

UNIVERSITI PUTRA MALAYSIA

PRODUCTION OF B -1,3 GLUCANASE FROM Penicillium oxalicum T3.3 FOR BIOCONTROL AGAINST Colletotrichum gloeosporioides AND Neoscytalidium dimidiatum

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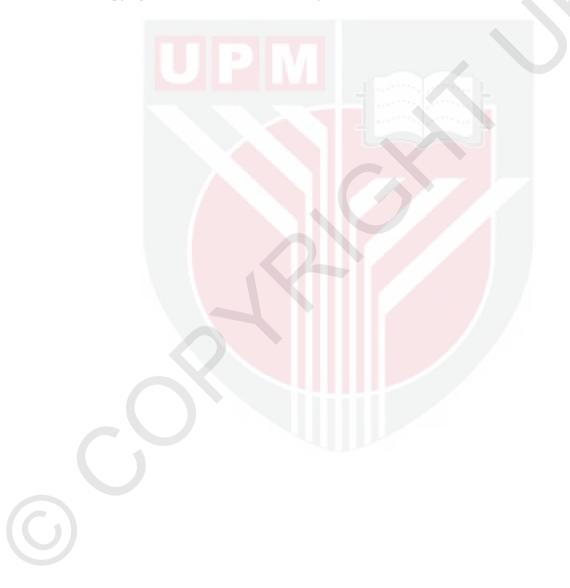
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March 2015

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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

PRODUCTION OF β-1,3 GLUCANASE FROM Penicillium oxalicum T3.3 FOR BIOCONTROL AGAINST Colletotrichum gloeosporioides AND Neoscytalidium dimidiatum

By

KHAIRUL ASMA SALSABILLA BT KHAIRUL IKHSAN



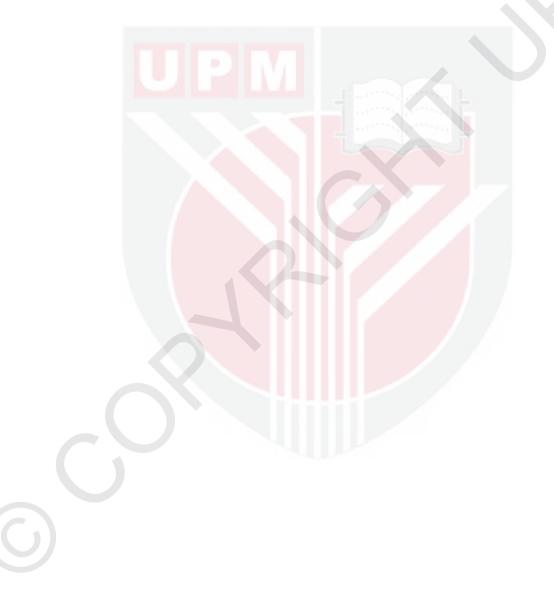
Chairman: Associate Professor Umi Kalsom bt Md Shah, PhDFaculty: Biotechnology and Biomolecular Sciences

 β -1,3 glucanase produced from fungi is an important hydrolytic enzymes which can been used as biocontrol agent against plant-pathogenic fungi. The objectives of this study are to determine the best substrates for β -1,3 glucanase production, to evaluate the cultural conditions which stimulate *in vitro* production of β -1,3 glucanase and to characterize the β -1,3 glucanase activity and then study the effect of crude β -1,3 glucanase towards fungal hypahe of C. gleosporioides and N. dimidiatum. β-1,3 glucanase production by *Penicillium* oxalicum T3.3 was investigated using shake flasks under various culture conditions such as different types of carbon and nitrogen sources, initial medium pH, agitation speed and different types of surfactants. The determination of best cultural conditions was carried out by varying and optimizing one variable at a time. The best cultural condition obtained from the shake flask experiment was used for β -1,3 glucanase production in a 2 L stirred tank fermenter where the effect of different impeller speed was investigated. Furthermore, the crude β -1,3 glucanase was characterized to study the enzyme stability. The role of this enzyme in antagonism action against Colletotrichum gloeosporioides and Neoscytalidium dimidiatum, a well-known fungal plant pathogens causing anthracnose disease in dragon fruit also was examined.

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In this study, seaweed *Undaria pinnatifida* was chosen as the best substrates for β -1,3 glucanase production. The highest production of β -1,3 glucanase of 84.73 U/mL was obtained at substrate concentration of 1% (w/v), 0.3% (w/v) peptone and 0.2% (w/v) yeast extract as nitrogen source, initial medium pH 5, temperature at 30°C, agitation speed at 200 rpm and with addition of sodium dodecyl sulfate as surfactant in shake flask condition. The β -1,3 glucanase activity was increased by 38.61% when optimized media and process parameters were used in shake flask culture. The

best cultural condition obtained from shake flask was further used for production β -1,3 glucanase in bioreactor. In bioreactor, the highest production of β -1,3 glucanase was obtained at 250 rpm. However, β -1,3 glucanase production in bioreactor was at par as compared with production in shake flask culture. The crude β -1,3 glucanase obtained was then concentrated by ammonium sulphate precipitation method. Concentrated enzyme was characterized and it was found that the optimum temperature and pH for the enzyme activity were 50°C and pH 5, respectively. This enzyme can also retain 93.4% and 83.6% of its activity after incubation for 2 h and 4 h, respectively, at 50°C and pH 5. In addition, fungal plant pathogen inhibition assay showed that the crude β -1,3 glucanase produced by *P. oxalicum* T3.3 was able to hydrolyze the fungal hyphae of *C. gloeosporioides* and *N. dimidiatum*.



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

PENGELUARAN β-1, 3 GLUCANASE OLEH Penicillium oxalicum T3.3 UNTUK KAWALAN BIOLOGI TERHADAP Colletotrichum gloeosporioides DAN Neoscytalidium dimidiatum

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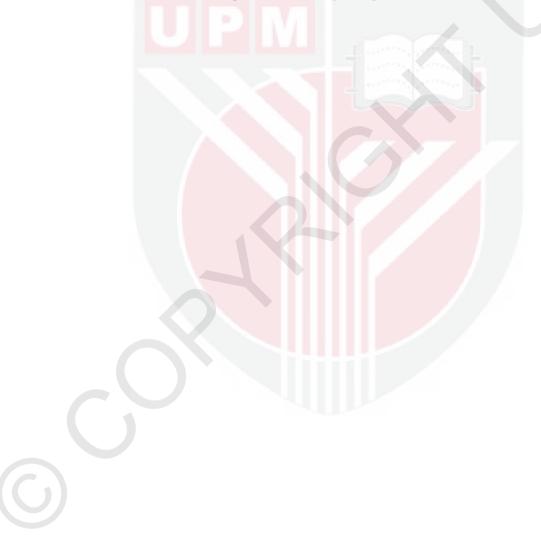


Pengerusi: Profesor Madya Umi Kalsom bt Md Shah, PhDFakulti: Bioteknologi dan Sains Biomolekul

 β -1,3 glukanase yang dihasilkan oleh kulat merupakan enzim hidrolitik penting yang boleh digunakan sebagai agen kawalan biologi terhadap kulat tumbuhan patogenik. Objektif kajian ini adalah untuk menentukan substrat yang terbaik untuk penghasilan β -1,3 glukanase, menentukan keadaan kultur terbaik untuk penghasilan β -1,3 glukanase secara in vitro dan untuk mencirikan aktiviti β-1,3 glukanase dan kemudian mengkaji kesan β -1,3 glucanase terhadap kulat C. gleosporioides dan N. dimidiatum. Penghasilan β -1,3 glukanase oleh *P. oxalicum* T3.3 telah dikaji dengan menggunakan kelalang bergoncang di bawah pelbagai keadaan kultur seperti jenis sumber karbon dan nitrogen, pH awal media, kelajuan goncangan dan jenis surfaktan. Penentuan keadaan kultur terbaik telah dijalankan dengan mengubah dan mengoptimumkan satu pemboleh ubah pada satu masa. Komposisi keadaan kultur terbaik yang yang diperolehi dari eksperimen kelalang bergoncang telah digunakan untuk penghasilan β-1,3 glukanase dalam bioreaktor 2L di mana kesan kelajuan putaran yang berbeza pada pengeluaran β -1,3 glukanase telah dikaji. Selain itu, β -1,3 glukanase dicirikan untuk mengkaji aktiviti enzim. Peranan enzim ini dalam kawalan terhadap C. gloeosporioides dan N. dimidiatum, patogen tumbuhan kulat yang terkenal dalam menyebabkan penyakit bintik berpusar di dalam buah naga juga telah dikaji.

Dalam kajian ini, rumpai laut *Undaria pinnatifida* telah dipilih sebagai substrat yang terbaik untuk penghasilan β -1,3 glukanase. Penghasilan paling tinggi β -1,3 glukanase (84.73 U/mL) dalam kelalang bergoncang telah diperolehi pada kepekatan substrat 1% (w / v), pepton dan ekstrak yis sebagai sumber nitrogen masing-masing pada 0.3% dan 0.2% (w/v), pH awal media 5, suhu 30°C, kelajuan goncangan pada 200 rpm dan dengan natrium sulfat dodecyl sebagai surfaktan. Ia juga menunjukkan

bahawa aktiviti β-1,3 glukanase meningkat sebanyak 38.61% dengan menggunakan keadaan kultur terbaik diperolehi dari eksperimen kelalang bergoncang. Keadaan kultur terbaik diperolehi daripada kelalang bergoncang kemudiannya digunakan untuk proses penghasilan β-1,3 glukanase dalam 2L bioreaktor. Di dalam bioreaktor, β-1,3 glukanase tertinggi telah diperolehi pada 250 rpm. Walau bagaimanapun, penghasilan β-1,3 glukanase dalam bioreaktor menunjukkan bacaan yang hampir sama dibandingkan dengan penghasilan di dalam kelalang bergoncang. β-1,3 glukanase diperolehi itu kemudian dipekatkan dengan menggunakan proses pemendapan ammonium sulfat. Suhu optimum dan pH aktiviti enzim adalah masingmasing pada 50°C dan pH 5. Aktiviti enzim ini juga boleh dikekalkan pada 93.4% dan 83.6% selepas diinkubasi selama 2 jam dan 4 jam, masing-masing, pada 50°C dan pH 5. Selain itu, ujian perencatan patogen tumbuhan kulat telah menunjukkan bahawa enzim β-1,3 glukanase yang dihasilkan oleh *P. oxalicum* T 3.3 dapat memusnahkan morfologi hifa kulat *C. gloeosporioides* dan *N. Dimidiatum*.



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This thesis was submitted to the Senate of the Universiti Putra Malaysia and has been accepted as fulfilment of the requirement for the degree of Master of Science. The members of the Supervisory Committee were as follows:

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LIST OF ABBREVIATIONS

СМС	Carboxylmethylcellulose
DO	Dissolved oxygen
DOT	Dissolved oxygen tension
FOL	F. oxysporum f. sp. lycopersici
LSD	Least significant differences
PDA	Potato dextrose agar
PDB	Potato dextrose broth
РРВ	Potassium phosphate buffer
SAS	Statistical Analysis Software
SDS	Sodium dodecyl sulphate
DCW	Dry cell weight

LIST OF SYMBOLS

	%	Percentage
	:	Ratio
	μL	micro liter
	μmol	micro molar
	CO ₂	carbon dioxide
	h	Hour
	h.	Hour
	H ⁺	Hydrogen ions
	H_2SO_4	Hydrogen Sulphate
	L	liter
	М	Molar
	mg/mL	milligram per mililiter
	min	minute
	mL	mililiter
	mM	mili Molar
	NaOH	Sodium Hydroxide
	NH4NO3	Ammonium nitrate
	nm	nanometer
	°C	Degree Celsius
	rpm	rotation per minute
	t	Time
	U/mL	Unit per mililiter
	U/mLd ⁻¹	Unit per mililiter per day
	β	Beta

CHAPTER 1

INTRODUCTION

1.1 Introduction

Dragon fruit plants in Malaysia have been reported to be infected by several bacterial and fungal diseases such as anthracnose, bacterial soft rot disease and necrotic lesion affected by Colletrotichum gloeosporioides (Masyahit et al. 2009a), Curvularia lunata (Masratul Hawa et al., 2009) and Enterobactor cloacae (Masyahit et al., 2009b) respectively. Besides that, several types of fungal pathogen have been reported to cause a disease in dragon fruit plant globally such as anthracnose infected by C. gloeosporioides (Takahashi et al. 2008), basal rot infected by Fusarium oxysporum (Wright et al. 2007), fruit rot, stem rot and fruit blotch infected by Bipolaris cactivora (Ben-Ze'ev et al., 2011). Furthermore, Neoscytalidium dimidiatum also has been reported causing stem canker on dragon fruit (Masratul Hawa et al., 2013). The pathogen has an ability of affecting at different plant parts such as leaves, blooms, root, twigs and fruit leading to a range of disease symptoms such as bloom blight, fruit rot, crow root rot and defoliation (Lubbe et al., 2006). Symptoms of the disease caused by this pathogen on the fruit were observed as hollow and water-soaked lesions that enlarge quickly on the fruit part (Voorrips et al., 2004). Fully extended lesions appear as soft, hollow and the range of colour changes from dark red to black which were known as anthracnose disease (Wharton and Dieguez Uribeondo, 2004).

In order to overcome these problems, chemical fungicides are generally used at high dosage. Excessive use of chemical fungicides has not only caused in the buildup of toxic compounds potentially hazardous to humans and environment but also resulted in resistance of the pathogens toward the fungicides. Furthermore, biological control of plant pathogen has received considerable attention and seems to be one of the effective alternatives to chemical control (Murali *et al.*, 2013). Antagonism is one of the mechanisms in biological control which may be achieved by parasitism, application of antibiotics, competition, or by a combination of these various modes of action. Moreover, a parasitism mode of action is hydrolytic enzymes production which was able to destroy cell walls of fungal pathogen. β -1,3-glucanase and chitinase play an important roles as enzymes responsible for fungal cell and sclerotial wall lysis and degradation (El Katatny *et al.*, 2000).

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 β -1,3 glucanases are semi-constitutive hydrolytic enzymes that can degrade glucan molecules embedded in the cell wall components of cereals and some species of fungi. This enzyme has an ability to hydrolyze glucan molecules and leading to the production of D-glucose, thus serving as carbon sources (Tang-Yao, 2002). β -1,3glucanases has been reported to be produced by a variety of organisms such as bacteria, fungi, and higher plants and many of them have been purified and characterized (Martin *et al.*, 2007). β -1,3-glucanase has been applied as biocontrol agent against plant-pathogenic and play an important role in alteration of cell wall of fungus and structure of β -1,3-glucan (Beshay *et al.*, 2003).

Penicillium sp. has been reported to produce lytic enzymes including chitinase and β -glucanase which are involved in degrading fungal cell wall (Patil *et al.*, 2013). In another study, it was suggested that *P. oxalicum* secretes chitinase and β -glucanases to degrade and penetrate into the conidiophores and spores of *Nigrospora oryzae* (Sempere and Santamrina, 2008). Chitinases and β -1,3 glucanase have been found to be directly involved in the mycoparasitism interaction between *Trichoderma* species and its hosts (Harman *et al.*, 2004).

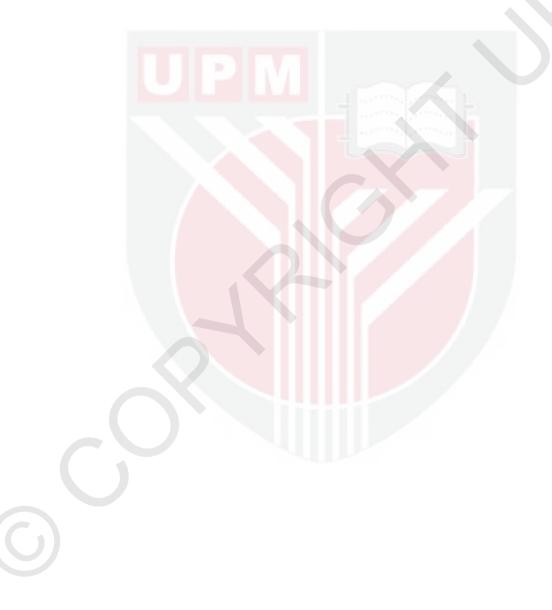
Commercial β -glucanases are reported to be produced from Aspergillus niger, Bacillus subtilis, Pseudomonas fluorescence, Disporotrichum dimorphosphorum and Penicillium species. β -glucanases from Penicillium species however, have been reported to have a greater benefit over glucanases from other sources. This is because Penicillium species are widely found in nature, spread most abundantly in soil and decaying fruits, not fastidious in their nutritional requirements and they can grow on different substrates and under a wide range of environmental conditions (Doughari and Hamuel, 2011).

To the best of our knowledge there are a little reports on the best cultural condition for β -1,3 glucanase production by *Penicillium* species. Thus a study on effect of cultural conditions is an important consideration to determine the best cultural condition for β -1,3 glucanases production by *P. oxalicum*. The most important aspects to decrease the production cost are optimization of media and process conditions (Goshal et al., 2011). On the other hand, reducing the cost for enzyme production is still needed in order to develop enzymatic treatment processes for different industrial and environmental applications, which might be more competitive than conventional and other novel treatment technologies. Another way, instead of recombinant DNA techniques, this might also be achieved by means of both process optimization using statistical experimental designs and the use of cheap growth substrates (Ikehata et al., 2004). Traditional methods of optimization involved changing one independent variable while fixing the others at a certain level. Optimization of one variable at a time techniques were applied in submerged fermentation to maximize β -1,3 glucanase by *Trichoderma harzianum* (El Katatny *et* al., 2000) and Aspergillus awamori (Nguyen and Quyen, 2010).

1.2 Objectives:

The specific objectives of this study are as follows:

- 1. To determine the best substrates for β -1,3 glucanase production.
- 2. To evaluate the best cultural conditions which stimulate *in vitro* production of β -1,3 glucanase produced by *P.oxalicum* T 3.3 in shake flask.
- 3. To characterize β -1,3 glucanase activity and study the effect of crude β -1,3 glucanase towards fungal hypahe of C. *gleosporioides* and N. *dimidiatum*.



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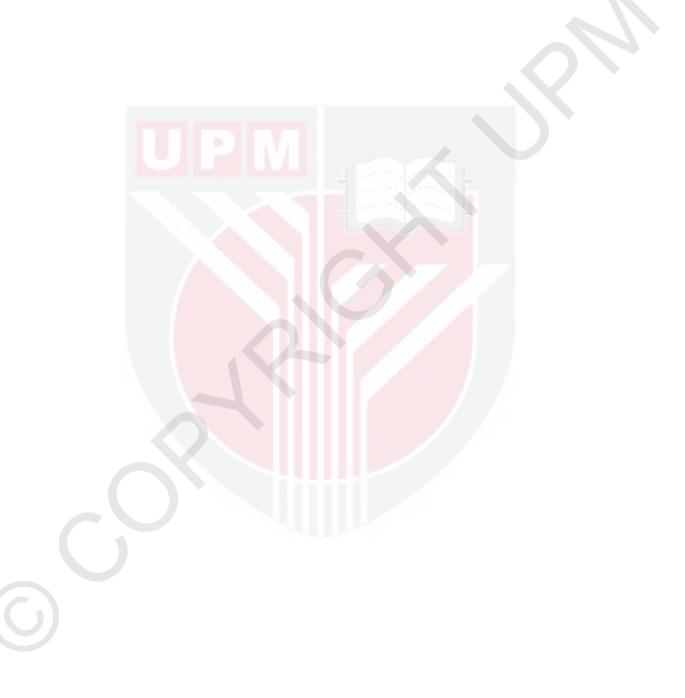
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BIODATA OF STUDENT

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After graduated her Master, she started her professional career in Market research Company as an executive. She is a passion learner, highly organized person, welldisciplined and multitasking team player to achieve individual and team goals. She is very dedicated and passionate in her work. She enjoyed travelling, doing outdoor activities and spending time with her families and friends. She is also an optimistic and positive person. Her life motto is "If you can dream it, you can do it".



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