UNIVERSITI PUTRA MALAYSIA

PRODUCTION OF PECTINASE BY LOCALLY-ISOLATED FUNGUS, Aspergillus fumigatus R6 IN SOLID STATE FERMENTATION FOR USE IN KENAF BIORETTING

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PRODUCTION OF PECTINASE BY LOCALLY-ISOLATED FUNGUS, *Aspergillus fumigatus* R6 IN SOLID STATE FERMENTATION FOR USE IN KENAF BIORETTING

By

WONG LI YIN

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

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DEDICATION

This dissertation is dedicated to my parents, family, lover, and friends.

Abstract of thesis presented to the Senate of Universiti Putra Malaysia in fulfilment of the requirement for the degree of Doctor Philosophy

PRODUCTION OF PECTINASE BY LOCALLY-ISOLATED FUNGUS, *Aspergillus fumigatus* R6 IN SOLID STATE FERMENTATION FOR USE IN KENAF BIORETTING

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August 2016

Chair: Wan Zuhainis binti Saad, PhD
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Kenaf fibres were produced by removing the pectin, a cementing material that binds the fibres together through the retting process. The traditional method of water retting requires long times and caused water pollution, while dew retting is weather and geographical dependence and produced fibres with low tensile strength. Retting using microbial pectinase is a promising approach as it reduces the times and produces fibres with high tensile strength, furthermore, it is environmental-friendly. The main objective of this study is to determine the efficacy of pectinase produced from a locally isolated fungus strain in kenaf bioretting. Pectinolytic fungi were isolated from the local sources and screened for their pectinase activity qualitatively and quantitatively. The selected pectinolytic fungus with the highest pectinase activity was identified by amplification of ITS region. The cultural conditions for maximum pectinase production from the selected strain were optimised using response surface methodology in solid state conditions, followed by purification using ammonium sulphate precipitation and gel chromatographic methods in order to characterise the pectinase produced. The pectinase produced was applied in kenaf bioretting to evaluate the possibility and efficiency of using pectinase in kenaf retting. A potential pectinolytic strain had been successfully isolated from the kenaf retting tank and was identified as *Aspergillus fumigatus* R6. Two types of pectinase were produced by *A. fumigatus* R6, which were polygalacturonase and pectin lyase using rice bran as the substrate in solid state conditions. The optimised cultural conditions for maximum production of polygalacturonase by *A. fumigatus* R6 was at an initial moisture level of 49.6%, 33°C, and 129 h of incubation time. *A. fumigatus* R6 polygalacturonase was purified using 60 – 80% ammonium sulphate precipitation and gel filtration chromatographic methods with a purification fold of 2.54 and polygalacturonase yield of 59.64%. The purified PgPl showed two bands on SDS-PAGE with a molecular weight of around 34 kDa and 95 kDa. The purified PgPl had an optimum temperature of 65°C. Two peaks were observed for pH optimal at pH 5 and pH 7, respectively. Pectinase produced by *A. fumigatus* R6 was stable at 40°C and covered a wide range of pH (pH 4 – 11). A 32 h treatment with *A. fumigatus* R6 crude pectinase solution produced the kenaf bast fibres with the highest tensile strength

(459 ± 166 MPa). Scanning electron microscopy (SEM) micrograph showed that the fibres fineness decreased and the surface of the kenaf fibres became smoother with a longer exposure of treatment. Crude pectinase produced from *A. fumigatus* R6 consisted of polygalacturonase with some pectin lyase and xylanase activities and low cellulase activity. Kenaf bast fibres tensile properties can improve by further optimisation of the enzyme formulation. A ratio of 3: 1 (v/w) of pectinase solution to kenaf bast produced kenaf fibres with the highest tensile strength. The enzyme formulation that produced kenaf bast fibres with the highest tensile strength was at 2 U/mL polygalacturonase activity supplemented with 50 mM of ethylenediaminetetraacetic acid (EDTA). In conclusion, *A. fumigatus* R6 pectinase shows potential to be used in kenaf bast bioretting process to produce strong kenaf fibres.

Abstrak tesis yang dikemukakan kepada Senat Universiti Putra Malaysia sebagai memenuhi keperluan untuk Ijazah Doktor Falsafah

PENGHASILAN PEKTINASE OLEH KULAT, *Aspergillus fumigatus* R6 DALAM PROSES FERMENTASI PEPEJAL UNTUK DIGUNAKAN DALAM PROSES PENGERETAN KENAF

Oleh

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Serat kenaf boleh dihasilkan dengan mengasingkan pektin, iaitu sejenis bahan yang mengikat serat kenaf dalam gulungan. Proses ini digelarkan sebagai proses pengeretan. Kaedah pengeretan tradisional dengan menggunakan air memerlukan masa yang lebih lama dan menyebabkan pencemaran air manakala pengeratan secara kering bergantung kepada cuaca dan kedudukan geografi dan serat yang dihasilkan mempunyai kekuatan tegangan yang rendah. Pengeretan menggunakan pektinase mikrob mengurangkan masa dan menghasilkan serat dengan kekuatan tegangan yang tinggi. Objektif utama kajian ini adalah untuk menentukan keberkesanan pektinase dihasilkan daripada kulat yang dipencilkan secara tempatan pada proses pengeratan kenaf. Dalam kajian ini, kulat pektinolitik telah dipencilkan dari sumber tempatan dan disaring untuk aktiviti pektinase secara kualitatif dan kuantitatif. Kulat yang mempunyai aktiviti pektinase tertingggi telah dipilih dan dikenal pasti. *Response surface methodology* telah digunakan untuk mengoptimisasikan keadaan kultur untuk pengeluaran pektinase yang tertinggi, diikuti dengan penulenan dengan pemendakan garam amonium sulfat pada kepekatan 60 – 80% dan kromatografi penurasan gel. Pektinase yang dihasilkan telah digunakan dalam proses pengeratan kenaf untuk menilai kemungkinan dan kecekapan pektinase untuk mengeret kenaf. Satu kulat yang berpotensi telah berjaya dipencilkan dari tangki pengeretan kenaf dan dikenal pasti sebagai *Aspergillus fumigatus* R6. Dua jenis pektinase telah dihasilkan oleh *A. fumigatus* R6 iaitu poligalakturonase dan pektin lyase dengan menggunakan dedak beras sebagai substrat dalam proses fermentasi keadaan pepejal. Keadaan kultur untuk penghasilan maksima pektinase daripada *A. fumigatus* R6 adalah pada tahap kelembapan sebanyak 49.6%, 33°C, dan 129 h masa inkubasi. Pektinase yang telah ditulenkan menunjukkan dua jalur pada SDS-PAGE dan mempunyai berat molekul kira-kira pada 34 kDa dan 95 kDa. Proses penulenan telah menghasilkan pektinase dengan tahap ketulenan 2.54 dan hasil sebanyak 59.64%. Suhu optimum untuk PgPl adalah pada 65°C. Dua suhu optimum telah diperhatikan pada pH 5 dan pH 7. PgPl yang ditulenkan daripada *A. fumigatus* R6 stabil pada 40°C dan pelbagai pH (pH 4 – 11). Rawatan 32 h dengan pektinase menghasilkan serat kenaf dengan kekuatan tegangan yang tertinggi (459 ± 166 MPa). Mikroskop imbasan elektron (SEM) mikrograf menunjukkan bahawa

kehalusan serat menurun dan permukaan serat kenaf menjadi lebih licin dengan pendedahan rawatan yang lebih lama. Pektinase *A. fumigatus* R6 terdiri terutamanya daripada poligalakturonase dan pektin lyase dengan xylanase dan aktiviti celulase yang sangat rendah. Kualiti serat kenaf boleh dipertingkatkan dengan mengoptimumkan formulasi enzim. Nisbah 3: 1 (v/w) pektinase dengan kulit kenaf menghasilkan serat kenaf yang kuat. Enzim formulasi yang optimum untuk penghasilan serat kenaf yang mempunyai kekuatan tegangan tinggi (490 ± 256 MPa) adalah pada aktiviti poligalakturonase 2 U/mL dan 50 mM asid atelindiamintetrasetik (EDTA). Pektinase daripada *A. fumigatus* R6 berpotensi untuk digunakan dalam proses pengeretan kenaf untuk menghasilkan serat panjang kenaf yang kuat.

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I certify that a Thesis Examination Committee has met on date 19 August 2016 to conduct the final examination of Wong Li Yin on her thesis entitled “Production of pectinase by locally-isolated fungus, *Aspergillus fumigatus* R6 in solid state fermentation for use in kenaf bioretting” 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 Degree of Doctor Philosophy.

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LIST OF ABBREVIATIONS

$(NH_4)_2SO_4$	Ammonium Sulphate
× g	Times gravity
°C	Degree Celsius
ANOVA	Analysis of Variance
AOAC	Association of Official Analytical Chemists
ASTM	American Society for Testing and Materials
A_w	Water Activity
CCD	Central Composite Design
CMCase	Carboxymethyl Cellulase
DNS	Dinitrosalicylic Acid
DP	Degree of Polymerisation
EDTA	Ethylenediaminetetraacetic Acid
$FeSO_4$	Iron(II) Sulphate
FTIR	Fourier Transform Infrared Spectroscopy
g	Gram
h	Hour
H_2O_2	Hydrogen Peroxide
HCl	Hydrochloric Acid
INTROP	Institute of Tropical Forestry and Forest Products
ITS	Internal Transcribed Spacer
kDa	Kilo Dalton
KH_2PO_4	Monopotassium Phosphate
L	Litre
M	Molar
mg	Milligram
mg/mL	Milligram per millilitre
$MgSO_4.7H_2O$	Magnesium Sulphate

min	Minute
mL	Millilitre
mM	Milimolar
N/tex	Newton per tex
NaCl	Sodium Chloride
NaOH	Sodium Hydroxide
nm	Nanometre
OFAT	One-factor-at-a-time
PDA	Potato Dextrose Agar
PE	Pectin Esterase
PG	Polygalacturonase
PNL	Pectin Lyase
psi	Pounds per Square Inch (pressure)
rpm	Rotation per Minute
RSM	Response Surface Methodology
SDS-PAGE	Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis
sec	Seconds
SEM	Scanning Electron Microscope
SmF	Submerged Fermentation
sp.	Species
SSF	Solid State Fermentation
U/g	Unit of Enzyme Activity per gram
U/mL	Unit of Enzyme Activity per millilitre
UPM	Universiti Putra Malaysia
v/v	Volume per Volume
v/w	Volume per Weight
w/v	Weight per Volume
w/w	Weight per Weight
µL	Microliter
µmol	Micromole

CHAPTER 1

INTRODUCTION

As a political consequence of the Kyoto protocol on global climate change, a transition to a sustainable bio-based economy has been emphasised, including the shifting of using feedstock for energy and chemical industry from petrochemical to a more eco-friendly renewable sources (Van Dam et al., 2008). Fibre crops such as kenaf, sisal, hemp, ramie, jute, and flax are non-food agricultural products that are cultivated for their content of fibrous materials. With the increasing of the social consciousness in preserving the environment, natural fibres are gaining interest as a substitution of synthetic fibres (Tahir et al., 2011). The ecological 'green' image of cellulosic fibres is the leading argument for innovation and development of fibre based products, resulting in emerging of new market potential.

Kenaf (*Hibiscus cannabinus*), belongs to the family of Malvaceae, is a fast growing annual crop plant that offers various usages from its leaves to stem (Ramesh et al., 2015). Kenaf fibre is applied in many industries such as news printing, textile industry, biocomposite materials in the automobile industry and structural applications (Baltina et al., 2012; Parikh et al., 2006; Mohta et al., 2004). Malaysia's government has declared kenaf as Malaysia's third commodity after rubber and oil palm in the year 2013. Advantages of kenaf include higher yields of fibres as compared to other natural fibre crops, fibres with low density and high toughness, low cost and most importantly, the biodegradability (Tahir et al., 2011; Song and Obendorf, 2007).

Kenaf bast fibres are found in the outer bast layer of the stalk. Kenaf fibres mainly consist of cellulose and bound together in the bundle form and with the inner core layer by pectin substances, which are the natural binding materials found in the plant (Tahir et al., 2011; Rowell and Stout, 2006). The removal of these mucilaginous materials exposes the fibre bundles and hence, kenaf single fibres are released (Othman et al., 2014). This process is known as retting and usually involves moisture with bacteria or chemicals (Rowell and Stout, 2006). Traditionally, water retting has been practised by the farmers where kenaf stems are immersed in stagnant water and fermented by anaerobic microbes. These microbes secrete an array of enzymes, especially pectinase to degrade pectin materials in the kenaf stem. Even though water retting can produce strong kenaf fibres; this method requires a long retting time and caused severe water pollution (Akin et al., 2007). Another retting method, dew-retting, where the kenaf stems are placed in an open place and colonised by filamentous fungi, gives rise to inconsistent fibres quality; moreover, this method is limited to the geographical region and weather dependence (Van Sumere, 1992). While fibres with a greater chemical and physical consistency can be obtained through the chemical retting process; however, the implementation of chemical retting process has been issued for its effect on the environment (Tamburini et al., 2004). Efforts have thus been made to find alternative retting methods, and the focus has been on bio-based retting technology.

Enzyme technology is gaining global recognition due to the fact that it is environmentally friendly and has a specific and focused performance (Hanana et al., 2015; Bledzki et al., 2010). Pectinase, which is responsible for the pectin hydrolysis, can be used in the retting process as the removal of pectin materials is the crucial step in producing natural fibres (Othman et al., 2014; Van den Brink and de Vries, 2011). The application of pectinase in retting process shortens the retting time as the enzyme acts specifically on the binding materials of kenaf stem to release the fibres. High strength fibres with consistent quality and various fineness can be produced via retting using microbial pectinase; however, the efficiency of pectinase in retting varied with the types of pectinase and the source of the microorganisms (Henriksson et al., 1999). Despite the advantages of retting using microbial pectinase, this method is costly and the dependence of commercial enzyme increases the production costs of kenaf fibres (Tahir et al., 2011). Hence, it is necessary to identify other sources of pectinase which has a potential to be applied in kenaf retting process.

The aim of this study is to assess and characterise the kenaf bast fibres retted with pectinase produced by a local isolated fungus. The following specific objectives were executed to achieve this crucial goal:

1. To isolate and identify a potential pectinolytic fungus from the local sources.
2. To optimise the cultural conditions for the maximum production of pectinase from the isolated fungus in solid state conditions using response surface methodology.
3. To purify the pectinase produced by the isolated fungus using ammonium sulphate precipitation and gel filtration chromatography for characterization.
4. To study the possibility of using the pectinase produced by the isolated fungus in kenaf bioretting.

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