UNIVERSITI PUTRA MALAYSIA

MOLECULAR CHARACTERISATION AND PROTEIN EXPRESSION OF SELECTED MARKERS FOR AGAR YIELD AND GEL STRENGTH OF Gracilaria SPECIES

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By

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

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Abstract of thesis presented to the Senate of Universiti Putra Malaysia in fulfillment of the requirement for the degree of Master of Science

MOLECULAR CHARACTERISATION AND PROTEIN EXPRESSION OF SELECTED MARKERS FOR AGAR YIELD AND GEL STRENGTH OF *Gracilaria* SPECIES

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April 2018

Chairman : Ho Chai Ling, PhD
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Gracilaria is a genus of economically important red algae that produce agar as their cell wall polysaccharides. The yield and quality of agar determine the commercial and industrial values of these seaweeds in the phycocolloid market. Traditional screening process of seaweed materials with good agar yield and gel quality is laborious, tedious, costly and requires huge amount of seaweed. The availability of molecular markers for agar yield and quality may offer a quick and accurate alternative for seaweed selection. The aim of this study was to develop protein markers that are related to agar yield and/or gel strength in *Gracilaria* species. The specific objectives were (1) to clone and express three candidate transcript markers identified from a previous study into *Esherichia coli* expression system, (2) to test and confirm the binding specificity of polyclonal antibodies to the recombinant proteins of candidate transcripts and seaweed proteins, and (3) to correlate the protein expression of candidate markers in different *Gracilaria* samples to their agar yield and gel strength. The three candidate markers chosen for this study were GcFBPA (putative fructose-bisphosphate aldolase), GcGALE (putative UDP-glucose 4-epimerase) and GcSMF (putative sulfatase-modifying factor 1) previously identified to be highly expressed in *G. changii* with good agar yield and gel quality. The open reading frame (ORF) of these three candidate markers that are 1,077, 1,038 and 1,251 bp, respectively, were successfully cloned into pET28(+) expression vector and transformed into *Escherichia coli* BL21 (DE3) pLysS strain. The conserved domains for GcFBPA (F_bP_aldolase, PF01116), GcGALE (Epimerase, PF01370; GDP_Man_Dehyd, PF16336; Polysacc_synt_2, PF02719) and GcSMF (FGE-sulfatase, PF03781) were identified from the Pfam database. The most abundant cis-acting regulatory elements present in the 1 kb promoter regions of the three candidate markers were those related to abiotic stress and hormone responsiveness. Recombinant proteins of GcFBPA and GcGALE were expressed as soluble proteins at both 30°C and 37°C, respectively, while recombinant GcSMF was expressed in the insoluble fraction at all temperatures tested (i.e. 20, 30 and 37°C) in auto-induction Luria Bertani medium for 16 hours. Polyclonal antibodies specific to these three candidate markers, were generated by immunizing rabbits with peptide antigen. Western blot showed that the custom made polyclonal antibodies were specific to GcFBPA, GcGALE and GcSMF recombinant proteins, however, only polyclonal antibodies against GcFBPA and GcGALE showed the expected protein band sizes when tested on *Gracilaria* samples. Trichloroacetic acid (TCA)-Phenol method was used for extraction of total protein samples from 20 different *Gracilaria*

samples with different agar yield and gel strength. The protein expression of FBPA and GALE on these samples were evaluated using western blot and the protein intensities were quantified with ImageJ software. Statistical analysis showed that protein accumulation of GcFBPA and GcGALE was significantly correlated ($P<0.01$) with agar gel strength and agar yield, respectively. Western blot analysis of SMF could not be performed due to the absence of expected proteins when tested on *Gracilaria* samples. In conclusion, GcFBPA and GcGALE have potential to be developed as protein markers for selection of seaweed materials with higher agar yield or gel strength for marine aquaculture exploitation.

Abstrak tesis yang dikemukakan kepada Senat Universiti Putra Malaysia sebagai memenuhi keperluan untuk ijazah Master Sains

PENCIRIAN MOLEKUL DAN PENGEKSPRESAN PROTEIN UNTUK PENANDA TERPILIH YANG BERKAITAN DENGAN HASIL AGAR DAN KEKUATAN GEL DARI SPESIS *Gracilaria*

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Gracilaria merupakan genus rumpair laut merah berkepentingan ekonomi yang menghasilkan agar sebagai polisakarida dinding sel. Hasil dan kualiti agar menentukan nilai komersial dan perindustrian rumpair ini di dalam pasaran fikokoloid. Proses penyaringan tradisional untuk bahan rumpair laut yang mempunyai hasil dan kualiti agar yang baik memerlukan tenaga manusia, melibatkan banyak langkah, berkos tinggi dan memerlukan rumpair laut yang banyak. Kewujudan penanda molekul untuk hasil dan kualiti agar akan menawarkan alternatif yang lebih pantas dan tepat untuk pemilihan bahan rumpair laut. Tujuan utama kajian ini adalah untuk membangunkan penanda protein yang boleh dikaitkan dengan hasil agar dan kekuatan gel dalam speses *Gracilaria*. Objektif khusus dalam kajian ini adalah (1) untuk mengklon dan mengekspresi tiga calon penanda ekspresi yang dikenalpasti dari kajian yang terdahulu ke dalam sistem ekspresi *Escherichia coli*, (2) untuk menguji dan memastikan kespesifikan pengikatan antibodi poliklon pada rekombinan protein bagi calon penanda ekspresi dan protein rumpair laut, dan (3) untuk mengkorelasikan ekspresi protein dari calon penanda dalam sampel *Gracilaria* dengan hasil agar dan kekuatan gel yang berbeza. Tiga calon penanda yang terpilih untuk kajian ini adalah GcFBPA (fruktosa bisfosfat aldolase putatif), GcGALE (glukosa 4-epimerase putatif) dan GcSMF (faktor pengubahsuaian sulfatase putatif) di mana pengeskpresannya adalah tinggi dalam *G. changii* yang mempunyai hasil agar dan kualiti gel yang baik. Rangka bacaan terbuka untuk tiga calon penanda tersebut iaitu 1,077, 1,038 dan 1,251 pasangan bes (pb), masing-masing, telah berjaya diklonkan ke dalam vektor ekspresi pET28(+) dan ditransformkan ke dalam strain *Escherichia coli* BL21 (DE3) pLysS. Domain yang terpelihara untuk GcFBPA (F_bP_aldolase, PF01116), GcGALE (Epimerase, PF01370; GDP_Man_Dehyd, PF16336; Polysacc_synt_2, PF02719) and GcSMF (FGE-sulfatase, PF03781) telah dikenalpasti berdasarkan pangkalan data Pfam. Elemen pengawalaturan cis yang paling kerap dijumpai di kawasan promoter yang bersaiz 1kb dalam ketiga-tiga calon penanda adalah berkaitan dengan tekanan abiotik dan gerak balas terhadap hormon. Protein rekombinan GcFBPA dan GcGALE telah diekspres sebagai protein terlarut pada suhu 30°C and 37°C, masing-masing, sementara rekombinan GcSMF telah diekspres di fraksi tidak terlarut pada kesemua suhu yang telah diuji (iaitu 20, 30 dan 37°C) dalam media Luria Bertani yang diinduksikan secara automatik selama 16 jam. Antibodi poliklon yang spesifik kepada calon penanda telah dihasilkan melalui pengimunan arnab dengan antigen peptida. Pemblotan western menunjukkan antibodi poliklon yang dihasilkan masing-masing

adalah spesifik kepada protein rekombinan GcFBPA, GcGALE and GcSMF masing-masing. Namum demikian, hanya antibodi terhadap GcFBPA dan GcGALE menunjukkan saiz protein yang dijangkakan apabila diuji terhadap sampel *Gracilaria* yang berlainan. Kaedah asid trikloroasetik (TCA)-fenol telah digunakan untuk memencilkan protein jumlah daripada 20 sampel *Gracilaria* yang mempunyai hasil agar dan kekuatan gel yang berbeza. Pengekspresan protein GcFBPA dan GcGALE dalam sampel-sampel tersebut telah dinilai dengan pemblotan western dan keamatan protein tersebut telah diukur dengan perisian ImageJ. Analisis statistik menunjukkan bahawa korelasi pengekspresan protein GcFBPA dan GcGALE dengan kekuatan agar dan hasil agar, masing-masing, adalah signifikasi ($P<0.01$). Analisis pemblotan western untuk GcSMF tidak dapat dilaksanakan kerana protein jangkaan tersebut tidak dapat dikesan dalam sampel *Gracilaria*. Kesimpulannya, GcFBPA dan GcGALE berpotensi tinggi untuk dibangunkan sebagai penanda protein untuk pemilihan bahan rumpair laut dengan hasil agar dan kekuatan yang tinggi untuk eksploitasi akuakultur laut.

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I certify that a Thesis Examination Committee has met on (date of viva voce) to conduct the final examination of Lim Yi Yi on his thesis entitled "Molecular characterisation and protein expression of selected markers for agar yield and gel strength of *Gracilaria* species" 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 Master of Science.

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LIST OF ABBREVIATIONS

aa	Amino acid
AI-LB	Auto-Induction Luria Bertani
ATP	Adenosine triphosphate
BLAST	Basic Local Alignment Search Tool
bp	Base pair
BSA	Bovine albumin serum
$CaCl_2$	Calcium chloride
cm	Centimeter
CREs	Cis-acting regulatory elements
DAB	3,3'-diaminobenzidine
DHAP	Dihydroxyacetone-3-phosphate
DNA	Deoxyribonucleic acid
dNTP	Deoxynucleotides
DTT	Dithiothreitol
dw	Dry weight
EDTA	Ethylenediaminetetraacetic acid
FAO	Food and Agriculture Organisation
FBP	Fructose-1,6-bisphosphate
FBPA	Fructose-bisphosphate aldolase
FDA	Food and Drug Administration
GAP	Glyceraldehyde-3-phosphate
g	Gram
g	Gravitational force
g/cm^2	Gram per square centimeter
GALE	UDP-galactose-4-epimerase
GALT	Galactose-1-phosphate uridylytransferase
GDP	Guanosine diphosphate
GMQ	Global model quality estimate
GRAVY	Grand average of hydropathicity
h	Hour
HCl	Hydrochloric acid
HRP	*Horseradish peroxidase*
H_2O	Water
ISSR	Inter Simple Sequence Repeat
KCl	Potassium chloride

kDa	Kilodalton
KH_2PO_4	Potassium potassium dihydrogen phosphate
kg	Kilogram
KLH	Keyhole limpet hemocyanin
LB	Luria Bertani
mA	Milliampere
MAE	Microwave assisted extraction
MEGA	Molecular Evolutionary Genetics Analysis
mg	Miligram
$MgCl_2$	Magnesium chloride
$MgSO_4$	Magnesium sulfate
mg/ml	Miligram per liter
min	Minute
ml	Mililiter
mM	Milimolar
$MnCl_2$	manganese chloride
MOPS	3-(N-morpholino) propanesulfonic acid
MW	Molecular weight
n	Haploid
NACA	Network of Aquaculture Centres in Asia-Pacific
NaCl	Sodium chloride
Na_2HPO_4	Disodium hydrogen phosphate
NCBI	National Center for Biotechnology Information
NEB	New England Biolabs
ng	Nanogram
nM	Nanomolar
NR	Non redundant
OD	Optical density
ORF	Open reading frame
PBS	Phosphate-buffered saline
PCR	Polymerase chain reaction
PEG	Polyethylene glycol
pI	Isoelectric point
PMSF	Phenylmethylsulfonyl fluoride
ppt	Part per thousand
PVDF	Immobilan-P polyvinylidene fluoride
PVP	Polyvinylpyrrolidone

RAPD	Random Amplified Polymorphic DNA
RbCl	Rubidium chloride
RE	Restriction enzyme
RNA	Ribonucleic acid
rpm	Rotation per minute
SAM	S-adenosyl-L-methionine
SDS	Sodium dodecyl sulfate
SDS-PAGE	Sodium dodecyl sulfate polyacrylamide gel electrophoresis
SMF	Sulfatase modifying factor
SNP	Single Nulceotide Polymorphism
TAE	Tris Acetate EDTA
T_a	Annealing temperature
TBST	Tris Buffered Saline with 0.1% (v/v) Tween 20
TCA	Trichloroacetic acid
TEMED	Tetramethylethylenediamine
UDP	Uridine diphosphate
URP	Universal Rhodophyta Primer
US$	United States Dollar
UTP	Uridine-5'-triphosphate
V	Volt
v/v	Volume per volume
W	Watt
w/v	Weight per volume
w/w	Weight per weight
2-ME	2-methanol
2n	Diploid
%	Percent
°C	Degree celcius
µg	Microgram
µg/ml	Microgram per mililiter
µl	Microliter
µM	Micromolar

CHAPTER 1

INTRODUCTION

1.1 Introduction

Gracilaria which belongs to the Gracilariaceae family, consists of edible species of red algae that are most important sources for commercial agar production (Lai and Lii, 1997). Approximately 91% of the agar in the world was derived from *Gracilaria* species (Porse and Rudolph, 2017). *Gracilaria changii* is an agarophytic seaweed which grows abundantly in the mangroves all around Malaysia (Phang, 1994; Phang et al., 1996) and had been used extensively in the food and phycocolloid industries (Norziah and Ching, 2000). Moreover, *G. changii* produces agar and agarose with higher gel strength compared to other locally found agarophytes (Phang et al., 1996; Lee et al., 2016), thus could be a potential resource for agar industry in Malaysia.

Agar is a polysaccharide extracted from the cell wall matrix of some red algae (Armisen and Galatas, 1987). The agar structure consists of α-1,4 linked L-galactose alternating with β-1,3 linked D-galactose (Araki, 1966). The hydroxyl groups of galactose in agar were commonly substituted by methoxyl, sulfate ester and pyruvate ketal group (Lahaye and Yaphe, 1988). The type, pattern and degree of substitution affect the quality of agar gel. Agar produced from *Gracilaria* species was found to be of low gel quality due to high sulfate content which can be improved using alkaline hydrolysis treatment (Armisen, 1995).

The market for agar is huge with an annual average growth rate of 9,600 tonnes in 2009 and 14,500 tonnes in 2015 (Bixler and Porse 2011; Porse and Rudolph, 2017). Among the seaweed phycolloids, agar has a higher retail price (US\$17 kg^{-1}) when compared to carrageenans (US\$9 kg^{-1}) and alginates (US\$14 kg^{-1}) (Rhein-Knudsen et al., 2015). Agar dominated the seaweed hydrocolloid industry with total sales of US$ 246 million in 2015 (Porse and Rudolph, 2017), and the demand for raw seaweeds for agar production is expected to increase (Santos and Melo, 2018).

The quantity and quality of agar can be affected by various factors such as the genetic and developmental stages, the environmental and physical conditions, and post-extraction treatment to the agar (Arvizu-Higuera et al., 2008; Gupta et al., 2011; Bunsom and Prathep, 2012). Traditional methods to evaluate the agar properties involve a series of tedious and time-consuming tasks (e.g. harvesting seaweeds starting materials, extraction of agar, and measuring the agar properties) (Coppen and Nambiar, 1991), which require a lot of technical replicates for accurate results. The high demand for agar affirms the needs for fast selection of *Gracilaria* with superior agar yield and gel strength accurately using molecular approach.

In a previous study (Lee, 2016), transcript markers were identified for screening of yield trait and gel quality of *Gracilaria* species using quantitative real time PCR (qRT-PCR). Three most promising transcript markers were identified, namely GcFBPA, GcGALE and GcSMF which were putative agar biosynthesis genes involved in carbon, galactose and sulfur metabolism, respectively. The gene expression of these markers showed high Pearson and Spearman correlations (i.e. P-value less than 0.05) with agar yield and/or gel strength. However, the qRT-PCR method requires high

technical skills, is limited to intraspecies gene expression analysis and difficult to be developed into user-friendly rapid detection kit.

Immunoassay which involves the hybridization of antibodies or antigen to protein of interest can be used to select seaweeds with desired traits, as it requires less technical skills, easy to use for quick detection and can be potentially suitable for cross species comparison. Thus, identification of protein markers based on the candidate transcript identified from Lee (2016) should assists the development of user-friendly detection kit. The general objective of this study is to develop protein markers based on three potential transcript markers (i.e. GcFBPA, GcGALE and GcSMF) identified by Lee (2016) for identification of seaweed samples/species with good agar properties.

1.2 Research objectives

The specific objectives of this study are:

1. To clone and express candidate transcript markers associated with agar yield and gel strength in *Escherichia coli* expression system;
2. To test and confirm the binding specificity of polyclonal antibodies to the recombinant proteins of candidate transcripts and total cellular seaweed proteins;
3. To correlate the protein expression of candidate transcripts to agar yield and gel strength in different *Gracilaria* samples.

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