium in autotrophic condition with light exposure (mg/g DW)	106
Figure 5.45 Total carotenoid content for <i>Chlorella fusca</i> that been cultivated in Bristol medium in mixotrophic condition (mg/g DW)	107
Figure 5.46 Total carotenoid content for <i>Chlorella fusca</i> that been cultivated in Bristol medium in heterotrophic condition (mg/g DW)	108
Figure 5.47 Violaxanthin content for <i>Chlorella fusca</i> that been cultivated in Bristol medium in autotrophic condition with light exposure (mg/g DW)	109
Figure 5.48 Violaxanthin content for <i>Chlorella fusca</i> that been cultivated in Bristol medium in mixotrophic condition (mg/g DW)	110
Figure 5.49 Violaxanthin content for <i>Chlorella fusca</i> that been cultivated in Bristol medium in heterotrophic condition (mg/g DW)	111
Figure 5.50 Zeaxanthin content for <i>Chlorella fusca</i> that been cultivated in Bristol medium in autotrophic condition with light exposure (mg/g DW)	112
Figure 5.51 Zeaxanthin content for <i>Chlorella fusca</i> that been cultivated in Bristol medium in mixotrophic condition (mg/g DW)	113
Figure 5.52 Zeaxanthin content for <i>Chlorella fusca</i> that been cultivated in Bristol medium in heterotrophic condition (mg/g DW)	114

Figure 5.53 Growth pattern for <i>Pseudanabaena</i> that been cultivated BBM medium with different nitrate concentrat	115
Figure 5.54 Lutein content for <i>Pseudanabaena</i> that been cultivated in BBM with different nitrate concentration (mg/g DW)	117
Figure 5.55 Growth pattern for <i>Chlorella fusca</i> that been cultivated BBM medium with different nitrate concentration	118
Figure 5.56 Lutein content for <i>Chlorella fusca</i> that been cultivated in BBM with different nitrate concentration (mg/g DW)	120
Figure 5.57 Growth pattern for <i>Chlorella fusca</i> that been cultivated BBM modified medium with different nitrate concentration	121
Figure 5.58 Lutein content for <i>Chlorella fusca</i> that been cultivated in BBM modified medium with different nitrate concentration (mg/g	123
Figure 5.59 Growth pattern for <i>Pseudanabaena</i> that been cultivated BBM modified medium with different nitrate concentration	124
Figure 5.60 Lutein content for <i>Pseudanabaena</i> that been cultivated in BBM modified medium with different nitrate concentration (mg/g DW)	126
Figure 5.61 Growth pattern for <i>Chlorella fusca</i> that been cultivate in Bristol medium with different nitrate concentration	127
Figure 5.62 Lutein content for <i>Chlorella fusca</i> that been cultivated in Bristol medium with different nitrate concentration (mg/g D	129
Figure 5.63 Growth pattern for <i>Pseudanabaena</i> that been cultivate in Bristol medium with different nitrate concentration	130
Figure 5.64 Lutein content for <i>Pseudanabaena</i> that been cultivated in Bristol medium with different nitrate concentration (mg/g D	132
Figure 6.1 Growth pattern for <i>Pseudanabaena</i> that been cultivate in BBM with different salicylic acid concentratio	147
Figure 6.2 Lutein content for <i>Pseudanabaena</i> that been cultivated in BBM medium with different salicylic acid concentration (mg/g D	149
Figure 6.3 Growth pattern for <i>Chlorella fusca</i> that been cultivate in BBM with different salicylic acid concentration	150
Figure 6.4 Lutein content for <i>Chlorella fusca</i> that been cultivated in BBM medium with different salicylic acid concentration (mg/g D	152
Figure 6.5 Growth pattern for <i>Chlorella fusca</i> that been cultivate in BBM with different PEG concentration	154

Figure 6.6 Lutein content for <i>Chlorella fusca</i> that been cultivated in BBM medium with different PEG concentration (mg/g DW)	156
Figure 6.7 Growth pattern for <i>Pseudanabaena</i> that been cultivate in BBM with different PEG concentration	157
Figure 6.8 Lutein content for <i>Pseudanabaena</i> that been cultivated in BBM medium with different PEG concentration (mg/g DW)	159
Figure 6.9 Growth pattern for <i>Chlorella fusca</i> that been cultivate in BBM with different NaCl concentration	161
Figure 6.10 Lutein content for <i>Chlorella fusca</i> that been cultivated in BBM medium with different NaCl concentration (mg/g DW)	163
Figure 6.11 Growth pattern for <i>Pseudanabaena</i> that been cultivate in BBM with different NaCl concentration	164
Figure 6.12 Lutein content for <i>Pseudanabaena</i> that been cultivated in BBM medium with different NaCl concentration (mg/g DW)	166

## LIST OF ABBREVIATIONS

$\zeta$	Zeta
$\alpha$	Alpha
$\beta$	Beta
g	Gram
cm	Centimetre
°C	Degree Celcius
ml	Mililiter
ANOVA	Analysis of varience
HPLC	High Performance Liquid Chromatography
BHT	Butylated hydroxy toluene
w/v	Weight solute per volume solvent ratio
BBM	Bold's Basal Medium
g/L	Gram per liter
mg/g DW	miligram per gram dry weight sample
SA	Salicylic acid
NaCl	Sodium Chloride
PEG	Polyethylene glycol

# CHAPTER ONE

## INTRODUCTION

### 1.1 BACKGROUND OF THE STUDY

In line with the fact that halal products are gaining wider recognition as a new benchmark for safety and quality assurance, there have been gains in the commercial development of microalgae. The precious bioconstituents of microalgae such as  $\beta$ -carotene, astaxanthin, docosahexaenoic acid, eicosahexaenoic acid, phycobilin pigments and microalgae have been exploited for cosmeceuticals and other products. Algal pigments are considered to have great potential for producing successful products exploited from algae (Koller, Muhr, & Braunegg, 2014). They are also the most interesting products that have been produced by microalgae thus far (Rodrigues, Menezes, Mercadante, Jacob-Lopes, & Zepka, 2015).

Global research on microalgae was initiated some 50 years ago. Studies on microalgae in Malaysia have been ongoing for the past 30 years and have found that they have great potential to produce highly valuable products for the pharmaceutical industry as well as for wastewater treatment and assessment of environmental toxicants. Hence, this microalgae biotechnology should be priority research in Malaysia. The aim of this research is to explore new sources of pigments to be used as halal food colourants and to find natural alternatives for synthetic dyes to fulfil the search for halal ingredients in daily used products. The adverse impact of synthetic food colourants on general human health reported by scientific findings makes it an urgent search for building a healthy generation. Thus, there is a solid need to investigate the potential of natural pigments particularly carotenoids in microalgae so that they can be utilised fully and commercialised especially in halal markets for

health advantages, food products and dye technology. The significant outcome of the research constitutes new findings of new natural carotenoid pigment sources as food colourants which cover not only the Shariah requirement but also the hygiene, sanitation, purification and safety aspects.

## **1.2 STATEMENT OF THE PROBLEM**

The global community has now begun to understand the importance of the Muslim requirement to consume halal food and products. The market for certified halal food and products is growing robustly, both domestically and internationally (Shaarani, 2004; Buang and Zakaria, 2004). Although the term halal has never attracted as much attention as in recent times, today, a halal food market exists wherever there are Muslim consumers whose tastes and preferences are governed by halal rules on food specification. Every Muslim must ensure that what they eat comes from a halal source. This does not mean only to check the ingredients per se, but also to ensure that the entire process is in accordance with Shariah principles.

Colourants are often added to food to enhance its visual aesthetics and to promote sales (Huang et al., 2002). Although the allowable number of synthetic colourants is reduced for consumer healthiness reasons in recent years, many kinds of synthetic food dyes are still widely used all over the world due to their low price, high effectiveness and excellent stability (Sadecka and Polonsky, 2000). Synthetic colourants can be classified into water-soluble and fat-soluble colourants based on their solubility. Most fat-soluble synthetic colourants present in the market are azo compounds, such as Sudan I, Sudan II, Sudan III, and Sudan IV. The genetic toxicity of some azo-dyes has been confirmed (IARC, 1975; Calbiani et al., 2004) and

structure-activity relationships have been assessed (Searly, 1976; Prival et al., 1988). Sudans (I–IV) have been classified as category three carcinogens to humans by the International Agency for Research on Cancer (Tateo and Bononi, 2004), and the use of Sudan I in foodstuff is forbidden in global food regulation acts (DiDonna et al., 2004). However, Sudan dyes are still found in food products exported to European countries and other parts of the world (Calbiani et al., 2004). Therefore, it is important to assure that the additives, colourants and Active Pharmaceutical Ingredients (API) are from acceptable sources and are processed according to halal requirements without the use of alcohol-based carriers for instance. In this situation, microalgae are still not a well-studied group from a biotechnological point of view.

Among the 10,000 species that are believed to exist, only a few thousand strains are kept in collections. On top of that, only a few hundred are investigated for chemical content and just a handful are cultivated in industrial quantities (Olaizola, 2003). Nature-identical colours are man-made pigments which are also found in nature. Examples are carotene, canthaxanthin and riboflavin. Synthetic colours are man-made colours which are not found in nature; these are often azo-dyes (Aberoumand, 2011). Examples of inorganic colours are titanium dioxide, gold and silver. Although structurally very diversified and from a variety of sources, natural food colourants can be grouped into a few classes, the three most important of which are: tetrapyrroles, tetraterpenoids and flavonoids. The most important member of the tetrapyrroles is chlorophyll, which is found in all higher plants. Carotenoids are tetraterpenoids that are as ubiquitous as chlorophyll since they too part of the photosynthetic apparatus. They also give the yellow–orange–red colour of many fruits. Anthocyanins are a group of flavonoids that provide the red–purple shade of

many fruits, in particular berries such as strawberries and black currants. Other important classes of colourants are the anthraquinones and the betalains.

The terms “pigment” and “dye” are often used interchangeably. A pigment is insoluble in the given medium, whereas a dye is soluble. Thus, carotenoids are dyes in oil but pigments in water (Alexandra et al., 2001; Beyer et al., 2002). Because of the deficiencies of existing natural food colourants, the demand for natural pigments is repeatedly raised by the food industry. This demand can be fuelled by research to offer a more natural and healthy way of colouring foods and provide a clean label declaration. Therefore, part of plant pigment research is seeking new sources of pigments. This will not only help find natural alternatives for synthetic dyes but also discover new procedures for the pigment production (Callebaut et al., 1997; Chen et al., 1995).

-

### **1.3 PURPOSE OF THE STUDY**

The focus of this study is to explore the potential to utilise the photosynthetic pigment of microalgae as a natural and halal food colourant. Carotenoids have been chosen to be as the alternative food the synthetic food colourant in the market. Food colourants were selected as the subject due to its common use in the industrial food company to attract the consumer to consume their products. The argument that the synthetic food colourants are carcinogenic to consumers had led to a search for alternatives for natural food colourants. In this regard, we have proposed the idea to manipulate the pigments of microalgae to produce food colourants.

In this study, freshwater green microalgae (Chlorophyta) and cyanobacteria was chosen as the candidates due to its resemblances of higher plants. Carotenoids