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, β-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 commersialised 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, Butyriciococcus sudeticus ,Chlorococcum, Pseudanabaena, Scenedesmus, Ankistrodesmus, and Butyrocicoccus Brauni. Chlorella fusca (chlorophyta) and Pseudanabaena(cyanobacteria)* and found three types of carotenoid out of them which are β-carotene, β- cryptoxanthin cryptoxanthin and lutein. Species that was having all three types of carotenoid was *Pandorina morum* (Figure 4.8) with lutein (54.64±3.11 mg/g DW), Beta cryptoxanthin (2.38±0.56 mg/g DW) and Beta carotene (1.39±0.38 mg/g DW). Two types of microalgae were chosen represent their division which are *Chlorella fusca* (Chlorophyta) and *Pseudanabena* (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 ± 240.61mg/g DW), violaxanthin (16299±423.09mg/g DW) and lutein (2335.53±69.82mg/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±40.13mg/g DW). *Chlorella fusca* and *Pseudanabena* 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±24.66mg/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 اكْتَسَبت منتجات الحلال اعترافاً ورواجاً واسعاً، باعتبارها معياراً جديداً لضمان السلامة والجودة. وبناءًا على ذلك تم تأسيس مشاريع تجارية لمنتجات الطحالب بسبب احتوائها على مواد ذات قيمة عالية مثل بيتا كاروتين، أستازانتين، وأصباغ فيكوبيلين ومستخلصات الطحالب التي تستخدم في منتجات ومستحضرات العناية بالبشرة التي تحتوي cosmeceuticals products).على مركبات نشطة حيوياً (بيولوجياً)، 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 للأصباغ المستخدمة كملونات غذائية أخرى ذات قيمة. حلال. هذا المسعى ليس موجها فقط في إيجاد بدائل طبيعية للأصباغ الاصطناعية، ولكن أيضا لاكتشاف أصناف أو ومن ثم، هناك الحاجة الماسة لفحص إمكانات الأصباغ (carotenoid) جديدة لإنتاج كاروتينات(taxons)أنواع الطبيعية خصوصا الكاروتينات في الطحالب لما لها من مزايا صحية كمنتجات غذائية يتم استخدامها بشكل كامل في تكنولوجيا الصبغ وتسويقها خصوصا في سوق المنتجات الحلال. لقد تم معملياً التقييم الكمي والنوعي لعشرة أنواع من الطحالب لإنتاج وتكوين الكاروتينات وهي :

Chlorella fusca, Chlorella vulgaris, Selenastrum capricornutum, Pandorina morum, Butyriciococcus sudeticus, Chlorococcum, Pseudanabaena, Scenedesmus, Ankistrodesmus, Butyrocicoccus 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 ± 3.11 مليجرام/جرام "جاف")، بيتا كريبتوزانثين Pandorina morumوجدت في تم اختيار نوعين (2.38 ± 0.56 مليجرام/جرام "جاف") وبيتا كاروتين (1.39 ± 0.38 مليجرام/جرام "جاف").) و *Chlorella fusca*) هما (*Cyanobacteria*) وال (Chlorophytaمن الطحالب يمثلان قسمي ال (تم تزريع كلاهما باستخدام بولد على التوالي. وهما يتعرضان لدراسة الإجهاد وتغيير المغذيات.*Pseudanabaena*) المتوسطة وأيضا المحورة وكانت هناك ثلاثة أنواع من الكاروتين قد (Bold's Basal Medium (BBMباصل ((16299 ± 423.09 violaxanthinأنتجت وهي زياكسانثين (21248.7 ± 240.61 مليجرام/جرام "جاف")، مليجرام/جرام "جاف") واللوتين (2335.53 ± 69.82 مليجرام/جرام "جاف" مليجرام/جرام "جاف") مرتبة تواليا من الأكبر إلى الأقل. تم التلاعب بتغيير تركيز النترات في جميع أنواع الطرق المستخدمة وجلها قد أنتجت اللوتين. تم إنتاج بواسطة مغذي بريستول مع 0.5 (نصف) تركيز النترات (Pseudanabaena 1317.91أكبر عدد اللوتين في تزريع مع إضافة BBM في Pseudanabena) و *Chlorella fusca*± 40.13 مليجرام/جرام "جاف"). وتم تزريع ( ) وكلوريد الصوديوم. وكلها PEG)، البولي ايثيلين جلايكول (SA المختلفة التي هي حمض الصفصاف (elicitors SA وجدت الدراسة أن آثار من 25 مليجرام/لتر من elicitorsبدأت بتنشيط إنتاج اللوتين. ومن بين تلك مخترعات أثمرت أعلى أنتاج حيث كان تركيز اللوتين (666.45 ± 24.66 مليجرام/جرام "جاف"). وتم أيضاً تسجيل تركيز وتعداد خلايا الطحالب الدقيقة لجميع الدراسات باستثناء التنميط. قدأجريت 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 binJamaludin
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 Kulliyyah 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.

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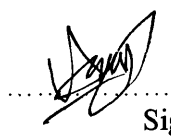

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ACKNOWLEDGEMENTS

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 appreaciation 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

LIST OF TABLES

LIST OF FIGURES

LIST OF ABBREVIATIONS

ζ	Zeta
α	Alpha
β	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 β-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).

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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