UNIVERSITI PUTRA MALAYSIA

CLONING, EXPRESSION AND CHARACTERIZATION OF ANTIFUNGAL PROTEIN GENE (ENDO-β-1,3-1,4-GLUCANASE) FROM Bacillus sp. STRAIN 289 AGAINST SHEATH BLIGHT DISEASE PATHOGEN, Rhizoctonia solani

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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 fulfilment of the requirement for the degree of Master of Science

CLONING, EXPRESSION AND CHARACTERIZATION OF ANTIFUNGAL PROTEIN GENE (ENDO-ɓ-1,3-1,4-GLUCANASE) FROM *Bacillus* sp. STRAIN 289 AGAINST SHEATH BLIGHT DISEASE PATHOGEN, *Rhizoctonia solani*

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Oktober 2015

Chair : Associate Prof. Mohd. Yunus Abd. Shukor, PhD
Faculty : Biotechnology and Biomolecular Sciences

Rhizoctonia solani is a destructive fungal that caused sheath blight disease in rice. Infection by *R. solani* has caused a serious threat and yield loss in rice industry worldwide. The management of sheath blight disease is mainly through chemical control but it is not considered as long term solution due to environmental and health concerns. Therefore, as an alternative option by using biological control agent, this study was done with the objective to isolate antagonist bacteria against *R. solani*. This research is also aimed to isolate, express and characterize potential antifungal protein, endo-β-1,3-1,4-glucanase (JTOX) from the best isolated antagonist bacteria using protein recombinant technology. A total of 390 pure culture bacteria were isolated from 60 soil samples collected from six different paddy field locations in Seberang Perai, Penang. Subsequently, the isolates were screened for growth inhibition activity on *R. solani.* There were 13 isolates exhibited antifungal activity with the highest inhibition zone was 22 ± 0.58 mm. Isolate with the highest inhibition zone was proceed for bacterial genus identification. Based on the biochemical profile identification results, 16S rRNA BLAST sequence analysis and phylogenetically related microorganism, the isolate SP 289 is in the genus of *Bacillus* and therefore is assigned tentatively as *Bacillus* sp. 289. Isolation of the JTOX showed an open reading frame of 720 bp in length which codes for 239 amino with molecular weight of 26.7 kDa. The gene was then cloned into pRSET A as expression vector and expressed in *E. coli* BL21. IPTG was used to induce the expression of the T7 RNA polymerase. The optimum time for the growth of *E. coli* BL21 to express the highest production of JTOX was at one hour after induction with the IPTG. The recombinant □*glu* was purified through affinity column using Ni-NTA resin. Characterization of the recombinant JTOX enzyme showed optimum activity at 50 ºC and optimum pH at pH 6. Enzyme activity was retained at almost 100 % after being preincubated for 30 minutes between 30 ºC to 50 ºC while pH stability profile showed the activity remained above 68 % at pH ranging from pH 5 to pH 10 upon treatment at 50 ºC for 30 minutes in various buffers. Initial rates of JTOX against lichenan concentration exhibited a K_m of 7.29 ± 2.57 mg/mL and a V_{max} of 68.16 ± 10.42 U/mg.

Bioassay test against the sheath blight pathogen was also done. The recombinant JIOX was found to inhibit the growth of *R. solani* mycelium and the inhibition was increased with the increased of enzyme concentration. With this finding, JIOX enzyme has potential as biological control for *R. solani* while the gene can be use in the development of transgenic rice resistant to sheath blight disease. This is the first report regarding the antifungal activity of endo-β-1,3-1,4-glucanase enzyme isolated from bacteria especially in inhibiting the growth of *R. solani*.

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

PENGKLONAN, PENGEKSPRESSAN DAN PENCIRIAN GEN ANTIFUNGUS (ENDO-ɓ-1,3-1,4-GLUCANASE) DARI *Bacillus* sp. STRAIN 289 ANTAGONIS TERHADAP PATOGEN PENYAKIT HAWAR SELUDANG, *Rhizoctonia solani*

Oleh

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Rhizoctonia solani adalah kulat perosak yang menyebabkan penyakit hawar seludang pada pokok padi. Jangkitan oleh kulat ini telah menyebabkan ancaman dan kerugian yang serius kepada industri beras di seluruh dunia. Pengurusan kawalan penyakit ini yang utama adalah secara kawalan kimia. Namun ia dianggap sebagai bukan penyelesaian untuk jangka masa panjang berikutan kebimbangan kesan racun terhadap alam sekitar dan kesihatan manusia. Oleh itu sebagai alternatif menggunakan kawalan secara biologi, kajian ini dijalankan dengan objektif untuk memencilkan bakteria yang bersifat antagonis terhadap *R. solani*. Kajian juga bertujuan untuk memencilkan, mengekspress dan melakukan pencirian terhadap protein, endo-β-1,3-1,4-glucanase (JIOX) yang berpotensi sebagai antikulat menggunakan kaedah teknologi protein rekombinan dari bakteria antagonis terbaik yang telah berjaya dipencilkan. Sebanyak 390 bakteria telah dipencilkan dari 60 sampel tanah sawah yang diambil dari enam lokasi penanaman padi di Seberang Perai, Pulau Pinang. Kesemua bakteria tersebut telah diuji keupayaan untuk merencat pertumbuhan miselium *R. solani*. Sebanyak 13 pencilan bakteria didapati mempunyai sifat antagonis terhadap *R. solani* dengan zon perencatan terbesar adalah 22 ± 0.58 mm. Bakteria yang menghasilkan zon perencatan terbesar telah dilakukan pengecaman secara biokimia, analisis 16S rRNA dan analisis filogenetik. Pencilan SP 289 telah di kenalpasti sebagai *Bacillus*. Oleh itu, ia di namakan sebagai *Bacillus* sp. 289. □*glu* telah berjaya dipencilkan dan gen mempunyai jujukan rangka terbuka 720 bp yang mengkodkan sebanyak 239 asid amino dengan berat molekul 26.7 kDa. Gen tersebut telah berjaya diklonkan ke dalam vektor pRSET A dan diekspresskan di dalam hos *E. Coli* BL21. IPTG digunakan untuk mengaruh pengekspressan T7 RNA polimerase. Tempoh masa optimum pengekspressan JIOX rekombinan oleh hos *E. Coli* adalah satu jam selepas diaruh menggunakan IPTG. JIOX kemudiannya ditulenkan melalui kolum afiniti menggunakan resin Ni-NTA. Pencirian biokimia protein JIOX menunjukkan aktiviti optimum pada suhu 50 ºC dan pH 6. Ujian stabiliti suhu pula menunjukkan aktiviti enzim kekal aktif hampir 100 % walaupun selepas dieram selama 30 minit pada suhu 30 hingga 50 ºC.

Kestabilan pH menunjukkan aktiviti yang kekal aktif melebihi 68 % pada pH 5 hingga pH 10 apabila dieram selama 30 minit dalam pelbagai larutan penimbal. Kadar awal JIOX terhadap kepekatan substrat lichenan mempamerkan nilai K_m 7.29 ± 2.57 mg/mL dan nilai V_{max} 68.16 ± 10.42 U/mg. Ujian bioasai terhadap *R. solani* mendapati, enzim JIOX berupaya merencat pertumbuhan *R. solani*. Perencatan juga didapati bertambah besar dengan peningkatan kepekatan enzim. Ini membuktikan bahawa protein JIOX bersifat antikulat dan berpotensi untuk digunakan sebagai agen kawalan biologi bagi merencat pertumbuhan *R.solani* manakala gen boleh digunakan untuk menghasilkan pokok padi transgenik yang rintang terhadap penyakit hawar seludang. Penemuan ini adalah yang pertama mengenai aktiviti antikulat oleh endo-β-1,3-1,4-glucanase yang dipencilkan dari bakteria yang berjaya merencat pertumbuhan *R. solani*.

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I certify that a Thesis Examination Committee has met on 26 October 2015 to conduct the final examination of Siti Noraini binti Bunawan on her thesis entitled "Cloning, Expression and Characterization of Antifungal Protein Gene (Endo-ß-1,3-1,4- Glucanase) from *Bacillus* sp. Strain 289 Against Sheath Blight Disease Pathogen, *Rhizoctonia solani*" 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

°C	Degree Celsius
JOX	endo-β-1,3-1,4-glucanase
µg	Microgram
µl	Microlitre
µM	Micromolar
Abs	Absorbance
Abs_{600}	Absorbance at 600nm
Abs_{560}	Absorbance at 560nm
Abs_{540}	Absorbance at 540nm
APS	Ammonium persulphate
BLAST	Basic local alignment search tool
bp	Base pair
BCA	Bicinchoninic acid
BSA	Bovine serum albumin
Da	Dalton
DEPC	Diethylpyrocarbonate
dH2O	Distilled water
DNA	Deoxyribonucleic acid
DNase	Deoxyribonuclease
dNTP	Deoxyribonucleotide triphosphate
EDTA	Ethylenediaminetetraacetic acid
et al.,	and others
EtOH	Ethanol
g	Gram
g/L	gram per liter
HCl	Hydrochloric acid
h	Hour
kDa	kilo dalton
min	Minute
ml	Millilitre
mm	Millimetre
mM	Millimolar
MW	molecular weight
NCBI	National Center for Biotechnology Information
NJ	Neighbor-joining
PCR	Polymerase chain reaction
pH	“Power (or potential) of hydrogen”
PVDF	Polyvinylidene fluoride
RNA	Ribonucleic acid
rpm	rotation per minute
RT-PCR	Reverse transcriptase polymerase chain reaction
RT	room temperature
SDS-PAGE	sodium dodecyl sulphate polyacrylamide gel electrophoresis
UV	ultraviolet
LB	Luria Bertani
TCA	trichloroacetic acid
ms	millisecond

Sec	second
SLS	sodium lauryl sulphonate
TEMED	N,N,N, N-Tetramethylenediamide
U/ml	unit per milliliter
v/v	volume per volume
w/v	weight per volume
x g	gravity
V	Volt
vol	volume
V/cm	volt per centimeter
kb	kilobase
U	unit
Ta	Annealing temperature
TAE	Tris, acetic acid, EDTA
Taq	*Thermus aquaticus*
Tm	Melting temperature
U	Units
UV	Ultraviolet
V	Voltage
v/v	Volume per volume
w/v	Weight per volume

CHAPTER 1

INTRODUCTION

Microbial antagonistic properties have created new opportunities in biological control technology. Several antagonist bacteria have been tested to have potential in inhibiting the growth of *Rhizoctonia solani*. *Bacillus* species are one of the most suitable bacterial candidates due to potent antifungal activity and their proven colonization aptitudes. The various suitable *Bacillus* species that have been reported include *B. subtilis* (Peng *et al*., 2013), *B. polymyxa* (Li *et al.,* 2015), *B. vallismortis* (Park *et al.,* 2006), *B. cereus* (Choudhary and Johri, 2009) and *B. megat*erium (Wiwattanapatapee *et al*., 2013).

R. solani is a fungal pathogen that caused sheath blight disease in rice. It has the capability to survive as mycelia within diseased plant material or as sclerotia for many years. The fungal can easily be transported into irrigation water, infested soil or through infected plant tissues during land preparation. Potential for seed-borne inoculums also exists (Taheri and Tarighi, 2011). In encouraging environmental conditions, especially in high moisture and temperature around 30 °C, the spread rate of the mycelia strands can be very fast. Moreover, the use of high rates of nitrogenous fertilizer, high rice plant population, double cropping and adaptation of high yielding rice plant through intensified rice production system recently have increased the incident and severity of the sheath blight disease (Wu *et al*., 2013). The disease contributes to the major yield loss of up to 50% in the rice industry worldwide (Liu *et al*., 2012, Taheri and Tarighi, 2011). In Malaysia, this disease was endemic in all major rice growing areas. It became the one of the worst diseases threatening the local rice industry after the seed broadcast system was adopted (Marzukhi, 2015). Apart from rice, this fungal pathogen also causes diseases to many other plant species such as lettuce, barley, maize, sorghum, bentgrass, bean, potato and tomato (Gkarmiri *et al*., 2015, Jeon *et al*., 2015, Solanki *et al*., 2012, Zhang *et al.,* 2009).

Control of sheath blight disease is not easy due to several factors such as broad host range of the fungal pathogen, low inherent resistance of rice cultivars (Marzukhi, 2015, Taheri *et al*., 2007), and the variability of its genetic. Furthermore, its capability to survive in soil for many years has also contributed to the difficulties in managing the disease. Currently, managing of rice sheath blight is achieved majorly through chemical control (Bhuvaneswari and Raju, 2012). However, the use of pesticides is not recommended as a long term solution due to several environmental and health concerns (Mishra *et al*., 2015).

These matters have led to an alternative control method by using biological agent. 6LQFH -glucanase FDQ LV WKH PD-glucanase FRPSR SOD\V DQ LPSRUWDQW UR-glucan DV D polymer by this enzyme has lead to fungal cell disruption by weakening the

mechanical strength of the cell walls. In the literature, antifungal activity was mainly observed in β-1,3-glucanases. These enzymes have been isolated from various sources including plants, fungi and bacteria. Previous studies also reported several number of β-1,3-1,4-glucanases which have been isolated and purified especially from bacteria. These enzymes are important in industrial applications especially in animal feeds production and brewing industries (Luo *et al.,* 2010, Qiao *et al.,* 2009, Beckmann *et al.,* 2006). Little is known about the antimicrobial activity of β-1,3-1,4-glucanase since there are not many researchers who made reports regarding the antifungal activity of the enzyme (Britto *et al.,* 2013, Luo *et al.,* 2010). In 2013, study by Britto *et al.,* has reported the antifungal activity of β-1,3-1,4-glucanase isolated from the cocoa plant *Theobroma cocao,* it is the first analysis shown antifungal activity of β-1,3-1,4-glucanase against *Moniliopthora perniciosa.*

Although a number of previous studies have reported the isolation of β-1,3-1,4-glucanase there are no recent reports regarding the antifungal activity of this enzyme especially in inhibiting the mycelial growth of *R. solani*. Therefore, this research is conducted with the following objectives:

1. To isolate and identify antagonist bacteria against sheath blight pathogen, *Rhizoctonia solani*
2. To isolate potential antifungal protein gene (endo-β-1,3-1,4-glucanase) from the antagonist bacteria
3. To clone and express the recombinant antifungal protein in *Escherichia coli*
4. To characterize the recombinant antifungal protein

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