

SYNERGISTIC FERULIC ACID PRODUCTION FROM
BANANA STEM WASTE BY CO-CULTURE

KAMALIAH BINTI ABDUL SAMAD

DOCTOR OF PHILOSOPHY (CHEMICAL ENGINEERING)

UNIVERSITI MALAYSIA PAHANG



SUPERVISOR'S DECLARATION

We hereby declare that we have checked this thesis and in our opinion, this thesis is adequate in terms of scope and quality for the award of the degree of Doctor of Philosophy in Chemical Engineering.

(Supervisor's Signature)

Full Name : ASSOC. PROF. IR. DR. NORAZWINA ZAINOL

Position : ASSOC. PROFESSOR

Date :

(Co-supervisor's Signature)

Full Name : DR. AZILAH BINTI AJIT @ ABD AZIZ

Position : SENIOR LECTURER

Date :



STUDENT'S DECLARATION

I hereby declare that the work in this thesis is based on my original work except for quotations and citations which have been duly acknowledged. I also declare that it has not been previously or concurrently submitted for any other degree at Universiti Malaysia Pahang or any other institutions.

(Student's Signature)

Full Name : KAMALIAH BINTI ABDUL SAMAD

ID Number : PKC13012

Date :

SYNERGISTIC FERULIC ACID PRODUCTION FROM
BANANA STEM WASTE BY CO-CULTURE

KAMALIAH BINTI ABDUL SAMAD

Thesis submitted in fulfillment of the requirements
for the award of the degree of
Doctor of Philosophy (Chemical Engineering)

Faculty of Chemical & Natural Resources Engineering
UNIVERSITI MALAYSIA PAHANG

MAY 2017

ACKNOWLEDGEMENTS

In the name of Allah, The Most Gracious and Merciful.

Grateful to The Divine, by His Grace, I managed to complete this thesis in pursuing my doctoral degree. On this winding journey, many people helped me in smoothing it. For that reason, I wish to deliver my sincere thanks to my supervisor Assoc. Prof. Ir. Dr. Norazwina Zainol for her valuable guidance, care and encouragement extended to me. Special thanks also go to my co-supervisor Dr Azilah Binti Ajit @ Abd Aziz for giving me a chance to be part of her research family.

Thanks a million to all my lab members, Shareena, Zulsyazwan, Siti Natrah, Faizan, Siti Mazlifah and Nor Hazwani for their friendship, support and knowledge sharing throughout this struggle. Unforgettable thanks to Nur Syahirah, an undergraduate student, who assisted me a lot and willing to stay overnight together in completing our lab work.

I also take this opportunity to thank you to all my friends, Aimi Syairah, Adila, Siti Hajar, Mah Kah Hong, Syazana, Fatin Syazwana, Nuri Adilah, Sabrina and others who directly or indirectly, have lent their helping. I place on record, my sense of gratitude to all administrative and technical staff of the Faculty of Chemical and Natural Resource Engineering, Universiti Malaysia Pahang for their guidance, co-operation and providing me all the necessary facilities.

Last but not least, a heartfelt thank you goes to my supportive family for their unceasing encouragement and inspiration. Thank you for always being there for me. I could not have done any of it without their prayer, support and love.

TABLE OF CONTENT

DECLARATION	
TITLE PAGE	
ACKNOWLEDGEMENTS	ii
ABSTRAK	iii
ABSTRACT	iv
TABLE OF CONTENT	v
LIST OF TABLES	xi
LIST OF FIGURES	xiii
LIST OF SYMBOLS	xv
LIST OF ABBREVIATIONS	xvi
CHAPTER 1 INTRODUCTION	1
1.1 Background	1
1.2 Problem Statement	3
1.3 Research Objectives	6
1.4 Research Scope	7
1.5 Overview of the Thesis	8
CHAPTER 2 LITERATURE REVIEW	10
2.1 Introduction	10
2.2 Ferulic Acid	10
2.2.1 Chemical Properties of Ferulic Acid	11
2.2.2 Application of Ferulic Acid	13

2.2.3	Mechanism Release of Ferulic Acid	14
2.3	Lignocellulosic Materials Composition	16
2.3.1	Cellulose	17
2.3.2	Hemicellulose	17
2.3.3	Lignin	19
2.4	Lignocellulosic Material from Agricultural Waste for Ferulic Acid Production	19
2.4.1	Potential Agricultural Waste for Ferulic Acid Production	20
2.4.2	Selection of Raw Material for Ferulic Acid Production	23
2.5	Role of Microorganism in the Fermentation Process	24
2.5.1	Soil Microbe as a Potential Ferulic Acid Esterase Producer	25
2.5.2	Microbial Enzymes for Ferulic Acid Production	26
2.6	Fermentation by Fermentative Microorganism	27
2.6.1	Pure Culture Fermentation	28
2.6.2	Co-Culture Fermentation	28
2.7	Factors Affecting Ferulic Acid Production	30
2.7.1	Temperature	30
2.7.2	pH	31
2.7.3	Rotation Rate	32
2.7.4	Substrate Concentration	33
2.7.5	Inoculum Size	34
2.7.6	Fermentation Time	35
2.8	Optimization of Ferulic Acid Production	35
2.8.1	Factorial Design	37
2.8.2	Central Composite Design	39
2.8.3	Application of Response Surface Methodology	40

2.9	Kinetic Model for Ferulic Acid Production and Substrate Utilization by Microbes	41
2.9.1	Michaelis-Menten Kinetics	42
2.9.2	Monod Kinetics	42
2.9.3	Haldane Kinetics	43
2.10	Numerical Solution for Differential Equation	44
2.10.1	Euler's Method	45
2.10.2	Runge-Kutta Methods	46
2.10.3	Heun's Method	46
2.11	Concluding Remarks	47
	CHAPTER 3 METHODOLOGY	49
3.1	Introduction	49
3.2	Source of Banana Stem Waste and Soil Culture	50
3.2.1	Preparation of Banana Stem Waste	50
3.2.2	Characterization of Banana Stem Waste	53
3.2.3	Acclimatization of Soil and Banana Stem Waste	55
3.3	Isolation of Bacterial Strain	56
3.3.1	Initial Isolation of Acclimatized Soil Microbe by Spread Plate Method	56
3.3.2	Purification of Microbe by Streak Plate Method	57
3.3.3	Culture Storage and Revival Technique	57
3.3.4	Morphology Observation and Gram Staining Technique	58
3.4	Screening Process for the Best Bacterial Strain for Ferulic Acid Production	58
3.4.1	Preparation of Inoculum	59

3.4.2	Fermentation Procedure	59
3.5	Identification of the Best Performer of Bacterial Strain	60
3.5.1	Bacterial Identification Using Biolog GEN III MicroPlate System	61
3.5.2	Molecular Identification by 16S rRNA Sequencing Method	62
3.5.3	DNA Extraction and Agarose Gel Electrophoresis	62
3.5.4	PCR Amplification	63
3.6	Development of Co-Culture	63
3.6.1	Determination of Pure Culture Growth Rate for Inoculum Preparation	64
3.6.2	Experimental Set-up for Co-Culture Evaluation	65
3.6.3	Development of Co-Culture Inoculum	66
3.7	Factorial Analysis for Selection of the Significant Factor	67
3.7.1	Preparation of Co-Culture Inoculum	68
3.7.2	Experimental Set-up for Factorial Analysis	68
3.8	Optimization of Ferulic Acid Production	71
3.8.1	Preparation of Co-Culture Inoculum	71
3.8.2	Experimental Set-up for Optimization of Ferulic Acid Production	71
3.8.3	Validation of the Model	73
3.9	Kinetic Study of Ferulic Acid Production	74
3.9.1	Batch Fermentation for Kinetic Study	74
3.9.2	Data Analysis from Batch Fermentation	75
3.9.3	Determination of Kinetic Constant	75
3.10	Mechanism of Ferulic Acid Production	77
3.10.1	Inoculum Preparation for Enzymatic Hydrolysis of Banana Stem Waste by Co-Culture	78
3.10.2	Batch Fermentation and Crude Enzyme Preparation	78

3.11	Analysis of Fermented Sample and Dry Weight Cell	78
3.11.1	Analysis of Ferulic Acid	79
3.11.2	Estimation Dry Weight Cell of Co-Culture	79
3.11.3	Ferulic Acid Esterase Assay	80
3.12	Concluding Remarks	80
CHAPTER 4 RESULTS AND DISCUSSION		82
4.1	Introduction	82
4.2	Characterization of Banana Stem Waste	83
4.3	Isolation of Bacterial Strain	84
4.3.1	Bacteria Strain in Acclimatized Soil	85
4.3.2	Morphology Observation on Isolated Bacterial Colonies	85
4.4	Screening for the Best Bacterial Strain for Ferulic Acid Production	86
4.5	Identification of the Best Ferulic Acid Producing Strains	96
4.5.1	Identification of Isolated Bacteria Based on Biolog GEN III MicroPlate System	96
4.5.2	Identification of Isolated Bacteria Based on 16S rRNA Sequencing	98
4.5.3	Ferulic Acid Production by Identified Bacterial Strain	101
4.6	Development of Soil Co-Culture	102
4.6.1	Growth Profile of Pure Culture for Co-Culture Preparation	103
4.6.2	Ferulic Acid Production by Co-Culture	104
4.6.3	Development of Co-Culture	105
4.7	Factorial Analysis for Selection of Significant Factors	107
4.7.1	Screening of Factors Affecting Production of Ferulic Acid	107
4.7.2	Analysis of Variance (ANOVA)	109

4.7.3	Main and Interaction Effects of the Factors	111
4.7.4	Selection of the Best Condition	116
4.8	Optimization Studies with Central Composite Design	117
4.8.1	Fitting the Model and Analysis of Variance (ANOVA)	118
4.8.2	Validation of the Model	123
4.8.3	Comparison with Previous Researches	124
4.9	Kinetic Modelling	126
4.9.1	Estimation by Lineweaver-Burk Plot	126
4.9.2	Substrate Utilization	127
4.9.3	Product Formation	130
4.9.4	Comparison of Kinetic Constant Values	132
4.10	Mechanism of Ferulic Acid Release by Enzymatic Hydrolysis	133
4.10.1	Production of Ferulic Acid Esterase by Co-Culture	134
4.10.2	Release of Ferulic Acid by Co-Culture	135
4.11	Concluding Remarks	137
CHAPTER 5 CONCLUSION		139
5.1	Introduction	139
5.2	Conclusions	139
5.3	Contribution of the Study	142
5.4	Recommendations for Future Research	143
REFERENCES		145
APPENDIX A EXPERIMENTAL PROCEDURE		170
APPENDIX B EXPERIMENTAL DATA		181
APPENDIX C LIST OF PUBLICATION		202

LIST OF TABLES

Table 2.1	Main components of lignocellulose wastes	21
Table 2.2	Number of microbial species at 0 to 6 inches (0 to 15 cm) depth of soil	26
Table 2.3	Application of response surface method in extracting phenolic compound	40
Table 3.1	Sets of inoculum of single and co-culture	66
Table 3.2	Variables and their coded and actual levels used in the method of 2^{7-3} fractional factorial experiments	70
Table 3.3	The design of the 2^{7-3} fractional factorial experiments (A, temperature in °C; B, pH; C, rotation rate in rpm; D, water-to-substrate ratio in w/w; E, volume of inoculum in %, v/v; F, fermentation time in h; G, type of co-culture)	70
Table 3.4	Experimental range and levels of the variables in the central composite design	72
Table 3.5	The table of central composite design	73
Table 4.1	Comparison of chemical composition of banana stem waste with other researchers	83
Table 4.2	Microscopic and macroscopic morphological characteristic of isolates	87
Table 4.3	Production of ferulic acid released by different bacterial strains	95
Table 4.4	The results from biochemical test by using Biolog Gen III MicroPlate	97
Table 4.5	Absorbance of each bacterial strain for genomic DNA purity	99
Table 4.6	Identification using 16S rRNA sequence system	101
Table 4.7	Comparison of the percentage of accuracy between two identification methods	102
Table 4.8	The design of the 2^{7-3} fractional factorial experiments (A, temperature in °C; B, pH; C, rotation rate in rpm; D, water-to-substrate ratio in w/w; E, volume of inoculum in %, v/v; F, fermentation time in h; G, type of co-culture)	108
Table 4.9	Test of significance for regression coefficient	109
Table 4.10	Statistics used to test goodness of fit of the models	110
Table 4.11	The contribution of effect list	113
Table 4.12	Experimental layout for optimization of FA production using central composite design	118
Table 4.13	ANOVA for response surface quadratic model	119

Table 4.14	Validation of model equation (A, rotation rate in rpm; B, volume of inoculum %, v/v)	124
Table 4.15	Comparison of FA production through fermentation or enzymatic hydrolysis by different type of microbes	124
Table 4.16	Comparison of FA production through different extraction method	125
Table 4.17	Comparison of kinetic constants (V_{\max} and K_m) for ferulic acid production	133

LIST OF FIGURES

Figure 2.1	Chemical structure and the pathway of ferulic acid and related compounds synthesis in plants	12
Figure 2.2	Schematic representation of two different isomeric forms of ferulic acid found in nature (a) <i>cis</i> conformation and (b) <i>trans</i> conformation of ferulic acid	13
Figure 2.3	Scheme of secondary plant cell wall. CA: <i>p</i> -coumaric acid; FA: ferulic acid; SA: sinapic acid	18
Figure 3.1	Experimental flow chart	51
Figure 3.2	Banana plantation at Jalan Gambang, Kuantan	52
Figure 3.3	Banana stem chopped up into cube before blend with water	53
Figure 3.4	Mixture of BSW and water under acclimatization process	56
Figure 3.5	Fermentation of BSW by co-culture	60
Figure 3.6	Sample of fermented supernatant after centrifugation for analysis	60
Figure 3.7	Stationary phase of co-culture inoculum used for further study	67
Figure 1.8	Relationship between kinetic parameters in Michaelis-menten	76
Figure 1.9	Trend of product formation and substrate concentration versus time	76
Figure 4.1	Genomic DNA of the bacterial isolates separated on a 1 % agarose gel. Lane 1-5: 1) MB2, 2) MB5, 3) WB8A, 4) WB1A and 5) UB9	99
Figure 4.2	PCR products separated on a 1 % agarose gel. Lane 1-5: 1) MB2, 2) MB5, 3) WB8A, 4) WB1A and 5) UB9	100
Figure 4.3	Growth curve of single culture at wavelength of 600 nm	103
Figure 4.4	Production of ferulic acid from five single culture and 26 types of co-culture	105
Figure 4.5	Growth curve of co-culture at wavelength of 600 nm	106
Figure 4.6	Pareto chart showing the relative effect of various factors on ferulic acid production	112
Figure 4.7	Interaction effect plot between factors of: (a) temperature and fermentation time (AF); (b) pH and water-to-substrate ratio (BD); (c) temperature and rotation rate (AC); (d) temperature and volume of inoculum (AE)	115
Figure 4.8	Comparison of predicted and experimental response of ferulic acid production	116
Figure 4.9	Normal probability plot of residuals (a) and correlation of actual conversion and values predicted by the model (b)	120
Figure 4.10	Perturbation plot for all factors	121

Figure 4.11	3D response surface (a) and contour plot (b) between reaction of rotation rate and volume of inoculum	123
Figure 4.12	Estimation of V_{max} and K_m value by using Lineweaver–Burk plot	127
Figure 4.13	Comparison of experimental (■) and predicted (line) data for substrate concentration using (a) Euler method and (b) RK-Fourth method	129
Figure 4.14	Biomass concentration based on dry cell weight vs time	130
Figure 4.15	Comparison of experimental (■) and predicted (line) data for product formation using (a) Euler method and (b) RK-Fourth method	132
Figure 4.16	Time course of co-culture AD (<i>Bacillus cereus</i> and <i>Bacillus thuringiensis</i>) growth pattern and ferulic acid esterase (FAE) production by co-culture AD grown on banana stem waste	135
Figure 4.17	Time course of ferulic acid production by co-culture AD (<i>Bacillus cereus</i> and <i>Bacillus thuringiensis</i>) from banana stem waste	136

LIST OF SYMBOLS

3-D	Three dimensional
β_0	Constant coefficient
β_i	Coefficient of the linear parameters
dS/dt	Rate of substrate utilization
dP/dt	Rate of product formation
$[S]$	Substrate concentration
V_{max}	Maximum rate of reaction
μ_{max}	Maximum cell growth rate
$[P]$	Product concentration
K_m	Michaelis constant
K_s	Substrate constant
R^2	Coefficient of determination
R_t	Retention time
g	Gram
h	Hour
L	Liter
min	Minute
mL	Milliliter
μm	Micrometer
M	Molar
mM	Milimolar
mol	Mole
μmol	Micromoles
pH	Potential Hydrogen
R^2	Coefficient of determination
%	Percentage
μL	Microliter
$^{\circ}\text{C}$	Degree Celsius

LIST OF ABBREVIATIONS

ADF	Acid detergent fiber
ADL	Acid detergent lignin
ANOVA	Analysis of variance
BBD	Box-Behnken Design
BLAST	Basic Local Alignment Search Tool
BSG	Brewer's spent grain
BSW	Banana stem waste
CCD	Central composite design
CV	Coefficient of variation
DAD	Diode array detector
DCW	Dry cell weight
DNA	Deoxyribonucleic acid
DO	Dissolved oxygen
DX	Design Expert
FA	Ferulic acid
FAE	Ferulic acid esterase
FFD	Fractional factorial design
GDP	Gross domestic product
GN	Gram negative
GP	Gram positive
HPLC	High performance liquid chromatography
IF-A	Inoculating fluid A
LB	Lineweaver Burk
LCC	Lignin-carbohydrate complex
NA	Nutrient agar
NB	Nutrient broth
NCBI	National Center for Biotechnology Information
NDF	Neutral detergent fiber
NP	Natural product
OD	Optical density
ODE	Ordinary differential equation

OFAT	One factor at a time
PAL	Phenylalanine ammonia lyase
PBD	Plackett-Burman Design
PCR	Polymerase chain reaction
PDE	Partial differential equation
PRESS	Prediction residual error sum of squares
RK	Runge-Kutta
rpm	Revolutions per minute
rRNA	Ribosomal ribonucleic acid
RSM	Response surface methodology
RV	Revolution
SBP	Sugar beet pulp
TAL	Tyrosine ammonia lyase
TLC	Thin layer chromatography
WB	Wheat bran