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*

SITI NORAINI BINTI BUNAWAN

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By

SITI NORAINI BINTI BUNAWAN

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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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*

By

SITI NORAINI BINTI BUNAWAN

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 (*βglu*) 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 *βglu* 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 *JOX* was at one hour after induction with the IPTG. The recombinant *βglu* was purified through affinity column using Ni-NTA resin. Characterization of the recombinant *JOX* enzyme showed optimum activity at 50 °C and optimum pH at pH 6. Enzyme activity was retained at almost 100 % after being preincubated for 30 minutes between 30 °C to 50 °C while pH stability profile showed the activity remained above 68 % at pH ranging from pH 5 to pH 10 upon treatment at 50 °C for 30 minutes in various buffers. Initial rates of *JOX* 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 \textit{JOX} was found to inhibit the growth of \textit{R. solani} mycelium and the inhibition was increased with the increased of enzyme concentration. With this finding, \textit{JOX} enzyme has potential as biological control for \textit{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-\textit{β}-1,3-1,4-glucanase enzyme isolated from bacteria especially in inhibiting the growth of \textit{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-\(\beta\)-1,3-1,4-GLUCANASE) DARI Bacillus sp. STRAIN 289 ANTAGONIS TERHADAP PATOGEN PENYAKIT HAWAR SELUDANG, Rhizoctonia solani

Oleh

SITI NORAINI BINTI BUNAWAN

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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-\(\beta\)-1,3-1,4-glucanase (\(\beta\)glu) 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. Pencilhan SP 289 telah di kenalpasti sebagai Bacillus. Oleh itu, ia di namakan sebagai Bacillus sp. 289. \(\beta\)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 dikelonkan 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 \(\beta\)glu rekombinan oleh hos \(E.\) Coli adalah satu jam selepas diaruh menggunakan IPTG. \(\beta\)glu kemudiannya ditulenkan melalui kolum afiniti menggunakan resin Ni-NTA. Pencirian biokimia protein \(\beta\)glu menunjukkan aktiviti optimum pada suhu 50 °C dan pH 6. Ujian stabilititi suhu pula menunjukkan aktiviti enzal aktif hampir 100 % walaupun selepas dieram selama 30 minit pada suhu 30 hingga 50 °C.
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 JOX 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 JOX berupaya merencat pertumbuhan R. solani. Perencatan juga didapati bertambah besar dengan peningkatan kepekatan enzim. Ini membuktikan bahawa protein JOX 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-β-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
Abs₆₀₀ Absorbance at 600nm
Abs₅₆₀ Absorbance at 560nm
Abs₅₄₀ Absorbance at 540nm
APS   Ammonium persulphate
BLAST Basic local alignment search tool
bp    Base pair
BCA   Bicinichonic 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”
PVPDF 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

xvi
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. megaterium* (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 -JOXFDQLVWKHPDLQFRPSRQHQWQLIXQJDOMHOEZBDO SODVQLPSRUWDQWUROHDVQWLIQJDOMSURLQ[WHQVLIQURGROYLVR] 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 antifungal 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 cacao. It was the first analysis showing antifungal activity of β-1,3-1,4-glucanase against Monilipthora 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
REFERENCES


Leelasuphakul, W., Sivanulsakul, P. and Phongpaichit, S. 2006. Purification, chaUDFWHULIDWLRQ DQG V\QiHUJLVWLF DP\Re]LQGI]U] and antibiotic extract from an antagonistic *Bacillus subtilis* NSRS 89-24 against rice blast and sheath blight. *Enzyme and Microbial Technology* 38: 990–997


Niu, Q., Zhang, G., Zhang, L., Ma, Y., Shi, Q. and Fu, W. 2015. Purification and characterization of a thermophilic 1,3-1,4-b-glucanase from *Bacillus methylotrophicus* S2 isolated from booklice. *Journal of Bioscience and Bioengineering* 1-6


pellet formulations to suppress sheath blight of rice caused by *Rhizoctonia solani*. *BioControl* 45: 245–256


Teng, D., Wang, J., Fan, Y., Yang, Y., Tian, Z., Luo, J., Yang, G. and Zhang, F. &ORQLQJRI  -1,3-1,4-glucanase gene from Bacillus licheniformis EGW039 (CGMCC 0635) and its expression in Escherichia coli BL21 (DE3). Applied Microbiology and Biotechnology 85: 1015-1023


megaterium glucanase in Streptomyces lydicus A02 enhanced its production of antifungal metabolites. *Enzyme and Microbial Technology* 81: 80–87


http://www.knowledgebank.irri.org/ipm/sheath-blight/symptoms.html