ISOLATION AND CHARACTERIZATION OF SOIL CHITINOLYTIC BACTERIA

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DECLARATION

The materials in this thesis are original except for quotations, excerpts, summaries and references, which have been duly acknowledged.

Ng Wui Ming @ Adrian Victor D'Ng
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ABSTRACT

ISOLATION AND CHARACTERIZATION OF SOIL CHITINOLYTIC BACTERIA

Chitinases (EC 3.2.1.14) hydrolyze the β-1-4-linkages of chitin, the second most abundant biopolymer on earth. Chitinases have high commercial value and their genes have great potential in the development of plant protection scheme against phytopathogenic attacks from fungi and insects. Bacteria are considered as major chitinase producers. Sabah with its megabiodiversity is hypothesized to harbour chitinolytic bacteria with optimal chitinase activity. Isolating local soil chitinolytic bacteria was initiated in which soil from mangrove areas at Sungai Merajah and Abai Bay of Kota Belud, Marudu Bay of Kota Marudu and Sungai Teri of Kimanis were sampled and screened. In addition, desolated normal garden soil enhanced with chitinous materials at Kota Belud was also sampled. Soil suspensions with pH adjusted to pH6.5 was used as inocula, which is the average pH value of soil. Five different solid media supplemented with cycloheximide, an antifungal agent were used for screening. Chitinase Detection Agar was found to be the most suitable screening medium. Selection of bacteria was based on their ability to grow and produce distinct halo on chitin containing medium within the initial five days of incubation. Five isolates designated as BRI 1, BRI 2, BRI 8, BRI 13 and BRI 36 were considered potential as they produce huge halos during the incubation period. Analysis on their partial 16S rDNA fragments revealed that BRI 1, BRI 2, BRI 13 and BRI 36 belong to the phylum Actinobacteria and are closely related to the genus Streptomyces while BRI 8 belong to the phylum Proteobacteria. However, all the test isolates sporulate indicating that they are actinomycete. Furthermore Gram staining showed that they are Gram positive bacteria, a characteristic of actinomycete. Therefore, a thorough study is proposed for the placing of BRI 8 into its proper taxon. PCR generated amplicon of around 350bp, confirming the presence of family 19 chitinase gene in the genome of all the test isolates. However, amplicon of around 400bp for family 18 chitinase gene was only successfully generated from the genome of BRI 1, BRI 8, BRI 13 and BRI 36. A reverse genetic is proposed to solve the problem encountered for BRI 2. Crude chitinase activity revealed that BRI 1 (8.61 Unit), BRI 2 (4.89 Unit), BRI 8 (4.17), BRI 13 (4.82 Unit) and BRI 36 (26.70 Unit) have higher activity compared to the commercially available chitinase from Streptomyces griseus, Sigma C6137 (2.54 Unit). BRI 36 showed the highest crude chitinase activity by 11 folds higher relative to that of Sigma C6137. Cloning of family 18 group A chitinase gene was initiated with BRI 13. However, problems were encountered during the preparation of DNA library. Proposed remedies were discussed. This opens a challenging experimental endeavour in isolating the open reading frame (ORF) of chitinase gene. ORF of chitinase genes can be used for various downstream applications such as in the development of transgenic crops with enhanced resistance towards fungal attacks, development of potent biopesticides and in the development of new strains of soil chitinolytic microbes to control soil fungal attacks on crops.

ABSTRAK

ISOLATION AND CHARACTERIZATION OF SOIL CHITINOLYTIC BACTERIA

Kitinase (EC 3.2.1.14) menghidrolisiskan ikatan β-1-4 pada kitin yang merupakan biopolimer kedua terbanyak di muka bumi. Kitinase mempunyai nilai komersil yang tinggi dan gennya berpotensi dalam pembangunan skim kawalan tumbuhan terhadap serangan kulat dan serangga. Bakteria dianggap sebagai penghasil kitinase yang baik. Sabah, dengan megabiodiversitinya, dihipotesiskan memiliki bakteria kitinolitik yang menghasilkan kitinase beraktiviti tinggi. Pemencilan bakteria kitinolitik dari tanah tempatan telah dimulakan di mana tanah-tanah dari kawasan paya bakau pada Sungai Merajah dan Kuala Abai di Kota Belud, Teuk Marudu di Kota Marudu dan Sungai Teri di Kimanis telah disampel dan disaring. Sebagai tambahan, tanah bercampur bahan berkitin terbiar di Kota Belud juga telah disampel. Ampaian tanah dengan nilai purata pH6.5 telah digunakan sebagai inokula. Lima media pepejal berlainan yang bercampur cycloheximide iaitu agen antikulat telah digunakan sebagai media penyaringan. *Chitinase Detection Agar* didapati merupakan media penyaringan terbaik. Pemilihan bakteria kitinolitik adalah berdasarkan kepada kebolehan sesuatu bakteria untuk tumbuh dan menghasilkan zon peluputan dalam tempoh eraman lima hari pertama. Lima isolat iaitu BRI 1, BRI 2, BRI 8, BRI 13 dan BRI 36 telah dikenalpasti dan dianggap berpotensi berdasarkan saiz zon peluputan yang dihasilkan dalam tempoh eraman tersebut. Analisa ke atas sebahagian daripada jujukan 16S rDNA menunjukkan bahawa BRI 1, BRI 2, BRI 13 dan BRI 36 merupakan anggota filum Aktinobacteria yang mana mereka menunjukkan hubungkait yang rapat dengan genus *Streptomyces*. BRI 8 dikenalpasti merupakan anggota filum Proteobakteria dalam analisa yang sama. Walau bagaimanapun, kesemua isolat itu menunjukkan ciri-ciri aktinomiset yang mana mereka menghasilkan spora dan bersifat Gram positif dengan menunjukkan warna unggu dalam ujian pewarnaan Gram. Oleh itu, kajian lebih mendalam telah dicadangkan dalam perletakkan BRI 8 ke dalam kumpulan taksonnya yang betul. PCR menghasilkan amplikon gen kitinase keluarga 19 (sekitar 350bp) untuk kesemua isolat dan ini sekaligus membuktikan kehadiran gen tersebut di dalam genom kesemua isolat itu. Namun demikian, amplikon gen kitinase keluarga 18 (sekitar 400bp) hanya berjaya dihasilkan dari genom BRI 1, BRI 8, BRI 13 dan BRI 36. Dengan itu, kajian genetik songsang dicadangkan untuk mengatasi masalah yang dialami oleh BRI 2. Aktiviti kasar kitinase menunjukkan BRI 1 (8.61 Unit), BRI 2 (4.89 Unit), BRI 8 (4.17 Unit), BRI 13 (4.82 Unit) and BRI 36 (26.70 Unit) mempunyai aktiviti kasar kitinase yang lebih tinggi berbanding dengan aktiviti kitinase dari *Streptmyces griseus*, Sigma C6137 (2.54 Unit). BRI 36 menunjukkan aktiviti kasar kitinase yang tertinggi iaitu 11 kali lebih tinggi daripada aktiviti kitinase dari Sigma C6137. Pengklonan gen kitinase keluarga 18 kumpulan A telah dimulakan dengan BRI 13. Walau bagaimanapun, kesukaran penghasilan perpustakaan DNA telah dialami. Cadangan kepada penyelesaian masalah ini telah dibincangkan. Pada masa yang sama, ini membuka peluang kepada kajian lanjutan dalam pengasingan *open reading frame* (ORF) gen kitinase. ORF gen kitinase boleh

dimanipulasikan dalam pelbagai aplikasi hiliran seperti pembangunan tumbuhan transgenik yang berdaya rintang tinggi terhadap serangan kulat, pembangunan racun–serangga-bio yang berdaya musnah tinggi dan pembangunan mikrob kitinolitik tanah aktif untuk mencegah serangan kulat tanah terhadap tumbuhan.

ABBREVIATION

~	Approximately
/	per
<	Less than
>	More than
≤	Less than or equal to
%	percent
α	alpha
β	beta
γ	gamma
λ	lamda
°C	degree celcius
AP	alkaline phosphatase
BLAST	Basic Local Alignment Search Tool
Bp	base pairs
BSA	Bovine Serum Albumin
cds	coding sequence
CFU	Colony Forming Unit
CIAP	Calf Intestinal Alkaline Phosphatase
cm	centimeter
DIG	digoxygenin
dH_2O	distilled water
DNA	deoxynucleic acid
dNTP	any deoxynucleoside
dUTP	deoxyuridine triphosphate
EC	Enzyme Class
EDTA	ethylenediaminetetra-acetate
EGC	ethylene glycol chitin
E-value	Expected value
g	gram
GlcNAc	N-acetyl-D-glucosamine
H_20	water
HCl	hydrochloric acid
h	hour
IDV	Integrated Density Value
IUBMB	International Union of Biochemistry and Molecular Biology
kb	kilobase pairs
kDa	kilodalton
K_m	Michaelis constant
LB	Luria-Bertani
M	Molar
mg	miligram
min	minutes
mL	mililitre
mM	milimolar
mm	milimeter
MUF	Methylumbelliferone
µL	microlitre
µM	micromolar

ABBREVIATION

~	Approximately
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BSA	Bovine Serum Albumin
cds	coding sequence
CFU	Colony Forming Unit
CIAP	Calf Intestinal Alkaline Phosphatase
cm	centimeter
DIG	digoxygenin
dH_2O	distilled water
DNA	deoxynucleic acid
dNTP	any deoxynucleoside
dUTP	deoxyuridine triphosphate
EC	Enzyme Class
EDTA	ethylenediaminetetra-acetate
EGC	ethylene glycol chitin
E-value	Expected value
g	gram
GlcNAc	N-acetyl-D-glucosamine
H_20	water
HCl	hydrochloric acid
h	hour
IDV	Integrated Density Value
IUBMB	International Union of Biochemistry and Molecular Biology
kb	kilobase pairs
kDa	kilodalton
K_m	Michaelis constant
LB	Luria-Bertani
M	Molar
mg	miligram
min	minutes
mL	mililitre
mM	milimolar
mm	milimeter
MUF	Methylumbelliferone
µL	microlitre
µM	micromolar

µm	micrometer
NCBI	National Center for Biotechnology Information
NEB	New England Biolabs
ng	nanogram
nm	nanometer
OD	Optical density
OH	hydroxyl group
PAGE	polyacrylamide gel electrophoresis
PCR	Polymerase Chain Reaction
pI	Isoelectric point
psi	pounds per square inch
rDNA	ribosomal DNA
RNA	ribonucleic acid
rpm	revolution per minute
rRNA	ribosomal RNA
sdH_2O	sterile distilled water
SDS	sodium dodecyl sulphate
sec	seconds
sp.	species
SSU	Small Subunit
TAE	Tris acetate EDTA
TBE	Tris borate EDTA
TE	Tris-EDTA
T_{hyb}	hybridisation temperature
T_m	melting temperature
tRNA	transfer RNA
UV	ultraviolet
V	voltage
w/v	weight per volume

International Union of Biochemistry genetic codes for mixed bases

r	a/g	**y**	c/t
m	a/c	**k**	g/t
w	a/t	**s**	g/c
h	a/t/c	**b**	g/t/c
v	g/a/c	**n**	a/g/c/t
d	g/a/t		

International Union of Biochemistry symbols for amino acids

Alanine	A	**Lysine**	K
Arginine	R	**Methionine**	M
Asparagine	N	**Phenylalanine**	F
Aspartic acid	D	**Proline**	P
Cysteine	C	**Serine**	S
Glutamine	Q	**Threonine**	T
Glutamic acid	E	**Tryptophane**	W
Glycine	G	**Tyrosine**	Y
Histidine	H	**Valine**	V
Isoleucine	I	**Unspecified**	X
Leucine	L		

The genetic code

5' base	Middle base				3' base
	U	C	A	G	
U	UUU Phe	UCU Ser	UAU Tyr	UGU Cys	U
	UUC Phe	UCC Ser	UAC Tyr	UGC Cys	C
	UUA Leu	UCA Ser	UAA Ter*	UGA Ter*	A
	UUG Leu	UCG Ser	UAG Ter*	UGG Trp	G
C	CUU Leu	CCU Pro	CAU His	CGU Arg	U
	CUC Leu	CCC Pro	CAC His	CGC Arg	C
	CUA Leu	CCA Pro	CAA Gln	CGA Arg	A
	CUG Leu	CCG Pro	CAG Gln	CGG Arg	G
A	AUU Ile	ACU Thr	AAU Asn	AGU Ser	U
	AUC Ile	ACC Thr	AAC Asn	AGC Ser	C
	AUA Ile	ACA Thr	AAA Lys	AGA Arg	A
	AUG Met†	ACG Thr	AAG Lys	AGG Arg	G
G	GUU Val	GCU Ala	GAU Asp	GGU Gly	U
	GUC Val	GCC Ala	GAC Asp	GGC Gly	C
	GUA Val	GCA Ala	GAA Glu	GGA Gly	A
	GUG Val	GCG Ala	GAG Glu	GGG Gly	G

* Termination codons have no amino acids assigned to them.

† The AUG codon is the usual initiation codon as well as that for methionine residues elsewhere.

The genetic code is almost universal but differences have been found in the DNA of mitochondria from a number of organisms. For example, in human mitochondria UGA codes for Trp and not for termination; AUA codes for Met and not for Ile; AGA and AGG are termination codons and do not code for Arg; AUA and possibly AUU act as initiation codons as well as AUG (Smith *et al.*, 2000).

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Table 4.42: Development of DNA library containing chitinase gene from BRI 13 through transformation of plasmid−chitinase fragment constructs with competent *Escherichia coli* JM109.

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