OPTIMIZATION OF FERMENTATION STRATEGY FOR ENHANCED PRODUCTION OF THERMOSTABLE XYLANASE BY RECOMBINANT *Escherichia coli*

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A thesis submitted in fulfilment of the
requirements for the award of the degree of
Doctor of Philosophy (Bioprocess Engineering)

School of Chemical and Energy Engineering
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AUGUST 2019

DEDICATION

This study is wholeheartedly dedicated to my beloved parents, wife, siblings, teachers and friends who have been the source of inspiration, continually provide their moral, spiritual, emotional, financial and technical supports.

ACKNOWLEDGEMENT

Primarily, I would like to thank my thesis supervisor Prof. Dr. rer. Nat. Hesham A. El Enshasy, the Director of Institute of Bioproduct Development (IBD), Universiti Teknologi Malaysia, for his esteemed scientific guidance and moral supports that helped me to complete this study with great passion and joy. My sincere thanks and gratitude go to Prof. Dr. Rajni Hatti Kaul, the head of biotechnology department, Lund University, Sweden, for the collaboration with Institute of Bioproduct Development (IBD), her kind supports and technical guidance throughout this study. I would also like to thank Prof. Ramlan b. Aziz, and Prof. Dr. Mohd Roji b. Sarmidi, of Institute of Bioproduct Development (IBD), Universiti Teknologi Malaysia, for their supports. To all my fellow researchers and friends at IBD, with a special mention to Ms. Roslinda bt. Abd. Malek, Mr. Solleh bin Ramli, Ms, Khairun Hani Natasya, and Mr. Abas b. Rahmat, let me take this opportunity to express my heartfelt thanks to you all for your encouragement and supports throughout my study. I had a great time with you all.

Finally, I must express my very profound gratitude to my beloved wife, parents, and to my siblings for providing me with unfailing support and continuous encouragement throughout my years of study and through the process of researching and writing this thesis. This accomplishment would not have been possible without them. Thank you all.

ABSTRACT

Xylan is the second most abundant polysaccharide in plant cell wall which is hydrolyzed by the group of enzymes called hemicellulase. β -1, 4 endo xylanase is considered as the most important among the xylanase enzymes, due to its wide industrial applications. *Escherichia coli* BL21 with a plasmid vector pET-22b (+) carrying xylanase coding gene, which was isolated from the extremely thermophilic bacterium called *Thermotoga neapolitana*, was used in the current study to enhance xylanase production. In phase 1 of this study, using the statistical approach called response surface methodology, the optimum media composition for enhanced xylanase production was successfully identified. Up to 800 IU mL^{-1} xylanase activity was observed in optimized media, which is around 3 folds higher compared to the activity achieved in unoptimized medium. In phase 2, optimization of lactose-based induction strategy was carried out to enhance the xylanase production. As a result of this induction optimization, the intracellular xylanase production was enhanced up to 2600 IU mL^{-1}. In phase 3, as a part of process scale up, the study was focused on developing suitable fed-batch fermentation conditions, by optimizing nutrients and inducer feeding strategy. With the optimized fed batch fermentation conditions in 16 L stirred tank bioreactor, the xylanase activity was enhanced up to 11000 IU mL^{-1}, which is 4 to 5 folds higher compared to activity reported in previous studies. During physicochemical characterization in phase 4 of the current study, the optimum temperature and pH of xylanse enzyme was found to be 80°C and 6.5, respectively. Among the metal ions and chelating agents tested, zinc sulfate and ethylenediaminetetraacetic acid were found to have the highest inhibitory effect on xylanase enzyme in this study.

ABSTRAK

Xilan merupakan polisakarida kedua terbanyak di dalam sel dinding tumbuhan yang dihidrolisis oleh kumpulan enzim hemicellulase. β -1, 4 endo xilanase dianggap sebagai enzim xilanase yang paling penting diantara xilanase enzim lain disebabkan oleh aplikasinya didalam perindustrian. *Escherichia coli* BL21 dengan vektor plasmid pET-22b (+) yang membawa gen pengekodan xilanase, yang mana telah dipencilkan daripada bakteria lampau termofilik, *Thermotoga neapolitana*, telah digunakan di dalam kajian semasa ini untuk meningkatkan pengeluaran xilanase. Di dalam fasa pertama kajian, penggunaan kaedah statistik dinamakan sebagai tindakbalas sambutan permukaan, pengoptimuman komposisi medium untuk meningkatkan pengeluaran xilanase telah berjaya dikenalpasti. Sebanyak 800 IU mL^{-1} aktiviti xilanase telah dicapai di dalam medium optimum, yang mana sekitar 3 kali ganda lebih tinggi berbanding aktiviti yang dicapai di dalam medium tanpa pengoptimuman. Di dalam fasa 2, pengoptimuman strategi induksi berasaskan laktosa telah dijalankan untuk meningkatkan penghasilan xilanase. Hasil daripada pengoptimuman induksi tersebut, penghasilan xilanase secara intrasel meningkat kepada 2600 IU mL^{-1}. Di dalam fasa 3, sebagai sebahagian daripada proses pengskalaan, kajian telah memfokuskan kepada pembangunan kondisi bagi fermentasi suapan kelompok yang sesuai, dengan mengoptimumkan nutrien dan strategi induksi suapan. Dengan pengoptimuman fermentasi suapan kelompok dalam bioreaktor teraduk 16 L, aktiviti xilanase telah meningkat kepada 11000 IU mL^{-1}, yang mana 4 hingga 5 kali ganda lebih tinggi berbanding aktiviti di dalam kajian-kajian terdahulu. Semasa pencirian fizikokimia di dalam fasa 4 kajian, suhu optimum dan pH xilanase masing-masing adalah 80 °C dan 6.5. Diantara kesemua ion logam dan agen penggabungan yang diuji, zink sulfat dan asid etilenadiaminatetraasetik didapati mempunyai kesan perencatan yang lebih tinggi terhadap enzim xilanase didalam kajian ini.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF ABBRIVIATIONS

Adj MS	-	Adjusted Mean Square
Adj. R^2	-	Adjusted R^2
Adj SS	-	Adjusted Sump of Squares
ANOVA	-	Analysis of variance
C	-	Carbon
CCD	-	Central Composite Design
CDW	-	Cell Dry Weight
CFU	-	Colony Forming Unit
CO_2	-	Carbon Dioxide
DF	-	Total Degrees of Freedom
DO	-	Dissolved Oxygen
DOE	-	Design of Experiment
E. coli	-	*Escherichia coli*
EC	-	Enzyme Commission
FDA-US	-	Food and Drug Administration - United States
GH	-	Glycoside Hydrolases
GM	-	Genetically Modified
HCD	-	High Dell Density Cultivation
HPLC	-	High Performance Liquid Chromatography
IBD	-	Institute of Bioproduct Development
IBS	-	Inclusion Bodies
IU	-	International Unit for enzyme activity
Lac	-	Lactose Promoter
LB	-	Luria Broth
N	-	Nitrogen
O_2	-	Oxygen
OD	-	Optical Density
OD 600nm	-	Optical Density at 600 Nano Meter
OFAT	-	One Factor at A Time

p -value		Probability Value
PBD	-	Placket Burman Design
PET	-	Plasmid of Expression by T7 polymerase
PI	-	Isoelectric Point
Pred. R^2	-	Predicted R^2
RNA	-	Ribonucleic Acid
rDNA	-	recombinant Deoxyribose Nucleic Acid
RSM	-	Response Surface Methodology
PAGE	-	Poly Acrylamide Gel Electrophoresis
Seq SS	-	Sequential Sum of Squares
SDS	-	Sodium Dodecyl Sulphate
SmF	-	Submerged Fermentation
sp.	-	Species
SSF	-	Solid State Fermentation
TAXI	-	Triticum Aestivum Xylanase Inhibitor
TES	-	Trace Element Solution
TCF	-	Total Chlorine Free
UTM	-	Universiti Teknologi Malaysia
MCB	-	Master cell bank
MW	-	Molecular Weight
WCB	-	Working Cell bank
WICC	-	Wellness Industry Culture Collection
XIP	-	Xylanase Inhibitor Protein

LIST OF SYMBOLS

$AlCl_3.6H_2O$	-	Aluminum Chloride Hexahydrate
Amp	-	Ampicillin
$BaCl_2$	-	Barium Chloride
bar	-	bar pressure
$CaCl_2.2H_2O$	-	Calcium Chloride di Hydrate
$CoCl_2.6H_2O$	-	Cobalt Chloride Hexahydrate
$CuSO_4$	-	Copper Sulphate
Df	-	Dilution factor
DNS	-	Dinitro Salicylic
EDTA	-	Ethylene Diamine Tetra acetic Acid
$FeSO_4.7H_2O$	-	Ferrous Sulphate Heptahydrate
$g\ h^{-1}$	-	Gram / hour
h	-	Hour
H_3BO_3	-	Boric Acid
IPTG	-	Isopropyl β-D-1-thiogalactopyranoside
K_2HPO_4	-	di Potassium Hydrogen Phosphate
kDa	-	Kilo Dalton
KH_2PO_4	-	Potassium di Hydrogen Phosphate
kHz	-	Kilo Hertz
L	-	Liter
M	-	Molar
$MgSO_4.7H_2O$	-	Magnesium Sulphate Heptahydrate
min	-	Minute
mL	-	Milli Liter
mM	-	Mille Molar
$MnCl_2.4H_2O$	-	Manganese Chloride Tetrahydrate
$MnSO_4.7\ H_2O$	-	Manganese Sulphate Heptahydrate
$Na_2MoO_4.2H_2O$	-	Sodium Molybdate di Hydrate
NaCl	-	Sodium Chloride

NaOH	-	Sodium Hydroxide
NH_4Cl	-	Ammonium Chloride
NH_4NO_3	-	Ammonium Nitrite
$(NH_4)\ H_2PO_4$	-	Ammonium di Hydrogen Phosphate
$(NH_4)_2\ SO_4$	-	di Ammonium Sulfate
$NiCl_2.6H_2O$	-	Nickel Chloride Hexahydrate
nm	-	Nanometer
P_{max}	-	Maximum Xylanase Activity (IU mL^{-1})
RPM	-	Rotation / Minute
t	-	Time of incubation (min^{-1})
TEMED	-	Tetramethylethylenediamine
TSB	-	Tryptone Soya Broth
U g^{-1}	-	Unit / gram
U mg^{-1}	-	Unit / Milligram
U mL^{-1}	-	Unit / Milli Liter
V	-	Volte
V	-	Volume of enzyme solution used (mL^{-1})
v/v	-	Volume / Volume
vvm	-	Volume /Volume /Minute
W	-	Weight of xylose (µmoles mL^{-1})
w/v	-	Weight / Volume
X	-	Cell Biomass (g L^{-1})
X_{max}	-	Maximum Cell Biomass (g L^{-1})
Xyl	-	Xylanase
$Y_{(pmax/x)}$	-	Specific Xylanase Activity (IU g^{-1})
$ZnSO_4.7H_2O$	-	Zinc Sulphate Heptahydrate
α	-	Alpha
β	-	Beta
λ	-	Lambda
-	-	Minus
%	-	Percentage
+	-	Plus
<	-	Less Than
>	-	Greater Than

±	-	Plus/Minus
°C	-	Degree Celsius
µ	-	Specific Growth Rate (h^{-1})
µg	-	Micro Gram
μ_{max}	-	Maximum Specific Growth Rate (h^{-1})

LIST OF APPENDICES

CHAPTER 1

INTRODUCTION

1.1 Background

Xylanase enzymes have been isolated and purified from a wide range of microorganisms such as bacteria, fungi and actinomycetes that are present in normal environmental conditions. However, most of these xylanase does not show any unique characteristics such as high alkaline and thermal stability. Therefore, the xylanase from microbes that grows in extreme environmental conditions such as low / high pH and low / high temperature have gained more attention for commercial applications, due to their novel characteristics. Simulating the extreme growing conditions of these microbial species in laboratory / industry set up, is found to be very difficult or extreemly expensive, to scale up the production of xylanase. Nowadays, the recombinant DNA technology brought a solution for this problem by introducing recombinant strains such as *Escherichia coli* BL21 (DE3) that can produce the xylanase enzyme with novel characteristics, under normal growing conditions. However, the expression level of xylanase gene in a recombinant strain depends on several aspects such as the cloning strategies used, gene copy number, plasmid stability and the host cell's metabolism.

To enhance the xylanase production, Mamo *et al.* (2007) constructed a clone of *Escherichia coli* BL21 (DE3) with a plasmid vector pET-22b(+) carrying xylanase coding gene, which is isolated from an extreme thermophilic bacteria, *Thermotoga neopolitana* DSM-4359. This has opened several new areas for further researches on

developing optimum fermentation and induction strategies to enhance the xylanase production.

1.2 Problem Statement

In submerged fermentation, the fermentation media composition plays important role in yield of xylanase production. An economically viable fermentation media formulation which can support the maximum xylanase production is necessary for industrial scale xylanase production. In such media, the nutritional sources are major and most important factor effecting xylanase production. Therefore, it is necessary to develop and optimize the fermentation medium, for enhanced production of recombinant. The conventional media optimization method is step by step process that involves varying one variable at a time while keeping the other variables constant which is tedious, time consuming and less reliable. Hence, it is ideal to apply Response Surface Methodology (RSM) based statistical approach, which is very reliable and less time consuming, in media optimization trials.

Xylanase expression in recombinant strain is greatly depending on the gene inducers present in the fermentation media. Isopropyl β-D-1- thiogalactopyranoside (IPTG) is the most widely used chemical inducer for *'lac'* based expression system. However, it is expensive and toxic in nature to the host cell at its higher concentration. Therefore, it is not recommended for the large scale production of recombinant proteins. Nowadays, researchers are using lactose as an alternative to IPTG. However, when compared to IPTG, lactose mediated induction was reported to be slightly slow and it is due to the catabolic repression by the expression host. Generally, the lactose-based induction carried out at log phase of cell growth, post achieving the high cell density, which results in relatively low product yield. The probable root cause for low product yield in above mentioned scenario is the poor intake of lactose at log phase of

cell growth. Therefore, it is important to develop a optimal lactose based induction strategy to enhance the xylanase production.

The conventional production strategies followed are found to be less effective to meet the growing need in the present xylanase market. Though, there are lots of researches going on in developing recombinant strains for xylanase production, a very limited number of these recombinant strains are used for the commercial scale production of xylanase due to the lack of industrially feasible production process. Therefore, it is important to investigate on alternative fermentation and induction strategies which enhances the xylanase production. For commercial applications, xylanase must be ideally produced in large quantities and simultaneously over a short period of time. Various fermentation strategies have been used to achieve the High Cell Density Cultivation (HCDC), as it is key to enhance the xylanase production. In a batch fermentation of *Escherichia coli,* it was found to be difficult to achieve the high cell density, due to several factors such as nutrient limitation, cell growth inhibition caused by secondary metabolites formation etc., Hence, fed batch fermentation with suitable feeding strategy have been used to overcome many of the above mentioned challenges. Substrate inhibition due to improper feeding and accumulation of acetate during high metabolic activity are the problems associated with fed batch fermentation. These factors may affect the plasmid stability also and results the low production. An optimized feeding strategy enables the control on specific growth rate in the fed batch fermentation shall results in better cell growths as well as xylanase production.

1.3 Aim

This study is aimed to enhance the production of Endo-1,4-β-Xylanase enzyme by recombinant *Escherichia coli* BL21 (DE3) at semi industrial scale (16 L bioreactor) using optimized fed batch cultivations system.

1.4 Objectives

The following objectives will be addressed to achieve the aim of the current research;

1.4.1 To optimize the production media for the enhanced production of xylanase enzyme through OFAT and DOE approach. Also, to optimize the lactose based induction strategy in optimized media for enhanced production of xylanase in shake flask culture.

1.4.2 To validate the optimized conditions at batch fermentation and to identify the optimum growing conditions of *Escherichia coli* BL21 (DE3) in 16 L stirred tank bioreactor.

1.4.3 To enhance the xylanase production in fed batch fermentation and to study and compare the effects of various feeding strategies on cell biomass and xylanase production. Also, to study the effects of lactose based induction on xylanase production in optimized fed batch fermentation conditions.

1.4.4 To partially purify and characterize the xylanase enzyme.

1.5 Scope

To achieve above mentioned objectives, this research is framed with the following five major scopes.

1.5.1 Media Optimization by One Factorial at Time (OFAT) approach followed by Response Surface Methodology (RSM) based statistical approach and then the validation of statistically predicted model at shake flask and 16 L stirred tank bioreactor (in batch fermentation).

1.5.2 Lactose based induction strategy (Inducer concentration, induction initiation time and post induction incubation temperature) optimization and comparison with IPTG based induction. Followed by, the evaluation of Optimized Induction Condition at 16 L stirred tank bioreactor

1.5.3 Batch cultivation with optimized growth parameters to determine the cell growth and xylanase production kinetics in 16 L stirred tank bioreactor and then the evaluation of impact of pH and dissolved oxygen level on cell growth and xylanase production.

1.5.4 Enhanced production of xylanase enzyme by fed batch fermentation using optimized media feeding (constant / pulse / stepwise increased) and inducer feeding (pulse / stepwise increased) strategies.

1.5.5 Partially purify the xylanase and characterize it by identifying the optimum temperature and pH, estimating thermal and alkali stability, molecular weight by SDS PAGE, impact of metal ions and chelating agents on enzyme activity and finally the substrate specificity.

1.6 Thesis Outline

In this thesis, chapter 1 describes the research background, problem statement, objective, scopes of current study. Chapter 2 covers the review of literatures related to xylanase enzyme, its commercial applications, approaches to enhance the production through various optimization studies and various production strategies followed. Chapter 3 describes the materials, methods and experimental designs used in current study for the optimization of xylanase production, scaling up to production, partial purification and physiochemical characterization. Chapter 4 discuss the results

of experiments carried out and it is also compared with the observations reported by other researchers in past. Chapter 5, which is the final chapter covers the conclusion and limitations of current study and it also details the recommendations for future exploration.

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