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ENHANCEMENT OF VERSATILE EXTRACELLULAR CELLULOLYTIC AND HEMICELLULOLYTIC ENZYME PRODUCTION BY Lactiplantibacillus plantarum RI 11

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By

NURSYAFIQAH ATHIRAH BT MOHAMAD ZABIDI

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February 2021

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Recently, lactic acid bacteria (LAB) isolated from Malaysian foods have been shown to have the capability to produce versatile extracellular proteolytic, cellulolytic and hemicellulolytic activities. Approximately 15% of agricultural waste rich in cellulosic biomass is generated annually, causing major disposal problem in Malaysia. Following the "waste-to-wealth" initiative, these inexhaustible renewable biomasses can be utilise for the production of cellulolytic and hemicellulolytic enzymes. These enzymes, in return, can be used to degrade the cellulosic biomass into high value-added products. Therefore, the main objective of this study is to enhance versatile extracellular cellulolytic and hemicellulolytic enzyme production by *L. plantarum* RI 11 using renewable agrowaste and to purify and characterise selected extracellular endoglucanase produced by *L. plantarum* RI 11.

Different agricultural wastes involved were molasses, rice straw and soybean pulp with glucose and yeast extract as control ingredients. Out of 6 lignocellulosed-based media, basal media consisted of the combination of molasses and yeast extract; M3 (log 10.51 CFU/mL) and combination between molasses and soybean pulp; M4 (log 10.51 CFU/mL) produced the highest cell biomass while producing the highest specific enzyme activities over a broad pH range. This indicate that the cell viability and the production of extracellular cellulolytic and hemicellulolytic enzymes were induced by different cellulosic substrates.

Subsequently, statistical optimisation approach of Fractional Factorial Design (FFD) was employed to enhance the cell biomass and extracellular cellulolytic and hemicellulolytic enzyme productions by *L. plantarum* RI 11. Out of the 16 formulated media, basal media with molasses, yeast extract and soybean pulp (F4 medium) and rice straw, yeast extract

and soybean pulp (F5 medium) produced the highest cell biomass of log 11.76 CFU/mL, whereas F12 medium supplemented with glucose, molasses and PKC enhanced extracellular endoglucanase (43.91 µg/min/mg), exoglucanase (26.10 µg/min/mg) and mannanase (10.26 µg/min/mg) specific activities significantly at various pH range.

Due to the highest specific activity of endoglucanase produced by using F12 medium, it was subsequently purified to apparent homogeneity by using Fast Protein Liquid Chromatography System. Strategy B was established as the best strategy as the purification fold of 2.17-fold at pH 5, 3.37-fold at pH 6.5 and 2.14-fold at pH 8. The native molecular mass of the purified endoglucanase was estimated to be 120 kDa by gel filtration chromatography, whereas 20 kDa peptide band was detected by Sodium Dodecyl Sulphate-Polyacrylamide Gel Electrophoresis (SDS-PAGE) inferring that the extracellular endoglucanase enzyme was probably comprised 6 subunits of 20 kDa peptide molecules.

In conclusion, *L. plantarum* RI 11 had the capability to produce versatile extracellular cellulolytic and hemicellulolytic enzymes by using various renewable polymers. Therefore, *L. plantarum* RI 11 is a promising and versatile bio-transformation agent to degrade lignocellulolytic biomass.

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

PENINGKATAN PENGELUARAN ENZIM SELULOLITIK DAN HEMISELULOLITIK EKSTRASELULAR YANG VERSATIL OLEH *Lactiplantibacillus plantarum* RI 11

Oleh

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Baru-baru ini, bakteria asid laktik (BAL) pencilan dari makanan Malaysia dilihat mempunyai keupayaan untuk menghasilkan aktiviti proteolitik, selulolitik dan hemiselulolitik ekstraselular yang versatil. Kira-kira 15% sisa pertanian kaya dengan biomas selulosa dihasilkan setiap tahun, menyebabkan masalah pembuangan utama di Malaysia. Mengikuti inisiatif "sisa-ke-kekayaan", biojisim boleh diperbaharui ini dapat digunakan untuk pengeluaran enzim selulolitik dan hemiselulolitik. Enzim ini, sebagai ganti, dapat digunakan untuk menukarkan biomas selulosa menjadi produk bernilai tinggi. Oleh itu, objektif utama kajian ini adalah untuk meningkatkan pengeluaran enzim selulolitik dan hemiselulolitik ekstraselular oleh *L. plantarum* RI 11 menggunakan sisa pertanian boleh diperbaharui dan untuk menulenkan dan mencirikan “endoglucanase” ekstraselular terpilih yang dihasilkan oleh *L. plantarum* RI 11.

Sisa pertanian yang berbeza adalah molase, jerami padi dan pulpa kacang soya dengan glukosa dan ekstrak ragi sebagai bahan kawalan. Daripada 6 media berasaskan lignoselulosa, media basal terdiri daripada gabungan molase dan ekstrak ragi; M3 (log 10.51 CFU / mL) dan gabungan antara molase dan pulpa kacang soya; M4 (log 10.51 CFU / mL) menghasilkan sel biomas tertinggi sambil menghasilkan aktiviti enzim spesifik tertinggi dalam julat pH yang luas. Ini menunjukkan bahawa daya hidup sel dan penghasilan enzim selulolitik dan hemiselulolitik ekstraselular disebabkan oleh substrat selulosa yang berbeza.

Seterusnya, pendekatan pengoptimuman statistik Fractional Factorial Design (FFD) digunakan untuk meningkatkan biomas sel dan pengeluaran enzim selulolitik dan hemiselulolitik ekstraselular oleh *L. plantarum* RI 11. Dari 16 media yang dirumuskan, media basal dengan molase, ekstrak ragi dan pulpa kacang soya (medium F4) dan jerami padi, ekstrak ragi dan pulpa kacang soya (medium F5) menghasilkan sel biojisim

tertinggi log 11.76 CFU / mL, sedangkan medium F12 dilengkapi dengan glukosa, molase dan PKC meningkatkan aktiviti ekstraselular *endoglucanase* (43.91 µg/min/mg), *exoglucanase* (26.10 µg/min/mg) dan *mannanase* (10.26 µg/ min/mg) dengan ketara di pelbagai pelbagai pH.

Oleh kerana aktiviti spesifik tertinggi *endoglucanase* dihasilkan dengan menggunakan medium F12, ia kemudian ditulenkan sehingga homogen yang jelas dengan menggunakan "Fast Protein Liquid Chromatography System". Strategi B ditetapkan sebagai strategi terbaik sebagai lipatan penulenan 2.17 kali lipat pada pH 5, 3.37 kali ganda pada pH 6.5 dan 2.14 kali ganda pada pH 8. Jisim molekul asli *endoglucanase* yang ditulenkan dianggarkan 120 kDa oleh gel kromatografi penapisan, sedangkan jalur peptida 20 kDa dikesan oleh "Sodium Dodecyl Sulphate-Polyacrylamide Gel Electrophoresis (SDS-PAGE)" menyimpulkan bahawa enzim endoglucanase ekstraselular mungkin terdiri daripada 6 subunit molekul peptida 20 kDa.

Kesimpulannya, *L. plantarum* RI 11 mempunyai kemampuan untuk menghasilkan enzim selulolitik dan hemiselulititik ekstraselular serbaguna dengan menggunakan pelbagai polimer yang boleh diperbaharui. Oleh itu, *L. plantarum* RI 11 adalah agen biotransformasi yang berpotensi dan serba boleh untuk menguraikan biomas lignoselulolitik.

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This thesis was submitted to the Senate of 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

%	Percentage
°C	degree Celcius
µL	microlitre
µg	microgram
α	alpha
ANOVA	analysis of variance
β	beta
BSA	bovine serum albumin
CFS	cell free supernatant
CFU	Colony forming unit
Da	Dalton
DNS	Dinitrosalicyclic acid
FPLC	Fast Protein Liquid Chromatography
FFD	Fractional Factorial Design
g	gram
GRAS	Generally Regarded As Safe
h	hour
kDa	kilodalton
L	litre
LAB	lactic acid bactera
M	molarity
mg	milligram
mL	millilitre

min	minute
mM	millimolar
nm	nanometer
nmol	nanomole
OD	optical density
PKC	palm kernel cake
RM	Ringgit Malaysia
Rf value	Relative migration distance
SDS	Sodium Dodecyl Sulphate
SDS-PAGE	Sodium Dodecyl Sulphate Polyacrylamide Gel Electrophoresis
SEM	Standard error of the mean
sp.	species
spp.	subspecies
USD	US Dollar
v/v	volume per volume
w/v	weight per volume

CHAPTER 1

INTRODUCTION

Agriculture is an important sector of Malaysia's economy due to its tropical climate with 3 main crops; rubber, palm oil, and cocoa that dominated the commodity exports. Approximately 15% of agricultural waste that are rich in cellulosic biomass is generated annually by the country, causing a major disposal problem. These biomass wastes are economically feasible, due to its high content of cellulose, hemicellulose and lignin. Following the "waste-to-wealth" initiative, these inexhaustible biomass waste can be utilised for the production of cellulolytic and hemicellulolytic enzymes. These enzymes, in return, can be used to degrade the cellulosic biomass into cellulolytic and hemicellulolytic enzymes.

Cellulolytic enzyme contributes 14% to the global market attributing to its extensive industrial applications (Rashid` *et al*. 2013; Wilson, 2009). Cellulolytic enzymes produced by various bacteria and fungi when they grow on lignocellulosic materials (Ponnambalam *et al*. 2011), which can be harnessed as a biotransformation agent for the degradation of agricultural biomass. Cellulolytic enzymes are usually accompanied with hemicellulolytic enzymes to ensure efficient biodegradation of lignocellulosic biomass. These enzymes can exist in a form of free extracellular enzymes and as multi-enzyme complexes, called cellulosomes.

The application of cellulolytic and hemicellulolytic enzymes in Malaysia includes food processing, paper manufacturing, bioethanol, animal feed production and textile industries (Kuhad *et al*. 2011). Despite various industries are generally utilised enzymes to increase and shorten the production rate and time, Malaysia has yet to produce enzymes locally due to its high capital investment and high production cost (Ibrahim, 2008). Nonetheless, enzyme importation is approximately RM 80 million annually, which affects its profit margin substantially (Lee *et al*. 2013). Thus, by producing industrial enzymes using indigenous microbial isolates and agricultural wastes, higher profit will be obtained since lower processing cost can be achieved. The waste disposal problem also can be resolved. Once the enzymes were produced, the purification strategy was developed to purify and study the characteristics of the enzyme produced. This is important to characterise the function, structure and interactions of the protein.

Lactic acid bacteria (LAB) are a group of bacteria, which was named after lactic acid that produces as the major fermented product from carbohydrate fermentation. They are characterised as non-spore forming gram-positive bacterium, acid-tolerant and strictly fermentative (Axelsson, 2004). LAB are well documented for their anti-inflammatory (Hevia *et al*. 2015), anti-oxidative (Ejtahed *et al.* 2012), anti-diabetic, anti-obesity (Kerry *et al*. 2018), and antimicrobial (Leroy & De Vuyst, 2004) properties, among many other beneficial effects. In addition, Taskila & Ojamo

(2013) reported the capability of LAB to degrade two major polysaccharide constituents of lignocellulolytic biomass: pentoses and hexoses via phosphoketolase pathway. This was later proven by Lee *et al*. (2019), in which LAB, especially *Lactiplantibacillus plantarum* have the capability to produce versatile extracellular cellulolytic and hemicellulolytic enzymes that active under broad pH conditions.

However microbial secondary metabolites that produce along with enzyme preparations such as mycotoxin and aflatoxin may cause chemical toxicity (Ibrahim, 2008). Hence, LAB having the GRAS status; non-toxicogenic and non-pathogenic (Sewalt *et al.* 2016), was chosen as the extracellular cellulolytic and hemicellulolytic enzyme producers in this study. The safety of the enzyme producers and its capability of producing various cellulolytic and hemicellulolytic enzymes in broad pH conditions are pragmatic as they can be applied to many different operational conditions require by various industries.

The hypothesis of this project was *L. plantarum* RI 11 can be extracellular cellulolytic and hemicellulolytic enzymes producer by using renewable agricultural waste as the growth medium. Therefore, the general objective of this project was to enhance versatile extracellular cellulolytic and hemicellulolytic enzymes production by *Lactiplantibacillus plantarum* RI 11 using renewable agrowastes and to purify and characterise selected extracellular endoglucanase produced by *L. plantarum* RI 11. The following were the specific objectives of this study:

(i) To determine the effects of carbon and nitrogen sources derived from renewable biomass on cell viability and production of extracellular cellulolytic and hemicellulolytic enzymes by *L. plantarum* RI 11.

(ii) To formulate agro-waste media by Fractional Factorial Design (FFD) for the enhancement of extracellular cellulolytic and hemicellulolytic enzyme productions by *L. plantarum* RI 11.

(iii) To purify and characterise selected extracellular endoglucanase enzyme produced by *L. plantarum* R11 using formulated agro waste medium.

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