الجامعة الإسلامية العالمية ماليزيا
INTERNATIONAL ISLAMIC UNIVERSITY MALAYSIA
يونيرسيتي إسلام انتارابغسا ملايسيا

THE SCREENING, IDENTIFICATION, OPTIMIZATION AND PARTIAL PURIFICATION OF INDUSTRIALLY IMPORTANT BACTERIAL β-ENDOGLUCANASE ISOLATED FROM SELECTED MALAYSIAN SOIL AND HOT SPRINGS

BY

ROHANI SALLEH

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Kulliyyah of Sciences
International Islamic University
Malaysia

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ABSTRACT

Bacteria isolated from different Malaysian soil and hot springs were screened for high level of β-endoglucanase activity. Two methods were used: agar diffusion assay and reducing sugar test. The screening processes showed that several isolates gave positive promises due to the high crude enzyme activity. The first three highest β-endoglucanase producers were identified using biochemical identifications, then confirmed with 16S rRNA sequencing method. All three isolates were identified as *Bacillus* strains; *Bacillus subtilis* and *Bacillus licheniformis*. *Bacillus subtilis* ASUIA 271A, which produced the highest β-endoglucanase activity, was further studied. The optimum growth conditions for culture media were studied by using statistical optimization method to find the best culture conditions that yield the highest β-endoglucanase. Thus, sufficient β-endoglucanase was available for next partial purification processes. The partial purification of *Bacillus subtilis* ASUIA 271A β-endoglucanase involved two-steps column chromatography: DEAE-Sepharose (ion-exchange) and Sephacryl S-200 (gel filtration). Active fractions were subjected to SDS-PAGE and the analysis of the separated bands revealed patterns with 37, 43, 46, 56, 62 and 75 kDa molecular weights. The chromatographic steps gave 63-fold purification with a recovery rate of 27.4%. The specific activity for partially purified enzyme was 21.4 U/mg. Furthermore, the activity of the β-endoglucanase was detected by a zymogram. Thus, this study has been successful in isolating high β-endoglucanase producing bacteria from selected Malaysian soil and hot springs.

خلاصة البحث

في هذه الدراسة تم فصل البكتيريا من عدة أنواع من التربة الماليزية والينابيع الساخنة لمعرفة النشاط العالي لبيتا- إندوغلوسانيس. تم إستخدام طريقتين وهما: طريقة إنتشارالاجاروفحص تقليل السكر. أوضحت عملية الفصل إنه ممكن الحصول على أنزيم ذو نشاط عالي في الكثير من المواد المفصولة. بإستعمال الطريقة البيوكيمياوية تم الحصول على أول ثلاثة أعلى أنواع من بيتا- إندوغلوسانيس، ثم تم التأكد بإتباع طريقة التتبع (16 أس آر آر أن أي). وتم تشخيص الثلاثة أنواع المفصولة كعروق باسيلوس، باسيلوس سيبتيليس، وباسيلوس ليجينيفورس. تم دراسة باسيلوس سيبتيليس (أي أس يو آي أي 271 أي) بالتفصيل لكونه يحمل أعلى نشاط بإستخدام الطريقة الاحصائية القصوى لمعرفة أفضل الظروف لاعطاء أعلى إنتاج. وبهذا كان هناك كمية كافية من بيتا- إندوغلوسانيس لعمل تجارب عمليات التنقية الجزئية. التنقية الجزئية لباسيلوس سيبتيليس تضمنت خطوتين في عمود الفصل: دي إي أي إي سيفاروس (عمود التبادل الآيوني) وسيفاكريل 200 أس (الفلترة بالجل). الاجزاء النشطة تم تعريضها الى (أس دي أس – بي أي جي إي) والتحليل أعطى الاحزمة المفصولة لمقاطع الوزن الجزيئي 37، 43، 46، 56، 62، و75 كيلودالتون. تم التنقية بحوالي 63 مرة بمعدل إنتاج 27.4%. والنشاط للانزيم الذي تم تنقيته بصورة جزئية كان 21.4 وحدة لكل ملغرام واحد. بالاضافة لذلك، تم معرفة نشاط بيتا- إندوغلوسانيس بواسطة زيموغرام. وبهذا، كانت هذه الدراسة ناجحة لفصل نسبة عالية من بيتا- إندوغلوسانيس وإنتاج بكتيريا من بعض الترب الماليزية والينابيع الساخنة.

APPROVAL PAGE

I certify that I have supervised and read this study and that in my opinion it conforms to acceptable standards of scholarly presentation and is fully adequate, in scope and quality, as a thesis for the degree of Master of Science

Ridzwan Hashim
Supervisor

Hamzah Mohd. Salleh
Supervisor

Ibrahim Ali Noorbatcha
Supervisor

Mohd Ramlan Mohd Salleh
Supervisor

I certify that I have read this study and that in my opinion it conforms to acceptable standards of scholarly presentation and is fully adequate, in scope and quality, as a thesis for the degree of Master of Science

Jamaluddin Mohd. Daud
Examiner

This thesis was submitted to the Department of Biotechnology and is accepted as fulfilment of the requirements for the degree of Master of Science

_____

Muhammad Nor Omar
Head, Department of Biotechnology

This thesis was submitted to the Kulliyyah of Sciences and is accepted as fulfilment of the requirements for the degree of Master of Science

_____

Ridzwan Hashim
Dean, Kulliyyah of Sciences

DECLARATION

I hereby declare that this thesis is the result of my own investigations, except where otherwise stated. I also declare that it has not been previously or concurrently submitted as a whole for any other degrees at IIUM or other institutions.

Rohani Salleh

Signature …… Date ……..

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Affirmed by Rohani Salleh.

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Signature Date

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Let me humbly express my praise and gratitude to Allah (s.w.t) our Merciful Creator, who sent a messenger, our beloved Prophet Muhammad s.a.w (peace be upon him), who had guided us the way to get the blessings of Allah. It is my pleasure to express my deepest gratitude and appreciation to my current main supervisor, Prof. Dr. Ridzwan Hashim, my previous main supervisor, Prof. Dr. Abd-Elaziem Farouk Gad and to all my co-supervisors, Dr. Hamzah Mohd. Salleh, Dr Ibrahim Ali Noorbatcha and Dr Mohammad Ramlan Mohammad Salleh for their continuous professional advices, help, support and encouragement to me in completing this project. My deepest appreciation also goes to Centre for Postgraduate Studies (CPS) and Research Centre, IIUM for their financial support in terms of scholarship and project grant. My special thanks and gratitude to my colleagues for their sincere help, useful suggestion and moral support. Last but not least, I would like to express my heartfelt thank to IIUM family especially the staff of both Kulliyyah of Sciences and Kulliyyah of Engineering.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF ABBREVIATIONS

mg	milligram
µg	microgram
ml	milliliter
µl	microliter
mm	millimeter
cm	centimeter
hr	hour
min	minute
U/ ml	units per milliliter
kDa	kiloDalton
temp	temperature
rpm	revolutions per minute
SDS	sodium dodecyl sulphate
PAGE	polyacrylamide gel electrophoresis
CBD	cellulose binding domain
EG	endoglucanase
CBH	exoglucanase
BSA	bovine serum albumin
LB	Luria Bertani
CMC	carboxymethyl cellulose
DNS	dinitrosalicylic acid
UV	ultravoilet
PCR	polymerase chain reaction
dNTP	deoxyribonucleotide 5'-triphosphate
ANOVA	analysis of variance
CFF	cross flow filtration
DEAE	diethylaminoethyl
IEX	ion exchange chromatography
Tris-HCl	tris-hydrochloric acid
GF	gel filtration
bp	base pair
RSM	response surface methodology

CHAPTER 1

INTRODUCTION AND THE AIM OF THE STUDY

1.1 INTRODUCTION

Life on earth depends on photosynthesis, which results in production of plant biomass having cellulose as the major component in plant cell wall (Lynd, Paul, Willem and Isak, 2002). Cellulose, which is the most abundant natural polymer in the biosphere, is at the present time an underutilized resource and is frequently a wasted byproduct of agricultural and industrial processes (Glick and Pasternak, 1989).

To date, plant biomass is the only foreseeable source of fuels and materials available to humanity (Lynd et al., 2002). Cellulosic materials are particularly attractive because of their relatively low cost and plentiful supply. The central technology impediment to more widespread utilization of this important resource is the general absence of low-cost technology for overcoming the recalcitrance of cellulosic biomass. A promising strategy to overcome this impediment involves the production of cellulolytic enzymes, hydrolysis of biomass, and the fermentation of resulting sugar to desired products in a single process step via a cellulolytic microorganism. In line with this need, it was found that a vast amount of research into the enzymology of cellulose degradation has occurred in the last 30 to 40 years. A significant portion of the recent research effort has been aimed at enzymatically hydrolyzing cellulose to glucose prior to converting it to other materials (Glick and Pasternak, 1989).

Cellulases representing the three enzyme groups (endoglucanases, exoglucanases and β-glucosidases) which act in a coordinated manner to efficiently hydrolyze cellulose (Lynd et al., 2002). Cellulases have attracted much interest because of the diversity of their applications, and also facilitating the understanding of mechanism of enzymatic hydrolysis of plant carbohydrate polymers. The major industrial applications of cellulases are in textile industry for 'bio-polishing' of fabrics and producing stonewashed look for denims, as well as in household laundry detergents for improving fabric softness and brightness. Besides, they are used in animal feeds for improving the nutritional quality and digestibility, in processing of fruit juices, and in baking, while de-inking of paper is yet another emerging application. A potential challenging area is where cellulases would have a central role in bioconversion of renewable cellulosic biomass to commodity chemicals.

The cellulases that are used so far for the above-mentioned industrial applications are those from fungal sources. Most commercial cellulases, currently, are produced by *Trichoderma* species and *Aspergillus* species (Zhang, Himmel and Mielenz, 2006). However, in the last 20 years, there has been increasing interest in cellulase production by bacteria (Robson and Chambliss, 1989). The great majority of cellulose hydrolysis research to date has focused on the genetics, structure, function, and the interaction of components of cellulase enzyme system (Lynd et al., 2002). There is a substantial effort into their cloning and expression as well as their study by site-directed mutagenesis (Martin, 2000). In addition, there is a general interest in obtaining new, more specific and stable enzymes for different applications. For this reason, this project is initiated to find new bacterial cellulases from local soil and hot springs.

1.2 PROBLEM STATEMENT

β-endoglucanase (endo-1,4-β-D-glucanase) is one of the three major enzymes which is essential for degradation of cellulose to produce glucose (Li, Ding, Wang, Xu and Zhao, 2006). β-endoglucanase is widely used in nearly every branch of industry (food, feed, textile, detergent etc.) and the demand of β-endoglucanase is steadily increasing. Thus, it would be a great achievement if β-endoglucanase can be produced from local microorganisms since Malaysia has been identified as one of the twelve countries having mega-biodiversity. Currently, the microorganisms present in Malaysia are poorly known as yet, and much study is necessary to obtain a comprehensive data on microorganism in Malaysia. To our knowledge, there is no publication on cellulolytic bacteria from Malaysian soil and hot spring. For production of these industrial enzymes locally and economically, our research efforts will screen for potential bacterial isolates from previous collection and to optimize the culture conditions for the bacterial isolate which give high β-endoglucanase activity. Malaysian soil and hot springs bacteria provide good opportunities for the screening of isolates for new industrial β-endoglucanase.

1.3 AIM OF STUDY

The aims of this study are:

1. To screen bacterial isolates from selected soil and hot springs in Malaysia for the production of industrially important cellulases (specifically β-endoglucanase) and to identify the high β-endoglucanase producing isolates
2. To optimize and partially purify β-endoglucanase from high producing bacteria isolate.

1.4 SIGNIFICANCE OF STUDY

The finding of this study will contribute to new bacterial strains, isolated locally and capable to produce β-endoglucanase. The optimum conditions for incubation temperature and initial pH was carried out for the highest β-endoglucanase producer. Thus, it will give a benefit for Malaysian documentation for Malaysian resources.

CHAPTER 2

LITERATURE REVIEW

2.1 CELLULOSE AND THE NEED FOR CELLULOLYTIC ENZYMES

A great deal of research on the enzymatic degradation of cellulose has been developed in the last 30 years. The practical goal of most of this work is to achieve efficient, inexpensive enzymatic conversion of cellulosic biomass to more useful products (Robson and Chambliss, 1989). Organic wastes from renewable forest and agricultural residues are rich sources of cellulose, hemicellulose and lignin, in an average ratio of 4:3:3 although the exact percentage of these components varies from source to source (Heck, Hertz and Ayub, 2002). Most of the cellulose is broken down by cellulolytic organisms that use it as a source of carbon but degradation of cellulose by cellulases occurs through other means in nature. Microbial cellulose utilization is responsible for one of the largest material flows in the biosphere and is of interest in relation to analysis of the carbon flux both local and global scales (Lynd et al., 2002).

Cellulose (Figure 2.1) is a major polysaccharide constituent of plant cell walls. It is an unbranched glucose polymer composed of anhydro-D-glucose unit linked by β-1,4-glycosidic bond. The natural degradation of its unit represents an important part of the carbon cycle within the biosphere**.** These glycosidic bonds can be hydrolyzed by cellulolytic enzymes. However, cellulose is very resistant to hydrolysis due to its high degree of crystallinity, the result of the unstrained linear conformation which allows strong hydrogen bonding between hydroxyl groups of the neighboring parallel chains.

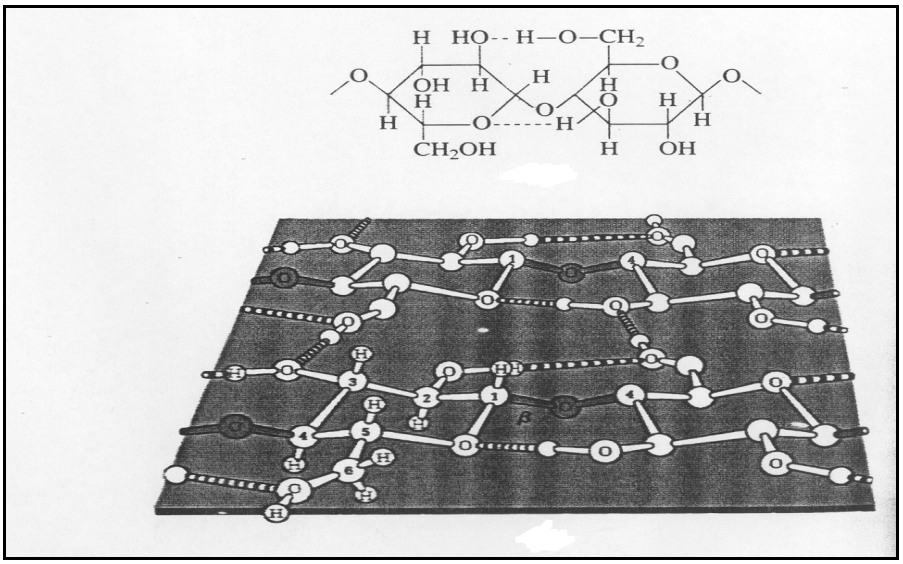

Figure 2.1
The chemical and three-dimensional structure of cellulose (Rodney, 1999).

In cellulose fiber, regions of the high crystallinity coexist with less order amorphous regions. The higher the ratio of crystalline regions to amourphous areas in a given cellulosic substrate, the more resistant it is to enzymatic attack (Robson and Chambliss, 1987). The ability to decompose the cellulosic biomass into glucose, which in turn can be converted into other valuable chemicals and energy, has made cellulases one of the most extensively investigated multicomponent enzyme systems (Andre, Rodrigo, Elba and Rosalie, 2005). Cellulose is the largest renewable carbon source available (approximately 150 billion tons of organic material is photosynthesized annually), is frequently found in close association with other compounds, such as hemicellulose, lignin and other polysaccharides, which make its bioconversion more difficult (Heck et al., 2002). Therefore, treatment of cellulose by cellulolytic enzymes for practical purposes has attracted the interest of researches in biotechnology (Andre et al.).

Cellulose is a chemically simple homopolymer but its physical state makes it a challenging substrate for enzymes. Single glucose polymers are packed onto each other to form highly crystalline microfibrils in which individual cellulose chains are held together by hydrogen bonds. Cellulose microfibrils also contain some amorphous regions. In wood fibers, the winding direction of cellulose microfibrils varies in different cell wall layers and this gives the fiber its unique strength and flexibility (Wolfgang, 2004).

For utilization of cellulosic substances as carbon and energy sources, many fungi and bacteria secrete enzymes as cellulase complex. The vital characteristics of this cellulase complex are:

1) The system is multienzymatic
2) At least three enzyme components are both physically and chemically distinct
3) All three components play essential roles in the hydrolysis of cellulose to glucose.

Components of cellulose systems were first classified based on their mode of catalytic action and have more recently been classified based on structural properties. Three major types of enzymatic activities are found:

1) Endoglucanases (EG, CMCase, 1,4-β-D-glucan-4-glucanohydrolases, EC 3.2.1.4)
2) Exoglucanases:
   2.1) 1,4-β-D-glucan glucanohydrolases (cellodextrinases, EC 3.2.1.74)
   2.2) 1,4-β-D-glucan cellobiohydrolases (cellobiohydrolases, CBH, Avicelase, EC 3.2.1.91)
3) β-glucosidases (β-glucoside glucohydrolase, cellobiases, EC 3.2.1.21)

Endoglucanases cut at random at internal amorphous sites in cellulose polysaccharides chain, generating oligosaccharides of various lengths and consequently new chain ends. Exoglucanases act in a processive manner on the reducing or nonreducing ends of cellulose polysaccharides chains, liberating either glucose or cellobiose as major products. Exoglucanases also can act on microcrystalline cellulose, presuming peeling cellulose chains from the microcrystalline structure. β-Glucosidases hydrolyze soluble cellodextrins and cellobiose to glucose (Mawadza, Hatti-Kaul, Zvauya and Mattiasson, 2000; Lynd et al., 2002; Wood and Bhat, 1988; Rajoka and Malik, 1997; Zhou, Chen and Li, 2004; Robson and Chambliss, 1989; Li et al., 2006; Andre et al., 2005; Huang and Mink, 2004; Bischoff, Rooney, Li, Liu and Hughes, 2006; Tang, He, Chan, Zhang and Ali, 2004; Glick and Pasternak, 1989). All enzymes appear to exist in multiple forms which differ in their relative activities on a variety of substrates (Wood and Bhat). Cellulase are distinguished from other glycoside hydrolases by their ability to hydrolyze β-1,4-glycosidic bonds between glycosyl residues. The enzymatic breakage of the β-1,4-glycosidic bonds in cellulose proceeds through an acid hydrolysis mechanism, using a proton donor and nucleophile or base (Lynd et al.).

The cellulase enzyme molecule is composed of up to three functionally different domains, as illustrated schematically in Figure 2.2:

1. The catalytically active core or catalytic domain, which is a large, spherical domain and responsible for hydrolysis of cellulose
2. A spherical cellulose-binding domain (CBD) that promotes adsorption onto the insoluble cellulose
3. The linker domain or linker peptide, which is an elongated and flexible spacer and joins the two domains together

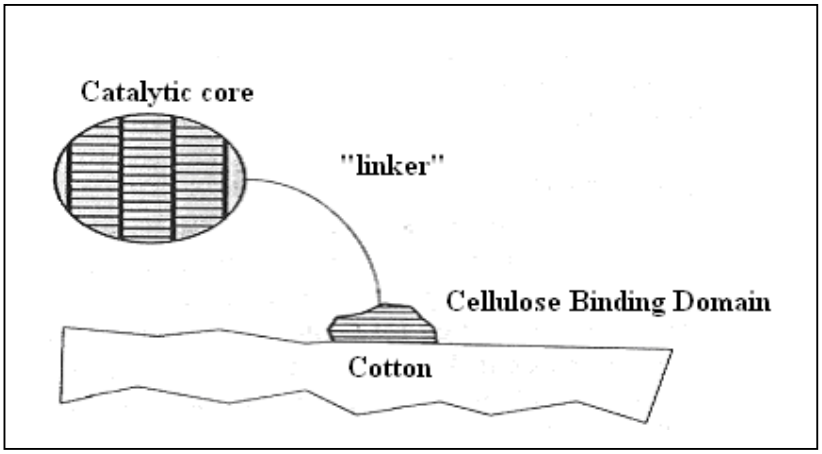

Figure 2.2
Schematic presentation of a multi-domain cellulase adsorb to cellulose substrate (Wolfgang, 2004).

Based on the amino acid sequence, catalytic active core or catalytic domains of cellulases can be grouped into families. Each family of catalytic domain is characterized by a conserved fold and different folds are expected in different families. This implies that enzymes belonging to the same family will show a similarity in folding pattern resulting in an overall conservation of active site topology. The endo- or exo mode of action of cellulases is determined by the specific details of 3D structure. Endo and exo cellulases in the same family have a similar global 3D fold but differing substrate specificity due to minor details of the structure. Endoglucanases of the same family were found to have similar overall fold but their active site was found to open into a cleft allowing random binding of the cellulose chains (Henrissat, 1994; Beguin and Aubert, 1994).

Meanwhile, Cellulose Binding Domains (CBDs) can be grouped based on amino acid sequence similarities with their catalytic domain. Most CBD's are structurally well defined domains linked to either the N- or C-terminus of the catalytic domains. CBDs binding to crystalline cellulose may differ in size and topology but