COMBINATION OF SECOND GENERATION FEEDSTOCKS AS A STRATEGY FOR BACTERIAL ENDOGLUCANASE PRODUCTION

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ABSTRACT

Environmental concerns and the instability associated with the use of fossils as sources of fuel and platform chemicals have prompted interest in the use of biomass as alternatives. Lignocellulosic biomass is cheap and abundant but its use is yet to be adopted widely because of the expensive processing requirements. Cellulases, enzymes required for the conversion of lignocellulosics into useful products, are very expensive because they are produced from purified commercial substrates. Researchers have thus explored the use of lignocellulosic substrates as cheaper alternatives. Efforts have so far focused mainly on the production of fungal cellulases from single feedstocks while the use of mixed feedstocks have been poorly explored, especially for bacterial cellulases. This project sought to investigate the feasibility of mixed lignocellulosic feedstocks application as a strategy for improving the production of bacterial endoglucanase, a cellulase type with wide industrial applications.

A sequential screening approach was used in selecting a bacterial strain that could efficiently utilize a mixture of oil palm empty fruit bunch (EFB), oil palm frond (OPF) and rice husk (RH) for endoglucanase production. Endoglucanase production ability of this strain on the single- (SS) and mixed substrates (MS), as well as on synthetic cellulosic substrates, was investigated. The possibility of reducing media supplementation cost by evaluating some agro-industrial wastes as supplements or substitutes to selected media components was explored. The effects of substrate pretreatment were also studied in order to identify an ideal pretreatment method conducive for enzyme production. The efficiency of joint/separate pretreatments and combination of the single substrates against their separate use for endoglucanase production was investigated. Response surface method (RSM) was applied in the optimization of selected fermentation process variables and substrate component proportions for maximum endoglucanase yield.

The first documentation of cellulolytic ability in a *Bacillus aerius* strain was presented in this study. This strain produced significantly higher yields of the enzyme on sequentially-pretreated MS than on the SS and synthetic substrates. *Leucaena leucocephala* (Petai belalang) as a medium supplement, increased endoglucanase production significantly compared to the control. Pretreatment studies showed that there was no significant difference in enzyme production between the pretreated and untreated MS. Higher pretreatment severity was detrimental to endoglucanase production. Pretreating the SS jointly favoured enzyme production than separate pretreatment approach. Combination of the SS had no negative effects on endoglucanase production. EFB showed a stronger effect towards enzyme production than the other mixture components. RSM optimization revealed that medium pH of 7.45, 184.8 rpm agitation speed, and 37 °C temperature were the most suitable conditions for endoglucanase production. Mixture designs showed that MS with higher amounts of EFB favoured enzyme production.

The study demonstrated that mixed feedstock strategy could help to realize enhanced and cost-effective production of *B. aerius* endoglucanase, provided that carefully selected, mildly pretreated mixture components, at appropriate ratios, and at optimal fermentation conditions are used. The study also provided insights into some aspects of *B. aerius* cellulolytic system that were previously unreported.

ABSTRAK

Isu alam sekitar dan ketidakstabilan yang berkaitan dengan penggunaan fosil sebagai sumber bahan api dan platform bahan kimia telah mendorong minat dalam penggunaan biomass sebagai alternatif. Biojisim lignoselulosa adalah murah dan banyak tetapi penggunaannya masih belum diguna pakai secara meluas kerana keperluan pemprosesan yang mahal. 'Cellulases', enzim yang diperlukan untuk penukaran bahan lignoselulosa kepada produk yang berguna, adalah sangat mahal kerana ia dihasilkan daripada substrat komersial. Para penyelidik telah menerokai penggunaan substrat lignoselulosa sebagai alternatif yang lebih murah. Usaha setakat ini tertumpu kepada pengeluaran cellulases kulat daripada bahan mentah tunggal manakala penggunaan bahan mentah campuran telah kurang diterokai, terutamanya bagi 'cellulases' bakteria. Projek ini bertujuan untuk menyiasat kebarangkalian penggunaan bahan mentah campuran lignoselulosa sebagai strategi untuk meningkatkan pengeluaran endoglukanase bakteria, sejenis selulase dengan aplikasi industri yang luas.

Pendekatan penyaringan berperingkat telah digunakan dalam memilih strain bakteria yang cekap menggunakan campuran tandan kosong buah kelapa sawit (EFB), pelepah kelapa sawit (OPF) dan sekam padi (RH) untuk pengeluaran endoglukanase. Keupayaan penghasilan endoglukanase menggunakan substrate tunggal (SS) dan campuran substrat (MS) dan juga substrat selulosa sintetik, telah dikaji. Kemungkinan untuk mengurangkan kos media suplemen dengan menilai beberapa sisa agro-industri sebagai suplemen atau pengganti kepada komponen media terpilih juga telah diterokai. Kesan substrat pra-rawatan substrat juga dikaji untuk mengenal pasti kaedah prarawatan kondusif yang ideal untuk pengeluaran enzim. Kecekapan pra-rawatan gabungan / berasingan serta substrat gabungan/ berasingan yang digunakan untuk pengeluaran endoglukanase juga telah disiasat. Kaedah 'Response surface methodology' (RSM) telah digunakan dalam

pengoptimuman pembolehubah proses penapaian terpilih dan bahagian komponen substrat untuk hasil endoglukanase yang maksimum.

Dokumentasi pertama dalam keupayaan cellulolytic strain *Bacillus aerius* telah dibentangkan dalam kajian ini. Strain ini menghasilkan enzim yang lebih tinggi dalam MS yang berpra-rawatan berperingkat daripada SS dan substrat sintetik. Penggunaan *Leucaena leucocephala* (Petai belalang) sebagai media suplemen, member peningkatan pengeluaran endoglukanase dengan ketara berbanding media kawalan. Kajian pra-rawatan menunjukkan bahawa tidak terdapat perbezaan yang ketara dalam pengeluaran enzim menggunakan MS yang telah dipra-rawat dan yang tidak dirawat. Tahap pra-rawatan yang lebih tinggi adalah menjejaskan pengeluaran endoglukanase. Penghasilan enzim lebih cenderung kepada gabungan pra-rawatan SS berbanding pra-rawatan tunggal Gabungan SS tidak mempunyai kesan negatif ke atas pengeluaran endoglukanase. EFB menunjukkan kesan yang lebih kukuh ke arah pengeluaran enzim berbanding komponen campuran lain. Pengoptimuman RSM mendedahkan pH media 7.45, kelajuan penggadukkan 184.8 rpm, dan suhu 37 ° C adalah keadaan yang paling sesuai untuk pengeluaran endoglukanase. Reka bentuk campuran menunjukkan bahawa MS dengan kandungan EFB yang lebih tinggi adalah yang paling sesuai untuk pengeluaran enzim.

Kajian ini menunjukkan bahawa strategi bahan mentah campuran boleh membantu untuk merealisasikan peningkatan pengeluaran dan kos yang efektif ke atas *B. aerius* endoglukanase, dengan syarat perhatian yang teliti diberikan ke atas komponen campuran dengan sedikit pra-rawatan, pada nisbah yang sesuai, dan pada keadaan penapaian yang optimum digunakan. Kajian ini juga memberikan wawasan dalam beberapa aspek sistem cellulolytic *B. aerius* yang tidak dilaporkan sebelum ini.

DEDICATION

I dedicate this thesis to my darling wife, Sherifat Buhari, for her love, patience,

understanding, support, and encouragement throughout the period of my studies.

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TABLE OF CONTENTS

CHAPTER 7: OPTIMIZATION OF *Bacillus aerius* S5.2 ENDOGLUCANASE PRODUCTION ON MIXED LIGNOCELLULOSIC SUBSTRATES USING RESPONSE SURFACE METHODOLOGY AND MIXTURE DESIGN 124

LIST OF FIGURES

LIST OF TABLES

LIST OF SYMBOLS AND ABBREVIATIONS

CMC : carboxymethyl cellulose

EFB : oil palm empty fruit bunch

OPF : oil palm frond

RH : rice husk

MS : mixed substrate

SS : single substrate

GH : glycoside hydrolase

CBH : cellobiohydrolase

EG : endoglucanase

βG : β-glucosidase

CBD : carbohydrate binding domain

CBM : carbohydrate binding module

CD : catalytic domain

MCC : microcrystalline cellulose

cAMP : cyclic AMP

HMF : hydroxymethyl furfural

OFAT : one-factor-at-a-time

DOE : design of experiment

RSM : response surface methodology

CMCA : carboxymethyl cellulose agar

TSB	:	Tryptic Soy Broth
TSA	:	Tryptic Soy Agar
OD	:	optical density
HC	:	hydrolytic capacity
rpm	:	revolutions per minute
DNS	:	3,5- dinitrosalicylic acid
pNPG	:	p-nitrophenyl-β-D-glucoside
pNP	:	p-nitrophenol
SSF	:	solid-state fermentation
FESEM	:	Field Emission Scanning Electron Microscope
CBP	:	consolidated bioprocessing
LL	:	*Leucaena leucocephala*
DC	:	oil palm decanter cake
NMMO	:	*N*-methylmorpholine-*N*-oxide
XRD	:	X-ray Diffraction
FTIR	:	Fourier transform infrared spectroscopy
CrI	:	crystallinity index
AARD	:	average absolute relative deviation
cfu	:	colony-forming unit

LIST OF APPENDICES

CHAPTER 1: INTRODUCTION

Problems associated with the use of fossils as raw materials for fuels and chemical commodities have motivated the search for alternatives. These problems include environmental pollution, depleting reserves, unstable prices, energy insecurity, and regional crises in resource rich nations (e.g. Nigeria and South Sudan). Biomass is considered the most viable alternative because of its abundance and the fact that it is the only carbon-rich resource on Earth (Cherubini, 2010). Hence the biorefinery concept has been pursued as a possible replacement to the petroleum refinery, seeking to overcome the problems associated with fossil exploitation by producing biofuels and biochemicals from biomass. Consequently, the consumption of biofuels has risen geometrically in recent times (Luque et al., 2008). Bioethanol has gained prominence among other biofuels owing to its use as a substitute or additive to gasoline as well as its other varied uses.

The production of biofuels and platform chemicals from food-based biomass such as starch, sugar or oil based crops, is considered unethical and unsustainable due to the competition with agricultural land and water, and its role in causing increases in food prices (Sims, Mabee, Saddler, & Taylor, 2010). These kinds of biomass are known as first generation biomass. Second generation biomass, mainly in the form of lignocellulosic feedstocks, are preferred because they are abundant, cheap, renewable, and their use has no conflicts with agricultural land, water or food. Lignocellulosic biomass are available in the form of agricultural and forest residues, dedicated energy crops and grasses, industrial wastes, and organic component of municipal solid wastes (Banerjee et al., 2010).

Lignocellulose is composed of cellulose, hemicellulose and lignin. Cellulose and hemicellulose are carbohydrate polymers which can be hydrolysed to their monomer sugars using chemicals or enzymes. The available sugars are then converted into several value-added products *via* microbial fermentation or by thermochemical conversion. The

lignin component cannot be utilized by microbes but it can be used for bioenergy applications or for the production of other valuable commodities. Cellulose is the most abundant component of lignocellulose (approximately 50 %) followed by hemicellulose and lignin, which may constitute different proportions depending on the nature of the biomass (Anwar, Gulfraz, & Irshad, 2014). Hemicellulose can be easily hydrolysed into its monomer units during pretreatment processes which are normally applied to fractionate lignocellulose. However, cellulose is more recalcitrant and requires further use of cellulase enzyme for hydrolysis. Thus, cellulases play an important role in the bioprocessing of lignocellulose.

Cellulases are a group of synergistic enzymes which attack β-1,4-glycosidic linkages of cellulose. Endoglucanases act randomly on the internal amorphous regions exposing shorter cellulose chains which are then cleaved by exoglucanases to generate cellobiose and shorter oligosaccharides. Beta-glucosidases complete the process by hydrolysing cellobiose to glucose (Chandel, Chandrasekhar, Silva, & Silverio da Silva, 2012). Endoglucanases are particularly important because they initiate cellulose hydrolysis. They also constitute a major part of commercial enzyme cocktails that are specially tailored towards bioenergy applications. Cellulases have other applications in industries such as paper, detergent, food/feed, textiles, and pharmaceutical industries.

Fungal cellulases have found more applications in commercial production due to the copious amounts of the enzyme produced by fungi. However, bacteria have recently attracted much attention due to their high growth rate, ease of genetic manipulation, the stability of their cells and enzymes under extreme conditions of bioprocessing, and their ability to produce cellulases in efficient complexes called cellulosomes (M. Maki, Leung, & Qin, 2009). Although, some cellulase-producing bacteria such as *Bacillus*, *Clostridium* and some actinomycetes are well known, bioprospecting for more efficient cellulolytic strains could lead to the discovery of novel genes which encode cellulases with better

properties. Such genes could be adapted into existing strains in order to achieve improved lignocellulosic bioprocessing (Chandel et al., 2012; M. L. Maki, Broere, Leung, & Qin, 2011).

Despite its advantages, lignocellulosic bioprocessing has not been deployed on a commercial scale because it is not currently considered as a cost-effective venture. This is mainly due to the high cost of feedstock supply and the technology bottlenecks (Banerjee et al., 2010; A.A. Rentizelas, Tatsiopoulos, & Tolis, 2009). Although lignocellulosic biomass by its nature is cheap, the logistics involved in getting the feedstocks available at the biorefinery are complicated and expensive. Activities such as harvesting, pre-processing, transportation, and storage attract high costs and make lignocellulosic biorefining expensive. Furthermore, the seasonal availability of the feedstocks and dependence of the supply on unstable weather conditions would hinder the continuous year-round running of the biorefinery (Athanasios A Rentizelas, Tolis, & Tatsiopoulos, 2009). Such scenarios would lead to periods of redundancy in the biorefinery thereby leading to considerable loss on capital investments.

Likewise, given the recalcitrant nature of lignocellulose, its utilization in a biorefinery would require complicated conversion technologies which are also expensive. For instance, pretreatments with expensive chemicals and energy-demanding thermochemical processes are required before lignocellulose can be separated into its individual components (Banerjee et al., 2010). In addition, such processes consume a lot of process water along with its concomitant expenses. More importantly, cellulases, which are expensive enzymes, are required in large amounts for the release of utilizable sugars from lignocellulose. In downstream fermentations, there is also the need for supplementation with additional nutrients since most lignocellulosics are nutritionally deficient, being plant wastes that are generated after the main nutritious parts have been

extracted. The challenges to the utilization of lignocellulosic feedstocks are summarized in Table 1.1.

Table 1.1: Factors hindering the commercial-scale deployment of lignocellulosic biorefinery and their implications.

Feedstock supply-related		Technology-related	
Factor	*Implications*	*Factor*	*Implications*
Feedstock supply logistics	Costs of harvesting, collection, handling, pre-processing, storage, transportation.	Biomass recalcitrance	Need for expensive pretreatments, detoxifying chemicals, process water, high energy demand, cost of cellulase
Seasonal availability	Fluctuating supply, redundancy, loss on capital investments	Low nutritional quality	Cost of extraneous nutrients
Unstable weather	Low crop yield, unstable supply	Low bulk density	Low product yields
Low bulk density	High cost of transportation		
Other competing uses of biomass	Limited supply		
Biomass degradation on storage	Increased costs		

The high cost of cellulases is one of the major bottlenecks in lignocellulose utilization. Cellulases constitute about 75 % of the total enzyme demands in biorefineries and comprised 40 – 49 % of the total cost of bioethanol production (Chandel et al., 2012). Cellulases are expensive because their large-scale production is carried out using expensive substrates such as lactose, Solka Floc, Avicel, carboxymethyl cellulose (CMC), and commercial cellulose pulp (Bigelow & Wyman, 2002; S. Chen & Wayman, 1991). Significant reduction in the cost of cellulase production would improve the economic feasibility of lignocellulosic bioprocessing (Gomes, Rodrigues, Domingues, & Gama, 2015). This can be achieved through the use of cheap substrates (such as lignocellulosics) as inducers in cellulase production (Bigelow & Wyman, 2002; S. Chen, 1993; Klein-Marcuschamer, Oleskowicz-Popiel, Simmons, & Blanch, 2012). Other ways of reducing

the cost of cellulase production are strain improvement, media formulation, and optimization of fermentation conditions (Chandel et al., 2012; S. Chen, 1993). Although a lot of advances have been made in strain improvement, the development of more efficient novel strains with abilities exceeding that of existing strains would require long-term efforts. In contrast, the use of novel carbon sources, media formulation strategies and optimization of fermentation process variables are measures that can be implemented more easily within a shorter time frame, and give immediate impacts on the industrial production of cellulases.

Lignocellulosic biomass have been widely investigated for cellulase production (Behera & Ray, 2015). However, most of these studies were based on single lignocellulosic biomass feedstocks. On the other hand, the use of mixtures of lignocellulosics in biorefining has been poorly explored. While a handful of reports are available regarding the use of mixed lignocellulosic feedstocks for production of ethanol (Elliston et al., 2015; Imamoglu & Sukan, 2014; Nguyen et al., 1999) and fermentable sugars (Brodeur-Campbell, Klinger, & Shonnard, 2012; K. H. Kim, Tucker, & Nguyen, 2005; R. D. O. Moutta, Ferreira-Leitão, & Bon, 2014), the use of such mixtures as carbon sources for microbial cellulase production is scarcely reported. Considering the challenges of feedstock supply explained earlier, dependence on a single feedstock for bioprocessing is risky and unsustainable. This problem could be managed if various feedstock types are processed together in the biorefinery. It has been shown that the use of mixed feedstocks has the potential to achieve 15 – 20% cost reductions (Nilsson & Hansson, 2001) as well reducing delivery costs to the biorefinery (Sultana & Kumar, 2011) when compared to single feedstock use. Furthermore, the use of such mixtures has been shown to be able to eliminate the need for extraneous nutrient supplementation in fermentations (Martín, Thomsen, Hauggaard-Nielsen, & BelindaThomsen, 2008). Thus, the use of mixed

lignocellulosics as carbon sources for cellulase production could be an interesting strategy for reducing the production cost of cellulases.

Lignocellulosic wastes such as oil palm and rice processing residues are abundant in Malaysia from the vibrant oil palm and agricultural industries (Abas, Kamarudin, Nordin, & Simeh, 2011). The disposal of these wastes through open burning and accumulation on land poses serious environmental and health hazards. The exploitation of these materials for cellulase production would be a double-pronged approach which could reduce environmental problems and provide additional source of income as well as encouraging the nascent biotechnological industry.

The foregoing analyses have motivated this study, which is primarily aimed at exploring the potential of mixed oil palm and rice lignocellulosic residues as substrates for bacterial endoglucanase production. The study was conducted to test the hypothesis that the use of a mixture of oil palm empty fruit bunch (EFB), oil palm frond (OPF), and rice husk (RH) as fermentation substrate could improve various aspects of bioprocessing of lignocellulose for endoglucanase production. The specific objectives of the study are:

1. To isolate an indigenous bacterial strain that could effectively utilize mixed substrate (MS) comprising EFB, OPF, and RH for cellulase production;

2. To compare endoglucanase production on single soluble- and insoluble substrates *vs.* MS by the selected microorganism;

3. To determine the effects of some agro-industrial wastes as nitrogen sources or MS supplements for endoglucanase production;

4. To determine the effects of pretreatment type and configuration (separate/joint) on endoglucanase production;

5. To optimize selected physico-chemical parameters of fermentation and substrate mixture ratios for endoglucanase production.

1.1 Thesis Structure

In Chapter 2, a review of literature relevant to the current study is presented. Chapters 3-7 are stand-alone chapters, each addressing different aspects of the specific objectives of this study. Each of these chapters has its own brief introduction and literature review, materials and methods, results and discussion, and conclusion. Chapters 3 to 7 address objectives 1 to 5 of this study in that (chronological) order. Chapter 8 contains the general conclusions and reflections on the findings of the study, as well as recommendations on future direction of the research. This is followed by a full list of references cited in this thesis. The supplementary section contains a list of publications which originated from this research and a list of papers presented at various conferences and fora during the period of study. An appendix section is also included.

CHAPTER 2: LITERATURE REVIEW

2.1 Mixed feedstock approach to lignocellulose bioprocessing

2.1.1 Mixed feedstock system and existing applications

The mixed biomass approach to biorefining entails the simultaneous utilization (processing and conversion) of two or more feedstocks of different types, in appropriate proportions, for the production of a commodity of interest, rather than utilizing a single feedstock (Fig. 2.1). The components of such mixtures may be of the same or different origin; they may have similar or contrasting characteristics; and they may require similar or different processing requirements. Generally, the mixed feedstock system has not been well explored because attention has been focused mainly on single feedstocks use (Athanasios A Rentizelas et al., 2009; Sultana & Kumar, 2011). Mixed feedstocks have been investigated for the production of bioethanol (Imamoglu & Sukan, 2014), biogas (Kalra & Panwar, 1986), microbial enzymes (Azin, Moravej, & Zareh, 2007; Roopesh, Ramachandran, Nampoothiri, Szakacs, & Pandey, 2006), polyhydroxyalkanoates (Sangkharak & Prasertsan, 2013), as well for power and heat applications (Arantes et al., 2008; Nilsson & Hansson, 2001). However, the number of such reports is limited compared to studies utilizing single feedstocks.
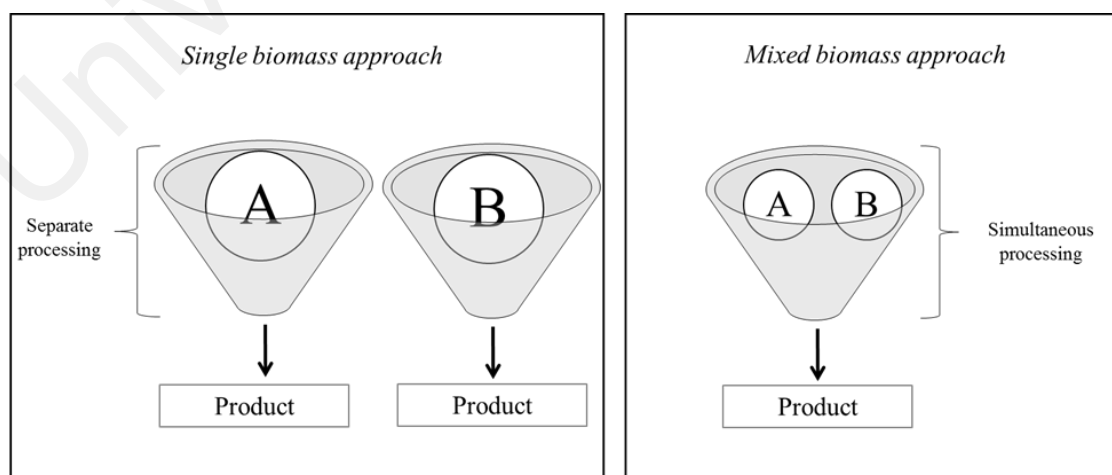
Figure 2.1: Differences between single and mixed feedstock processing.

Diverse combinations of feedstocks have been used in bioprocessing. While some are entire mixtures of second generation feedstocks, others combine first and second generation feedstocks. Among the complete lignocellulosic mixtures category, there are two types of combinations. First, biomass of the same category may be combined, such as complete mixtures of agricultural residues (e.g. wheat bran and rice bran), dedicated energy crops (e.g. mixed Prairie grasses), or forest residues (e.g. mixed hardwood). In this type, parts of the same plant or of different plants may be combined; it may also involve whole plant biomass utilization. The second type of combination involves biomass from different categories e.g. forest residues and agricultural residues, energy crops and agricultural residues, etc. For first/second generation mixtures, the components may originate from the same plant or may be from different plants. Examples of different biomass feedstock combinations are presented in Table 2.1.

Table 2.1: Feedstock mixture combinations across various categories of biomass.

Biomass feedstock mixture combination				Examples	Ref.
Complete second generation feedstock mixture	Components from same biomass category	Parts from same plant		Wheat bran + wheat straw. Sugarcane bagasse + sugarcane straw.	(Azin et al., 2007; Jecu, 2000). (R. D. O. Moutta et al., 2014; R. D. O. Moutta, R, Silva, Reis Corrales, Santos Cerullo, & Ferreira-Leitão, 2013).
		Different plants	Agricultural residues	Rice straw + wheat bran. Sugar cane bagasse + wheat bran. Mustard stalk and straw + wheat bran + rice straw.	(Kang, Park, Lee, Hong, & Kim, 2004; Qi, Yaoa, Yua, & Chena, 2007; Shamala & Sreekantiah, 1986; Sherief, El-Tanash, & Atia, 2010). (Camassola & Dillon, 2007). (Pal, Banik, & Khowala, 2013).
			Forest residues	Bark-rich sawmill residues. Mixed hardwood.	(K. H. Kim et al., 2005). (Jin, Yang, Jameel, Chang, & Phillips, 2013; Lim & Lee, 2013; L. Lynd & Grethlein, 1987; Deborah L Sills & James M Gossett, 2012)
			Grasses	Prairie grasses	(Deborah L Sills & James M Gossett, 2012; Deborah L. Sills & James M. Gossett, 2012)
		Whole plant biomass		Sugarcane straw + bagasse + tops	(Pereira, Maehara, Machado, & Farinas, 2015)

Table 2.1 (continued): Feedstock mixture combinations across various categories of biomass.

Biomass feedstock mixture combination			Examples	Ref.
Complete second generation feedstock mixture	Components from different biomass categories	Agricultural residue + forest residue + grass	Corn stover + switch grass + lodge pole pine + eucalyptus	(Shi et al., 2013)
		Forest residue + grass	Aspen + balsam + switch grass	(Brodeur-Campbell et al., 2012)
			Aspen/basswood/red maple/ balsam + switch grass	(J. Jensen, Morinelly, Aglan, Mix, & Shonnard, 2008)
		Agricultural residue + forest residue	Sawdust of rubber wood + mixed hardwood + oil palm trunk	(Chin, H'ng, Wong, Tey, & Paridah, 2011)
		Grass + legume	Clover + ryegrass	(Martín et al., 2008)
		Municipal solid wastes + agricultural residue	Municipal solid wastes + wheat straw	(Lissens, Klinke, Verstraete, Ahring, & Thomsen, 2004; Nguyen et al., 1999)
		Municipal solid wastes/ Mixed solid wastes		(J. W. Jensen, Felby, Jørgensen, Rønsch, & Nørholm, 2010; A. Li, Antizar-Ladislao, & Khraisheh, 2007; Mtui & Nakamura, 2005)
First/second generation feedstock combination	Components from same plant		Wheat straw + wheat meal	(Erdei et al., 2010; Erdei, Hancz, Galbe, & Zacchi, 2013)
	Components from different plants		Spruce chips + wheat flour hydrolysate	(Brandberg, Karimi, Taherzadeh, Franzén, & Gustafsson, 2007)

2.1.2 Criteria for selection of biomass types as feedstock mixtures

Henry (2010) suggested fifteen general criteria for the selection of plant species for bioenergy production. They are: (1) high biomass accumulation, (2) high harvest index, (3) high fraction of biofuel in harvested biomass, (4) nutrients partition to non-harvested parts, (5) able to be grown on marginal lands, (6) harvested material able to be stored in the field, (7) high bulk density, (8) high water use efficiency, (9) high nitrogen use efficiency, (10) potential as a weed, (11) co-product potential, (12) biomass composition, (13) scale of potential production, (14) cost of harvest, (15) suitability for genetic improvement. However, for mixed feedstocks, some other considerations could influence the selection of biomass types. Since the main motivation for mixed feedstock utilization is the high cost of feedstock supply and processing, the ease of processing and economic feasibility of joint utilization of such feedstock are of utmost importance. Proximity of the feedstocks to each other and to the point of collection or processing facility is an important factor to consider. Feedstocks which are located close to each other may be given priority over those that are located farther apart. This would make the logistics easier and reduce transportation costs. Feedstocks with similar characteristics would also be preferred over those with contrasting characteristics due to the possibility of processing them using the same equipment. Abundance and cheap cost of feedstocks may also determine their selection for combined utilization because of lower costs and continuous running of the biorefinery. Lastly, feedstocks may be selected due to the anticipated benefits during downstream processes such as fermentation. An example of this is elimination of the need for additional nutrient supplementation (Martín et al., 2008) and synergistic detoxification (Elliston et al., 2015).

2.1.3 Advantages of mixed feedstock approach to lignocellulose utilization

The benefits associated with the use of mixed feedstocks for various applications have been highlighted in a number of published literature. These advantages are related to logistics, technological aspects, and environmental aspects.

2.1.3.1 Improved logistics and associated cost reductions

Mixed feedstocks utilization has been shown to have potential for reduction of costs related to some upstream processes as well being able to bring about total cost reduction. According to Murphy et al. (2015), the use of mixed feedstocks for regional production of biofuel could ensure sustainability of biomass supply. This is because mixed feedstocks use will eliminate the risks (e.g. climatic unpredictability, pests, diseases, etc.) that are associated with single biomass types by providing a buffering effect during periods of interrupted supply. Furthermore, the use of mixed feedstocks would reduce storage requirements in the case of seasonal feedstocks (A.A. Rentizelas et al., 2009). When seasonal biomass types are used, there is usually the need for extensive storage of huge amounts in order to compensate for periods of unavailability; but with multiple biomass being used, the same space can be used for other biomass when the first option is not available (Nilsson & Hansson, 2001; Athanasios A Rentizelas et al., 2009). This could help reduce storage costs. The use of mixed feedstocks has also been shown to have the potential for cost reduction when they are delivered to the biorefinery in appropriate forms and proportions. Sultana and Kumar (2011) assessed the delivery costs of different forms of mixed agricultural residues and woody feedstocks to a biorefinery. They found that delivery cost was lowest when the feedstocks were delivered as 70% wood chips and 30% agricultural biomass bales. This cost was lower than the cost of delivering 100% of each single feedstock. The authors attributed this to lower transportation costs as a result of conveying the biomass in higher bulk density forms. Nilsson and Hansson (2001) studied the possibility of achieving total cost reductions when reed canary grass (RCG)

is co-fired with wheat straw (WS) for district heating application. They found that up to 15-20% cost reductions are possible if appropriate proportions of RCG and WS are used. The fact that the two biomass are of similar characteristics and can be processed with the same equipment and stored in the same facility also improves the chances of cost reductions. Additionally, the authors further projected that the lowest possible cost reductions could be achieved if wood chips are used together with WS and RCG. These examples indicate that considering upstream activities, adoption of mixed feedstock utilization could be beneficial for the biorefinery and other biomass-exploitation platforms.

2.1.3.2 Technological benefits

Several studies have demonstrated that the use of mixed feedstocks in the production of some bioproducts can improve product yields as well having potential for cost reductions. It has been shown that the need for extraneous nutrient supplementation in downstream fermentations can be eliminated or reduced with the use of substrate combinations. Thomsen and Haugaard-Nielsen (2008) reported that the supplementation of wheat straw with high fractions of clover-rye grass resulted in high ethanol yields (80% of theoretical) despite the fact that no nitrogen supplementation was added. The nutrient composition of the clover-rye grass was sufficient for the fermenting yeast. Similarly, Martín et al. (2008) reported that the addition of urea as nitrogen supplementation during the fermentation of wet oxidation pretreated clover-rye grass mixtures did not have any significant effect on ethanol yield as the nitrogen content of the mixture hydrolysate was sufficient. This shows that costs arising from additional nutrient supplements could be reduced in the biorefinery if mixed feedstocks are used.

The use of mixed feedstocks in the fermentation of lignocellulosics could also reduce or eliminate the need for detoxification. For some types of biomass, the pool of hydrolysates after pretreatment can neutralize or dilute the generated inhibitors. The detoxifying effect

of co-pretreatment of wheat straw (WS) and waste paper (WP) was demonstrated by Elliston et al. (2015). The amount of inhibitors in the mixture hydrolysate was found to be lower than those in the single substrates' hydrolysates. Furthermore, when the hydrolysates were fermented in SSF, ethanol yield was higher on the mixture than on either WS or WP, and the lag period was significantly reduced. The authors also showed that incorporation of WP with WS had shown similar effects as $CaCO_3$ would have on filter paper.

The mixture approach has also been shown to improve product yields. Roopesh et al. (2006) reported that phytase production by *Mucor racemosus* NRRL 1994 during solid state fermentation of a mixture of wheat bran (WB) and sesame oil cake was higher than the amount produced on either of the single substrates. Under optimized conditions, the enzyme yield was 1.5 to 4-fold higher than what was obtained on any of the single substrates. For enzymatic hydrolysis and ethanol production, while some studies (R. D. O. Moutta et al., 2014; R. D. O. Moutta, R et al., 2013; Pereira et al., 2015) reported that mixtures gave intermediate yields compared to single substrates, others (Elliston et al., 2015; Erdei et al., 2010; Erdei et al., 2013; Imamoglu & Sukan, 2014) reported higher yields on mixtures than on single substrates. This suggests that the combination of substrates does not exhibit observable detrimental effects on these processes.

With a combination of first and second generation feedstocks, higher concentration of fermentation products is possible since starch-based biomass usually contains higher sugar content, which can make up for the lower sugar concentrations in the lignocellulosic biomass. According to Erdei et al. (2010), the higher product concentration could help reduce energy demand during product distillation. This combination can also give more co-products (e.g. protein-rich residues) after distillation (Erdei et al., 2013).

2.1.3.3 Environmental benefits

Accumulation of municipal wastes is a big problem globally. Greater portion of these wastes are lignocellulosic in nature and are available in mixed forms. They are usually disposed through landfilling or open burning. The recent restrictions on landfilling (A. Li et al., 2007) (e.g. the European Union) and the increase in landfilling costs (Elliston et al., 2015), as well as regional bans e.g. in Malaysia (Siddiqui, Meon, Ismail, & Rahmani, 2009) on open burning of wastes has placed a lot of constraints on the efficient disposal of such wastes. Exploitation of these wastes in bioconversion processes is a viable alternative to landfilling. Consequently, researchers have explored the feasibility of utilizing municipal solid waste (MSW) for bioethanol production (A. Li et al., 2007; Lissens et al., 2004; Mtui & Nakamura, 2005).

The use of mixed lignocellulosic feedstocks could lead to a better energy balance. This is because combined processing of such feedstocks in appropriate proportions has been shown to improve process efficiency and better use of machinery (Nilsson & Hansson, 2001). However, this possibility needs to be scrutinized from empirical studies and life cycle assessments as there is a dearth of literature in this aspect.

2.2 Pretreatment of lignocellulosic mixtures

The general goal of pretreatment is to alter the properties of lignocellulose so as to improve its enzymatic digestibility for subsequent microbial utilization. This is achieved through reduction of cellulose crystallinity, increasing its accessible surface area, lignin removal and/or modification, and hemicellulose solubilisation (Alvira, Tomás-Pejó, Ballesteros, & Negro, 2010; Taherzadeh & Karimi, 2008). An ideal pretreatment is expected to meet certain requirements for it to be considered effective and economical. The requirements that are relevant to microbial utilization of lignocellulosic biomass include ability to produce a highly digestible solid, generation of high sugar concentration, preventing the degradation of sugars, and avoiding the production of toxic

chemicals that could inhibit enzymes and fermenting microorganisms (Alvira et al., 2010; Taherzadeh & Karimi, 2008).

Pretreatment methods are classified as physical, chemical, biological, or a combination of either of the three methods. Physical pretreatments apply heat, milling or irradiation to increase the accessible surface area of cellulose, decrease its crystallinity, and reduce the degree of polymerization. Chemical pretreatments involve the use of chemicals like acids, alkali, and ionic liquids to increase cellulose accessibility by removing lignin and solubilizing hemicellulose. Biological pretreatments employ the use of fungi to degrade lignin and hemicellulose through their repertoire of hydrolytic enzymes. Extensive reviews of lignocellulose pretreatments have been presented by Mosier et al. (2005), Taherzadeh and Karimi (2008), Alvira et al. (2010), and Xuebing Zhao, Zhang, and Liu (2012).

2.2.1 Selection of optimal pretreatment conditions for lignocellulosic mixtures

The choice of pretreatment method is dependent upon the nature of the biomass. Due to the variability in the characteristics of different lignocellulosic biomass, no single pretreatment can work best for all biomass types. For mixed feedstocks, the choice of a suitable pretreatment is difficult to make as the feedstock components are of different origin and high structural variability. A severe pretreatment might be effective for one component while destroying the sugars in the other component of the mixture (Nguyen et al., 1999). Thus, a compromise has to be made by choosing pretreatment conditions that are as close as possible to the optimum for each mixture component. This is depicted in Figure 2.2 which illustrates the selection of optimum pretreatment conditions for a hypothetical biomass mixture.
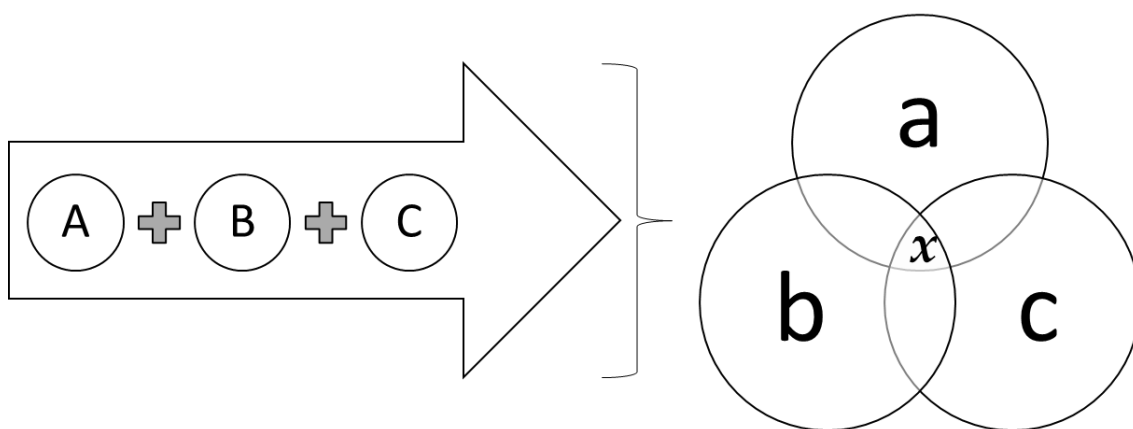
Figure 2.2: Selection of ideal pretreatment conditions for a hypothetical three-component lignocellulosic biomass mixture. In this figure, *a, b* and *c* represent the individual optimum conditions for feedstocks A, B and C respectively. The ideal pretreatment condition for the mixture of A, B and C would be *x,* which signifies the set of conditions closest to the optimum for each single feedstock component.

Researchers have adopted different approaches in selecting optimal pretreatment conditions for biomass mixtures. One common approach among studies of mixed feedstocks is subjecting the whole mixture to different pretreatment types and choosing the one that gives the best results (in terms of sugar yields, low inhibitors, etc.). This is often done without considering the optimum conditions for the individual feedstocks. For example, Lim and Lee (2013) investigated the optimum pretreatment parameters for production of glucose from mixed hardwood by subjecting the mixture to three acids (sulphuric, maleic, and oxalic acids) under varying temperatures, pH, and residence times. They found that maleic acid at 160 °C, pH 1.38, and residence time of 118 min produced the highest glucose yield, while lower yields were obtained with the other conditions. In a recent study (Shi et al., 2015), blended forms (flour/pellet) of a mixture of corn stover, switchgrass, lodgepole pine, and eucalyptus were subjected to ionic liquid, dilute acid, and aqueous ammonia pretreatments. The pretreatments were compared in terms of their effects on saccharification yields and isopentenol fermentation and ionic liquid was found to be most favourable. This approach could also involve the optimization of pretreatment parameters of a single method for the mixture. Jin et al. (2013) pretreated a mixture of hardwoods with a 1:1 sodium sulphite-formaldehyde mixture as solvent. They varied the

sulphite charge and pretreatment temperature in order to identify the best conditions for subsequent enzymatic hydrolysis of the pretreated pulp. This approach was similarly applied in other earlier studies (Lissens et al., 2004; L. Lynd & Grethlein, 1987; Martín et al., 2008).

Another approach, less adopted in studies, but possibly similarly effective, is the selection of pretreatment conditions based on the optimal parameters for the single feedstocks. In this approach, the set of conditions that are closest to the best for the individual feedstocks are used for the mixture. Thomsen and Haugaard-Nielsen (2008) pretreated wheat straw and clover-grass mixtures using wet oxidation pretreatment parameters that were previously reported (Bjerre, Olesen, Fernqvist, Plöger, & Schmidt, 1996; Martín et al., 2008) as being optimal for each of the single substrates. They obtained high ethanol yields in subsequent simultaneous saccharification and fermentation (SSF) experiments. Using this approach, A. Li et al. (2007) applied more rigorous measures by first subjecting the individual components of MSW to various single and combined pretreatments, and later applying the best of them on the mixture. This produced the highest glucose yield from the mixed substrate after enzymatic hydrolysis.

2.2.2 Separate and combined pretreatment of lignocellulosic mixtures

Besides identifying the optimal pretreatment conditions for a lignocellulosic mixture, the choice of pretreatment configuration, in terms of either pretreating the components separately or jointly, is another important consideration. In many studies involving whole lignocellulosic mixtures, the individual components are usually pretreated together simultaneously in the same vessel. Following this method, Elliston et al. (2015) achieved higher ethanol concentration and lower inhibitors formation by co-pretreating wheat straw and waste paper instead of pretreating them separately. The advantage of this method is that the use of the same equipment and chemicals for all the mixture components could bring about cost savings. However, this simultaneous pretreatment of

mixtures has been shown to be suitable only for biomass of a similar nature (e.g. hardwood mixtures, softwood mixtures, or agricultural residue mixtures) because they are more likely to have similar dissolution kinetics (J. Jensen et al., 2008). Pretreating widely contrasting biomass types simultaneously would require harsher and more energy-demanding pretreatments. Whole lignocellulosic mixtures may also be pretreated simultaneously but in a sequential manner with different pretreatments. Each pretreatment is targeted at particular components of the mixture in order to achieve optimal yields of sugars from each component. For example, Nguyen et al. (1999) pretreated a solid waste mixture in sequence using dilute acid and steam pretreatments and obtained 80% yield of ethanol after fermentation of the hexose sugars. In another study, a sequential two-stage dilute acid pretreatment was also applied on a mixture of hog fuel and pin chips and this allowed for high sugar recoveries (K. H. Kim et al., 2005).

Conversely, each single feedstock can be pretreated separately using conditions that are best suited for it. Thereafter, the individual hydrolysates are mixed together before hydrolysis and/or fermentation. This approach is more commonly adopted with mixtures of first generation and second generation feedstocks (Brandberg et al., 2007; Erdei et al., 2010; Erdei et al., 2013; M. Yang et al., 2015) whereby appropriate physicochemical pretreatments are applied to the lignocellulosic biomass while the starch-based biomass is liquefied and saccharified using appropriate enzymes. This method has also been applied to a mixture of cotton stalks and rice hulls, forming an entire second generation feedstock mixture (Imamoglu & Sukan, 2014). One advantage of separate pretreatment, as demonstrated by Huang, Wang, Ren, Chen, and Xu (2013) , is that pretreatment of the single substrates with different chemicals (e.g. acid and alkali) could eliminate the need for detoxification due to the mutual neutralization effect of mixing the hydrolysates together after pretreatment. That is, an acid pretreated-hydrolysate would naturally neutralize the high pH of an alkali-pretreated hydrolysate. The drawback of this method

however, is the need for multiple pretreatment equipment and chemicals, especially when the individual feedstocks require completely different setups.

2.2.3 Effects of mixtures on material recovery and yields during pretreatment

In an effective pretreatment process, all the valuable biomass components should be recovered without much loss so that they can be available for further utilization (Rocha, Martín, da Silva, Gómez, & Gonçalves, 2012). For mixed feedstocks, the biomass components are structurally heterogeneous and the interaction between them could affect the overall material yields and recoveries. Interestingly, available data from relevant studies showed that in most cases, mixing different feedstocks together has no negative effects on material recovery and carbohydrate yields. Mixtures usually present recoveries and yields that are intermediate in value or similar to those of single feedstocks. In a recent study, Chenlin Li et al. (2015) reported that a mixture of switch grass and eucalyptus gave intermediate material recovery and carbohydrate yields as compared to the single feedstocks during ionic liquid pretreatment. A higher glucan recovery was even recorded for the mixture. J. Jensen et al. (2008) reported a slight rate enhancement in experimental xylose recovery for mixtures as compared to model predicted values (derived from single feedstock data) during the dilute acid pretreatment of a five-component mixture of hardwood, softwood and switch grass. The experimental xylose yields for mixtures were generally similar to those of single feedstocks. Material recovery and lignin solubilization for sugarcane bagasse-sugarcane straw mixture were found to be intermediate in values compared to the single species for dilute acid pretreated feedstocks and there was no significant difference in hemicellulose removal for both setups (R. D. O. Moutta et al., 2014). However, in a similar study (R. D. O. Moutta, R et al., 2013), insoluble solids recovery for sugarcane bagasse-straw mixture was reported to be lower than that of the single feedstocks for hydrothermally pretreated bagasse and straw, although the recovery for the mixture was still within an acceptable range. The authors

attributed this to the heterogeneity of the mixture. However, the hemicellulose removal and level of inhibitors generated for the mixture were of intermediate range as compared to the single feedstocks.

2.3 Effects of mixture ratios on product yields during bioprocessing of lignocellulosic mixtures

Optimum proportion of mixture components are necessary for high product yield during various stages of bioconversion processes involving mixed lignocellulosics. Since each component has its own unique characteristics which may differ from other components in terms of suitability for processing and microbial utilization, each component needs to be used at an appropriate level to ensure optimal results. Thomsen and Haugaard-Nielsen (2008) studied the effects of different ratios of wheat straw (WS) and clover-grass (CG) mixtures on sugar recovery and ethanol yield during SSF of the substrates with *S. cerevisiae.* Mixtures with higher proportions of CG had lower overall sugar recoveries due to the formation of Maillard compounds from the free sugars and amino acids of CG during pretreatment. However, the mixture with the highest CG content (CG:WS ratio 85:15) gave the highest ethanol yield. This was attributed to the higher amounts of nutrients in CG and the positive effects of the presence of low levels of inhibitors on the yeast which led to increased ethanol production as compared to mixtures with higher WS content. R. D. O. Moutta et al. (2014) reported that mass solubilization and enzymatic hydrolysis yield increased with increasing content of sugarcane straw in the mixtures during acid pretreatment of sugarcane bagasse and straw. This was attributed to the higher amount of hemicellulose in the straw which is known to be more susceptible to acid pretreatment. When different ratios of cassava residues (CR) and furfural residues (FR) were used in SSF with *S. cerevisiae*, L. Ji, Yu, Liu, Jiang, and Sun (2015) found that mixtures with higher proportions of CR gave higher ethanol yield and lower side product (glycerol) formation than those with higher FR (with higher lignin content). The higher

CR fractions diluted the lignin content of the mixtures and allowed for better hydrolysis of cellulose by reducing non-productive adsorption of the enzymes to lignin during SSF. Furthermore, CR provided additional nutrients for the fermenting organism. Imamoglu and Sukan (2014) studied the effects of different ratios of rice husk (RH) and cotton stalks (CS) on ethanol production in SSF with *E. coli* KO11. They reported that mixtures with higher proportions of RS had higher ethanol yield due to the fact that dilute acid hydrolysed RH more easily than CS, hence the higher amount of fermentable sugars in higher RS fractions.

With respect to the use of mixed lignocellulosics for microbial enzyme production, studies have shown that appropriate ratios are essential for maximal enzyme production. Azin et al. (2007) studied the effects of different proportions of wheat bran (WB) to wheat straw (WS) on xylanase production by *Trichoderma longibrachiatum* PTCC 5140 during solid substrate fermentation. They found that WB:WS ratio of 7:3 supported higher xylanase production than all other ratios tested, and produced 14.9% increase in enzyme yield compared to WS alone. However, when multiple enzymes (cellulases and hemicellulase) were produced on the same mixed substrates (Qi et al., 2007; Sherief et al., 2010), it was found that the optimal ratio for one enzyme was unfavourable for the production of the other enzymes. This disparity could be due to the varied effects of lignocellulose components (e.g. lignin) on the metabolism of the fermenting microorganism for the enzyme of interest.

From the foregoing, it can be inferred that the efficiency of bioconversion processes involving lignocellulosic mixtures is dependent on the following factors:

1. Relative contribution of each component to the total available carbohydrates/sugars, nutrients and pretreatment-generated inhibitors as determined by the substrate proportions in the mixture;

2. Response and susceptibility of each mixture component to the applied pretreatment conditions;

3. Interactions between the mixture components (or their hydrolysates) and the effects of such interactions on the metabolism of the fermenting organism.

2.4 Cellulase system

2.4.1 Cellulose

Cellulose is the most abundant source of renewable carbon on Earth. It is found predominantly in plants where it constitutes about 35-50 % of most plant cell walls and almost 100 % in cotton. It is also found to a smaller extent in some bacteria, fungi, algae, and animals (L. R. Lynd, Weimer, Zyl, & Pretorius, 2002). Cellulose is composed repeating units of *D*-glucopyranose linked by β-1,4-glycosidic bonds (O'Sullivan, 1997). The smallest unit of the homopolysaccharide is cellobiose which can be further converted into glucose (M. Maki et al., 2009). Cellulose contains crystalline phases and amorphous phases as a result of the differential aggregation of hydroxyl groups on glucose which form dense regions of hydrogen bonding (crystalline) and other regions with lesser degree of hydrogen bonding (amorphous). It is usually embedded in a matrix along with lignin and hemicellulose. Cellulose was isolated for the first time by Anselme Payen, a French chemist, in 1839 after he treated timber alternately with nitric acid and sodium hydroxide solution. At normal temperatures, cellulose is insoluble in water, dilute acids and dilute alkaline solutions (H. Chen, 2014b). Microbial utilization of cellulose is a crucial part of the carbon cycle (L. R. Lynd et al., 2002).
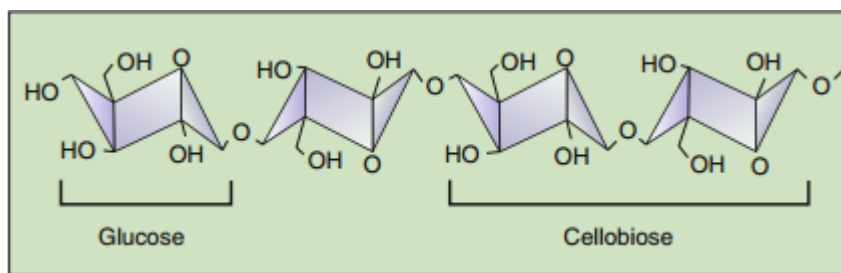
Figure 2.3: Molecular chain structure of cellulose (H. Chen, 2014b).

2.4.2 Diversity of cellulolytic ability among living organisms

Microorganisms were once thought to be the only organisms with the ability to utilize cellulose for generation of energy (D. B. Wilson, 2011). However, it is now known that some animals possess this ability as well, which is distinct from that of their symbiotic microflora. Evidence of cellulolytic ability has been demonstrated in nematodes (Smant et al., 1998), termites (Tokuda et al., 1999), earthworm (Nozaki, Miura, Tozawa, & Miura, 2009), clam (Sakamoto, Touhata, Yamashita, Kasai, & Toyohara, 2007), sea urchin (Nishida et al., 2007), and beetle (Sugimura, Watanabe, Lo, & Saito, 2003). Cellulases are also produced by plants, where they perform cell wall modifying functions (Hartati et al., 2008). Cellulase production is common among different groups of bacteria such as the aerobic, anaerobic, mesophilic and thermophilic bacteria (H. Chen, 2014a). The aerobic fungi and some anaerobes among them are also good cellulase producers (Kuhad et al., 2016). The fungi are the most prolific cellulase producers known so far with the genera *Trichoderma* and *Aspergillus* being the most extensively studied (M. Wang, Li, Fang, Wang, & Qu, 2012).

2.4.3 Nature of cellulases

Cellulases are enzymes which catalyse the hydrolysis of β-1,4-glycosidic bonds that link the glucosyl residues in cellulose (D. B. Wilson, 2011). They belong to the glycoside hydrolase (GH) families in the Carbohydrate-Active enZYmes database (CAZy; www.cazy.org)(Cantarel et al., 2009). While other GH members might hydrolyze

glycosidic bonds between carbohydrate and non-carbohydrate moieties, cellulases act specifically on bonds linking glucosyl residues (L. R. Lynd et al., 2002). There are three main types of cellulases which act synergistically to completely hydrolyze cellulose. Endoglucanases (E.C. 3.2.1.4) catalyse the cleavage of glycosidic bonds at the amorphous regions of cellulose, thereby liberating long chains of oligosaccharides. Exoglucanases (cellobiohydrolases), act on the crystalline regions, hydrolyzing the shorter chains either from the reducing (E.C. 3.2.1.176; CBH I) or non-reducing ends (E.C. 3.2.1.91; CBH II) to produce cellobiose. β-glucosidases (E.C. 3.2.1.21) then hydrolyze the cellobiose to glucose (Juturu & Wu, 2014; M. Maki et al., 2009; M. Wang et al., 2012). Recently, lytic polysaccharides monooxygenases (LPMO) which participate in cellulose degradation in an oxidative manner have been discovered. CBM33 and GH61, the two LPMOs identified so far, bind to substrates' crystalline surfaces and cause disruption of the crystal packing thereby enhancing accessibility. In this manner, they facilitate the action hydrolytic cellulases (Horn, Vaaje-Kolstad, Westereng, & Eijsink, 2012). The mechanism of action of cellulases in hydrolyzing cellulose is shown in Figure 2.4.
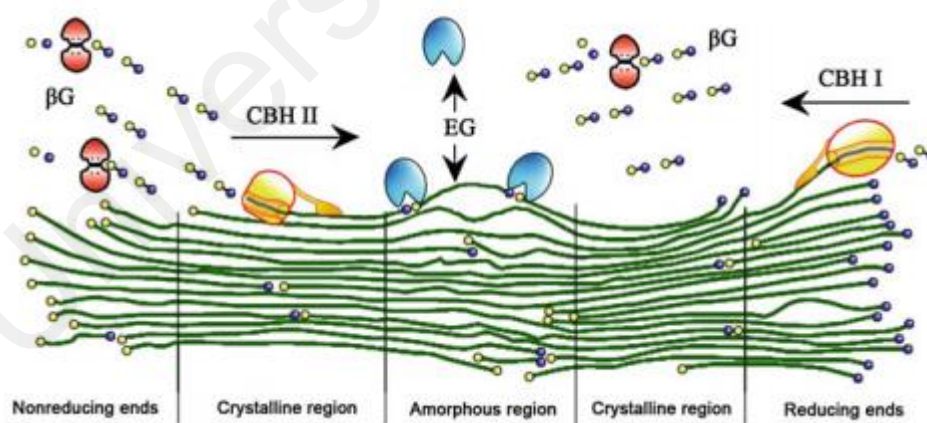
Figure 2.4: Mode of action of cellulases in hydrolyzing cellulose. *CBH* cellobiohydrolase (exoglucanase), *EG* endoglucanase, β*G* β-glucosidase.(M. Wang et al., 2012)

Cellulases are described as exhibiting synergism in their action because the sum of the activities of individual cellulases is less than the activity when they act jointly. Hence

different types of synergism between cellulases have been reported: synergism between (1) endoglucanase and exoglucanase, (2) exoglucanase and exoglucanase, and (3) exoglucanase and β-glucosidase (L. R. Lynd et al., 2002). Another type of synergism between the domains of an enzyme (intramolecular) has also been reported (Din et al., 1994). Synergism between endoglucanases is not common but processive endoglucanases have been shown to act in synergy with all other types of cellulases. However, cellulases from different organisms can also act in synergy (David B. Wilson, 2009). Processivity of cellulases indicates the ability of cellulases to remain bound to cellulose until it is hydrolyzed. Exoglucanases are processive while endoglucanases can be processive or non-processive (Sukharnikov, Cantwell, Podar, & Zhulin, 2011).

Unlike the case of soluble substrates where the substrate diffuses to the enzyme, cellulose hydrolysis requires that the enzyme attaches itself to the insoluble cellulose. For this reason, cellulase has a modular structure which is composed of the carbohydrate binding domain/module (CBD or CBM) which is connected to the catalytic domain (CD) *via* a flexible linker peptide (D. B. Wilson, 2011). The CBD binds to the surface of cellulose and brings the CD into close contact with the substrate. The CD then cleaves the cellulose chains, releasing cellobiose in the process. Besides the binding function, CBD has also been shown to initiate hydrolysis by disrupting the hydrogen bond network on the surface of cellulose (L. Wang, Zhang, & Gao, 2008; Xiao, Gao, Qu, & Wang, 2001) as well as increasing the concentration of the enzyme on the surface of the substrate (M. Wang et al., 2012). The intramolecular synergy between the CBD, CD and the linker facilitates efficient digestion of cellulose.

2.4.4 Complexed and non-complexed cellulase systems

Microorganisms have evolved two major systems for utilizing cellulose. Aerobic bacteria and fungi secrete cellulases extracellularly in a "non-complexed" system. Here, the enzymes act synergistically to hydrolyze the cellulose; attachment to the substrate is not

required. The most extensively studied cellulase in this system is that of *Trichoderma reesei*. This fungus produces at least five endoglucanases, two exoglucanases, and β-glucosidases (H. Lin, Li, Guo, Qu, & Ren, 2011). The aerobic bacterium, *Thermobifida fusca*, also produces a complete set of non-complexed cellulases which act synergistically (Chir, Wan, Chou, & Wu, 2011). Cellulose degradation by aerobic bacteria is similar to, but nonetheless, distinct from that of aerobic fungi. While cell-free preparations of cellulolytic bacteria exhibit low specific activities compared to their whole cultures, the case is not so with aerobic fungi like *T. reesei* (Kuhad et al., 2016; Tomme, Warren, & Gilkes, 1995). Furthermore, purified bacterial cellulases display comparable or even higher specific activities compared to those of fungi, although lower quantities of cellulase are produced by cellulolytic bacteria (Robson & Chambliss, 1989).

In the "complexed" system which is utilized by anaerobic bacteria and fungi, a multi-enzyme structure known as cellulosome is present on the surface of the cell and attaches the cell to the substrate in order to effect hydrolysis. The structure of cellulosome and its mechanism of cellulose hydrolysis is shown in Fig. 2.5. The scaffoldin, a non-catalytic component of the cellulosome, anchors the various enzymatic subunits. Each scaffolding has a series of cohesins which bind to the enzyme subunits *via* the dockerins. Each dockerin is attached to the enzyme on one side and to the cohesin at the other end. The scaffoldin also has a CBM which binds it to the cellulose substrate. Surface layer homology domains (SLHs) bind the cellulosome to the cell wall. Cellulosome composition differs among different bacterial species due to species-specific variations in scaffoldin structure (Bayer, Henrissat, & Lamed, 2009; Juturu & Wu, 2014). The cellulase system of *Clostridium thermocellum* is the most studied in this category (Rani Singhania, 2011).
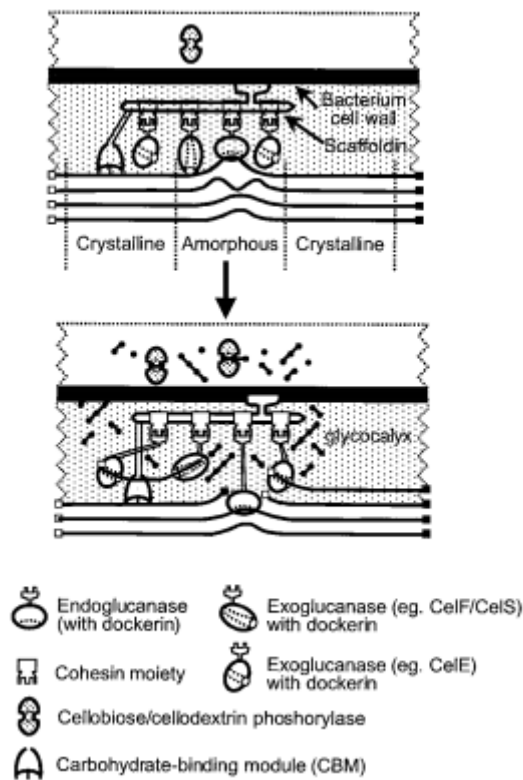
Figure 2.5: Schematic representation of the hydrolysis of cellulose in a complexed (cellulosomal) cellulase system.(L. R. Lynd et al., 2002)

2.4.5 Industrial applications of cellulases

Cellulases have a wide range of existing and potential applications in industries such as food processing, animal feed, brewing, detergents, textile, paper and pulp, and medical industry. They are used either solely or in conjunction with other enzymes (e.g. proteases, xylanases, etc.) in some of the industries. Cellulases accounted for about 20 % of global enzyme market in 2010, but this share is expected to increase drastically, possibly becoming the largest industrial enzyme by volume, if biofuels become the major form of transportation fuels in the future (Rani Singhania, 2011). Applications of cellulases in various industries are summarized in Table 2.2.

Table 2.2: Some industrial applications of cellulases.

Industry	Applications
Textile	Bio-stoning of denim garments, replacing the use of pumice stones; bio-polishing of fabrics; softening and defibrillation of fabrics.
Laundry and detergent	Used as detergent additive for improved colour, texture, and dirt removal; used to prevent re-deposition of removed dirt from fabric; removing detached microfibrils; smoothening of textiles.
Food processing	Clarification of fruit juices; used for improving texture, aroma and taste of fruit and vegetables; improvement of olive oil extraction.
Animal feed	Improvement of nutritional value of grain feed and silage; elimination of anti-nutritional factors in feed.
Brewing	Reducing the viscosity of wort, improving filterability, and glucan hydrolysis; improvement of wine aroma; improving wine must extraction and filtration, decreasing must viscosity, improving wine stability.
Paper and pulp making	Used for bio-mechanical pulping; de-inking or recycled paper fibers; improving drainage and free flow in paper mills; used in making biodegradable cardboards and packaging materials.
Biofuel production	Conversion of cellulosic biomass to fermentable sugars for production of single cell proteins, bioethanol, and other fermentation products.
Medical	Treatment of phytobezoars; used as antibiofilm agent in medical implants and prosthetics; treatment of chitosan for antitumor and antibacterial activities.

Compiled from Kuhad, Gupta, and Singh (2011); Menendez, Garcia-Fraile, and Rivas (2015); Rani Singhania (2011)

2.4.6 Factors affecting cellulase production

While research on cellulases has advanced appreciably, earlier studies focused on the various factors affecting cellulase production by microorganisms. The understanding of these factors have allowed for improvements in yields of cellulase enzymes from various microbial fermentations. The yields and properties of cellulases from different microorganisms are mainly influenced by the type of carbon source, nutrients and surfactants, and fermentation conditions (S. Chen, 1993).

2.4.6.1 Nature of carbon source

Not only do carbon sources serve as sources of energy for microbial growth during fermentation for cellulase production, they also act as inducers or repressors for cellulase synthesis (S. Chen, 1993). The carbon sources that have been used as substrates in cellulases production studies are generally available in three forms: soluble carbon

sources (e.g. simple sugars, CMC, short oligosaccharides), insoluble pure cellulosics (Avicel, cotton, Solka Floc, microcrystalline cellulose), and lignocellulosics (pretreated or untreated). The effects of the various types of carbon sources on cellulase production depends on the type of microorganism and the type of cellulase being produced. Among the fungi, insoluble pure cellulosics are most favourable for cellulase production (S. Chen, 1993). On the other hand, soluble carbon sources, especially simple sugars, tend to give lower enzyme production. Glucose represses cellulase production according to some studies (Narasimha, Sridevi, Buddolla, Subhosh, & Rajasekhar, 2006; Niranjane, Madhou, & Stevenson, 2007; Nwodo-Chinedu et al., 2007; Stewart & Parry, 1981), while lactose, sophorose, and cellobiose have a better effect on production (Dashtban, Buchkowski, & Qin, 2011; El-Hadi, El-Nour, Hammad, Kamel, & Anwar, 2014; Narasimha et al., 2006). Carboxymethyl cellulose (CMC), a soluble form of cellulose, has been reported to give higher cellulase production compared to other carbon sources (Ahmed, Bashir, Saleem, Saadia, & Jamil, 2009; Narasimha et al., 2006; Niranjane et al., 2007; Tong & Rajendra, 1992). In some cases, pretreated lignocellulosics may produce comparable enzyme titers with pure cellulose (Nwodo-Chinedu et al., 2007), however, the presence of toxic pretreatment-generated inhibitors may result in lower enzyme production. The type of pretreatment used and the extent of washing of the pretreated solid would determine the suitability of the substrate for enzyme production. Dashtban et al. (2011) studied the effects of various forms of carbon sources on cellulase production by one wild type and two mutant strains of *T. reesei*. The authors found that insoluble pure cellulose (Avicel and microcrystalline cellulose) gave the highest cellulase activities while the lignocellulosic materials gave the least cellulase activities. Cellulase production was only slightly higher on the soluble carbon sources (highest from lactose) than on the lignocellulosics. The findings of these researchers is a reflection of what is generally obtainable in most fungal cellulase studies.

Unlike the fungi, insoluble pure celluloses usually give lower cellulase production as compared to soluble carbon sources in bacteria. This observation has been reported by several researchers (Abou-Taleb, Mashhoor, Nasr, Sharaf, & Abdel-Azeem, 2009; Chan & Au, 1987; Shankar & Isaiarasu, 2011). However, Chan and Au (1987) reported higher avicelase production by *Bacillus subtilis* on Sigmacell and filter paper, which are pure insoluble forms of cellulose. Contradictory results with regards to the effect of glucose have been reported from different researchers. While some reported that it repressed cellulase production (Abou-Taleb et al., 2009; Au & Chan, 1986; Fukumori, Kudo, & Horikoshi, 1985), others reported an improvement in production (Mawadza, Boogerd, Zvauya, & van Verseveld, 1996; S. Sethi, Datta, Gupta, & Gupta, 2013). This disparity seems to be due to strain differences. A conclusive statement about the relative suitability of lignocellulosic substrates compared to pure cellulosics as carbon sources for bacterial cellulase production is difficult to make as studies comparing bacterial cellulase production on these substrates are limited. W. Yang et al. (2014) compared CMCase production by *B. subtilis* BY-2 on 1% of each of CMC, microcrystalline cellulose (MCC), rice hull, wheat bran, and corn powder. CMCase production on the substrates ranged as follows: corn powder > wheat bran > (rice hull, CMC) > MCC. MCC produced very low CMCase titers while rice hull and CMC produced similar results. Bai et al. (2012) reported higher cellulase production by a strain of *B. pumilus* on lactose (23.96 U/mL) than on wheat bran (20.96 U/mL). However, it was not mentioned in these studies whether the lignocellulosic substrates used were pretreated. When Harun et al. (2013) compared the production of cellulases on MCC and alkali- and heat-pretreated EFB samples, they reported that MCC supported higher cellulase production than the pretreated EFB samples. Thus, it seems that for lignocellulosic substrates, the nature of the substrate, in terms of post-pretreatment modifications, as well as residual pretreatment inhibitors, would determine the extent of cellulase production by the bacteria.

Substrate concentration also affects cellulase production. Concentrations in the range of 1-2 % (w/v) are commonly reported as being optimal for cellulase production in the literature. Higher concentrations create problems of mixing, oxygen transfer and foaming in batch fermentations. Also, higher concentrations usually result in longer fermentation times (S. Chen, 1993).

2.4.6.2 Nutrients

Adequate supply of nutrients is crucial for growth and enzyme production in cellulolytic microorganisms. A wide variety of organic (e.g. peptone, urea, yeast extract, casaminoacids, etc.) and inorganic substances (ammonium salts, nitrates, etc.) have been investigated as suitable nitrogen sources for cellulase production. Such substances provide a good supply of required nutrients and growth factors for the microorganisms. Generally, organic nitrogen sources have been found to favour cellulase production in many microorganisms better than the inorganic ones (Abou-Taleb et al., 2009; Garcia-Martinez, Shinmyo, Madia, & Demain, 1980; Magnelli, Ramos, & Forchiassin, 1996; Narasimha et al., 2006). However, in some cases, inorganic sources were found to be more favourable (S. Sethi et al., 2013; Tong & Rajendra, 1992). Due to the high cost of peptone, cheaper alternatives such as corn steep liquor and peanut cake have been sought as a replacement (S. Chen, 1993).The effects of different nitrogen sources on enzyme production is strain- and substrate-specific (Kachlishvili, Penninckx, Tsiklauri, & Elisashvili, 2006). Thus, it is important to select an economical and effective medium that works best for the microorganism of interest.

The inclusion of surfactants in culture media has been shown to promote growth and cellulase production. Shahriarinour, Wahab, Mohamad, Mustafa, and Ariff (2011) showed that the addition of 2 mL/L of Tween 80 to the fermentation media resulted in a two-fold increase in cellulase production by *Aspergillus terreus* compared to the media without surfactant added. Shankar and Isaiarasu (2011) also reported that Tween 20

promoted cellulase production by *B. pumilus*. Other studies have also shown that inclusion of natural oils or fatty acids and non-ionic surfactants helped to promote cellulase production (Suha Sukan, Güray, & Vardar-Sukan, 1989; Yazdi, Woodward, & Radford, 1990). However, Amat, Arora, Nain, and Saxena (2014) recently reported that Tween 20, Tween 80, and Triton X-100 did not promote cellulase production in *Xanthomonas axonopodis pv. punicae.* This may be due to use of sub-optimal concentrations of the surfactants as other researchers have shown that the effect of surfactants on cellulase production is related to their concentration (Pardo, 1996; Shankar & Isaiarasu, 2011). It has been suggested that the positive effect of surfactants on cellulase production could be as a result of increased cell permeability and/or the release of cell-bound enzymes (Pardo, 1996; Reese & Maguire, 1969).

The presence of metal ions also influences cellulase production. This was demonstrated by Chan and Au (1987) where they showed that different concentrations of metallic ions in the fermentation led to a significant increase in the yield of cellulase produced by *B. subtilis* as compared to media without metallic ions. Saxena, Bahadur, and Varma (1992) also showed that cobalt and nickel selectively promoted CMCase production in *Cellulomonas* spp. but not xylanase production. Depending on the microbial strain and other media components, different metal salts have been reported to exert superior influence on cellulase production over other metals in several studies (Bansal, Soni, Janveja, & Soni, 2012; Shankar & Isaiarasu, 2011). Generally, an adequate balance of metal ions is required for cellulase production and this seems to be more important than their individual concentrations (Mandels & Reese, 1957).

2.4.6.3 Fermentation conditions

Aeration and agitation: - the availability of oxygen is important for cell growth and metabolism. The amount of dissolved oxygen as controlled by the level of agitation exerts an important influence on cellulase production by microorganisms. Agitation has been

shown to promote cellulase production in bacteria. Cellulase production by *B. amyloliquefaciens* under agitated conditions (100 rpm) was found to be two-fold higher than under static conditions (Khan & Husaini, 2006). F. J. Stutzenberger (1972) reported that at 180 rpm agitation, C_1 and C_x (cellulase components) production by *Thermomonospora curvata* increased by 2.7- and 4.2-fold respectively as compared to results obtained under static conditions. Similarly, Abou-Taleb et al. (2009) obtained maximum cellulase activities by two bacterial strains (*B. alcalophilus* S39 and *B. amyloliquefaciens* $C2_3$) at 150-200 rpm speeds as compared to lower speeds. Generally, at speeds close to 200 rpm, difference in cellulase production became insignificant. The converse seems to be the case with regard to fungi as lower cellulase production is commonly reported at higher agitation speeds. Ahamed and Vermette (2010) reported higher enzyme titers and productivity when *T. reesei* was cultivated with no mechanical agitation as compared to agitated conditions. An increase of agitation speed from lower intensities (100, 300 rpm) to higher intensity (500 rpm) was found to have caused a decline in FPase activity of *Pycnoporus sanguineus* (Fadzilah & Mashitah, 2010). Similar observations were reported by Mukataka, Kobayashi, Sato, and Takahashi (1988) and R. Lejeune and Baron (1995) in the production various cellulases by *T. reesei* QM 9414. These observations were mainly attributed to the destruction of fungal cells/hyphae at higher speeds. However, Shahriarinour et al. (2011) reported four times higher cellulase production under agitated shake flask condition (200 rpm) than under static conditions, and they attributed this to the increase in dissolved oxygen concentration. This disparity can be explained by the fact that the other examples mentioned used bioreactors with impellers which could injure the cells at higher speeds. On the other hand, this would not be the case in shake flasks as the agitation is rather mild due to rotary motion.

Initial pH of medium: - microbial growth and yield of cellulase is highly dependent on the initial pH of the medium. Bacterial cellulase production occurs over a wide pH range

but initial pH values in the range of 6 - 8 have been reported as optimal for several species (Amat et al., 2014; Chan & Au, 1987; Garcia-Martinez et al., 1980; F. J. Stutzenberger, 1972). Significant decline in enzyme production is often observed outside this pH range for most bacteria. For fungi, the optimum pH range for the production of various cellulase types is typically around 4.0 – 6.0 (Abo-State, Swelim, Hammad, & Gannam, 2010; Shahriarinour et al., 2011; Tong & Rajendra, 1992), although there have been reports of optimal production around pH 6.5 – 7.0 as well (El-Hadi et al., 2014; Magnelli et al., 1996).

Temperature: - temperature of cultivation is one of the most important factors influencing cellulase production. Different values of temperature have been reported as optimal for cellulase production in bacteria, often reflecting the conditions of the original source of the organism (Abou-Taleb et al., 2009) or coinciding the with the optimum temperature for growth (Amat et al., 2014; Mawadza et al., 1996). Hence, optimum cellulase production around 28 – 37 °C for mesophilic bacteria (Amat et al., 2014; Shankar & Isaiarasu, 2011) and around 40 – 50 °C for thermophilic bacteria (Abou-Taleb et al., 2009; Chan & Au, 1987; Mawadza et al., 1996) have been reported. The trend is similar for fungal cellulase producers although the optimal temperatures for growth and enzyme production of *T. reesei* were reported as 32 – 35 °C and 27 – 28 °C respectively (S. Chen, 1993).

Besides the aforementioned factors, the inoculum level as well as period of incubation have also been identified as influential factors affecting cellulase production (Chahal, 1982; Coutts & Smith, 1976; El-Hadi et al., 2014; Shankar & Isaiarasu, 2011). Since the interaction between these factors is complex, it is important to adopt efficient optimization techniques in order to obtain maximal yield of the enzyme in a particular fermentation process.

2.5 Bacterial cellulase production

2.5.1 Cellular location and distribution of cellulases in bacteria

Cellulases in bacteria can be located intracellularly, extracellularly, and they can be cell-membrane bound. Earlier studies on localization of cellulase activity in bacteria used the term "cell-bound" rather ambiguously and it was difficult to ascertain whether they referred to the intracellular or cell-membrane locations. An example of bacteria that produces only cell-bound cellulases is *Cytophaga* (Chang & Thayer, 1977; X. Ji et al., 2014). Those that produce only extracellular cellulase include *Cellvibrio vulgaris* (Oberkotter & Rosenberg, 1978), *Thermoactinomyces* (Hägerdal, Ferchak, & Pye, 1978) and *Clostridium* sp. (B. H. Lee & Blackburn, 1975). Both cell-bound and extracellular cellulases are produced by *Cellulomonas* (Lo, Saratale, Chen, Bai, & Chang, 2009), *Pseudomonas fluorescens* (Yamane, Yoshikawa, Suzuki, & Nisizawa, 1971), and *Cellvibrio fulvus* (Berg, 1975).

The location of cellulase in bacteria is often determined by the environment/substrate in which it is grown as well as the age of the culture. Berg (1975) reported that cellulase production was predominantly cell-bound when *C. fulvus* was grown on glucose or cellobiose but was extracellularly produced when the strain was grown on cellulose. The enzyme became cell-free when the cells underwent lysis during the stationary phase. Lo et al. (2009) also reported that cellulases were differentially expressed in various cellular locations when *Cellulomonas* spp. were grown on xylan, rice straw and rice husk.

2.5.2 Regulation of cellulase production in bacteria

Cellulase production is generally believed to be controlled by catabolite repression and induction mechanisms. An additional type of control at the level of secretion has been suggested but many aspects of the control of cellulase secretion are not clearly understood at the moment (Coughlan, 1985; Yan & Wu, 2013).

Regulation by catabolite repression involves the repression of cellulase synthesis when readily metabolizable carbon sources such as glucose are present. Addition of such easily utilizable substrates to cultures grown solely on cellulose blocks the synthesis of cellulase by the organism. Derepression of cellulase synthesis then occurs on exhaustion of the simple substrate (Bisaria & Mishra, 1989). In such cases, there is usually an inverse relationship between growth rate and cellulase production (Robson & Chambliss, 1989). Catabolite repression of cellulase synthesis has been shown to be regulated by cyclic AMP (*c*AMP) levels in *Thermomonospora curvata* (Fennington, Neubauer, & Stutzenberger, 1984; F. Stutzenberger, 1985; W. E. Wood, Neubauer, & Stutzenberger, 1984), *Pseudomonas fluorescens* (A. Lejeune, Courtois, & Colson, 1988), and *Acidothermus cellulolyticus* (Shiang, Linden, Mohagheghi, Grohmann, & Himmel, 1991). Cellulases are also susceptible to end-product inhibition. Endoglucanases and exoglucanases are inhibited by cellobiose, while β-glucosidases are inhibited by glucose (Bisaria & Mishra, 1989).

In the regulation of cellulase synthesis by induction, cellulose or its derivatives such as cellobiose induce the production of cellulase when they are present in the culture media. Sophorose and lactose are also known to induce cellulase synthesis. While induction by cellobiose and sophorose are dependent on concentration, with high concentrations repressing cellulase synthesis, high concentrations of lactose continue to induce synthesis and do not cause repression (Bisaria & Mishra, 1989). It is believed that in this system, some basal (constitutive) level of cellulase production occurs which causes the hydrolysis of insoluble cellulose to its smaller derivatives e.g. cellobiose. These in turn induce greater cellulase production (L. R. Lynd et al., 2002). Cellobiose continues to induce cellulase production and the increased cellulose hydrolysis generates more cellobiose until it reaches a concentration at which repression sets in. This creates a balance between repression and induction (Robson & Chambliss, 1989). Inhibition by increased levels of

cellobiose is as a result of the accumulation of intracellular glucose due to the action of cell-bound β-glucosidases on cellobiose (Gong & Tsao, 1979). This regulatory system has been demonstrated by Rodríguez, Alea, and Kyslíkova (1996) with respect to *Cellulomonas*.

2.5.3 Bioprospecting cellulolytic bacteria

The cost of cellulase production is high and its utilization is expected to increase when biofuels gain more relevance in the transport sector. For these reasons, the quest for cellulolytic microorganisms producing novel cellulases and with higher enzyme productivity is an imperative task. An important way to accomplish this is the screening and isolation of cellulolytic organisms from nature. Bacteria that exhibit unique cellulolytic properties have been isolated from various environments such as compost and soil (Okeke & Lu, 2011), paper mill sludge (M. L. Maki et al., 2011), termite gut (Wenzel, Schönig, Berchtold, Kämpfer, & König, 2002), animal dung (Shuchi Singh, Moholkar, & Goyal, 2013), bovine rumen (Halliwell & Bryant, 1963), hot springs (Mohagheghi, Grohmann, Himmel, Leighton, & Updegraff, 1986), and human faeces (Robert & Bernalier-Donadille, 2003).

Screening for cellulase production can be done by enrichment on media containing insoluble cellulosic material as the sole carbon source followed by subculturing of cellulolytic strains on agar medium incorporated with cellulose as carbon source (Rastogi et al., 2009). Cellulosic substrates that can be used in enrichment for screening of cellulolytic microorganisms include filter paper and ball-milled cellulose, acid-treated cellulose, dewaxed cotton string, bacterial cellulose, Avicel, and soluble cellulosics such as CMC and cellobiose (McDonald, Rooks, & McCarthy, 2012). The most commonly adopted strategy of screening for cellulolytic bacteria is plate-screening on agar containing CMC. In this method, the bacteria are grown on the CMC-incorporated media for a certain period after which the plates are then stained with a dye such as 1%

hexadecyltrimethyl ammonium bromide (HAB) (Hankin & Anagnostakis, 1977) or 0.1% Congo red (Teather & Wood, 1982). Hendricks, Doyle, and Hugley (1995) developed a medium which had the dye incorporated into the agar rather than staining with dye after incubation. This allowed for rapid and easier identification and enumeration of cellulolytic strains as well as eliminating the concern of cross-contamination associated with the staining methods. Kasana, Salwan, Dhar, Dutt, and Gulati (2008) later devised a more efficient process with the use of Gram's iodine which allowed for a rapid and easier visualization of cellulolytic activity in less than five minutes. The use of short cello-oligosaccharide substrates with chromogenic or fluorogenic properties has been suggested (M. Maki et al., 2009). However, the limitation with these substrates is their unfavourable diffusibility in agar.

Generally, a major weakness in the use of plate screening methods for enzyme production is that they are not always reliable. Several studies have shown lack of correlation between the halo zones produced on agar media and the actual enzyme production in liquid medium (Ajijolakewu, Leh, Wan, & Lee, 2015; Sridevi & Charya, 2011; Teather & Wood, 1982). Besides, in some situations, strains that produced halo zones on agar did not produce any detectable enzyme activity in liquid media (Tseng, Fang, & Tseng, 2000). Consequently, more efficient and reliable strategies are needed to enable the detection/selection of cellulolytic bacteria.

2.6 Cellulase production on mixed lignocellulosics

Although there is a general dearth of literature regarding the utilization of mixed lignocellulosic substrates as compared to single substrates, a handful of studies are available with respect to cellulase production. However, all of these reports are focused on cellulase production by fungi. To the best of the knowledge of this author, no published report of bacterial cellulase production on mixed lignocellulosic substrates is available. Moreover, almost all of the mentioned reports, with the exception of the work of Olsson

and her colleagues (Olsson, Christensen, Hansen, & Palmqvist, 2003), utilized solid-state fermentation, presumably because this system is generally believed to be more effective for fungal enzyme production (Behera & Ray, 2015; Kuhad et al., 2016). However, considering the advantages associated with the use of bacteria for cellulase production (high growth rate, amenability to genetic manipulation, production of cellulosomes, etc.), and the unique properties of bacterial cellulases (e.g. tolerance of relatively harsh conditions) (M. Maki et al., 2009), it is necessary to explore cellulase production from mixed substrates using bacteria. Besides, the submerged fermentation system is more suitable for bacterial cultivation and it is easier to control process conditions for optimization of enzyme yields under this system (Kuhad et al., 2016; Singhania, Sukumaran, Patel, Larroche, & Pandey, 2010).

The effects of several factors on the production of different kinds of cellulases on mixed lignocellulosics have been studied. Jecu (2000) investigated the effects of fermentation conditions on endoglucanase production by *A. niger* on wheat bran (WB)-wheat straw (WS) mixtures and found that maximum endoglucanase production (14.81 UI) was obtained in 96 hours under optimal conditions of 74 % moisture content, pH range of 4.5 – 5.5, temperature 30 °C, and WB:WS ratio of 1:9. Sherief et al. (2010) reported that the optimal conditions for the production of CMCase, β-glucosidase, exoglucanase, endoglucanase, and xylanase by *A. fumigatus* which was grown on a rice straw-wheat bran mixture were: 75 % moisture level, initial pH 5 – 6, 40 °C, and with $NaNO_3$ as the nitrogen source. Equal ratios of rice straw (RS) to wheat bran (WB) was found to be optimal for all the enzymes except for β-glucosidase whose optimal ratio was 7 RS: 3 WB. Shamala and Sreekantiah (1986) showed that an optimal balance between nutrient levels and the mixed substrate is essential for maximum cellulase yields. Fujian, Hongzhang, and Zuohu (2002) and Mo, Zhang, and Li (2004) studied the effects of different forms of heat and mass transfer control of SSF on cellulase production from

mixed WB/WS substrates and reported that dynamic systems favoured cellulase production than the static system in the studied organism (*Penicillium decumbens*).

The proportions of the individual components of mixed substrates have been found to exert a strong influence on cellulase production. This relationship is dependent on the kind of enzyme and the microorganism. While particular components promoted the production of certain cellulase types in higher proportion, the opposite was observed for other types of cellulase. For example, different proportions of RS and WB have been found to exert different effects on cellulase production by some fungi. Qi et al. (2007) reported that *Trichoderma viride* ZY-01 produced higher FPase on substrates containing higher proportions of RS but found that such mixtures resulted in lower β-glucosidase production. Interestingly, CMCase production was not affected by the ratio of RS-to-WB. Similarly, Kang et al. (2004) reported that while *Aspergillus niger* KK2 produced higher FPase on RS only (and lower FPase on mixtures), CMCase and β-glucosidase production were similar irrespective of the substrate combinations. Xia and Cen (1999) studied the effects of different dosages of WB (20 – 50 %) on cellulase production during the growth of *T. reesei* ZU-02 on corncob residue. At 20 % WB dosage, low fungal growth and low cellulase production was observed while maximum production was obtained with 30 % WB. At higher dosages (> 30 %), cellulase production declined. Thus, an optimal ratio of the individual substrates is crucial for obtaining optimal cellulase production on mixed substrates as far as fungi are concerned. In addtion, it must be ensured that any substrate to be used in mixtures must be favourable for the organism. It would be interesting to observe whether this same phenomenon is applicable when bacteria are used.

It is interesting to note that despite the importance of pretreatment in the bioconversion of lignocellulosics, very few researchers have studied the role of pretreatment on cellulase production from lignocellulosic mixtures. Untreated substrates are used in most cases. It was only in a recent study (Scholl et al., 2015) that an attempt was made to investigate

the role of pretreatment in cellulase production on mixed substrates. However, only one of the mixture components was pretreated. This group found that with mixtures of elephant grass and WB, production of cellulases by *Penicillium echinulatum* S1M29 was generally higher with mixtures containing pretreated and washed grass than those with pretreated but unwashed grass. This observation was attributed to the release of enzyme inducers in the pretreated samples and the removal of inhibitors during washing. Table 2.3 shows a summary of relevant studies involving the use of mixed lignocellulosic substrates for cellulase production.

Table 2.3: Summary of studies utilizing mixed substrates for cellulase production.

Substrates	Organism	Mode of fermentation	Objective(s)	Result	Ref.
WS and WB	*A. niger* 38	SSF	Effects of fermentation conditions and mixture ratios on endoglucanase production.	Best conditions: 74 % moisture, pH 4.5 – 5.5, 30 °C, ratio WS:WB of 9:1.	(Jecu, 2000)
RS and WB	*A. fumigatus*	SSF	Effects of culture conditions on CMCase, exoglucanase, endoglucanase, and β-glucosidase.	Best conditions: 75 % moisture, pH 5-6, 40 °C, NaNO$_3$ as N source, RS: WB of 1:1 for CMCase and endoglucanase, RS:WB of 7:3 for β-glucosidase and exoglucanase.	(Sherief et al., 2010)
Corncob residue and WB	*T. reesei* ZU-02	SSF	i. effects of moisture level, WB dosage, and initial pH on cellulase production. ii. effects of repeated batch process on cellulase production. iii. effects of enzyme dosage on saccharification yield.	i. Best conditions: 70 % moisture level, 30 % WB, pH 4.5 – 6.0, ii. cellulase production highest during second batch fermentation. iii. up to 85% yields obtained at enzyme loadings above 15 IFPU/g substrate.	(Xia & Cen, 1999)
RS and WB	*T viride* ZY-01	SSF	Effects of mixture ratios on production of cellulases.	Optimal RS:WB ratios: 3:2 for FPase; 1:4 for β-glucosidase; 4:1 for CMCase.	(Qi et al., 2007)
RS and WB	*A. niger* KK2	SSF	Effects of mixed substrate compositions on production of cellulases.	FPase higher on RS alone than on mixtures; CMCase and β-glucosidase not affected by mixture composition.	(Kang et al., 2004)

Note: Only aspects related to cellulase production and mixed substrate experiments are presented. SSF- solid-state fermentation; SmF-submerged fermentation.

Table 2.3 (continued): Summary of studies utilizing mixed substrates for cellulase production.

Substrates	Organism	Mode of fermentation	Objective(s)	Result	Ref.
Cellulose (Solka floc), sugar beet pulp and alkaline extracted sugar beet pulp	*T reesei* Rut C-30	SmF	Effects of substrate combinations on endoglucanase production.	Higher endoglucanase production recorded on cellulose or cellulose-containing mixtures.	(Olsson et al., 2003)
RS and WB	*A. ustus,*	SSF	Effects of substrate composition and substrate-minerals ratio on production of cellulases.	Optimal WB:RS ratios: 1: 9 for FPase and CMCase; 9:1 for β-glucosidase.	(Shamala & Sreekantiah, 1986)
Steam-exploded WS and WB	*Penicillium decumbens* JUA10	SSF	Effects of periodically dynamic change of air for heat and mass transfer control on cellulase production.	Higher cellulase production in dynamic culture than in static culture.	(Fujian et al., 2002)
Steam-exploded WS and WB	*Penicillium decumbens* no. 1	SSF	Effects of periodic pressure combined with forced aeration for heat control on cellulase production.	Combined method gave higher cellulase production than static system and single methods of heat control.	(Mo et al., 2004)
Elephant grass (EG) and WB	*Penicillium echinulatum* S1M29	SSF	Effects of pretreatment and washing of EG in mixed substrate on production of cellulases.	Mixtures with pretreated and washed EG favoured higher enzyme production than those with pretreated but unwashed EG.	(Scholl et al., 2015)
Sugar cane bagasse and WB	*Fusarium chlamydosporum*	SSF	Properties of cellulases produced on mixed substrate.	β-glucosidase and CMCase exhibited high pH and temperature stability.	(Qin, He, Li, Ling, & Liang, 2010)

Substrates	Organism	Mode of fermentation	Objective(s)	Result	Ref.
WS, WB, corn cobs and sweet sorghum stalks	*Neurospora crassa*	SSF	i. Effects of substrate composition (single *vs.* mixed) on production of cellulases. ii. optimization of culture conditions for enzyme production.	WS:WB ratio 25:5 (w/w) favoured highest enzyme production than single substrates and higher ratios of WS/WB. Optimal conditions: $NH_2(SO_4)$ as N source; initial pH 5.0 for endo- and exoglucanase, pH 4.0 and 5.0 for β-glucosidase; 70.5 % moisture content.	(Dogaris, Vakontios, Kalogeris, Mamma, & Kekos, 2009)
RS, WB, cauliflower waste, kinnow pulp, and pea-pod waste.	*A. niger* and *T. reesei*	SSF	i. effects of mono- and mixed cultures on cellulase production. ii. effect of mixed substrate composition on cellulase production.	Mono cultures produced higher enzyme activities. WS/WB combination in ratio 3:2 supported higher enzyme production.	(Dhillon, Oberoi, Kaur, Bansal, & Brar, 2011)
RS, WB, (RS + WB), WS, corn cob, bagasse, newspaper, and saw dust	*Scytalidium thermophilum* (*Humicola insolens*)	SSF	i. Effects of carbon sources on cellulase production. ii. Statistical optimization of cellulase production.	RS/WB ratio 1:3 supported higher enzyme production. Optimal conditions: 0.39 % $NH_2(SO_4)$, pH 5.75, and 2 mL inoculum.	(Jatinder, Chadha, & Saini, 2006)

2.7 Substrate features affecting cellulase production

Besides acting as an inducer for cellulase synthesis, the substrate characteristics are important because they affect microbial utilization. Although cellulose is the main component of lignocellulosic biomass which serves an inductive function for cellulase synthesis, the other major components which embed it (lignin and hemicellulose), other superficial simple carbohydrates, non-carbohydrate components, and the physicochemical properties of the substrate exert influences on the ease with which the cellulolytic organism would utilize the substrate. While the effects of soluble low molecular weight carbohydrates (e.g. glucose, sorbose, sophorose, cellobiose, and lactose), soluble cellulosics (e.g. CMC) and insoluble pure cellulosics (e.g. Solka Floc, Avicel, etc.) are relatively well understood (Béguin & Aubert, 1994; James & Ming, 1991; Suto & Tomita, 2001; Tomme et al., 1995), the effects of the unique features of lignocellulosic substrates on cellulase production have not been well articulated in the literature. More attention has only been paid to the role of such features on cellulose enzymatic hydrolysis. However, these two processes (*viz* microbial cellulase production and enzymatic hydrolysis of cellulose) are quite different. For example, while the yield of sugars during enzymatic hydrolysis of lignocellulosic biomass is known to be dependent on the cellulose content of the substrate (Ioelovich & Morag, 2012), cellulase yield on such substrate is not always dependent on the amount of cellulose (Bigelow & Wyman, 2002). Nonetheless, cellulose accessibility is important in both processes. The formation of an enzyme-substrate complex is necessary in the case of constitutive cellulase production which initiates the generation of inducers (see Section 2.5.2 on regulation of cellulase). For cellulosomal organisms, the formation of an enzyme-substrate-microbe complex is equally necessary for cellulase production (L. R. Lynd et al., 2002). Therefore, substrate features that affect the physical contact of the

microorganism to the substrate and those which affect its growth and metabolism would all determine the extent of cellulase production. Some of the substrate characteristics that affect cellulase production are examined below.

2.7.1 Chemical composition

The relative percentage of cellulose, hemicellulose and lignin in a substrate do influence cellulase production by the fermenting microorganism. Each of these components has its own impact on microbial utilization. Cellulose accessibility is also dependent on the lignin and hemicellulose content. Studies have shown that substrates with higher lignin composition, such as acid-pretreated substrates, usually give lower cellulase yields (Bigelow & Wyman, 2002). Besides, lignin has been shown to be unfavourable for growth and cellulose digestibility in some bacteria (Camp et al., 1988; Dehority & Johnson, 1961; Han & Callihan, 1974). Basu and Ghose (1960) showed that the removal of lignin and the presence of hemicellulose led to increase in cellulase production by selected fungi.

2.7.2 Pretreatment-generated inhibitors

When lignocellulosic substrates are pretreated using thermochemical means, chemical compounds called inhibitors are generated. Examples are acetic acid, formic acid, levulinic acid, furfural, and hydroxymethyl furfural (HMF) (Jönsson & Martín, 2016). Substrate-bound inhibitors if not thoroughly washed, could negatively affect cellulase production by the microorganism. Such inhibitors also sometimes exert synergistic effects on cellulase production. Szengyel and Zacchi (2000) reported that different concentrations of acetic acid and furfural affected the production of cellulases by *T. reesei* RUT C30 growing on steam-pretreated willow. Culture media containing the highest concentrations of both inhibitors resulted in complete inhibition of cell growth. Media containing only furfural inhibited the production of cellulases but the inhibitory effect of acetic acid was found to be dependent on the presence of furfural in the medium. Bigelow

and Wyman (2002) showed that *T. reesei* RUT C30 growing on hot water pretreated bagasse liquid hydrolysate displayed much reduced growth rate compared to its growth on thoroughly washed pretreated solids. Furthermore, more extensive washing resulted in higher enzyme production. This observation was attributed to the presence of inhibitory compounds in the pretreated material.

2.7.3 Crystallinity

Evidences from literature suggest that the degree of crystallinity of the substrate affects not only the microbial utilization of the substrate, but also the quantity and quality of cellulases produced. Baker, Quicke, Bentley, Johnson, and Moxon (1959) showed that the digestibility of various substrates by rumen microorganisms was directly related to the degree of crystallinity. More crystalline substrates were less digestible than the amorphous ones. The effect of the crystalline nature of substrates on the quality of the secreted cellulase complex was demonstrated by Evans, Wales, Bratt, and Sagar (1992). They found that *T. reesei* cellulase complex induced by Solka floc was deficient in an endoglucanase compared to that induced by scoured cotton. However, reports about the effects of crystallinity on cellulase production are conflicting. While some researchers claimed that no relationship exists between substrate crystallinity and cellulase production (Han & Callihan, 1974; Mes-Hartree, Hogan, & Saddler, 1988), Brijwani and Vadlani (2011) showed that a relationship actually exists, but such relationship depends on the type of enzyme produced and the culture used for enzyme production. They cultivated mono- and mixed cultures of *T. reesei* and *A. niger* on soybean hulls which had varying crystallinity index as a result of varying degrees of pretreatment. Production of different kinds of cellulases was then assessed under the various conditions. With *T. reesei*, it was found that production of cellulolytic enzymes increased with increasing crystallinity, but with *A. niger*, only endoglucanase production followed this trend. With the mixed cultures, decrease in FPase and endoglucanase was recorded with increase in

crystallinity. The confusion concerning the effects of crystallinity on cellulase production can be attributed to the differences in the methods used by different researchers in preparing substrates of varying degrees of crystallinities and the differences in the methods of measuring crystallinity index (Foston, 2014). For correct conclusions to be made about the role of crystallinity on cellulase production, the methods used in obtaining substrates of varying crystallinity should be such that allow for other substrate features to be controlled. This is because the methods used in published studies so far end up altering other features which also affect enzyme production (Foston, 2014).

2.7.4 Particle size

Reduction of substrate particle size would make it easier for microorganisms and their cellulases to attach to the substrate for the initiation of cellulose utilization or cellulase synthesis. This is due to the increased surface area. Han and Callihan (1974) reported that cellulose digestibility (as indicated by cell growth and substrate loss) increased when mixed cultures of *Cellulomonas* sp. and *Alcaligenes faecalis* were grown on sugarcane bagasse with much smaller particle size (< 60 mesh) compared to that observed on bagasse and computer paper with much larger particle sizes. Dehority and Johnson (1961) also reported that ball-milling of forage resulted in increased cellulose digestibility and that more extensive milling (i.e. smaller particle sizes) produced even higher digestibilities.

2.8 Strategies for improving bacterial cellulase production

Bacteria generally produce lower titers of cellulases as compared to fungi although their cellulases have more unique properties which make them promising for industrial applications. A lot of efforts have been made in order to enhance the natural ability of bacteria to produce cellulases. These measures range from strain improvement efforts to optimization of media and culture conditions.

2.8.1 Bioprospecting hyper-producing strains from various sources

Isolation and screening techniques have been quite useful in the discovery of novel cellulolytic bacteria with either enhanced production abilities or secreting enzymes with unique properties. A novel thermophilic *Ureibacillus terrenus* strain was recently isolated from compost of oil palm empty fruit bunch. Crude enzyme of this strain contained cellulase which was active at both mesophilic and thermophilic temperatures. This strain was reported to be promising for optimising composting processes due to its possession of dual temperature active cellulases and ligninases (Ting, Tay, Peh, Tan, & Tee, 2013). X. H. Li, Bhaskar, Yang, Wang, and Miao (2009) used plate screening technique with CMC-Na agar to isolate a unique *Escherichia coli* strain from a soil sample. This strain was able to produce all the major cellulases and its CMCase showed maximum activity at 60 °C and pH 6.0. Furthermore, this enzyme could tolerate temperatures of up to 70 °C for at least 20 minutes. Numerous reports of isolation of efficient cellulolytic bacteria from unique environments are continuously published. This shows that bioprospecting remains a valuable way of improving cellulase production and discovering biotechnological strains despite recent advancements in genetic engineering and recombinant DNA technology (Labeda, 1990).

2.8.2 Strain improvement

Strain improvement techniques such as mutagenesis, genetic engineering, and heterologous gene expression have been used to develop bacterial strains with improved cellulase production or ability to overcome catabolite repression and end-product inhibition. Sadhu, Ghosh, Aditya, and Maiti (2014) subjected a *Bacillus* strain to treatment with *N*-methyl-*N*'-nitro-*N*-nitrosoguanidine and obtained a mutant with increased production of CMCase, FPase, Avicelase and β-glucosidase compared to the wild type. Kotchoni, Shonukan, and Gachomo (2003) used ethyl methanesulphonate as a mutagenic agent to obtain a catabolite repressed *B. pumilus* mutant strain which produced

up to four times higher cellulase yield than the wild type. Several researchers have used heterologous gene expression to achieve increased cellulase production in *E. coli* and other microorganisms (Chuan Wei, Teoh, Koshy, Salmah, & Zainudin, 2015; S. Gupta, Adlakha, & Yazdani, 2013; Pandey et al., 2014; Zafar, Ahmed, Khan, & Jamil, 2014; X. H. Zhao, Wang, Wang, & Wei, 2012). Metabolic engineering has been applied as well to develop improved cellulolytic strains (Mazzoli, Lamberti, & Pessione, 2012). However, these avenues of improving cellulolytic strains necessitate repeated trials and screening/selection of mutants which require significant time investment and effort.

2.8.3 Optimization of culture media and fermentation conditions

Since cellulase production is inducible and is influenced by the physicochemical conditions of fermentation (L. R. Lynd et al., 2002), optimization of these factors is a good strategy to boost cellulase yields in cellulolytic bacteria. Factors such as carbon and nitrogen sources, and other minerals have been optimized to identify those which promote cellulase production. Cultural conditions such as incubation temperature, initial medium pH, agitation speed, etc. have also been optimized for several cellulolytic bacterial species. Several researchers have optimized media components and process parameters for bacterial cellulase production using the traditional "one-factor-at-a-time (OFAT)" methodology which involves holding some factors constant while studying the effect of another on cellulase production (Abou-Taleb et al., 2009; S. Sethi et al., 2013; Shankar & Isaiarasu, 2011). However, considering the multitude of influential factors involved and the tediousness of investigating the effect of each before performing optimization, statistical methods of optimization using design of experiment (DOE) have been suggested as a superior alternative. DOE techniques such as response surface methodology (RSM) take into consideration the interaction of factors, assist to identify important factors, and allow for conducting optimization experiments using minimal number of experiments (Mandenius & Brundin, 2008). Y.-J. Lee, Kim, Gao, Chung, and

Lee (2012) applied RSM in optimizing carbon sources, nitrogen sources, medium initial pH for cell growth and CMCase production by a recombinant *E. coli* strain expressing CMCase of *Bacillus amyloliquefaciens*. The final CMCase production was three times higher than that obtained under un-optimized conditions. A 33 % improvement in CMCase production (compared to un-optimized system) by *B. subtilis* was also reported after physical parameters for cellulase production were optimized using central composite design (Deepmoni Deka et al., 2013).

CHAPTER 3: ISOLATION AND SEQUENTIAL SCREENING OF BACTERIA WITH POTENTIAL FOR ENDOGLUCANASE PRODUCTION ON MIXED LIGNOCELLULOSIC SUBSTRATES

3.1 Introduction and literature review

Environmental sustainability and economic concerns that accompany the use of fossil resources for the production of fuels and platform chemicals have spurred global interest in better alternatives. Lignocellulosic biomass comprising mostly agricultural and forest residues, dedicated energy crops and municipal solid wastes are considered a viable choice because they are abundant, cheap and their use is environmentally benign. Lignocellulose is a complex matrix that is made up of predominantly cellulose (35-50%), hemicellulose (20-35%), and lignin (5-30%) (L. R. Lynd et al., 2002). Cellulose, a major part of plant cell walls, is a homo-polymer of glucose containing repeating glucopyranoside units linked by β-1,4-glycosidic bonds (O'Sullivan, 1997). Cellulases, a group of synergistic enzymes, hydrolyze the glycosidic bonds in cellulose to produce glucose which can be converted to several value-added products *via* biochemical or thermochemical routes. There are three types of cellulases, namely endoglucanases (acting on internal amorphous regions), exoglucanases (acting on reducing and non-reducing ends of crystalline regions), and β-glucosidases (liberating glucose from cellobiose) (M. Wang et al., 2012). Endoglucanases are of special interest because they initiate cellulose hydrolysis and constitute major part of commercial enzyme cocktails. Cellulases are widely applied in several industries such as textiles, detergent, paper and pulp, medical, food processing, animal feed, brewing, and biofuel industries. Cellulases are expected to constitute a significant share of industrial enzymes (20% as at 2010) if biofuels become the major transportation fuels in the near future (Rani Singhania, 2011).

Cellulases are produced by fungi, bacteria, plants, and some animals. However, commercial production of cellulases is done using fungi due to the production of high

titers of the enzyme by several fungal species. Although bacteria produce lower titers of cellulases, their high growth rate which results in higher enzyme production rate (H Ariffin, Abdullah, Umi Kalsom, Shirai, & Hassan, 2006), and the stability of bacterial cells and enzymes under harsh conditions of bioprocessing makes them a good source of novel cellulases, which can be applied under industrial conditions (M. Maki et al., 2009). Besides, bacteria are more amenable to genetic manipulation that are targeted at improving cellulase production and/or enzyme properties. Although modern strain improvement techniques and protein engineering technology have been used to produce improved cellulolytic strains and cellulases with enhanced properties, traditional screening and isolation is still highly relevant. Isolation of cellulolytic microorganisms from various sources could lead to the discovery of novel cellulase genes which could be adapted into existing strains or which could serve as framework for modifying known cellulase genes. Furthermore, such strains could act as hosts for existing cellulase genes (M. L. Maki et al., 2011).

Various carbon sources have been used as inducers for cellulase production. Some of these include soluble sugars (e.g. cellobiose, lactose, sophorose), pure soluble cellulosics (e.g. carboxymethyl cellulose- CMC), and pure insoluble cellulosics (e.g. Avicel, Solka floc, cotton, microcrystalline cellulose) (Chandel et al., 2012; L. R. Lynd et al., 2002). However, the cost of these substrates has made cellulase production expensive (Umikalsom et al., 1997; Wayman & Chen, 1992). Consequently, the use of cheap lignocellulosic biomass such as oil palm residues (Harun et al., 2013), banana fruit stalk (Krishna, 1999), wheat bran (Da Vinha et al., 2011), and bagasse (Rajoka & Malik, 1997) as substrates for bacterial cellulase production has been explored. In a practical sense, a biorefinery would have to utilize mixed streams of lignocellulosic feedstocks due to the seasonality of biomass supply and other logistics issues that affect the supply of feedstocks (Athanasios A Rentizelas et al., 2009; Shi et al., 2013). More so, the use of

substrate mixtures has been shown to eliminate or reduce the need for additional nutrient supplementation in microbial fermentations (Martín et al., 2008; Thomsen & Haugaard-Nielsen, 2008). Hence, the use of mixed lignocellulosics for cellulase production is still a largely unexplored option for reducing the cost of cellulase production. While mixed lignocellulosics have been investigated for fungal cellulase production (Jecu, 2000; Scholl et al., 2015; Sherief et al., 2010), such reports for bacterial cellulase production are extremely rare, if available at all. To date, only single lignocellulosics have been exploited for the production of bacterial cellulases.

Bioprospecting of bacteria for cellulase production has traditionally been done using screening and isolation on CMC-containing agar plates, followed by flooding of the plates with certain dyes for visualization of cellulolytic activity (Hankin & Anagnostakis, 1977; Kasana et al., 2008; Teather & Wood, 1982). With these techniques, the strain of choice is selected based on the diameter of halo zones produced in the agar. While this method has allowed for the discovery of efficient cellulolytic bacteria, studies have shown that relying solely on plate screening technique may not always be reliable (Ajijolakewu et al., 2015). Poor correlation between halo diameter and true enzyme activity has been reported (Sridevi & Charya, 2011; Teather & Wood, 1982). Also, some enzyme producing strains may not show any detectable halo zone on agar (Tseng et al., 2000). For this reason, an additional level of screening may be necessary to prevent inadvertent exclusion of potential strains with the desired characteristics.

The objective of this study was to isolate a bacterial strain which could effectively utilize mixed lignocellulosic substrates (MS) for the production of cellulases generally, but with special interest in endoglucanase production. An additional level of screening using MS comprising oil palm empty fruit bunch (EFB), oil palm frond (OPF), and rice husk (RH) was introduced following plate screening in order to refine the selection of the strains based on the performance on the targeted substrate.

3.2 Materials and Methods

3.2.1 Collection and preparation of lignocellulosic samples

Fresh OPF samples were obtained from Malaysian Palm Oil Board (MPOB), Bangi, Malaysia. The leaflets were removed and the fresh petioles were cut into smaller pieces and dried under sunlight. Only petioles were used in this study because the leaflets of OPF have other important uses in an oil palm plantation. The petiole has been shown to have higher content of sugars which makes it more desirable for use as feedstock for biofuels and other bioproducts (Zahari et al., 2012). Dried and shredded EFB fibres were obtained from Biocomposting Pilot Facility, UPM, Malaysia. Rice husk (RH) was collected from a paddy field in Kedah, Malaysia. The three biomass samples were reduced to smaller particle size using a Rapid granulator (GK 205-K, Terramar, Hamburg, Germany). The samples were mixed in equal proportions (1:1:1) and the mixture was kept in a dry airtight container until the sample was ready for use. This mixed substrate (MS) was used in the enzyme production experiments.

3.2.2 Isolation of cellulolytic bacteria

Bacteria were isolated from samples of decomposing lignocellulosic residues and their adjacent soils. The residues were oil palm empty fruit bunch, oil palm frond, rice husk, rice straw, coconut husk, and solid paper mill effluent. Ten grams of each sample were suspended into 90 mL of sterile distilled water and stirred at 200 rpm for 30 minutes at room temperature (28 ± 2 °C). The resulting suspension was serially diluted and aliquots of appropriate dilutions were inoculated on CMC agar (CMCA) which is a modified medium of Dickerman and Starr (1951) with the following composition (w/v): K_2HPO_4 (0.08 %), KH_2PO_4 (0.02 %), $MgSO_4 \cdot 7H_2O$ (0.02 %), NaCl (0.02 %), $NaNO_3$ (0.1 %), $CaCO_3$ (0.001 %), yeast extract (0.05 %), CMC (0.5 %; medium viscosity), and agar (2 %). The pH of the medium was adjusted to 7.0 by the addition of 2 M NaOH or HCl and was autoclaved before use. Following a 24-hour incubation period, colonies obtained

were repeatedly subcultured on CMCA until pure cultures were obtained. Pure cultures of the isolates were stored on nutrient agar slants at 4 °C and were subcultured at regular intervals.

3.2.3 Screening of bacteria for cellulolytic ability

Two levels of screening were performed in order to select the most efficient cellulolytic strains.

3.2.3.1 Inoculum preparation

Standardized inoculum of each isolate was prepared as follows: each isolate was grown to its late log phase in Tryptic Soy Broth (TSB). Fifty millilitre of each broth culture was then centrifuged at 6000 rpm for 10 minutes at 4 °C (Sorvall ST 16R, Thermo Fisher Scientific Inc., Germany). The supernatant was discarded and the cell pellet was resuspended in sterile 0.1 % peptone. The cell suspension was adjusted to approximately $OD_{600} = 1.0$ and was used as inoculum in the screening experiments.

3.2.3.2 Primary screening (plate screening on CMC agar)

Five microlitre of the standardized inoculum of each isolate was spot-plated onto fresh CMCA plate in triplicate (Kasana et al., 2008). The plates were incubated for 48 hours at 30 °C. Each plate was then flooded with 0.1 % Congo Red dye for 30 minutes, followed by another flooding with 1 M NaCl for 30 minutes (Teather & Wood, 1982). The dyes were discarded following each flooding of the plates. Isolates that produced zones of hydrolysis (halos) in the agar after Congo Red staining were selected for further studies. The ratio of the halo diameter to the colony diameter was recorded and the mean of triplicate readings was taken as the hydrolytic capacity (HC) ratio of each isolate (Hankin & Anagnostakis, 1977). Isolates with the highest HC values were selected for the second stage of screening.

3.2.3.3 Secondary screening (targeted screening on MS)

In order to identify the isolated strains that could effectively utilize the MS for cellulase production, those isolates with the highest HC values were further cultivated on culture medium containing pretreated MS as the sole carbon source and their culture supernatants were assayed for various cellulolytic activities. MS used in this screening was first subjected to sequential pretreatment with NaOH and autoclaving following the method of Umikalsom et al. (1997) with slight modification by extending the duration of autoclaving to 15 minutes. The washed pretreated solid was dried in an oven at 60 °C for at least 12 hours.

Culture medium containing pretreated MS in place of CMC (and without agar) in the CMCA agar was used in the screening of the selected isolates for cellulolytic activity (total cellulase activity -FPase, endoglucanase, exoglucanase, and β-glucosidase). Standardized inoculum (5 µL) of each isolate was inoculated into triplicate 250 mL flasks, each containing 50 mL of media with 2 % (w/v) pretreated MS. The flasks were incubated at 30 °C for up to 72 hours at 200 rpm. Aliquots of culture supernatant from each flask, collected at 24-hour intervals, were centrifuged at 6000 rpm for 10 minutes at 4 °C. The cell-free supernatant obtained was filtered through a 0.45 µm filter to remove suspended MS particles and the filtrate was used as crude enzyme sample for cellulase activities assay. For each isolate, the highest titer produced for each enzyme from the samples collected (24 – 72 hours) was recorded as the value of cellulase activity for that particular enzyme. The isolate that produced the highest endoglucanase activity from the pretreated MS was chosen as the best strain and was used in the later stages of this work. Cellulolytic activities were also compared across the isolates.

3.2.4 Identification of selected isolates

The isolates that gave some of the highest HC values during primary screening were identified. Identification of the isolates was done by 16S rRNA gene sequencing. Few

colonies of each isolate were inoculated into TSB and grown till the OD_{600} was about 1.0. Genomic DNA was then extracted using i-Genomic BYF DNA Extraction Mini kit (Intron Biotechnology Inc., Korea) following the manufacturer's instructions. The extracted DNA was used as a template for the PCR reaction which contained the following mixture: 27F (5'-AGAGTTTGATCMTGGCTCAG) and 1492R (5'-CGGTTACCTTGTTACGACTT) primers (Frank et al., 2008) (10 mM, 1 µL each), $10 \times$ buffer (12.5 µL), Taq polymerase (0.5 µL), dNTP (10 mM, 1 µL), $MgCl_2$ (10 mM, 1 µL), DNA template (30 ng, 1 µL) and ddH_2O (7.5 µL). The following protocol was applied for PCR: initial denaturation 95 °C, 5 minutes and 35 cycles of denaturation 95 °C, 1 minute; annealing 55 °C, 1 minute; and extension 72 °C, 1 minute 30 seconds. Final extension was done at 72 °C for 5 minutes. The final PCR product was sequenced using ABI 3730*xl* Genetic Analyzer (Applied Biosystems, USA). Similarity search for each isolate was done by checking the sequence against the 16S ribosomal RNA sequences (Bacteria and Archaea) database using the BLAST tool on the NCBI website (http://www.ncbi.nlm.nih.gov/blast/Blast.cgi?PROGRAM=blastn&PAGE_TYPE=Blast Search&LINK_LOC=blasthome). The sequences of the strains with their closest ID matches were submitted to Genbank and accession numbers were obtained for the sequences.

3.2.5 Cellulase assays

3.2.5.1 Total cellulase activity (FPase)

Total cellulase activity assay was performed according to the method of Y. H. P. Zhang, Hong, and Ye (2009). This was done by measuring the amount of reducing sugars liberated from the reaction of 0.5 mL of the crude enzyme preparation with a 1×6 mm filter paper strip that was suspended in 1.0 mL of 0.05 M phosphate buffer (pH 7.0). The reaction mixture was incubated for 2 hours at 50 °C. The reaction was stopped by the addition of 3.0 mL of 3,5- dinitrosalicylic acid (DNS) reagent and boiling for 5 minutes

in a water bath. The amount of reducing sugars released were determined as glucose equivalents using the DNS reagent (Miller, 1959). One unit (U) of enzyme activity was defined as the amount of enzyme that liberated 1 µmol of reducing sugar per mL per minute from the substrate.

3.2.5.2 Endoglucanase assay

Endoglucanase activity was determined by measuring the reducing sugars released after the reaction of 200 µL of the enzyme preparation with 200 µL of 2 % CMC in 0.05 M phosphate buffer (pH 7.0) (Y. H. P. Zhang et al., 2009). The mixture was incubated for 30 minutes at 50 °C and the reaction was stopped by the addition of 800 µL of DNS reagent followed by boiling for 5 minutes. Released sugars were measured as glucose equivalents using the DNS reagent (Miller, 1959). One unit (U) of enzyme activity was defined as the amount of enzyme that liberated 1 µmol of reducing sugar per mL per minute from the substrate.

3.2.5.3 Exoglucanase assay

Exoglucanase activity was determined by measuring the total soluble sugars released from Avicel using a modified method of Y. H. P. Zhang et al. (2009). The enzyme (0.4 mL) was mixed with 1.6 mL of 1.25 % (w/v) Avicel (in 0.05M phosphate buffer, pH 7.0) and the mixture was incubated for 2 hours at 50 °C. The reaction was halted by transferring the reaction tube to an ice-cold water bath. The reaction mixture was then centrifuged at 13000 rpm for 3 minutes. Total soluble sugar content of the supernatant as glucose equivalents was determined using the phenol-H_2SO_4 method (Dubois, Gilles, Hamilton, Rebers, & Smith, 1956). One unit (U) of enzyme activity was defined as the amount of enzyme that liberated 1 µmol of glucose per mL per minute from the substrate.

3.2.5.4 β-glucosidase assay

β-glucosidase activity was determined following the method of T. M. Wood and Bhat (1988). The enzyme (200 µL) was added to a test tube containing 1 mL of 5 mM *p-*

nitrophenyl-β-D-glucoside (*p*NPG) in 1.8 mL of 0.05 M phosphate buffer (pH 7.0). The mixture was incubated for 30 minutes at 50 °C and the reaction was stopped by the addition of 4 mL of 0.4 M glycine buffer (pH 10.8). The *p*-nitrophenol (*p*NP) released was measured at 430 nm. Concentration of *p*NP was extrapolated from standard calibration of µmol *p*NP against absorbance (430 nm). One unit (U) of enzyme activity was defined as the amount of enzyme that liberated 1 µmol of *p*NP per mL per minute from the substrate.

3.3 Results and Discussion

A total of twenty-five bacterial isolates showed good growth on CMCA during initial isolation from various decomposing lignocellulosic biomass samples. These isolates were purified by repeated subculturing on CMCA and stock cultures were kept on nutrient agar slants.

3.3.1 Plate screening on CMC agar and identification of isolates

Each of the twenty-five isolates was screened for cellulolytic ability on CMCA plates by spot-plating and Congo Red staining. Out of these, five isolates showed some of the highest HC values (largest halo zones) and were selected for identification and secondary screening. Identification of the isolates through 16S rRNA gene sequencing revealed the closest identities of the strains as *Klebsiella pneumoniae* (strain M1), *Bacillus aerius* (strain S4F), *Bacillus aerius* (strain S5.2), *Bacillus anthracis* (strain S5) and *Bacillus subtilis* (strain S8.2) with ID values ranging from 99% (M1, S4F, S5.2 and S5) to 100% (S8.2). Table 3.1 shows the closest identities and 16S rDNA GenBank accession numbers for the selected strains. Although strains S5.2 and S4F had very similar identity from BLAST results, their enzyme production characteristics differed significantly; hence they were used as separate strains in these studies. The strains were deposited at the Microbial Culture Collection Unit (UNiCC), Institute of Bioscience, Universiti Putra Malaysia. Accession numbers were obtained for the deposited strains (Table 3.1).

Table 3.1: Identification of selected cellulolytic bacterial isolates.

Isolate	Source	Closest ID	Identity		GenBank Accession Number	UNiCC Accession Number
M1	Paper mill solid effluent	*Klebsiella pneumoniae*	99%		KP178218	UPMC 1183
S4F	Sugarcane bagasse	*Bacillus aerius*	99%. score total 2619; cover 99%	Max 2619; score query	KP178214	UPMC 1180
S5.2	EFB residues	*Bacillus aerius*	99%. score total 2625; cover 100%	Max 2625; score query	KP178216	UPMC 1179
S8.2	EFB adjacent soil	*Bacillus subtilis*	100%		KP178217	UPMC 1182
S5	Paddy straw residues	*Bacillus anthracis*	99%		KP178215	UPMC 1181

Four of the highly cellulolytic strains isolated in this study are species of the genus *Bacillus*. Members of this genus (e.g. *B. subtilis*) are known for cellulase production (Y.-K. Kim, Lee, Cho, Oh, & Ko, 2012). However, reports of cellulase production by *B. anthracis* are not common, probably due to its pathogenic nature. The relatively high cellulase activity exhibited by *B. anthracis* S5 in this study could indicate the presence of novel cellulase genes in the isolate which could be adapted into non-pathogenic microorganisms for improved cellulose hydrolysis. Cellulase production has not been reported for *B. aerius* prior to this study. This bacterium was first announced as a novel species of *Bacillus* in 2006 after it was isolated from cryogenic tubes used for air sampling at high altitudes (Shivaji et al., 2006). Since then, the most commonly reported enzyme of biotechnological importance in this organism is lipase (Narwal, Saun, Dogra, Chauhan, & Gupta, 2015; Saun, Mehta, & Gupta, 2014; Saun, Narwal, Dogra, Chauhan, & Gupta,

2014). Furthermore, a search of NCBI (*http://www.ncbi.nlm.nih.gov/*) and CAZy (*www.cazy.org*) databases for proteins produced by this specie and its available genome data revealed no information for cellulase. Thus, the strains isolated in this study are potentially useful for further studies. *K. pneumoniae* has been reported to produce cellulase in previous studies (Anand et al., 2010; Ng et al., 2013), and like *B. anthracis*, its cellulase genes could be of potential biotechnological interest despite being classified as a pathogenic organism.

Results of the plate assays for the isolates are shown in Figure 3.1 (*A-E*). The halo zones produced and their HC values are indicated. The highest cellulolytic activity as indicated by HC values was displayed by *B. subtilis* S8.2 (4.22 ± 0.11) while the lowest was recorded for *K. pneumoniae* M1 (1.63 ± 0.11). *B. aerius* S4F and *B. aerius* S5.2 presented different HC values of 2.53 ± 0.17 and 4.06 ± 0.18 respectively. These were higher than that recorded for *B. anthracis* S5. The HC value is a measure of assessing microbial cellulolytic activity through the hydrolysis of CMC in the agar (Hankin & Anagnostakis, 1977).

| (A) *K. pneumoniae* M1 (HC = 1.63 ± 0.11) | (B) *B. anthracis* S5 (HC = 2.53 ± 0.17) | (C) *B. aerius* S4F (HC = 3.61 ± 0.24) | (D) *B. aerius* S5.2 (HC = 4.06 ± 0.18) | (E) *B. subtilis* S8.2 (HC = 4.22 ± 0.11) |
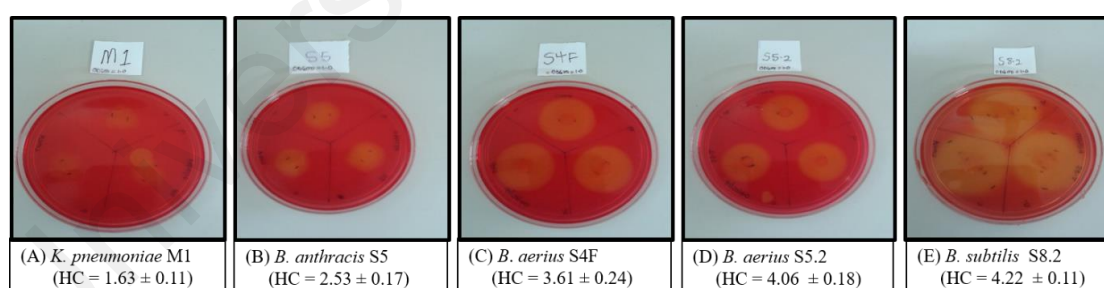
Figure 3.1: Plate screening of bacteria for cellulase activities: (*A-E*) halo zones produced by cellulolytic bacteria on CMCA with their HC values.

3.3.2 Targeted screening on pretreated MS

Since it was the goal of this study to isolate a prospective bacterial strain for the production of cellulase from MS, a second level of screening applying this substrate, was conducted. The titers of various cellulase activities produced by the isolates during their growth on pretreated MS were determined. Result of the second screening are presented

in Figure 3.2. *B. aerius* S5.2 produced the highest titers of FPase (0.03 U/mL), endoglucanase (0.05 U/mL), and exoglucanase (0.09 ± 0.02 U/mL). *B. aerius* S4F produced the highest amount of β-glucosidase (0.03 U/mL). *B. anthracis* S5 and *K. pneumoniae* M1 produced the least amount of all the enzymes assayed.
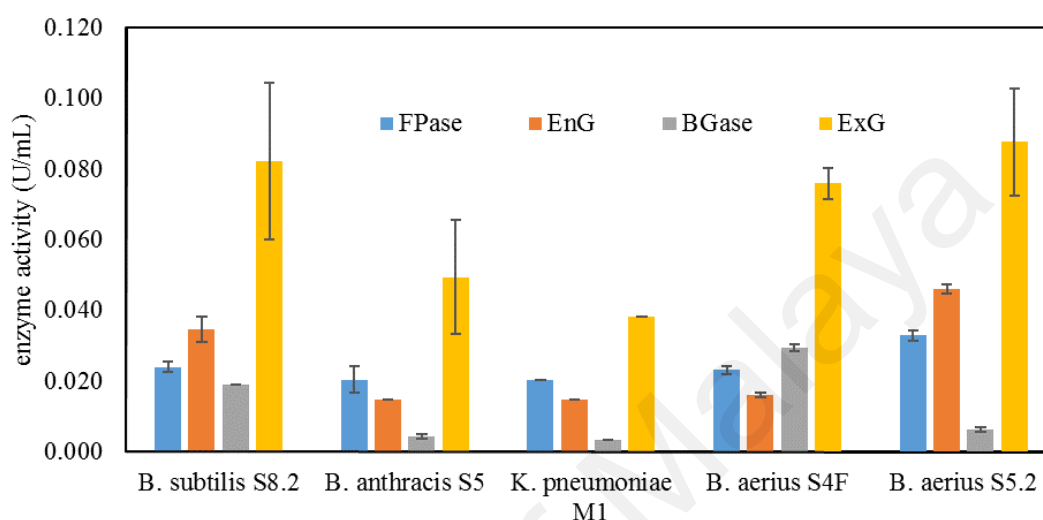
Figure 3.2: Maximum titers of cellulases produced by selected isolates during targeted screening on pretreated MS. *FPase*, total cellulase activity; *EnG*, endoglucanase; *ExG*, exoglucanase; *BGase*, β-glucosidase. Error bars represent standard deviation of three replicates.

Contrary to what was observed during plate screening where *B. subtilis* S8.2 had the highest HC value, *B. aerius* S5.2 showed superior cellulolytic ability than the rest of other strains during targeted screening. This observation underscored the importance of applying sequential and targeted screening in selecting cellulase producers. If the plate screening alone was relied upon, *B. subtilis* S8.2 would have been selected as the most productive strain whereas, in actual fact its cellulase production on the MS was actually lower compared to *B. aerius* S5.2. The limitation of using zone of clearance on agar plate as a means of selecting enzyme-producing strain has been highlighted in several studies. Ajijolakewu et al. (2015) compared fungal xylanase production on agar plates with its production in solid state fermentation (SSF). Very poor correlation between the two methods of evaluating enzyme production was observed. The halo zone diameters

produced on agar medium were not consistent with titers recorded in SSF. In fact, some strains which produced no halo zones had shown higher enzyme activities. This disparity can be attributed to the differential growth and enzyme production of different species on agar as well as differences in rates of diffusion of the enzymes on solid media (Teather & Wood, 1982).

Most studies involving screening and isolation of cellulolytic strains almost rely solely on plate screening with CMC agar for selecting the desired organisms. This procedure is most often not performed in parallel with other methods. Furthermore, the actual substrate to be used for cellulase production is not always the one utilized in the screening exercise. The use of substrates with practical relevance (rather than synthetic ones) during screening would help to increase the chances of discovering the enzymes and biocatalysts that are more process-relevant (Dowe, 2009; Himmel, Decker, & Johnson, 2012; Y.-H. P. Zhang, Himmel, & Mielenz, 2006).

3.4 Conclusions

Continuous bioprospecting for efficient cellulase-producing microorganisms is essential for lowering the cost of cellulase production and discovery of novel genes which encode enzymes with better properties. Bacterial cellulases have attracted much interest due to the high growth and enzyme production rates and the stability of bacterial cells and enzymes under harsh conditions. The use of mixed lignocellulosic biomass as substrates for bacterial cellulase production has been scarcely explored. In this study, a two-step sequential strategy using plate screening on CMCA and targeted screening on alkali-pretreated MS was applied in obtaining potential strains that could utilize MS for cellulase production. A *Bacillus aerius* strain with strong cellulolytic ability was isolated. The strain showed the highest FPase, endoglucanase and exoglucanase activities on MS amongst other strains tested. The strategy employed in this study highlighted the

inadequacy of applying plate screening method alone for bioprospecting of cellulolytic strains.

CHAPTER 4: EFFECTS OF SINGLE AND MIXED CARBON SOURCES ON THE PRODUCTION AND LOCALIZATION OF ENDOGLUCANASE IN *Bacillus aerius* S5.2

4.1 Introduction and literature review

Lignocellulosic biomass is an abundant natural resource which is considered a viable replacement for fossil resources in the production of fuels and platform chemicals. Cellulose, the major structural polysaccharide of lignocellulose, is made up of glucose units which can be converted into value-added products through various means. For this to be done, cellulases, a group of synergistic enzymes which hydrolyze β-glycosidic bonds in cellulose, are required. Cellulases are categorized into three major groups: endoglucanases, exoglucanases, and β-glucosidases (L. R. Lynd et al., 2002). Although the concerted action of the three cellulase types leads to efficient cellulose hydrolysis, this feature may be undesirable in some situations due to the varied activities and substrate specificities of the individual cellulase components (Puranen et al., 2014). Hence, it may be necessary to focus on the production of certain cellulase types whose properties are more suitable for specific applications. Endoglucanases are of special interest because they initiate cellulose hydrolysis and their action on amorphous regions of crystalline cellulose matrix is the rate-limiting step of cellulose utilization (Malherbe & Cloete, 2002). Furthermore, endoglucanases have special applications in textile and food processing industries (Juturu & Wu, 2014; Puranen et al., 2014).

Commercial production of cellulases has been carried out predominantly using fungi. This is because of the higher titers produced by fungi compared to bacteria. However, considering the high growth rate of bacteria, the stability of their cells and enzymes under relatively harsh conditions, their easier genetic amenability, and their production of efficient multienzyme complexes (cellulosomes), bacterial cellulases may be competitive for commercial production (M. Maki et al., 2009; M. L. Maki et al., 2011).

The quantity and quality of cellulase produced by a microorganism has been shown to be strongly dependent on the nature of the carbon source (Dashtban et al., 2011; Evans et al., 1992) and is controlled by repression and induction mechanisms (Bisaria & Mishra, 1989; James & Ming, 1991). The carbon source also influences the location of the cellulases in the cell. Studies have shown that cellulases are expressed differentially in various cell locations when grown on different substrates (Berg, 1975; Lo et al., 2009). Various carbon sources such as sugars (e.g. cellobiose, lactose, and sophorose) and pure cellulosics (carboxymethyl cellulose -CMC, Avicel, Solka floc, etc.) are good inducers of cellulase but the current use of such substrates on a commercial scale is a contributing factor to the high cost of cellulase because they are expensive (S. Chen & Wayman, 1991). In fact, sensitivity analysis has shown that the carbon source is the major cost factor in cellulase production (Ryu & Mandels, 1980). Hence, researchers have explored the use of lignocellulosic biomass as substrates for cellulase production since they are cheap and abundant.

The preponderance of studies concerning the use of lignocellulosics for bacterial cellulase production have focused mainly on the use of single substrates (Assareh, Shahbani Zahiri, Akbari Noghabi, Aminzadeh, & Bakhshi Khaniki, 2012; Da Vinha et al., 2011; Harun et al., 2013; Krishna, 1999). However, this approach might be unsustainable in a real biorefinery situation as feedstock supply is subject to seasonal variation and other logistic problems (Allen, Browne, Hunter, Boyd, & Palmer, 1998; A.A. Rentizelas et al., 2009; Sokhansanj & Hess, 2009). Already, studies have shown that the use of mixed substrates in biorefineries can help lower logistic costs as well as eliminate the need for extraneous nutrient supplementation (Martín et al., 2008; Sultana & Kumar, 2011; Thomsen & Haugaard-Nielsen, 2008). Consequently, the use of mixed substrates would be an interesting option to explore for lowering cellulase production cost.

Some members of the genus *Bacillus* are known to be good cellulase producers (Acharya & Chaudhary, 2012; Asha & Sakthivel, 2014; Balasubramanian & Simões, 2014; Gaur & Tiwari, 2015). Some *Bacillus* spp. are also known to produce cellulases that are stable under extreme conditions (Annamalai, Thavasi, Vijayalakshmi, & Balasubramanian, 2011; Rastogi et al., 2010; Trivedi et al., 2011). However, cellulase production has not been reported previously for *B. aerius*. This specie was first classified as a novel *Bacillus* specie in 2006 after Shivaji et al. (2006) isolated it from cryogenic tubes used for sampling air at high altitudes. At the time of writing this report, the only enzyme of biotechnological interest reported for this organism is lipase (Narwal et al., 2015; Saun, Mehta, et al., 2014; Saun, Narwal, et al., 2014). *B. aerius* S5.2 used in this study was isolated from decomposing oil palm empty fruit bunch (EFB) samples in Malaysia. This strain showed unique ability to produce endoglucanase from mixed rice and oil palm residues. Since little is known at present regarding the cellulolytic system of *B. aerius*, this study was conducted to study the effects of various single and mixed carbon sources on its endoglucanase production as well as investigating the location(s) of its endoglucanase when grown on different substrates. The manner of utilization of the mixed substrate by this bacterium was also studied by determining the extent of degradation after seven days of cultivation.

4.2 Materials and methods

4.2.1 Bacterial strain

Bacillus aerius S5.2 used in this study was isolated from decomposing EFB residues that were collected from an oil palm plantation in Kuala Selangor, Malaysia. This strain produced high titers of endoglucanase on a mixed substrate (MS) that comprised EFB, oil palm frond (OPF), and rice husk (RH). It was identified as *B. aerius* following sequencing of the 16S rRNA gene and sequence similarity check using the BLAST tool on the NCBI database. The sequence was submitted to GenBank and an accession number (KP178216)

was obtained. The cell morphology was observed using Field Emission Scanning Electron Microscope (FESEM) (JSM-7001F, JOEL, Tokyo, Japan). Gram staining and the non-staining KOH method (Buck, 1982) were used to determine the Gram reaction of the strain. Stock cultures of the strain were stored on nutrient agar slants and kept at 4 °C with regular subculturing.

4.2.2 Carbon sources for endoglucanase production

The carbon sources used for induction of cellulase production comprised two pure cellulosics: insoluble (microcrystalline cellulose-MCC), and soluble (CMC); single pretreated lignocellulosics (EFB, OPF, and RH); and pretreated MS (Table 4.1). Medium containing no cellulosic carbon source was used as control in the experiments.

Table 4.1: Carbon sources used for endoglucanase production by *B. aerius* S5.2.

	Pure cellulosic substrate	Lignocellulosic substrate
Soluble	CMC	-
Insoluble	Microcrystalline cellulose (MCC)	EFB, OPF, RH, MS

Fresh OPF samples were obtained from Malaysian Palm Oil Board (MPOB), Bangi, Malaysia. The leaflets were removed and the fresh petioles were cut into smaller pieces and dried under sunlight. Only the petioles were used in this study because the leaflets of OPF have other important uses in an oil palm plantation. The petiole has been shown to possess higher content of sugars which makes it more desirable for use as feedstock in biofuels and other bioproducts processes (Zahari et al., 2012). Dried and shredded EFB fibres were obtained from Biocomposting Pilot Facility, UPM, Malaysia. Rice husk (RH) was collected from a paddy field in Kedah, Malaysia. The three biomass samples were reduced to small particles (300 – 425 µm) using a Rapid granulator (GK 205-K, Terramar, Hamburg, Germany). The MS carbon source was prepared by mixing EFB, OPF, and RH in equal proportions (1:1:1). The mixture was kept in a dry airtight container until the sample was ready for use.

The lignocellulosic substrates used in this study were pretreated sequentially with NaOH and autoclaving according to the method of Umikalsom et al. (1997) with slight extension of the autoclaving duration to 15 minutes. The washed pretreated solids were dried in an oven at 60 °C for at least 12 hours.

4.2.3 Culture media

Modified version of the medium for cellulolytic microorganisms described by Dickerman and Starr (1951) containing 2 % (w/v) of the respective carbon sources was used for endoglucanase production. Other components of the medium are (w/v): K_2HPO_4 (0.08 %), KH_2PO_4 (0.02 %), $MgSO_4.7H_2O$ (0.02 %), NaCl (0.02 %), $NaNO_3$ (0.1 %), $CaCO_3$ (0.001 %), and yeast extract (0.05 %). The medium pH was adjusted to 7.0 using 2 M NaOH or HCl. Sterilization of the media was done at 121 °C for 15 minutes in an autoclave.

4.2.4 Endoglucanase production

B. aerius S5.2 was cultivated in nutrient broth until the late log phase (12 h) was reached. Aliquots (containing approximately 10^7 cfu/mL) from this culture were used as inocula in the experiments. Five percent inoculum (v/v) was inoculated into 250 mL Erlenmeyer flasks containing the culture media with the respective carbon source. Each flask was incubated at 30 °C and at 200 rpm agitation speed for 72 h. Triplicate flasks were used for each carbon source. Aliquots of culture sample were initially collected after 6 h, and at 12-h intervals subsequently. Collected samples were centrifuged at 6000 rpm for 10 minutes at 4 °C. The cell-free supernatant was used as the crude enzyme preparation for the endoglucanase assay.

4.2.5 Growth measurement

Growth of the organism at each sampling period was monitored by estimating total cfu count in the culture supernatant using the drop plate technique (Herigstad, Hamilton, & Heersink, 2001).

4.2.6 Reducing sugar measurement

Amount of reducing sugars in the culture supernatant a result of the bacterial cellulolytic activities at each sampling period was determined using the DNS method (Miller, 1959).

4.2.7 Localization of endoglucanase

To determine the localization of endoglucanase in the cell, *B. aerius* S5.2 was grown in the culture media with 2 % (w/v) of either CMC or pretreated MS as carbon source at 30 °C for 36 hours and 200 rpm. The culture broth (30 mL) was centrifuged at 8000 rpm for 10 minutes at 4 °C and the supernatant was used as the extracellular enzyme sample. The cell pellet was washed twice with 10 mL of 0.05 M phosphate buffer (pH 7.0) and later resuspended in 15 mL of the same buffer. It was then kept on ice to preserve enzyme activity. The intracellular and membrane-bound fractions of the enzyme were prepared by sonication (Lo et al., 2009). A preliminary experiment was conducted to determine the appropriate sonication amplitude to use. Aliquot samples of the cell pellet suspension were sonicated at varying amplitudes (10, 30, 50, 70 and 90 %) using a probe type sonicator (Branson Ultrasonics Corp., USA). A control was set up without sonication. The samples were placed on ice and pulse sonication was performed for 10 minutes with 30/10 seconds on and off intervals, respectively. Endoglucanase activity was then determined for each sample. It was found that the samples corresponding to 30 % amplitude treatment gave the highest endoglucanase activities (see Appendix A). This amplitude was subsequently used in later experiments. The cell pellet suspension kept on ice was sonicated as described above but at 30% amplitude. This was followed by centrifugation at 8000 rpm for 10 minutes at 4 °C. The supernatant obtained was used as

the intracellular enzyme sample. The cell pellet was resuspended in 5 mL of buffer (in order to concentrate the sample) and was used as the membrane-bound enzyme sample. All the enzyme fractions were analysed for endoglucanase activity and protein concentration. Enzyme activity in the various fractions was expressed as enzyme units per µg protein because different volumes of buffer were used in resuspending the samples.

4.2.8 Protein concentration measurement

Protein concentration was determined using the Bradford assay (Bradford, 1976). The enzyme suspension (100 µL) was mixed with 5 mL of Bradford reagent (Sigma-Aldrich, USA). The mixture was kept at room temperature for 10 minutes after which the absorbance was read against reagent blank (100 µL buffer + 5 mL reagent) at 595 nm. The protein concentration was determined by extrapolation from a standard calibration of different concentrations (µg/mL) of bovine serum albumin suspended in 0.05 M phosphate buffer *versus* absorbance at 595 nm (Appendix B).

4.2.9 Endoglucanase assay

Endoglucanase activity was determined by measuring the reducing sugars released after the reaction of 200 µL of the enzyme preparation with 200 µL of 2 % CMC in 0.05 M phosphate buffer (pH 7.0) (Y. H. P. Zhang et al., 2009). The mixture was incubated for 30 minutes at 50 °C and the reaction was stopped by the addition of 800 µL of DNS reagent followed by immersing the reaction tubes in boiling water for 5 minutes. Released sugars were measured as glucose equivalent using the DNS reagent (Miller, 1959). One unit (U) of enzyme activity was defined as the amount of enzyme that liberated 1 µmol of reducing sugar per mL per minute from the substrate.

4.2.10 Degradation of MS by *B. aerius* S5.2

Five millilitres of *B. aerius* S5.2 inoculum from late log phase was transferred into a 250-mL conical flask that contained 50 mL of medium with 1 g of MS as the carbon source.

The flask was incubated for seven days at 30 °C with 200 rpm agitation. A control was prepared with another flask, containing the same amount of substrate, but with no inoculum. All the experiments were conducted in triplicates. The extent of degradation of MS was determined by calculating the dry weight loss of the substrate. After the cultivation period, the entire content of each flask was poured onto a dry Whatman No.1 filter paper (initial weight W_0). The liquid was allowed to drain completely, and the residue with the filter paper was dried in an oven at 70 °C until constant weight (W_1). The extent of degradation of MS was expressed as percentage dry weight loss of the substrate as follows:

$$1.0 - (W_1 - W_0) \times 100\% \qquad \text{(Eq. 4.1)}$$

The numerical value 1.0 represented the initial amount of MS (1 g) used in the degradation experiment. The mean of three replicates was recorded as the final dry weight loss.

4.3 Results and Discussion

This study aimed to determine the effects of selected soluble, insoluble, single and mixed cellulosic carbon sources on endoglucanase production by *B. aerius* S5.2. The localization of endoglucanase activities when grown on some of the carbon sources was also determined.

4.3.1 Morphology of *B. aerius* S5.2

The morphology of *B. aerius* S5.2 was observed using FESEM. Its Gram reaction was also confirmed using normal Gram staining as well as non-staining KOH method. *B. aerius* S5.2 cells were found to be Gram-positive and rod-shaped. Micrographs of the cell

morphology are presented in Figure 4.1 (*a & b*). The cell size was 0.40 – 0.50 by 1.22 –

2.49 µm, while its endospores ranged between 0.23 and 0.26 µm in size.
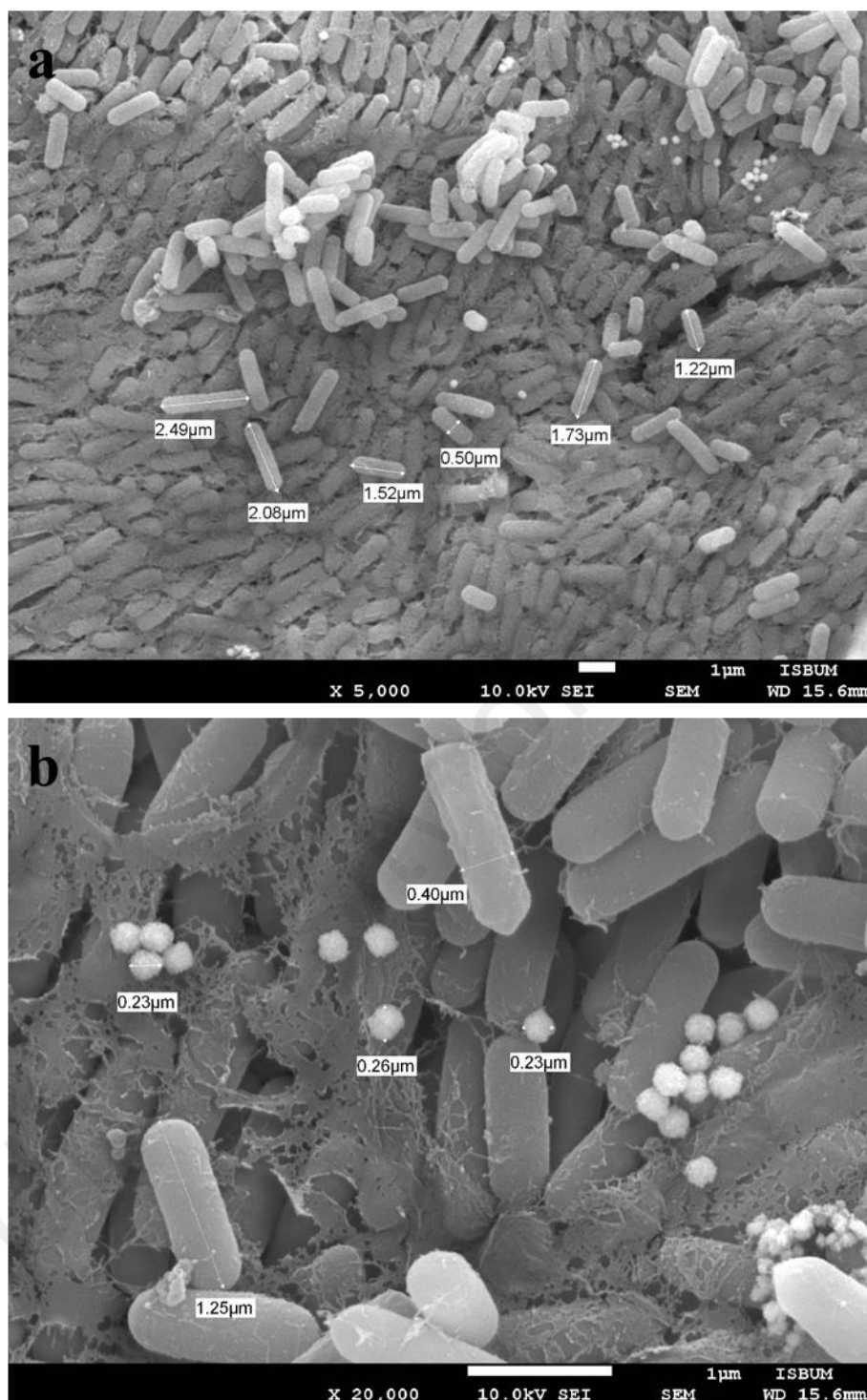
Figure 4.1: Morphology of *B. aerius* S5.2 cells as seen under field emission scanning electron microscope. (a) cells (× 5,000); (b) cells and endospores (× 20,000).

4.3.2 Growth and endoglucanase production on various cellulosic carbon sources

The growth curve and endoglucanase production profile of *B. aerius* S5.2 during growth on various cellulosic carbon sources are presented in Figures. 4.2 and 4.3 respectively. The organism showed better growth on the lignocellulosic substrates (single and mixed) than on pure cellulosic substrates (CMC and MCC). The least suitable substrate for growth was MCC, followed by CMC as the maximum growth values (9.07 log cfu for MCC and 11.52 log cfu for CMC) obtained on these substrates were inferior. The highest growth was recorded on EFB (max. 16.38 log cfu) and OPF (max. 15.46 log cfu). Comparable maximum growth values were recorded on MS (12.96 log cfu) and RH (12.92 log cfu) although growth of *B. aerius* S5.2 was observed to be faster on the latter.

A similar trend was also observed for endoglucanase production. MCC and CMC produced the least enzyme titers while higher titers were recorded on the lignocellulosic substrates. The maximum endoglucanase titer recorded on MCC was 0.108 ± 0.050 U/mL while that of CMC was 0.305 ± 0.063 U/mL. On the other hand, the maximum endoglucanase titers recorded on MS (0.787 ± 0.062 U/mL) and RH (0.658 ± 0.019 U/mL) were the highest among all the carbon sources investigated. Endoglucanase production on EFB and OPF was very similar, with maximum titers of 0.47 ± 0.056 U/mL and 0.463 ± 0.007 U/mL respectively. Based on the maximum enzyme titers obtained on each substrate, it was observed that MS supported endoglucanase production 7.3, 2.6, and 1.2 times better than MCC, CMC, and RH respectively. Also, MS produced 1.7-fold higher endoglucanase titers than OPF and EFB.

From Figs. 4.2 and 4.3, it was observed that endoglucanase production correlated with the growth pattern of the bacterial culture on the substrates. This is in agreement with a previous study which showed that cellulase production is growth-related (H Ariffin et al., 2006). The higher cell growth and enzyme production recorded on the lignocellulosic

substrates could be due to the availability of more growth-promoting substances in them. Lignocellulosic biomass materials have in addition to cellulose, hemicellulose, and lignin, other components such as proteins (A Sluiter et al., 2008). These may have improved the metabolism of the bacterium as compared to the pure cellulosic carbon sources. W. Yang et al. (2014) compared CMCase production on CMC, MCC, rice hull, and wheat bran by *B. subtilis* BY-2. They reported that the highest enzyme production was obtained on wheat bran while rice hull and CMC gave lower enzyme yields. Very low CMCase titer was recorded on MCC. Similar observation was reported by Chan and Au (1987) where *Bacillus subtilis* AU-1 produced lower cell yields and CMCase when grown on pure cellulosics such as Sigmacell 20 and filter paper compared to other carbon sources that were tested. In contrast, Harun et al. (2013) reported that microcrystalline cellulose supported higher cellulase production by *Thermobifida fusca* than pretreated EFB used in their study. However, this could be attributed to strain differences and the fact that the authors dried their substrates at 105 °C after pretreatment while the substrates used in this study were dried at 60 °C. The high temperature used for drying the EFB may have destroyed protein components of the substrate, thereby reducing the nutrients for growth and enzyme production.

One important observation from the results is that maximum endoglucanase titer on MS was significantly higher ($p < 0.05$) than on the pure cellulosics, and on EFB and OPF. The only exception was RH, which showed similar titers with MS. This was possibly due to the combination of the favourable characteristics (e.g. nutrients, cellulose accessibility, etc.) of each individual lignocellulosic in the mixture. This strategy is a worthwhile route to reducing the cost of cellulase production which is currently based on expensive synthetic substrates. Furthermore, the use of mixed lignocellulosics would make it easy to handle feedstock supply fluctuations to the biorefinery (Nilsson & Hansson, 2001). The use of mixed feedstocks has also been shown to be able to bring about reduced

delivery costs to the biorefinery compared to the use of single type feedstocks (Sultana & Kumar, 2011). Studies in other applications of lignocellulosic mixtures have demonstrated that combining substrates usually has no negative effects on product yields, more often than not higher yields were obtained on mixtures than on the single substrates. Such observations have been reported with respect to pretreatment and hydrolysis of mixed substrates (J. Jensen et al., 2008; R. D. O. Moutta et al., 2014; R. D. O. Moutta, R et al., 2013; Pereira et al., 2015), bioethanol production (Erdei et al., 2010; Pereira et al., 2015), and fungal cellulase production (Olsson et al., 2003).
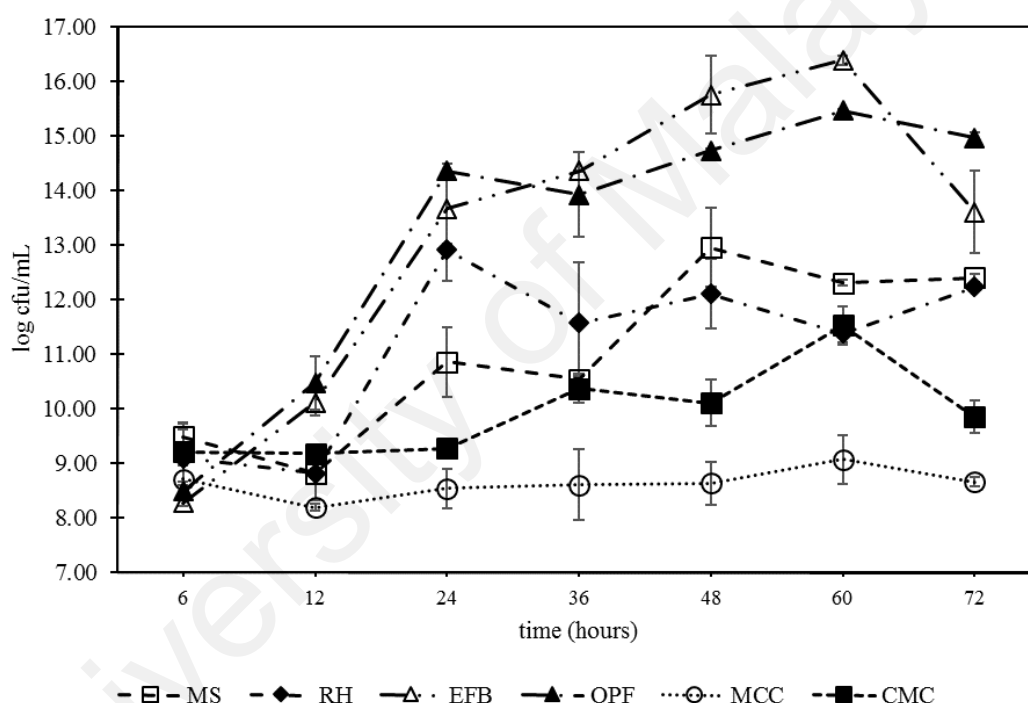
Figure 4.2: Growth curve of *B. aerius* S5.2 on various cellulosic substrates. Error bars represent standard deviation of three replicates.
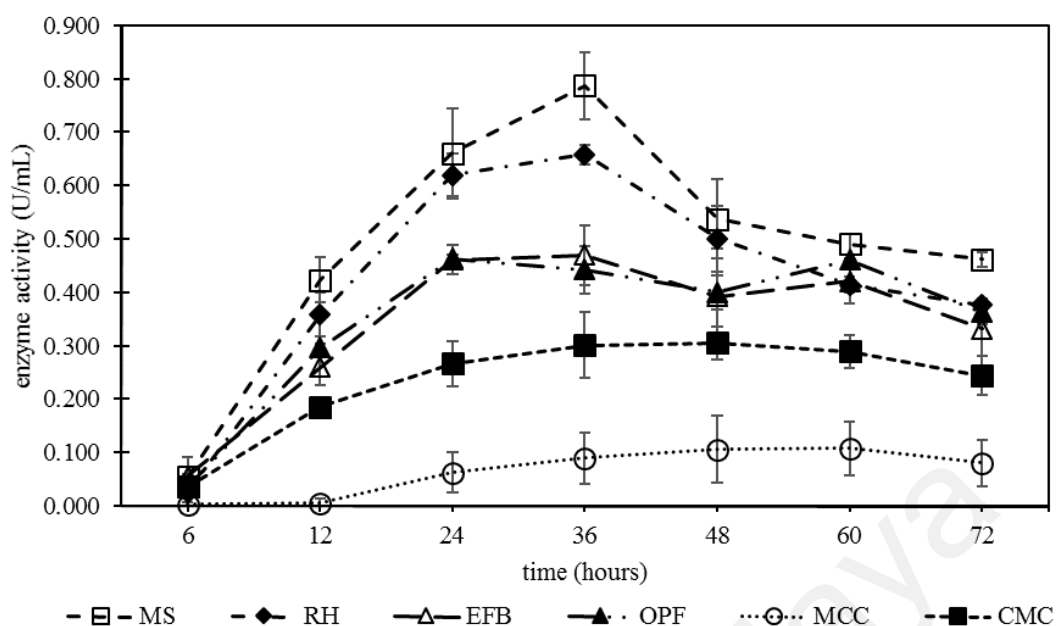
Figure 4.3: Endoglucanase production by *B. aerius* S5.2 on various cellulosic substrates. Error bars represent standard deviation of three replicates.

The reducing sugar profile of the culture supernatants during growth on the carbon sources is presented in Fig. 4.4. Unlike what was observed in Figs. 4.2 and 4.3, where higher growth and endoglucanase production were obtained on the lignocellulosic substrates than on MCC and CMC, no such trend was observed with respect to residual reducing sugar in the culture supernatants. The amount of reducing sugar released into the medium is an indication of the relative digestibility of a substrate and also an indication of the bacterium's cellulolytic ability (Han & Callihan, 1974). Hence, reducing sugars in the culture medium are generated as a result of the hydrolytic activity of the enzymes secreted by the bacterium. Extremely low levels of reducing sugar were detected in the MCC medium because it was a poor carbon source for growth and enzyme production (Figs. 4.2 and 4.3). Interestingly, the highest amount of sugar was produced in the CMC medium despite the fact that lower endoglucanase titers were produced on this substrate. An explanation for this observation was the increased level of hydrolysis of CMC compared to the other substrates. Endoglucanase is known to have high substrate specificity for CMC and lower specificity for crystalline cellulose (Dobrev & Zhekova,

2012; C. H. Kim, 1995; Miotto et al., 2014). The complex nature of the lignocellulosic substrates used in this study may have resulted in lesser affinity for the enzyme as a result of inhibitory effects of lignin on cellulase action (D. Gao et al., 2014; Rahikainen et al., 2013). Hence, the enzyme produced into the medium must have hydrolysed the CMC better, thus producing higher amount of reducing sugar than was obtained on the other substrates. The differences in the levels of reducing sugars produced in the media of the lignocellulosic substrates are probably due to the differences in the structural and physicochemical characteristics of the substrates as a result of the pretreatment. Pretreatment creates differences in the properties (e.g. chemical composition, crystallinity, accessible surface area, porosity, etc.) of lignocellulosic substrates which consequently result in varying degrees of digestibility with cellulase (Meng & Ragauskas, 2014).
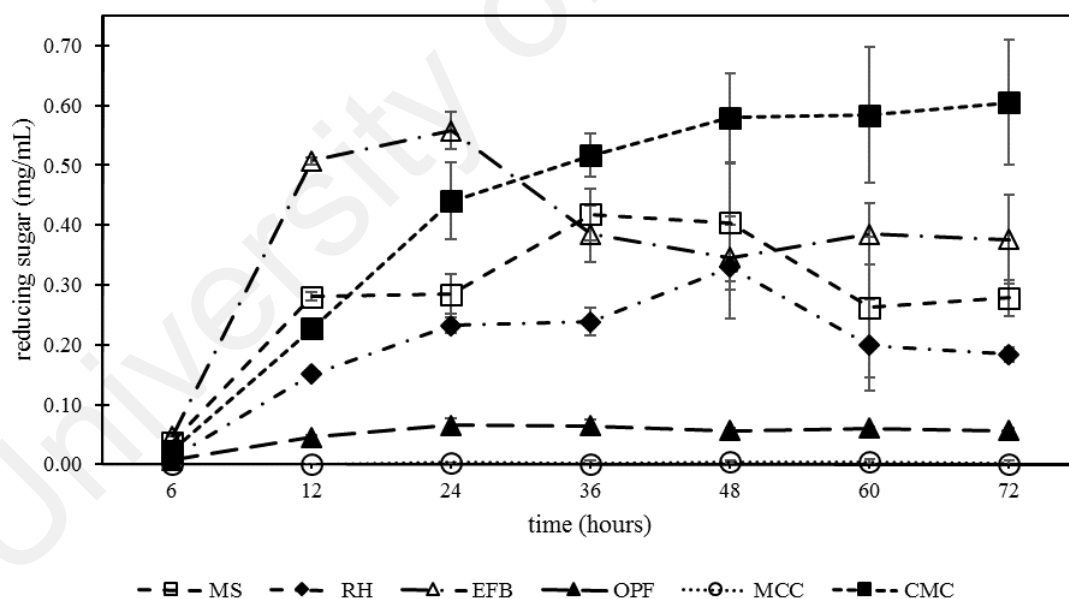
Figure 4.4: Reducing sugar profile in culture supernatants of *B. aerius* S5.2 during growth on cellulosic substrates. Error bars represent standard deviation of three replicates.

4.3.3 Localization of endoglucanase on CMC and MS

The localization of endoglucanase activities in *B aerius* S5.2 grown on a soluble pure cellulosic carbon source and a lignocellulosic substrate was investigated. CMC was

chosen as the pure cellulosic since higher endoglucanase activity was obtained on it than on MCC. Likewise, MS was chosen as the lignocellulosic substrate because it supported the highest endoglucanase production by the species. This was done in order to investigate the expression pattern of the enzyme in relation to cellular location when the bacterial strain is grown on different substrates. Results of the investigation are presented in Fig. 4.5.

Endoglucanase production was predominantly extracellular irrespective of the substrate solubility. In agreement with the earlier carbon source experiments, endoglucanase production on MS was significantly higher ($P < 0.05$) than on CMC extracellularly. These observations are in agreement with the report of Kricke et al. (1994) who reported that CMCase was produced both extracellularly and intracellularly by a *Bacillus* sp. isolated from termite mount soils. Extracellular production of enzymes among *Bacillus* spp. is common (Priest, 1977). Some amount of endoglucanase activity was also detected as intracellular and membrane-bound when *B. aerius* S5.2 was grown on both substrates, but these were much lesser compared to extracellular. Cell-bound (intracellular and membrane-bound) cellulase activity in bacteria is believed to represent a basal level of expression of the enzyme. This is because synthesis of cellulase proceeds by induction, transcription, and translation within the cell. Hence, cellulases are initially cell-bound before being secreted into the medium (Gong & Tsao, 1979). The higher extracellular expression of endoglucanase by this bacterium on MS and CMC is a reflection the nature of the substrates. The pretreated substrates and CMC both contain amorphous region which is known to be susceptible to endoglucanase action. Cellulases that are required for the hydrolysis of a particular substrate are usually expressed extracellularly when the bacterium is grown on that substrate (Ramasamy & Verachtert, 1980). Therefore, it is expected that *B. aerius* S5.2 should express higher amount of extracellular endoglucanase when grown on MS and CMC.
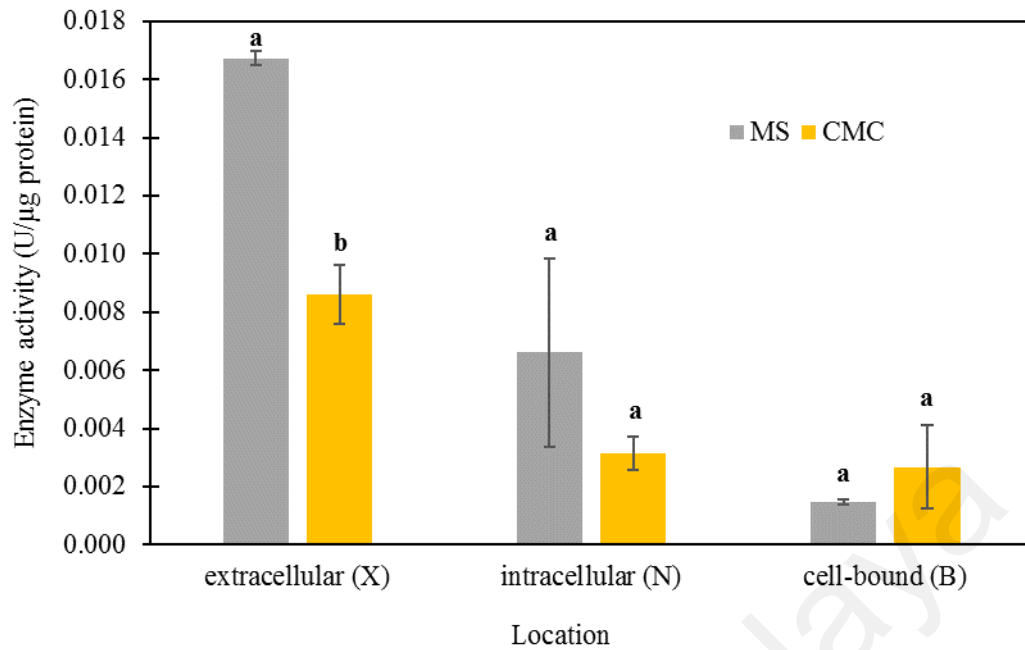
Figure 4.5: Cellular location of *B. aerius* S5.2 endoglucanase during growth on MS and CMC. Error bars represent standard deviations. Within the same location, bars that share the same letters are not significantly different ($P > 0.05$).

Extracellular production of enzymes has promising implications because they are easier to purify and can be less prone to proteolytic attack compared to intracellular enzymes (Dongfang Gao, Wang, Li, Yu, & Qi, 2015). Furthermore, extracellular secretion of cellulase by any strain indicates that such strain would be useful in developing consolidated bioprocessing (CBP) systems where cellulase production, cellulose hydrolysis, and fermentation occur simultaneously (Dongfang Gao et al., 2015; L. R. Lynd et al., 2002).

4.3.4 Degradation of MS

The ability of *B. aerius* to utilize MS was assessed by substrate gravimetric dry weight loss after some period of cultivation. The gravimetric method has been used by researchers to evaluate the cellulolytic ability of several bacteria on different lignocellulosic substrates (P. Gupta, Samant, & Sahu, 2012; M. Maki, Iskhakova, Zhang, & Qin, 2014). This method has the advantage of being a simple and easy assessment of microorganisms' suitability for bioprocessing of lignocellulosics, as the weight loss is

brought about by the synergistic action of their repertoire of enzymes such as cellulases, hemicellulases and ligninolytic enzymes.

It was found that 25.3 ± 2.5 % substrate dry weight loss occurred with MS compared to the control flask with 19 % weight loss. This difference was statistically significant (t-test, $p = 0.049$). The weight loss recorded with the control was primarily attributed to the dissolution of part of the substrate into the medium due to continuous agitation. From Table 4.2, it is shown that the culture was able to attain high cell growth on the MS under 72 hours with cell yield reaching 12.96 log cfu/mL. However, at the end of the seven days cultivation, it was found that growth had declined to 9.88 log cfu/mL. It is likely that the active growth part of the culture was supported almost wholly by the soluble compounds of the MS, thus resulting in the slight difference between the test and the control. It appears that the nature of lignocellulosic biomass could affect the utility of gravimetric method to assess cellulolytic ability of bacterial culture on certain of these substrates, as shown in this study.

4.4 Conclusions

In this study, selected carbon sources comprising soluble, insoluble, pure cellulosics and single/mixed lignocellulosic substrates, were evaluated for growth and endoglucanase production by *B. aerius* S5.2. Superior endoglucanase production was recorded on the lignocellulosic substrates than on CMC and MCC. A mixture of EFB, OPF, and RH supported higher enzyme production than the single lignocellulosic substrates. When the culture was grown on CMC and MS, endoglucanases production was predominantly extracellular. After seven days of fermentation, approximately 25 % of MS dry weight loss was recorded. The findings showed that mixed lignocellulosics are promising for commercial production of endoglucanase where the cost of cellulase production might be reduced. Higher amount of extracellular endoglucanase by *B. aerius* S5.2 is an advantage

in terms of enzyme recovery and for use in CBP. This study also provided insights into cellulolytic system of *B. aerius* which have not been reported previously.

CHAPTER 5: ENDOGLUCANASE PRODUCTION BY *B. aerius* S5.2 ON MIXED LIGNOCELLULOSIC SUBSTRATE: EFFECTS OF SUPPLEMENTATION WITH UNTREATED AGRO-INDUSTRIAL RESIDUES

5.1 Introduction and literature review

Cellulases (endoglucanase, exoglucanase, and β-glucosidase) are a group of synergistically-acting enzymes which hydrolyze glycosidic bonds of cellulose into glucose and shorter cello-oligosaccharides. Cellulases have wide applications across various industries (Kuhad et al., 2011). Endoglucanases, a group of cellulases that act on the internal amorphous regions of cellulose, are used in stonewashing of denim jeans (Clarkson, Swanson, & Winetzky, 2002) and also in some food processing applications (Tahir-Nadeem, Butt, Anjum, & Asgher, 2009). Cellulases are currently the third largest industrially important enzymes and their share of the global enzyme market is expected to increase with the projected worldwide adoption of biofuels as the main form of transportation fuel (Rani Singhania, 2011; Saloheimo, Pakula, Aro, & Joensuu, 2014). Fungi are more commonly used in commercial cellulase production because they produce copious amounts of the enzyme. However, bacterial cellulases are now attractive because of the high growth rate of bacteria, the tolerance of their cells and enzymes under relatively harsh conditions, production of efficient multienzyme complexes (cellulosomes) by bacteria, and the fact that bacteria are more amenable to genetic manipulation (M. Maki et al., 2009; M. L. Maki et al., 2011). Although members of *Bacillus* genus are known to produce unique cellulases (Deka, Jawed, & Goyal, 2013), there has been no report of endoglucanase production in *Bacillus aerius* till date.

The high cost of cellulases is one of the greatest bottlenecks in the biofuel industry and other industries where these enzymes are utilized (Banerjee et al., 2010; Chandel et al., 2012). Sensitivity analysis has shown that the carbon source is the major cost factor in cellulase production (Ryu & Mandels, 1980). Cellulases are produced commercially

using substrates such as lactose, Solka Floc, Avicel, and other pure cellulosics, which are expensive to use on a large scale (S. Chen & Wayman, 1991; Wayman & Chen, 1992). Furthermore, industrial fermentations for the production of such enzymes require the use of nitrogen sources such as yeast extract and peptone, which when used on a large scale, incur high cost. Thus, the use of cheaper substrates and medium optimization using alternative nitrogen sources have been advocated as a way of reducing the production costs (Chandel et al., 2012; S. Chen, 1993). Consequently, research efforts have focused on the use of cheap and widely abundant lignocellulosic biomass as substrates for cellulase production (H Ariffin et al., 2006; Bigelow & Wyman, 2002; Da Vinha et al., 2011; Harun et al., 2013; Jo et al., 2008; Krishna, 1999).

Majority of the studies related to the use of lignocellulosics for cellulase production have focused mainly on single feedstock types. However, in a practical situation, the use of single feedstock types cannot be sustained due to the seasonality of feedstock supply and other logistic challenges (A.A. Rentizelas et al., 2009; Athanasios A Rentizelas et al., 2009; Sultana & Kumar, 2011). Hence, there has been increased interest in the use of mixed lignocellulosics for various biotechnological applications in the last five years (Imamoglu & Sukan, 2014; L. Ji et al., 2015; R. D. O. Moutta et al., 2014; R. D. O. Moutta, R et al., 2013; Pereira et al., 2015; Shi et al., 2015). Mixed feedstock provides an opportunity for the combination of favourable characteristics of different biomass types in obtaining a product of interest. For example, it has been shown that supplementation of wheat straw with clover-rye grass resulted in high ethanol yields (80 % of theoretical) during simultaneous saccharification and fermentation with *Saccharomyces cerevisiae* without the need for additional nutrient supplementation (Thomsen & Haugaard-Nielsen, 2008). The clover-rye grass was able to provide sufficient nutrients needed for the fermentation. Similarly, Martín et al. (2008) reported that the addition of urea as nitrogen source during fermentation of wet oxidation pretreated clover-rye grass mixtures did not

result in any significant increase in ethanol yield as the mixture already contained enough nitrogen needed for the yeast metabolism during fermentation. These studies showed that combining different feedstocks may reduce or eliminate the need for extraneous nutrient supplementation, thereby leading to cost reduction.

Conversion of agricultural and agro-industrial wastes to obtain value-added products is a way of ensuring environmental safety and sustainability. Malaysia generates up to 70 million tonnes of biomass annually, and 85.5 % of this originates from the oil palm industry (Shuit, Tan, Lee, & Kamaruddin, 2009). Decanter cake (DC) is one of the major wastes that are generated during the processing of oil palm. This material has been demonstrated to have potential value for cellulase production (Zanirun, Bahrin, Lai-Yee, Hassan, & Abd-Aziz, 2013). *Leucaena leucocephala* (LL) (known as "*Petai belalang*" in Malaysia) is a fast-growing, multi-purpose tree which is widely used in Malaysia as a shade tree in plantations (Rahman, Rahman, Kasim, Japarudin, & Pailing, 2014). The trees are often left to decay or are burnt when the plantation crops mature. Its seeds are rich in protein and other nutrients (Alabi & Alausa, 2006), and are thus suitable for use in fermentation. Although seeds of this plant have been used for animal feeds (Sotolu & Faturoti, 2008), it has not received much attention for use in microbial enzyme production. These two highlighted agro-industrial materials are available in huge amounts in Malaysia and can be readily utilized as medium components for cellulase production.

The objective of this study was to evaluate the feasibility of using DC and LL as medium supplements (carbon source adjuncts or nitrogen sources) for the production of endoglucanase by *B. aerius* S5.2 on a mixed substrate (MS) comprising oil palm empty fruit bunch (EFB), oil palm frond (OPF), and rice husk (RH). Since pretreatment cost is one of the major obstacles obstructing the successful bioconversion of lignocellulosics (Banerjee et al., 2010), the supplements used in this study were not pretreated. The assumption here was that such a strategy, in a real biorefinery scenario, would save costs

and reduce the amounts of toxic inhibitors and/or process water needed to remove pretreatment-generated inhibitors. To the best of the author's knowledge, this is the first report of the use of LL in cellulase production.

5.2 Materials and methods

5.2.1 Bacterial strain

Bacillus aerius S5.2 used in this study was isolated from decomposing EFB residues that were collected from an oil palm plantation in Kuala Selangor, Malaysia. This strain produced high titers of endoglucanase on a mixed substrate (MS) that comprised EFB, oil palm frond (OPF) and rice husk (RH). It was identified as *B. aerius* following sequencing of the 16S rRNA gene and sequence similarity check using the BLAST tool on the NCBI database. The sequence was submitted to GenBank and an accession number (KP178216) was obtained. The strain was maintained on nutrient agar slants at 4 °C.

5.2.2 Collection and preparation of mixed substrate (MS) and supplements

Fresh OPF samples were obtained from Malaysian Palm Oil Board (MPOB), Bangi, Malaysia. The leaflets were removed and the fresh petioles were cut into smaller pieces and dried under sunlight. Only the petioles were used in this study because the leaflets of OPF have other important uses in an oil palm plantation. The petiole has been shown to possess higher content of sugars which makes it more desirable for use as feedstock in biofuels and other bioproducts processes (Zahari et al., 2012). Dried and shredded EFB fibres were obtained from Biocomposting Pilot Facility, UPM, Malaysia. Rice husk (RH) was collected from a paddy field in Kedah, Malaysia. The three biomass samples were reduced to small particles (300 – 425 µm) using a Rapid granulator (GK 205-K, Terramar, Hamburg, Germany). The MS was prepared by mixing EFB, OPF and RH in equal proportions (1:1:1). MS used in this study was subjected to sequential pretreatment with NaOH and autoclaving according to the method of Umikalsom et al. (1997), with the slight extension of the autoclaving duration to 15 minutes. The washed pretreated solids

were dried in an oven at 60 °C for at least 12 hours. The sample was kept in a dry airtight container prior to use.

DC used in this study was obtained from an oil palm mill in Kuala Lumpur, Malaysia. Fresh *Leucaena leucocephala* (LL) pods (Appendix D) were obtained from a LL tree around the University of Malaya campus. The seeds (Appendix E) were removed from the pods and were washed repeatedly with distilled water. They were then sun-dried and ground into powdered form. The powder was sieved with a 0.5 mm mesh sieve to remove the larger particles. All samples used in the supplementation experiments were kept in an air-tight container at room temperature before use. The samples were analysed for Nitrogen, Calcium, Phosphorus, Potassium, Magnesium, Sodium, and Sulphur content at the Soil Chemistry Laboratory, Forestry Research Institute of Malaysia (FRIM), Kepong, Malaysia.

5.2.3 Culture media

Modified version of the medium for cellulolytic microorganisms described by Dickerman and Starr (1951) was used for endoglucanase production. The medium had the following composition (w/v): K_2HPO_4 (0.08 %), KH_2PO_4 (0.02 %), $MgSO_4 \cdot 7H_2O$ (0.02 %), NaCl (0.02 %), $NaNO_3$ (0.1 %), $CaCO_3$ (0.001 %), and yeast extract (0.05 %). For the experiments involving the use of the agro-industrial residues as carbon source adjuncts, each supplement was mixed with MS in 1:3 proportion and the mixture was used as the carbon source at 2 % (w/v) concentration. In the experiments involving the use of the supplements as nitrogen sources, each supplement was used in place of yeast extract and $NaNO_3$ in the original medium at 0.15 % (w/v) concentration i.e. replacing 0.1 % $NaNO_3$ and 0.05 % yeast extract (Table 5.1). The original medium with 2 % MS and without any supplement added, was used as control. The medium pH was adjusted to 7.0 using 2 M NaOH or 2 M HCl. Sterilization of the media was done at 121 °C for 15 minutes in an autoclave.

Table 5.1: Media composition used in supplementation experiments with DC and LL.

Medium component	Supplement (DC/LL) *	
	As carbon source adjunct	As nitrogen source
K$_2$HPO$_4$	+	+
KH$_2$PO$_4$	+	+
MgSO$_4$·7H$_2$O	+	+
NaCl	+	+
NaNO$_3$	+	-
CaCO$_3$	+	+
Yeast extract	+	-

*Control medium had MS only as carbon source, with NaNO$_3$ and yeast extract as nitrogen sources. No supplement was added.

5.2.4 Endoglucanase production

B. aerius S5.2 was cultivated in nutrient broth until the late log phase (12 h) was reached. Aliquots (containing approximately 10^7 cfu/mL) from this culture were used as inocula in the experiments. Ten percent inoculum (v/v) was inoculated into 250 mL Erlenmeyer flasks containing the respective culture media. Each flask was incubated at 30 °C and at 200 rpm agitation speed for 36 h. Previous experiments had revealed that the bacterium produced maximum endoglucanase titer at 36 h on MS media. Triplicate flasks were used for each experiment. Culture samples were collected and centrifuged at 6000 rpm for 10 minutes at 4 °C. The cell-free supernatant was used as the crude enzyme preparation for the endoglucanase assay.

5.2.5 Cell growth measurement

The growth of the bacterial culture on each substrate at a specific period of sampling was determined by estimating total cfu count in the culture supernatant using the drop plate technique (Herigstad et al., 2001).

5.2.6 Endoglucanase assay

Endoglucanase activity was determined by measuring the reducing sugars liberated after the reaction of 200 µL of the enzyme preparation with 200 µL of 2 % CMC in 0.05 M phosphate buffer (pH 7.0) (Y. H. P. Zhang et al., 2009). The mixture was incubated for

30 minutes at 50 °C and the reaction was stopped by the addition of 800 µL of DNS reagent followed by immersing the reaction tubes in boiling water for 5 minutes. Released sugars were measured as glucose equivalent using the DNS reagent (Miller, 1959). One unit (U) of enzyme activity was defined as the amount of enzyme that liberated 1 µmol of reducing sugar per mL per minute from the substrate.

5.2.7 Statistical analysis

All statistical analyses were carried out using IBM SPSS Statistics, Version 22 (IBM Corp., Armonk, New York).

5.3 Results and discussion

5.3.1 Elemental composition of DC and LL

LL and DC were investigated as carbon source adjuncts and nitrogen sources for the production of endoglucanase by *B. aerius* S5.2. Samples of these materials were analysed for their elemental composition and the results are presented in Table 5.2. From the results, it can be seen that LL contained significantly higher ($p < 0.05$) amounts of all the mineral elements than DC, except for calcium and magnesium which were higher in DC. Sodium was high in DC (59.47 %) but was not detected in LL. Sotolu and Faturoti (2008) reported very low levels of sodium (0.01 – 0.03 %) in samples of LL seed meal prepared by different methods as compared to other minerals. The amounts of mineral elements found in this study for DC are similar to those reported by Nutongkaew, Duangsuwan, Prasertsan, and Prasertsan (2014). Seeds of *Leucaena* have been reported to be rich in nutrients and minerals. Besides the nutrients analysed in this study, LL seeds are also known to be rich in protein, carbohydrate, and lipids (Alabi & Alausa, 2006; P. Sethi & Kulkarni, 1995).

Table 5.2: Elemental composition of DC and LL used in the study.

Element	Composition (% oven dry weight)	
	Leucaena leucocephala	Decanter cake
N	5.28 ± 0.05^a	2.32 ± 0.04^b
Ca	0.36 ± 0.01^a	1.22 ± 0.01^b
P	0.26 ± 0.00^a	0.17 ± 0.00^b
K	1.35 ± 0.02^a	0.81 ± 0.01^b
Mg	0.19 ± 0.00^a	0.22 ± 0.00^b
Na	ND	59.47 ± 6.10
S	0.79 ± 0.01^a	0.23 ± 0.01^b

Presented values are means of three replicates $\pm$ standard deviation. Values within the same row and having same superscript letters are not significantly different ($p > 0.05$). ND - Not detected.

5.3.2 Cell growth on supplemented media

Table 5.3 shows the growth (in cfu) attained by *B. aerius* S5.2 when it was grown on MS media supplemented with LL and DC as carbon source adjuncts and as nitrogen source. When the bacterial culture was grown on media containing the respective supplements as carbon source adjuncts, growth on the two supplemented media was significantly higher ($P < 0.05$) than on the control which had MS only as the carbon source. Growth on medium containing LL was significantly higher ($P < 0.05$) than that recorded on the medium with DC. When these materials were used as nitrogen sources, replacing yeast extract and $NaNO_3$ in the control medium, significantly higher ($P < 0.05$) growth was recorded with LL compared to the control. However, there was no significant difference between the growth values recorded on LL and DC. Similarly, DC supported growth of the culture as much as the control as there was no significant difference ($P > 0.05$) in values recorded on both media.

Table 5.3: Growth attained by *B. aerius* S5.2 on MS media with DC or LL as carbon source adjuncts or nitrogen source.

Medium	Cell growth (log cfu)	
	As carbon source supplement	**As nitrogen source**
Control	8.90 ± 0.05^c	8.90 ± 0.05^b
DC	9.13 ± 0.14^b	$9.05 \pm 0.19^{a, b}$
LL	9.38 ± 0.03^a	9.24 ± 0.09^a

Presented values are means of three replicates $\pm$ standard deviation. Values within the same column and having same superscript letters are not significantly different ($p > 0.05$).

The higher growth observed with LL as carbon source adjunct can be attributed to its lower lignin content besides the additional nutrients it must have contributed to the medium. The MS being a lignocellulosic substrate, has some amount of lignin. DC also being a residue of the processing of a lignocellulosic biomass, has been reported to contain about 30 % lignin (Razak, Ibrahim, Yee, Hassan, & Abd-Aziz, 2012). Lignin has been reported to inhibit the growth of bacteria on cellulosic substrates (Camp et al., 1988; Dehority & Johnson, 1961; Han & Callihan, 1974). The fact that the DC used here was untreated makes its lignin content a significant factor to consider. On the whole, the total amount of lignin present in the medium with LL is presumably lower than in the DC medium and the control since LL seeds are known to have low lignin content, around 4.2 % (Sultan Singh et al., 2002). The comparable growth obtained on LL and DC media as nitrogen sources, despite LL higher nitrogen content, could mean that the amount of nitrogen in the DC medium was sufficient for the bacterial culture growth. So, the additional nitrogen in the LL medium did not result in significantly higher growth. However, the higher growth on LL compared to the control could be due to the relatively higher nutrient composition of LL.

5.3.3 Endoglucanase production on supplemented media

Results of endoglucanase production when DC and LL were used as carbon source adjuncts and nitrogen sources are presented in Figure 5.1. The highest endoglucanase titer (0.650 U/mL) was recorded when LL was used as carbon source adjunct, and this was

significantly higher ($p < 0.05$) than titers obtained with DC (0.520 U/mL) or the control (0.439 U/mL). There was no significant difference ($P > 0.05$) in endoglucanase production between DC and the control when DC was used as carbon adjunct. There was no significant difference ($P > 0.05$) in endoglucanase titers obtained on the three media when the supplements were used as nitrogen sources. LL (0.511 U/mL) produced the highest titer as nitrogen source but this was not significantly higher ($P > 0.05$) than titers obtained with DC (0.388 U/mL) or the control (0.439 U/mL). Generally, higher endoglucanase titers were obtained when the supplements were used as carbon source adjuncts than when they were used as nitrogen sources.
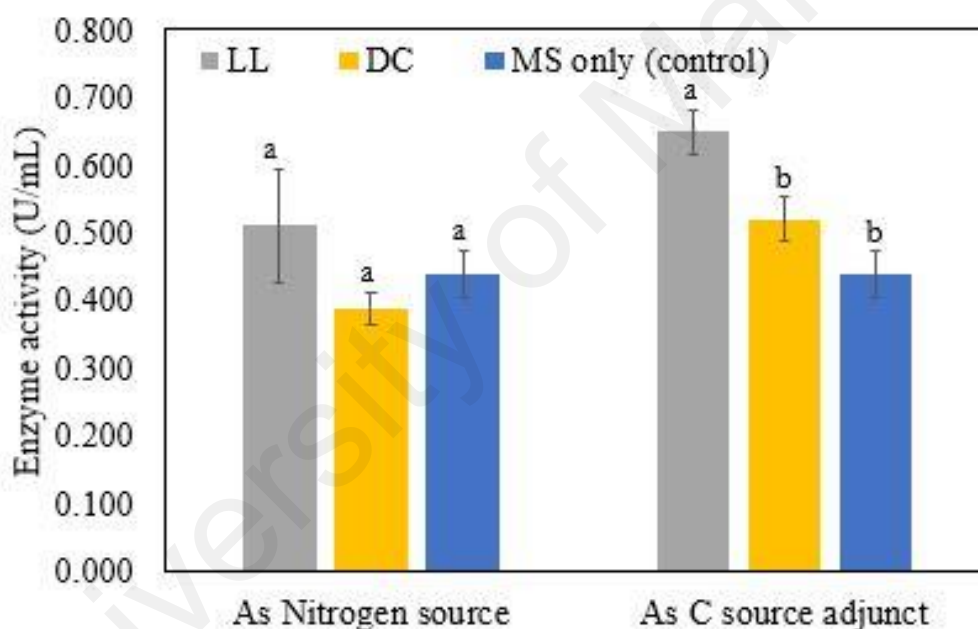
Figure 5.1: Endoglucanase production by *B. aerius* S5.2 on supplemented media. Error bars represent standard deviation of three replicates. Bars having the same letters for carbon or nitrogen source are not significantly different ($P > 0.05$).

A possible reason for the higher endoglucanase production obtained when DC and LL were used as carbon source adjuncts is the amount of each of them used in the media. Higher concentration of each supplement was present in the media when used as carbon source adjuncts (0.5 % w/v, i.e. ¼ of 2 % substrate concentration) than as nitrogen sources

(0.15 % w/v). This explained the significantly higher enzyme production on LL as a result of its higher nutrient composition (Table 5.2). The lower titers recorded when they were used as nitrogen sources showed that higher concentrations of DC and LL than were used in this study would be needed to obtain titers significantly higher than that obtained on the control medium.

As in the case of growth (Table 5.3), the lower endoglucanase titers recorded on DC compared to LL in the experiments can be attributed to the presence of lignin in the untreated DC used in this study. Besides inhibiting bacterial growth, lignin is also known to have negative effects on cellulase production. Lignin limits the accessibility of cellulose for cellulase induction (L. Zhang, Liu, Niu, Liu, & Liao, 2012), and it also causes irreversible adsorption of cellulase leading to enzyme loss (Bigelow & Wyman, 2002). However, given the complex nature of lignocellulosic biomass, the nutritional composition and lignin content cannot be used solely to explain the observed endoglucanase production pattern. Other factors influence the production of cellulases on such substrates; some of them are chemical composition, crystallinity, residual pretreatment inhibitors, and substrate particle size (Brijwani & Vadlani, 2011; Han & Callihan, 1974; Szengyel & Zacchi, 2000). Pretreatment of the DC could produce better results, but this would lead to increased costs in practical situations.

Previous studies have demonstrated that the utilization of agro-industrial wastes through the mixed substrate system as investigated in this study, could bring about improvements in the production of value-added bioproducts. Mladenović, Djukić-Vuković, Kocić-Tanackov, Pejin, and Mojović (2015) showed that combined use of distillery stillage and sugar beet molasses as a substrate provided important nutrients for the production of lactic acid by selected bacterial strains. The incorporation of ricotta whey, a potentially hazardous industrial waste, with sugarcane bagasse, was also demonstrated to be able to increase ethanol production than when bagasse alone was used in the fermentation

(Ferreira et al., 2015). Thus, a hypothetical biorefinery in Malaysia producing cellulases from oil palm residues, would benefit immensely from utilizing oil palm processing waste (DC) and an under-utilized agro-residue (LL) as media supplements.

5.4 Conclusions

The findings from this study showed that DC and LL contained adequate nutrients and minerals to support the growth and endoglucanase production by *B. aerius* S5.2 when used as supplements in a mixture of EFB, OPF, and RH. LL and DC can be used to replace yeast extract and $NaNO_3$ without significant decrease in endoglucanase production, although higher concentration of both supplements would be needed to achieve significantly higher titers than the control medium (MS only) when they are used as nitrogen sources. LL as carbon source adjunct produced significantly higher endoglucanase titers than DC and the control. These results showed that the utilization of cheap and abundant agro-industrial residues as applied in this study could help to pave a more cost-effective route to endoglucanase production.

CHAPTER 6: ENDOGLUCANASE PRODUCTION BY *Bacillus aerius* S5.2 ON

MIXED LIGNOCELLULOSIC SUBSTRATES: EFFECTS OF

PRETREATMENT AND SUBSTRATE COMBINATION

6.1 Introduction and literature review

The cheap and abundant nature of lignocellulosic biomass makes it a preferred alternative to fossils as raw material for production of fuels, platform chemicals and other commodities (Anwar et al., 2014). Cellulases are essential for the conversion of the cellulose component of lignocellulosic biomass into biofuels and value-added bioproducts. The three major types of cellulases (endoglucanases, exoglucanases, and β-glucosidases) act in synergy to produce glucose from cellulose by hydrolyzing the β-glycosidic bonds of cellulose (M. Wang et al., 2012). Cellulases have varied applications in several industries but endoglucanases are of particular interest because their action on the amorphous regions of cellulose is considered the rate limiting step in cellulose utilization (Malherbe & Cloete, 2002). These class of cellulases also have specific applications in the textile (Clarkson et al., 2002) and food processing industries (Tahir-Nadeem et al., 2009). Fungi are the main species used in the commercial production of cellulases primarily because they secrete copious amounts of these enzymes. However, bacterial cellulases have received much attention lately due to their high growth rate, their ease of genetic manipulation for strain improvement, their production of efficient multienzyme complexes (cellulosomes), and the fact that their cells and enzymes are relatively more stable under extreme conditions that may be encountered during bioprocessing (M. Maki et al., 2009; M. L. Maki et al., 2011). Some bacterial genera that have been reported for cellulase production include *Cellulomonas*, *Streptomyces*, *Thermobifida*, *Acetivibrio, Ruminococcus, Bacillus,* and *Clostridium* (Sadhu & Maiti, 2013). *M*embers of *Bacillus* genus are known to produce cellulases with unique properties

(D. Deka et al., 2013). However, cellulase production has not been reported in *B. aerius* until now.

High cost of cellulases is considered as one of the major bottlenecks facing the lignocellulosic biorefinery (Banerjee et al., 2010). This situation has been attributed to the cost of substrates used in commercial cellulase production. Sensitivity analysis revealed that the major cost factor in cellulase production is the carbon source (Ryu & Mandels, 1980). So far, cellulase is produced commercially using carbon sources such as lactose, Solka floc, Avicel, cotton, and microcrystalline cellulose, thus attracting high cost when used on a large scale (S. Chen & Wayman, 1991; Wayman & Chen, 1992). The use of cheap substrates has therefore been suggested as one of the ways of reducing the cost of cellulase production. Consequently, researchers have explored the use of lignocellulosic materials as substrates for cellulase production (Hidayah Ariffin et al., 2008; Bigelow & Wyman, 2002; Da Vinha et al., 2011; Jo et al., 2008; Krishna, 1999). Unfortunately, most of these researchers have focused mainly on the use of single substrates while the use of mixed substrates has received very little attention. Owing to seasonality of feedstock supply and logistic issues associated with lignocellulosic biomass utilization, the use of single feedstocks is not sustainable in the long run (Murphy et al., 2015; A.A. Rentizelas et al., 2009; Sultana & Kumar, 2011). A biorefinery would have to utilize mixed streams of varied feedstocks in order to be able to run all-year round and avoid loss of capital. Studies have shown that significant cost savings can be achieved when mixed feedstocks are used in a biorefinery (Nilsson & Hansson, 2001; Sultana & Kumar, 2011). While the use of mixed lignocellulosics for the production of bioethanol (Nguyen et al., 1999), fermentable sugars (Vera, Bura, & Gustafson, 2015), fungal enzymes (Qi et al., 2007), and polyhydroxyalkanoates (Sangkharak & Prasertsan, 2013) has been reported, no such study has been reported for bacterial cellulases.

Another important aspect of lignocellulose bioconversion is pretreatment. Given the recalcitrant nature of lignocellulose, physicochemical treatments are usually required in order to open up the cellulose-hemicellulose-lignin matrix and to improve cellulose digestibility (Taherzadeh & Karimi, 2008). In the case of cellulase production from lignocellulosic substrates, pretreatments may be needed to alter the substrate physicochemical characteristics in order to allow for microbial utilization and induction of cellulase synthesis (Brijwani & Vadlani, 2011). While studies on pretreatment of lignocellulosic biomass are numerous (Alvira et al., 2010; Hendriks & Zeeman, 2009; Taherzadeh & Karimi, 2008; X. Zhao et al., 2012), very few of these studies focused on the pretreatment of substrates for cellulase production. Even fewer reports on cellulase production from mixed lignocellulosics in the literature investigated the effects of pretreatments. Furthermore, the effects of pretreatments with respect to the combination of similar or varied substrate types have not been well explored.

Lignocellulosic biomass wastes such as empty fruit bunch (EFB) and oil palm frond (OPF) from the vibrant oil palm industry are in abundance in Malaysia. Up to 70 million tonnes of biomass are generated annually in Malaysia. About 85.5 % of this are from the oil palm industry (Shuit et al., 2009). Rice husk (RH) is equally abundant in the country (Shafie, Mahlia, Masjuki, & Ahmad-Yazid, 2012). These wastes are usually disposed of through accumulation on land or by open burning which contributes to environmental pollution. Utilization of these wastes for the production of value-added bioproducts such as cellulases is a way of ensuring environmental health, sustainability and additional income.

This study aimed to compare the effect of four pretreatment methods in altering the characteristics of single and mixed lignocellulosic substrates for the production of endoglucanase by *B. aerius* S5.2. Changes in the chemical composition, microstructure, functional groups, and crystallinity of the substrates were investigated in order to relate

the specific effects of each pretreatment to the observed endoglucanase production patterns. The effectiveness of joint- versus separate pretreatment of the single substrates (SS) was also compared using the pretreatment method which gave the highest endoglucanase productivity. Lastly, the effectiveness of combining substrates for endoglucanase production was evaluated by comparing enzyme activities obtained on untreated single- against mixed substrates (MS).

6.2 Materials and methods

6.2.1 Bacterial strain

Bacillus aerius S5.2 used in this study was isolated from decomposing EFB residues that were collected from an oil palm plantation in Kuala Selangor, Malaysia. This strain produced high activity of endoglucanase on a mixed substrate (MS) that comprised EFB, oil palm frond (OPF), and rice husk (RH). It was identified as *B. aerius* following sequencing of the 16S rRNA gene and sequence similarity check using the BLAST tool on the NCBI database. The sequence was submitted to GenBank and an accession number (KP178216) was obtained. The strain was maintained on nutrient agar slants at 4 °C.

6.2.2 Collection and preparation of substrates

Fresh OPF samples were obtained from Malaysian Palm Oil Board (MPOB), Bangi, Malaysia. The leaflets were removed and the fresh petioles were cut into smaller pieces and dried under sunlight. Only the petioles were used in this study because the leaflets of OPF have other important uses in an oil palm plantation. The petiole has been shown to possess higher content of sugars which makes it more desirable for use as feedstock in biofuels and other bioproducts processes (Zahari et al., 2012). Dried and shredded EFB fibres were obtained from Biocomposting Pilot Facility, UPM, Malaysia. Rice husk (RH) was collected from a paddy field in Kedah, Malaysia. The three biomass samples were reduced to small particles (300 – 425 µm) using a Rapid granulator (GK 205-K, Terramar,

Hamburg, Germany). The MS was prepared by mixing EFB, OPF and RH in equal proportions (1:1:1). The sample was kept in a dry airtight container prior to use.

6.2.3 Substrate pretreatments

The three single substrates (SS) used in this study (EFB, OPF and RH) were mixed in a 1:1:1 ratio, and the resulting mixture (MS) was used as substrate in the pretreatment studies. MS was subjected to dilute acid, dilute alkali, hydrothermal and organic solvent pretreatments with 1 % (v/v) H_2SO_4, 1 % (v/v) NaOH, distilled water, and 85 % (w/w) *N*-methylmorpholine-*N*-oxide (NMMO), respectively as pretreatment solvents. MS was suspended in the respective solvents of each pretreatment method in 500 mL bottles to obtain a solid loading of 10 % (w/v) on dry weight basis. The suspension was heated in an autoclave at 121 °C, 15 psi for 1 h. After cooling, the liquid fraction was separated from the slurry by vacuum filtration. The solid fraction was washed with deionized water until neutral pH. For the NMMO pretreatment, 150 mL of hot distilled water was added as an anti-solvent to recover the dissolved materials before separation (Kabir, Niklasson, Taherzadeh, & Horvath, 2014). Washing of the solid was repeated until a clear filtrate was obtained. The washed pretreated solids were freeze-dried (Freezone 7670530, Labconco, Kansas City, MO, USA) and kept at 4 °C until they were ready for use.

6.2.4 Endoglucanase production on pretreated MS samples

B. aerius S5.2 was cultivated in nutrient broth and the culture was allowed to reach late log phase (12 h). Aliquot (containing approximately 10^7 cfu/mL) from this culture was used as inoculum in the experiments. Modified medium of Dickerman and Starr (1951) with 2 % (w/v) of each respective pretreated MS as carbon source was used for endoglucanase production. Medium pH was adjusted to 7.0 with 1.0 M NaOH or 1.0 M HCl. Fifty ml of media in 250 mL Erlenmeyer flask was inoculated with 10 % (v/v) of inoculum. Each flask was incubated at 30 °C and at 170 rpm agitation speed for 72 h. The experiments were conducted in triplicates for each pretreatment method. Culture samples

were collected at 6-h intervals initially, and at 12-h intervals subsequently. These were centrifuged at 6000 rpm at 4 °C for 10 minutes. The cell-free supernatant obtained was used as crude preparation in the enzyme assay.

6.2.5 Effects of joint/separate pretreatment on endoglucanase production

In order to determine whether joint- or separate substrate pretreatment favoured endoglucanase production, each SS was pretreated separately using the pretreatment method which supported the highest endoglucanase production by *B. aerius* S5.2 on MS. The pretreatment conditions (substrate loading, temperature, residence time, and solvent concentration) and fermentation conditions as used for MS were applied for SS. Culture samples were collected at 6-h intervals initially, and at 12-h intervals subsequently and were analyzed for endoglucanase activities.

6.2.6 Effects of substrate combination on endoglucanase production

To test the effects of combining the SS on endoglucanase production, untreated SS and untreated MS were used as substrates, applying the fermentation conditions described earlier.

6.2.7 Analytical methods

6.2.7.1 Compositional analysis

All substrates (raw and pretreated) in this study were used on a dry weight basis after the determination of total solids of each material. Total solids was determined by monitoring the difference in dry weight of each material following drying to constant weight at 105 °C in an oven (A. Sluiter et al., 2008). Cellulose, hemicellulose and lignin in the pretreated substrates were determined using the National Renewable Energy Laboratory (NREL) protocols (A Sluiter et al., 2008).The biomass (300 mg) was hydrolyzed with 3 mL of 72 % H_2SO_4 at 30 °C for 60 min. The acid was then diluted to 4 % by the addition of deionized water and the sample was heated at 121 °C for 60 min in an autoclave for a

second hydrolysis. Then, the sample was vacuum-filtered. Sugars in the filtrate were analyzed by HPLC (Waters 2695, Waters Corporation, Milford, USA). A lead based column (Aminex HPX-87P, Bio-Rad, Hercules, USA) was used at 85 °C and 0.6 mL/min flow rate of ultrapure water as eluent. Detection was done using a refractive index (RI) detector (Waters 2414). The monomer sugars concentrations were used in calculating the amount of cellulose and hemicellulose in the biomass. Acid soluble lignin in the filtrate was determined using a UV spectrophotometer (Libra S60, Biochrom, England) at 320 nm and ε value of 30 L/(g.cm). Acid insoluble lignin was determined gravimetrically after heating the solid residue in a muffle furnace at 575 °C for 24 h and deducting the ash content.

6.2.7.2 Field emission scanning electron microscopy (FESEM) analysis

The effects of pretreatment on the surface morphology of the substrates were observed with a scanning electron microscope (JSM-7001F, JOEL, Tokyo, Japan). Images were taken at 5-15 kV and at different magnifications. For the MS samples, images of the different portions were repeatedly taken to ensure that each SS was captured. The SS were identified in the mixtures by making comparisons with micrographs of pure SS samples.

6.2.7.3 X-ray Diffraction (XRD) analysis

Changes in the crystallinity of the samples were determined using a PANalytical Empyrean Multipurpose X-ray diffractometer (PANalytical BV, Netherlands). Scans were taken at 4 s per step from $2\theta = 5 – 60$ °C with a step size of 0.03°. Relative degree of crystallinity (*CrI*) of the samples was calculated according to the method of Segal, Creely, Martin, and Conrad (1959), using the equation:

$$CrI = (I_{002} − I_{am})/I_{002} * 100 \qquad \text{(Eq. 6.1)}$$

Here, I_{002} is the maximum intensity of the 002 lattice diffraction around $2\theta = 22.8°$ (corresponding to the crystalline region), and I_{am} is the intensity of diffraction around $2\theta = 18°$ (corresponding to the amorphous region).

6.2.7.4 Fourier transform infrared spectroscopy (FTIR) analysis

Changes in the functional groups of the substrates were monitored using FTIR. FTIR analysis was carried out using a Perkin-Elmer FTIR spectrum-400 spectrometer (Perkin-Elmer Inc., Wellesley, MA, USA) and the spectra were obtained in the range of 500 – 4000 cm^{-1}.

6.2.7.5 Endoglucanase assay

Endoglucanase activity was determined by measuring the reducing sugars liberated after the reaction of 200 µL of the enzyme preparation with 200 µL of 2 % CMC in 0.05 M phosphate buffer (pH 7.0) (Y. H. P. Zhang et al., 2009). The mixture was incubated for 30 minutes at 50 °C and the reaction was stopped by the addition of 800 µL of DNS reagent followed by immersion of the reaction tubes in boiling water for 5 minutes. Released sugars were measured as glucose equivalent using the DNS reagent (Miller, 1959). One unit (U) of enzyme activity was defined as the amount of enzyme that liberated 1 µmol of reducing sugar per mL per minute from the substrate.

6.2.8 Statistical analysis

All statistical analyses were carried using IBM SPSS Statistics, Version 22 (IBM Corp., Armonk, New York).

6.3 Results and discussion

Lignocellulosic biomass are cheap carbon sources for cellulase production. However, they are not easily utilizable by bacteria in their natural untreated form. Pretreatments are necessary to modify the physicochemical characteristics of these substrates to make them suitable for cellulase induction (Brijwani & Vadlani, 2011). In this study, the

characteristics of a mixture of EFB, OPF, and RH were altered using different pretreatments in order to determine the effects of these changes on endoglucanase production by *B. aerius* S5.2. Changes in the substrates' chemical composition, microstructure, functional groups, and crystallinity were monitored and were used to deduce the specific effects of the pretreatments on endoglucanase activities obtained.

6.3.1 Effects of pretreatments on chemical composition of MS

Cellulase induction is strongly dependent on the nature of the carbon source (L. R. Lynd et al., 2002). Hence, it is necessary to investigate the effects of substrate complexities (as reflected in the chemical composition) on endoglucanase production. The chemical compositions of the untreated and pretreated MS samples are presented in Table 6.1.

Table 6.1: Chemical composition of MS from various pretreatments used in this study.

Treatment	Chemical composition (%)				
	Cellulose	**Hemicellulose**	**Lignin**	**Ash**	**Others**
Untreated MS	31.97 ± 0.30^a	15.07 ± 0.24^b	20.88 ± 0.10^a	4.70 ± 0.19^a	27.38 ± 1.63^a
Acid	48.65 ± 0.39^c	6.88 ± 0.97^a	30.87 ± 1.45^b	5.62 ± 3.22^a	7.98 ± 1.90^b
Alkali	37.84 ± 1.62^b	19.04 ± 0.87^c	23.36 ± 0.52^a	3.78 ± 1.27^a	15.98 ± 3.18^b
Hydrothermal	38.31 ± 0.56^b	19.80 ± 0.47^c	23.73 ± 1.86^a	2.25 ± 1.99^a	15.91 ± 3.27^b
NMMO	40.20 ± 1.35^b	20.07 ± 0.57^c	22.29 ± 0.27^a	1.83 ± 2.65^a	15.60 ± 3.95^b

Data are expressed on a dry weight basis. [a-c] Values represented means of   two (untreated MS) or three (pretreated MS) replicates $\pm$ standard deviation (SD). Values within the same column and having same superscript letters are not significantly different ($p > 0.05$).

Compared to the untreated MS, all pretreated MS samples showed significantly higher cellulose and hemicellulose compositions, with the exception of the acid-pretreated MS, which had significantly lower amount of hemicellulose (6.88 %). This could be due to the high amount of other components present in the untreated mixture. The amount of other components in the untreated single substrates varied between 20 – 24 % (Appendix G), and this explained the high value obtained for the mixture following pretreatment. These other components may include protein and extractives (A Sluiter et al., 2008) but they were not individually analyzed in this study. Acid-pretreated MS also had the highest composition of cellulose (48.65 %) and lignin (30.87 %). This can be attributed to the removal of a greater portion of hemicellulose by the acid, which caused the increase in the relative proportion of the other two components in the sample (X. Zhao et al., 2012). There was no significant difference in the chemical composition among the alkali-, hydrothermal- and NMMO-pretreated substrates. This observation suggested that the acid treatment exerted stronger effects compared to the others, despite the fact that similar conditions (*viz.* 1 % (v/v/) solvent concentration, 121 °C, and 15 psi) were applied in all the pretreatments investigated.

6.3.2 Effects of pretreatment on physicochemical characteristics of MS and SS

Since the chemical composition of lignocellulose alone does not completely account for cellulase production patterns (Rodriguez-Zuniga, Bertucci Neto, Couri, Crestana, & Farinas, 2014), the substrates were further analyzed using FESEM, FTIR and XRD in order to gain better understanding of the relationship between the changes which occurred at the structural and molecular level of the substrates and the observable enzyme production pattern.

6.3.2.1 FESEM

Due to the heterogeneous nature of mixtures, it is difficult to monitor changes to individual components following pretreatments due to the irregular size, structure and distribution of the diverse components (R. D. O. Moutta, R et al., 2013). In order to get around this, images of the SS were taken separately before capturing those of the MS. When images of the MS were captured, repeated different shots were taken to ensure each SS appeared in the MS micrographs. The untreated SS samples had relatively unruffled microstructure (Fig. 6.1 *a-d*) with EFB showing intact microfibrils with embedded silica deposits. Untreated OPF had a fairly intact mesh-like inner surface structure and a smooth outer fibrillar surface. Similarly, untreated RH had a well-ordered surface with embedded silica bodies. However, RH in all the pretreated MS (Fig. 6.1 *e, f, g* and *h*) samples seemed to have undergone very little changes when compared to the untreated RH. This may be due to the natural recalcitrance of the RH biomass as a result of abundant silica bodies present. The surface of the EFB component of MS from all the pretreatments were altered with exposed microfibrils and silica bodies. Exposure of the microfibrils was more evident in the acid (Fig. 6.1 *e, i,* and *m)* and NMMO-pretreated MS (Fig. 6.1 *h, l,* and *p)* while removal of silica bodies was more pronounced in the NMMO-pretreated MS. Disruption of the EFB component was least obvious in the hydrothermally-pretreated MS (Fig. 6.1 *g, k,* and *o*). Distortion of the smooth OPF component outer surface could be observed in the acid-pretreated MS while the disruption of the mesh-like network of the OPF was most obvious in the alkali- (Fig. 6.1 *f, j,* and *n*) and hydrothermal-pretreated samples. Generally, there were not many differences among MS from all the pretreatments with respect to their microstructure. This is consistent with the chemical composition data where all the MS, with the exception of the acid-pretreated, had similar composition. The observation was attributed to the relatively mild conditions applied in

the pretreatments. The higher severity of the acid treatment was indicated by its relatively higher solubilization of hemicellulose.
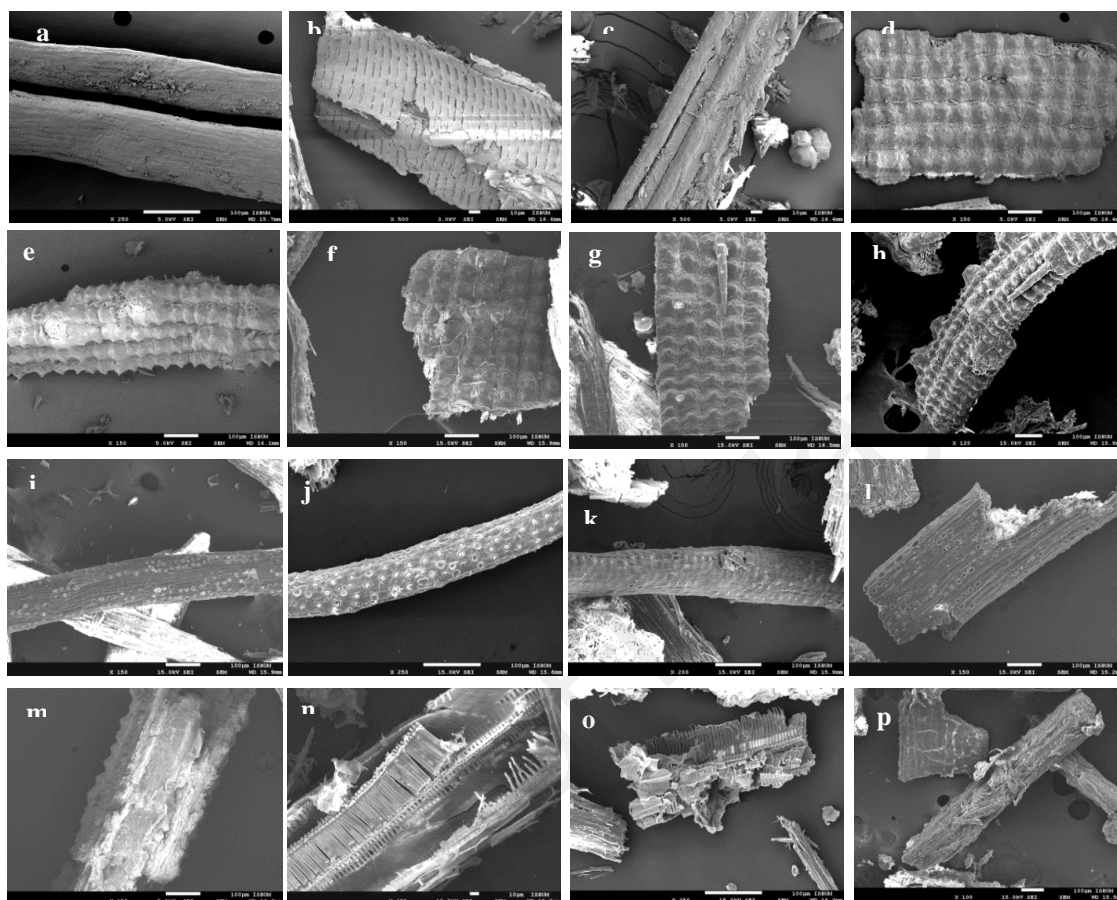
Figure 6.1: Micrographs of untreated SS and individual components of pretreated MS samples. Untreated samples: *a*- EFB × 250; *b*- OPF × 500 (inner surface); *c*- OPF × 500 (outer surface); *d*- RH × 150. Pretreated MS samples: acid (*e*- RH × 150, *i*- EFB × 150, *m*- OPF × 150); alkali (*f*- RH × 150, *j*- EFB × 250, *n*- OPF × 400); hydrothermal (*g*- RH × 150, *k*- EFB × 200, *o*- OPF × 250); NMMO (*h*- RH × 120, *l*- EFB × 150, *p*- OPF × 100). SS were identified in the MS micrographs by comparing MS images with those of pure untreated SS.

6.3.2.2 FTIR and XRD

FTIR and XRD are useful tools for monitoring molecular and crystallinity changes in pretreated lignocellulosic substrates (Xu, Shi, & Wang, 2013). These techniques have been previously applied in the analysis of lignocellulosic mixtures (R. D. O. Moutta, R et al., 2013). However, from the observations made in this study, it seemed that these

techniques are not entirely applicable for lignocellulosic mixtures. It was observed that replicated (5) FTIR spectra and XRD patterns of each pretreated mixture gave inconsistent and dissimilar spectra. This made it difficult to ascertain the actual nature of the changes in each pretreated MS. Thus, any comparison between MS from different pretreatments would be inconclusive. FTIR and XRD measurements require very small amounts of samples, which in the case of mixtures, may not be representative of the actual mixed material that was used in the experiments. Such mixtures are heterogeneous and there is no assurance that the sample used for the measurement contains the exact proportions of the components in the actual mixture. In this regard, other methods need to be explored in order relate the observable enzyme production pattern to the structural and molecular changes occurring in the mixtures.

6.3.3 Effects of different pretreatments on endoglucanase production on MS

The profiles of endoglucanase production by *B. aerius* S5.2 on the MS from the various pretreatments and the untreated MS is shown in Fig. 6.2. A sharp rise in enzyme production was observed between 6- and 12 h on the untreated substrate and the pretreated substrates except for the acid-pretreated substrate, which showed some lag in enzyme production. Maximum enzyme production was reached on all the substrates at 48 h except for the hydrothermally-pretreated MS (60 h). Interestingly, the untreated MS supported the highest endoglucanase production (0.677 U/mL) although this difference was not statistically significant ($P > 0.05$) when compared to the alkali-pretreated and NMMO-pretreated MS. Next to it was the alkali-pretreated MS which gave maximum enzyme titer of 0.630 U/mL. The NMMO-pretreated MS and the hydrothermally-pretreated MS showed similar enzyme production profiles but enzyme activities reached peak value on NMMO-pretreated MS (0.557 U/mL at 48 h) faster than the hydrothermally-pretreated MS (0.549 U/mL at 60 h). There was no significant difference ($P > 0.05$) in the enzyme production on the alkali-, NMMO- and hydrothermally-pretreated MS samples. Enzyme

production on the untreated MS was significantly higher ($P < 0.05$) than on the hydrothermally-pretreated and acid-pretreated MS samples. The acid-pretreated MS produced the least amount of enzyme with a maximum titer of 0.305 U/mL, which was significantly lower ($P < 0.05$) than activities obtained on all other substrates.
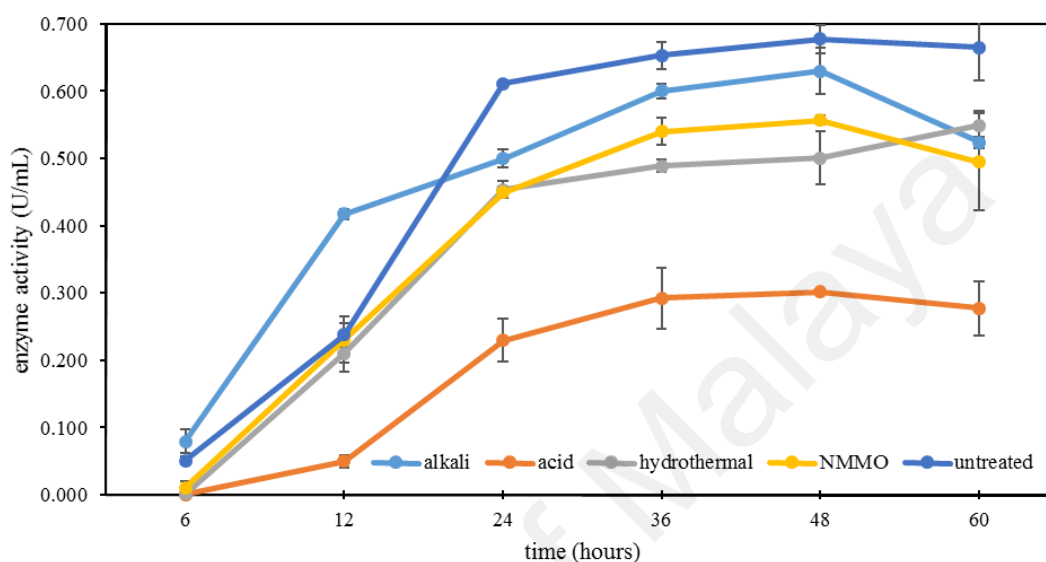
Figure 6.2: Endoglucanase production by *B. aerius* S5.2 on pretreated and untreated MS samples.

Several reasons could be offered for the higher enzyme production recorded on the untreated MS in this study (Fig. 6.2). Firstly, the milling process used in obtaining the small particle sizes is a form of pretreatment itself. Milling brings about increased surface area, reduced crystallinity and provides greater accessibility (X. Zhao et al., 2012). These characteristics may have been altered in the pretreated substrates due to the negative effects of the treatments. Olsson et al. (2003) reported that the removal of some parts of pectin and hemicellulose from sugar beet pulp as a result of pretreatment caused a lag in the growth of *Trichoderma reesei* as compared to the untreated substrate. This is an indication that in some instances, pretreatment could make a substrate less accessible and less suitable for microbial growth and utilization when compared to the untreated one. Acid treatment can also cause lignin to condense on the surface of crystalline cellulose

(C. Li et al., 2010) thereby limiting substrate accessibility for cellulase induction. As the untreated MS used was not washed, the presence of free sugars on the surface of the untreated MS could also have led to the higher endoglucanase production. It was found that un-inoculated fermentation media containing the untreated MS had reducing sugar concentrations of 0.63 mg/mL while those of the pretreated substrates were between 0.01 and 0.06 mg/mL. Cellulase production is normally induced by the presence of soluble cellulose derivatives and other low molecular weight carbohydrates such as cellobiose, xylose, sophorose, and lactose (James & Ming, 1991). Furthermore, the removal of these substances and other water-soluble micronutrients from the MS during the pretreatments might have made the substrate less favourable for the organism's metabolism (Basu & Ghose, 1960). Higher enzyme production on untreated substrates have been previously reported (Rodriguez-Zuniga et al., 2014; Sharma et al., 2015).

The acid-pretreated MS gave the least enzyme production despite its higher cellulose content (Fig. 6.2). It also had the highest amount of lignin and the lowest amount of hemicellulose as compared to the other pretreated MS samples (Table 6.1). Although the extent of cellulase production/induction is dependent on the accessibility and exposure of cellulose in the substrate, previous studies have shown that amount of cellulose is not the sole determinant of cellulase production in microbial fermentation. The substrate's physicochemical and structural characteristics also influence cellulase production (Brijwani & Vadlani, 2011; Umikalsom et al., 1997). Bigelow and Wyman (2002) reported that increased cellulose level of hot water-pretreated bagasse had little effect on cellulase production by *T. reesei* C30. This trend was not observed with similar concentrations of Solka floc which is almost entirely composed of cellulose. The authors therefore suggested that other inhibitory effects inherent in the pretreated substrates were responsible for this observation. Similarly, Sharma et al. (2015) found no direct relationship between cellulose content and cellulase production by *Penicillium*

janthinellum EMS-UV-8 on wheat straw samples which had been subjected to varying degrees of pretreatments. However, the significantly higher lignin content (Table 6.1) could be the major reason for the low endoglucanase production recorded on the acid-pretreated MS in this study. Acid pretreatment is known to preferentially solubilize hemicellulose and less ordered forms of cellulose, thereby leaving a lignin-rich residue behind (X. Zhao et al., 2012). It has been well established from previous studies that lignin plays an inhibitory role towards cellulose accessibility. The effects of lignin on microbial cellulase production can be summarized as follows: (1) inhibition of microbial growth and cellulase production (Bigelow & Wyman, 2002), (2) irreversible adsorption and cellulase loss (Bigelow & Wyman, 2002), and (3) limiting exposure of cellulose thereby reducing availability for enzyme induction (L. Zhang et al., 2012). Lower cellulase production on acid-pretreated substrates as compared to other pretreatment methods have been reported previously (Salihu, Abbas, Sallau, & Alam, 2015; L. Zhang et al., 2012).

Despite the similarity in the chemical composition of the alkali-, hydrothermal- and NMMO-pretreated MS samples (Table 6.1), alkali-pretreated MS supported significantly higher ($P < 0.05$) endoglucanase production than the hydrothermally-pretreated MS but had similar enzyme activity to the NMMO-pretreated MS. This can be attributed to the unique effect of alkali and NMMO on lignocellulose which altered the characteristics of the substrate in a more favourable manner than the hydrothermal pretreatment. Alkali pretreatment causes the swelling of cellulose fibrils and increased internal surface area thereby making the cellulose accessible for enzyme induction (L. Zhang et al., 2012). NMMO causes reduction in surface lignin, reduced crystallinity and increased porosity of the substrate microstructure (Shafiei, Karimi, Zilouei, & Taherzadeh, 2014). These effects might not have been as pronounced in the hydrothermal pretreatment applied in this study.

6.3.4 Chemical composition of alkali-pretreated SS

Since the alkali-pretreated MS supported the highest endoglucanase production by *B. aerius* S5.2, the same pretreatment was subsequently applied on each SS. The pretreated SS were used as carbon source for endoglucanase production by the strain. This was done in order to ascertain whether pretreating the SS separately was more favourable than pretreating them jointly for enzyme production. Compositional analysis of the alkali-pretreated SS samples was conducted so as to relate their compositional changes to the observed enzyme production pattern. Results of the compositional analysis are presented in Table 6.2. Alkali-pretreated EFB had the highest cellulose and hemicellulose content while alkali-pretreated RH had the highest lignin content and the least amount of hemicellulose.

Table 6.2: Composition of alkali pretreated single substrates.

Substrate	Chemical composition (%)				
	Cellulose	Hemicellulose	Lignin	Ash	Others
EFB	32.83 ± 0.08^a	21.97 ± 2.42^a	15.21 ± 0.52^b	0.72 ± 0.15^a	29.28 ± 1.80^b
OPF	26.49 ± 0.88^c	17.76 ± 0.44^a	13.60 ± 0.60^b	-	42.15 ± 1.04^a
RH	28.49 ± 0.73^b	16.65 ± 2.62^a	23.01 ± 3.39^a	0.48 ± 0.16^a	28.75 ± 0.70^b

Data are expressed on a dry weight basis. [a-c] Values represented means of two (EFB and OPF) or three (RH) replicates ± standard deviation (SD). Values within the same column and having same superscript letters are not significantly different ($p > 0.05$).

6.3.5 Physicochemical characteristics of alkali-pretreated SS

6.3.5.1 FESEM

As observed in the MS pretreatments, alkali-pretreated RH (Fig. 6.3 *b*) showed very little changes compared to its untreated counterpart (Fig. 6.1 *d*). EFB was visibly altered due to alkali pretreatment as can be seen in the exposed microfibrils and dislodged silica bodies (Fig 6.3 *a*). Disruption of the surface of the alkali-pretreated OPF fibers could also been seen (Fig 6.3 *c*). These changes could play an important role in the uptake and

utilization of the substrate by the organism and its subsequent use for endoglucanase production.
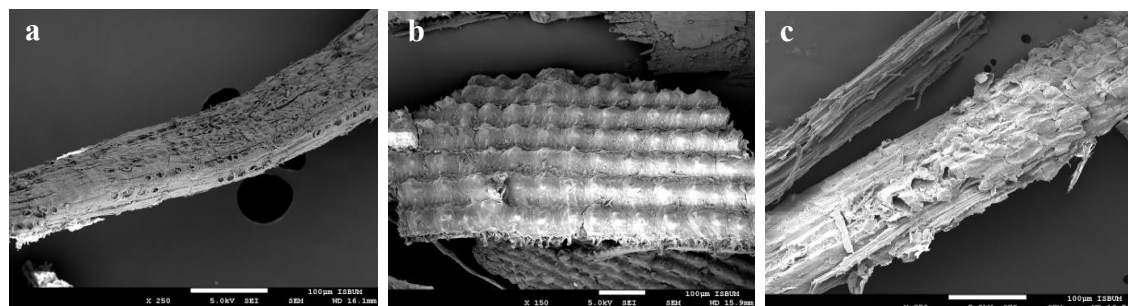
Figure 6.3 FESEM micrographs of alkali-pretreated SS samples. *a-* EFB × 250; *b-* RH × 150; *c-* OPF × 250.

6.3.5.2 FTIR

The extent of alteration of each pretreated SS from its untreated form was determined by the difference in intensities at frequencies of prominent band changes of its spectrum. These are presented in Table 6.3 along with the band assignments. Comparison of the FTIR spectra showed that OPF had the most significant alteration from its untreated form (Fig. 6.4 *b*). It had the highest changes in band intensities at 1032 cm^{-1} and around 2920 – 2900 cm^{-1}. This showed greater degradation of the major lignocellulose components in the alkali-pretreated OPF. The highest change in band intensity at 3336 cm^{-1} was seen in the alkali-pretreated EFB (Fig. 6.4 *a*). This suggested that higher amount of delignification occurred in the EFB due to the increased presence of the OH groups associated with cellulose. The spectrum of the alkali-pretreated RH was very similar to that of the untreated sample except for the reduced band intensity at 1032 cm^{-1} (Fig. 6.4 *c*). This was consistent with observation from the FESEM analysis which showed that very little changes occurred in the pretreated RH.

Table 6.3: Band intensity changes in FTIR spectra of alkali-pretreated SS.

Wavenumber (cm^{-1})	Band assignment	Source component	Difference in band intensity			Ref
			EFB	OPF	RH	
1033 – 1030	C–O, and C–C, and C–O–C stretching	Cellulose, hemicellulose, lignin	0.1986	0.3978	0.1644	(Deborah L Sills & James M Gossett, 2012)
2920 – 2900	Methylene C-H stretching	Cellulose	0.0545	0.0992	0.0293	(Hsu, Guo, Chen, & Hwang, 2010)
3336 – 3330	O–H stretching of hydrogen bonds	Cellulose	0.2325	0.2072	0.033	(Hsu et al., 2010)
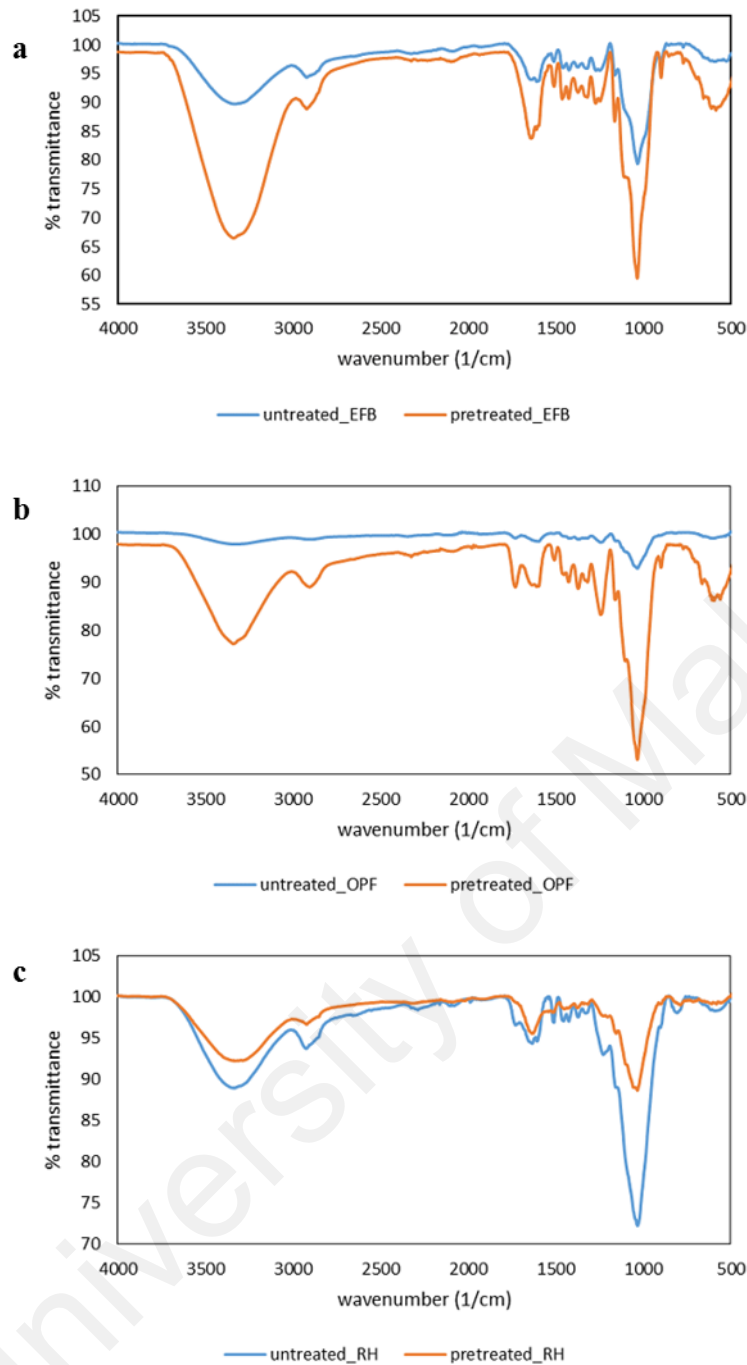
Figure 6.4: FTIR spectra of alkali-pretreated SS samples: EFB (*a*). OPF (*b*) and RH (*c*).

6.3.5.3 XRD

Based on the XRD data (Fig. 6.5), the *CrI* values of the alkali-pretreated SS used in this study were 44.61, 50.08 and 41.10 for EFB, OPF and RH respectively. The high *CrI* value

of the OPF was due to the severity of the pretreatment on this substrate as compared to EFB and RH. Higher *CrI* values are consistent with lesser abundance of amorphous portions (amorphous cellulose, hemicellulose and lignin) of biomass following their removal during pretreatment (Rodriguez-Zuniga et al., 2014). This was supported by data from the chemical composition of the substrates (Table 2) that showed the samples with higher *CrI* had lower amorphous components (EFB- 37.18%, OPF- 31.36% and RH- 39.66%) *viz.* hemicellulose and lignin.
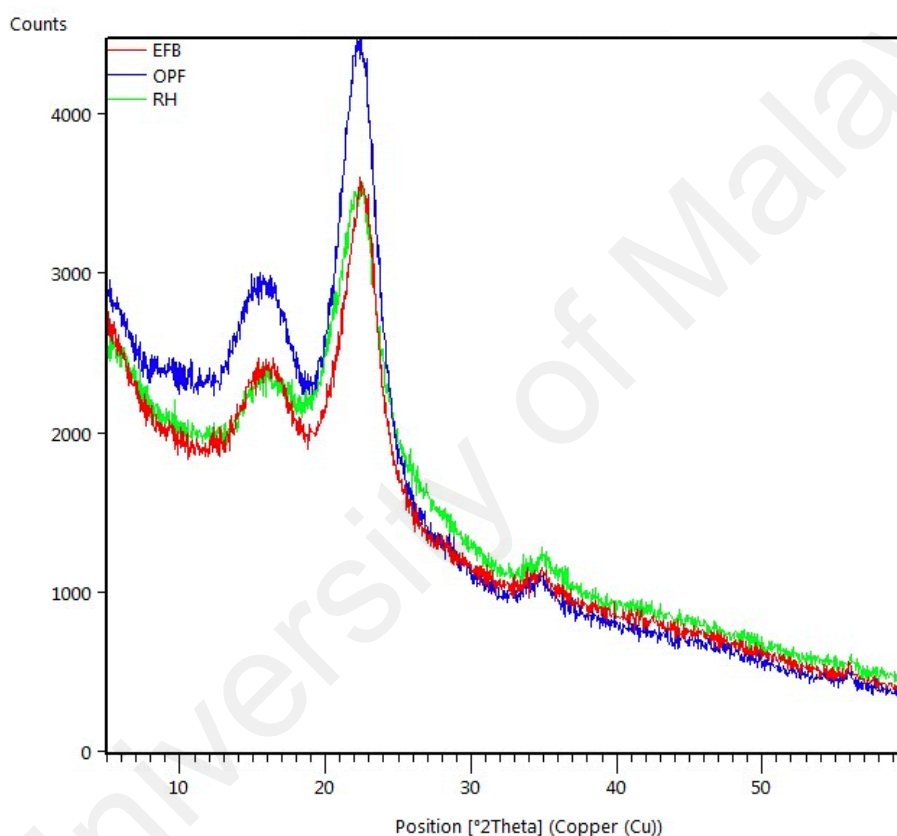
Figure 6.5: XRD diffraction patterns of alkali-pretreated EFB, OPF and RH.

6.3.6 Effects of separate/joint pretreatment on endoglucanase production

The endoglucanase production profile of *B. aerius* S5.2 on the alkali-pretreated SS is presented in Fig. 6.6. Endoglucanase production data on the alkali-pretreated MS was also included for comparison. There was a sharp increase in enzyme production on the SS similar to that observed in the MS. Enzyme production peaked at 48 h on all the substrates. Enzyme titer was highest on alkali-pretreated EFB but was not significantly

higher than ($P > 0.05$) that recorded on alkali-pretreated MS. The enzyme production profile on the two substrates was very similar. Although enzyme production was considerably higher on alkali-pretreated RH in the first 36 h of fermentation than was obtained on alkali-pretreated OPF, there was no significant difference ($P > 0.05$) in their maximum enzyme activities at 48 h.
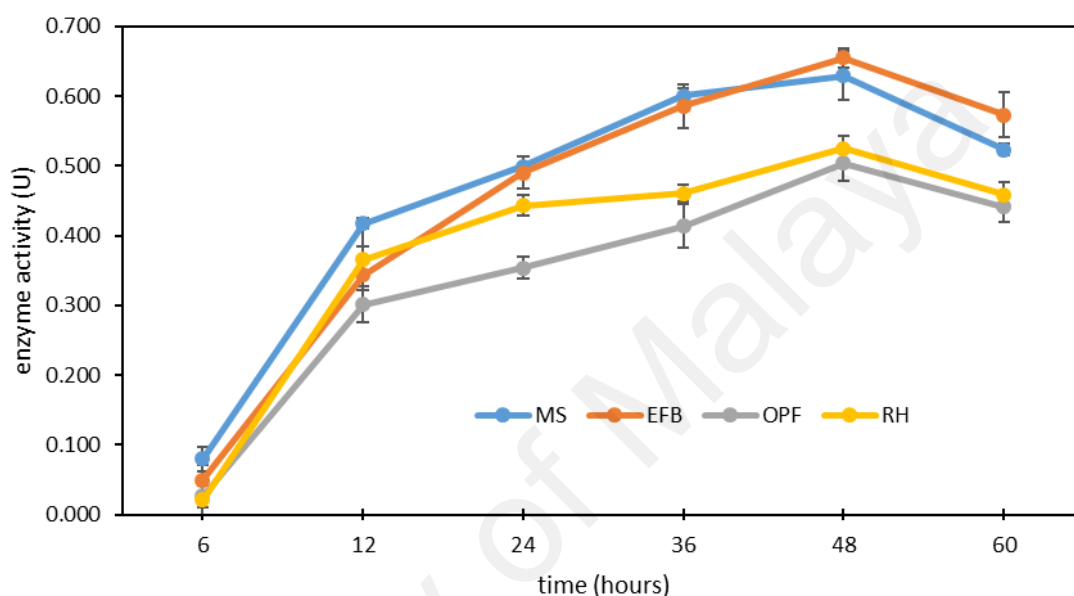
Figure 6.6: Endoglucanase production by *B. aerius* S5.2 on alkali-pretreated single and mixed substrates. Error bars represent standard deviation of three replicates.

The greater severity of alkali pretreatment on OPF as indicated by FESEM (Fig. 6.3), FTIR (Fig. 6.4, Table 6.3), and XRD (Fig. 6.5) data may have caused the low enzyme production recorded on this substrate. Severe pretreatment conditions erode amorphous portions of biomass thereby rendering the substrate unfavourable for microbial uptake and enzyme production. In a recent study, Sharma et al. (2015) reported higher cellulase production with increasing amorphous nature of the substrate when *Penicillium janthinellum* EMS-UV-8 was grown on wheat straw which had been subjected to varying levels of pretreatment severity. As for the RH, FESEM and FTIR data showed that it underwent very little change after pretreatment as the silica bodies were still intact (Figs. 6.1 & 6.3). Retention of silica bodies in this substrate may have made it unfavourable for

the organism. Silica bodies have been reported to prevent bacterial attachment to plant biomass and are also inhibitory to cellulolytic microorganisms (Bae et al., 1997).

Thus, it seemed that EFB had significant contribution to the mixed substrate properties that made the mixture more favourable for endoglucanase production than the other SS. This suggestion was supported by the observed similarity in enzyme production on the alkali-pretreated MS and alkali-pretreated EFB. Previous studies have shown that individual components vary in their contribution to enzyme production on mixtures with some favouring enzyme production than the others. Jecu (2000) reported that wheat straw (WS) used singly or in higher proportion supported higher endoglucanase production than sole wheat bran (WB) or WS:WB mixtures with higher WB proportions. Similar findings have also been reported for RS:WB mixtures (Sherief et al., 2010). Therefore, it may be necessary to optimize the ratio of the mixture components used in order to achieve significantly higher enzyme production on the MS. Although these studies used fungi and solid substrate fermentations, it is generally known that cellulase production is inducible and substrate-dependent in most microorganisms (L. R. Lynd et al., 2002). The higher cellulose and hemicellulose content was also influential in making EFB more favourable for enzyme production since hemicellulose content is known to influence cellulase production (Basu & Ghose, 1960).

Findings from this study thus showed that combining all the SS together under a single pretreatment was more favourable for endoglucanase production than pretreating them separately since the enzyme titer obtained on the alkali-pretreated MS was higher than that obtained on most of the alkali-pretreated SS (Fig. 6.6). The difference in enzyme production between the alkali-pretreated MS and alkali-pretreated EFB was also not significant ($p > 0.05$). These results are in agreement with the findings of Olsson et al. (2003) who reported that higher levels of endoglucanase, endoxylanase and polygalacturonase were obtained on mixtures of cellulose and pretreated sugar beet pulps

than on the single substrates when *T. reesei* Rut C-30 was used. It is however necessary to optimize MS pretreatment conditions so as to obtain enzyme titers higher than those on the SS or at least comparable to any of the SS. This would ensure that the use of MS would be economically advantageous.

6.3.7 Effects of untreated SS and MS (substrate combination) on endoglucanase production

As shown in Fig. 6.7, endoglucanase production on the untreated SS and untreated MS followed a similar pattern except for untreated RH. Maximum endoglucanase production on untreated OPF (0.660 ± 0.008 U/mL) and untreated RH (0.543 ± 0.025 U/mL) was reached at 36 h, while untreated EFB (0.687 ± 0.052 U/mL) was reached at 60 h. Endoglucanase production peaked at 48 h on untreated MS (0.697 ± 0.018 U/mL). There was no significant difference ($p > 0.05$) in endoglucanase activities obtained on untreated-EFB, untreated-OPF, and untreated-MS. However, endoglucanase titer was significantly lower ($P < 0.05$) on untreated-RH compared to the other substrates. These results showed that combining the SS had no deleterious effects on endoglucanase production. The results are in agreement with the observations recorded for alkali-pretreated SS and MS.
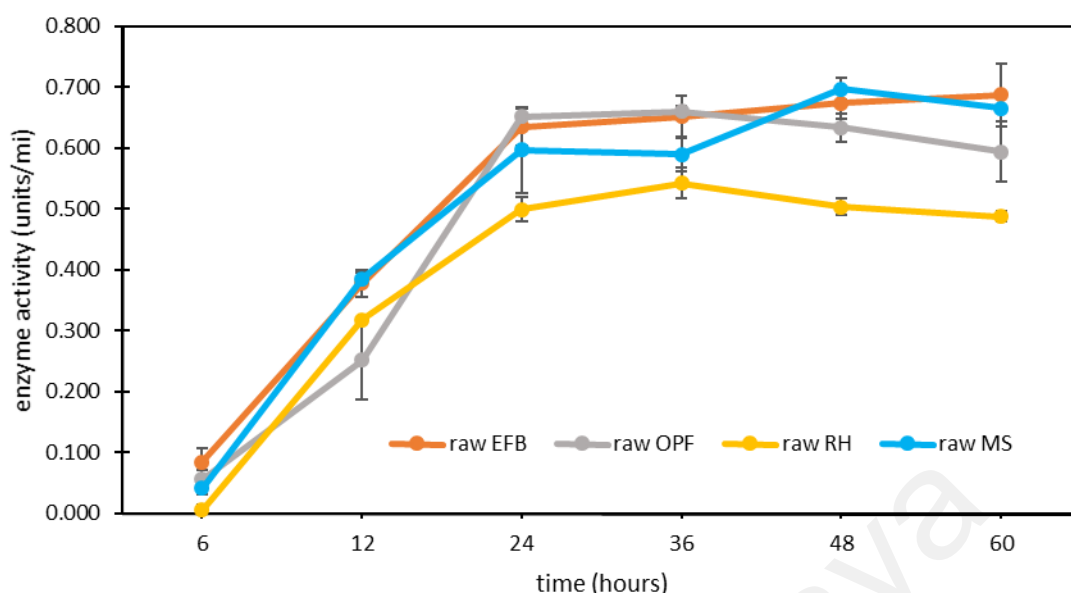
Figure 6.7: Endoglucanase production by *B. aerius* S5.2 on untreated single and mixed substrates.

6.4 Conclusions

It can be inferred from the results that the more severe pretreatments were unfavourable for endoglucanase production by *B. aerius* S5.2. Thus, thermochemical pretreatment of lignocellulosic substrates is deemed unsuitable for particle size range of 300 – 425 µm as used in the study. Pretreating the SS jointly gave better endoglucanase production than pretreating them separately. Furthermore, it was observed that combining the SS as a mixture for endoglucanase production had no negative effects compared to their separate use. Optimization of mixture proportion using statistical tools could help to enhance endoglucanase titers. Unique characteristics of the single substrates should be considered before selecting them as mixture components. The ones with more favourable features for the intended application should be in higher proportion in such mixtures.

CHAPTER 7: OPTIMIZATION OF *Bacillus aerius* S5.2 ENDOGLUCANASE PRODUCTION ON MIXED LIGNOCELLULOSIC SUBSTRATES USING RESPONSE SURFACE METHODOLOGY AND MIXTURE DESIGN

7.1 Introduction and literature review

Cellulases are a group of synergistic enzymes which catalyse the hydrolysis of cellulose into glucose. The three main types are: endoglucanases (acting on internal amorphous regions), exoglucanases (acting on crystalline regions at reducing- and non-reducing ends), and β-glucosidases (hydrolyzing cellobiose to glucose) (L. R. Lynd et al., 2002). Cellulases have varied applications in several industries but endoglucanases are of special interest because their action is considered the rate limiting step in cellulose utilization (Malherbe & Cloete, 2002). These class of cellulases also have specific applications in the textile (Clarkson et al., 2002) and food processing industries (Tahir-Nadeem et al., 2009).

Since cellulases are inducible enzymes (L. R. Lynd et al., 2002), the nature of the carbon sources which act as inducers for enzyme synthesis is very important. Sensitivity analysis has shown that the carbon source is the major cost factor in cellulase production (Ryu & Mandels, 1980). Commercial cellulase production is usually carried out using carbon sources such as lactose, Solka floc, Avicel, microcrystalline cellulose, and other pure cellulosic substrates, which are expensive to use on a large scale (S. Chen & Wayman, 1991; Wayman & Chen, 1992), which contributed to the high cost of cellulases. The use of cheap substrates has therefore been advocated as a means of reducing the cost of cellulase production (Klein-Marcuschamer et al., 2012). Consequently, lignocellulosic biomass materials have been explored as substrates for cellulase production since they are cheap and abundant (Hidayah Ariffin et al., 2008; Da Vinha et al., 2011; Jo et al., 2008). Lignocellulosic biomass in the form of oil palm and rice wastes are produced in large amounts in Malaysia from the vibrant oil palm and rice industry (Shafie et al., 2012;

Shuit et al., 2009). These wastes are usually disposed of through open burning or by accumulation on land, both of which are harmful to the environment. Utilization of these wastes for the production of value-added bioproducts such as enzymes provide avenues for additional income and ensure a safer environment.

Most of the available literature concerning the use of lignocellulosic substrates for cellulase production focused mainly on single feedstock (substrate) types. However, this approach cannot be considered very practical, considering the challenges of feedstock supply and logistics confronting the lignocellulosic biorefinery (Murphy et al., 2015; A.A. Rentizelas et al., 2009). The seasonality of agricultural biomass and its concomitant effect of dwindling feedstock supply to the biorefinery necessitate a biorefinery to utilize mixed streams of varied biomass types. This would ensure a smooth year-round running of the facility and avoid losses on investment. Hence, it is necessary to combine different substrates for use in lignocellulosic bioconversion processes. It has been shown that the use of mixed lignocellulosics could bring about significant cost reduction (Nilsson & Hansson, 2001; Sultana & Kumar, 2011), improve product yields in bioconversion processes (Imamoglu & Sukan, 2014; Pereira et al., 2015), and eliminate/reduce the need for extraneous nutrient supplementation (Martín et al., 2008; Thomsen & Haugaard-Nielsen, 2008) and detoxification (Elliston et al., 2015) in downstream fermentations.

While mixed lignocellulosics have been used in some studies for the production of fungal cellulases (Jecu, 2000; Qi et al., 2007; Shamala & Sreekantiah, 1986), the use of such mixtures for bacterial cellulase production has not been reported. Furthermore, the use of lignocellulosic mixtures for cellulase production has been studied predominantly in solid-state fermentations (SSF). Reports of cellulase production from mixed lignocellulosics in submerged fermentations are extremely rare. However, considering the advantages associated with the use of bacteria for cellulase production (high growth rate, amenability to genetic manipulation, production of cellulosomes, etc.), and the unique properties of

bacterial cellulases (e.g. tolerance of relatively harsh conditions) (M. Maki et al., 2009), it is necessary to explore cellulase production from mixed substrates using bacteria. Besides, the submerged fermentation system is more suitable for bacterial cultivation and it is easier to control process conditions for optimization of enzyme yields under this system (Kuhad et al., 2016; Singhania et al., 2010).

Cellulase production is controlled by a complex interaction between numerous culture media-related variables (carbon, nitrogen sources and their concentrations, and trace minerals) and environmental factors (temperature, pH, aeration, etc.) (Biswas, Persad, & Bisaria, 2014). For this reason, significant improvements in cellulase titer and productivity can be achieved through systematic optimization of relevant media components and process (fermentation) variables (Biswas et al., 2014; Ryu & Mandels, 1980). In the case of mixed substrates utilization, the relative proportion of the mixture components also influences cellulase production. Qi et al. (2007) studied the effects of different ratios of rice straw (RS) and wheat bran (WB) on the production of cellulases by *Trichoderma viride* ZY-01 in SSF. They found that while higher proportions of RS favoured the production of FPase and CMCase, the reverse was the case for β-glucosidase. The optimal ratios (RS:WB) for the enzymes were 3:2 for FPase, 4:1 for CMCase, and 1:4 for β-glucosidase. In another study, Jecu (2000) investigated the effects of fermentation conditions and ratios of wheat straw (WS) and WB on endoglucanase production by *Aspergillus niger* 38 in SSF. They reported that the best conditions were 74 % moisture, pH 4.5 – 5.5, and 30 °C. The optimal ratio of the mixed substrate was WS:WB = 9:1. In these studies and others of their kind (Shamala & Sreekantiah, 1986; Sherief et al., 2010), selection of the mixture ratios to be studied were made arbitrarily by random variation of mixture proportions. Furthermore, not all possible combinations of the components were tested.

The approach adopted in the studies mentioned above (one-factor-at-a-time -OFAT methodology) has been shown to be inefficient, unreliable, time consuming, laborious, and sometimes misleading (Antony, 2003). The use of statistical optimization tools such as screening designs, response surface methodology (RSM), and design of experiment (DoE) principles, assists in identifying important factors, takes into consideration the interaction among factors, and allows for optimization using minimal number of runs (Biswas et al., 2014; Mandenius & Brundin, 2008). If all other influential factors are adequately controlled, the relative proportion of the substrate components becomes the sole factor determining the yield/productivity of the enzyme in cellulase production from mixed substrates. This is true, provided that the total amount of the mixture (substrate concentration) is held constant (Cornell, 2002; Prakasham et al., 2009). In such a situation, mixture designs of RSM would be suitable for optimizing the response (Cornell, 1973). In mixture design, the components of the mixture are the factors influencing the response and the relative proportions are regarded as the factor levels. Unlike factorial experiments, the factors in mixture experiments are not independent since they all add up to 100 % and the amount of one component determines the amount of another. To the best of this author's knowledge, there has been no report on the use of mixture design for the optimization of cellulase production from mixed substrates.

The objective of this study was to optimize process conditions and substrate component proportions for maximal endoglucanase production by *B. aerius* S5.2 on a mixed substrate (MS) comprising oil palm empty fruit bunch (EFB), oil palm frond (OPF), and rice husk (RH). Selected process variables were screened for their influence on enzyme yields using fractional factorial design. These were then optimized using Box-Behnken design. Under the optimal levels of the process variables, substrate component proportions for maximal endoglucanase titers were optimized using two forms of mixture designs (simplex

centroid and extreme vertices). This was done in order to determine which of the designs is superior in predicting and optimizing endoglucanase production on the MS.

7.2 Materials and methods

7.2.1 Bacterial strain

Bacillus aerius S5.2 used in this study was isolated from decomposing EFB residues that were collected from an oil palm plantation in Kuala Selangor, Malaysia. This strain produced high endoglucanase activity on a mixed substrate (MS) that comprised EFB, OPF, and RH. It was identified as *B. aerius* following sequencing of the 16S rRNA gene and sequence similarity check using the BLAST tool on the NCBI database. The sequence was submitted to GenBank and an accession number (KP178216) was obtained. The strain was maintained on nutrient agar slants at 4 °C.

7.2.2 Substrates collection and preparation

Fresh OPF samples were obtained from Malaysian Palm Oil Board (MPOB), Bangi, Malaysia. The leaflets were removed and the fresh petioles were cut into smaller pieces and dried under sunlight. Only the petioles were used in this study because the leaflets of OPF have other important uses in an oil palm plantation. The petiole has been shown to possess higher content of sugars which makes it more desirable for use as feedstock in biofuels and other bioproducts processes (Zahari et al., 2012). Dried and shredded EFB fibres were obtained from Biocomposting Pilot Facility, UPM, Malaysia. Rice husk (RH) used in the process variables screening and in the simplex centroid design experiments was collected from a paddy field in Kedah, Malaysia. The RH used in the extreme vertices design experiments was obtained from Bernas Rice Mill, Sekinchan, Selangor, Malaysia. The biomass samples were reduced to small particles (300 – 425 µm) using a Rapid granulator (GK 205-K, Terramar, Hamburg, Germany) and were kept in a dry environment prior to their usage.

7.2.3 Endoglucanase production

Modified medium of Dickerman and Starr (1951) with 2 % (w/v) of untreated MS as carbon source was used for endoglucanase production. Other components of the medium were (w/v): K$_2$HPO$_4$ (0.08 %), KH$_2$PO$_4$ (0.02 %), MgSO$_4$·7H$_2$O (0.02 %), NaCl (0.02 %), NaNO$_3$ (0.1 %), CaCO$_3$ (0.001 %), and yeast extract (0.05 %). MS used in the process variables optimization experiments contained EFB, OPF, and RH in equal proportions, while that used in the mixture design experiments contained the substrate proportions of the respective design points used in the study. Medium pH was adjusted to the respective levels of the experimental design with 1.0 M NaOH or 1.0 M HCl. Two percent of *B. aerius* S5.2 inoculum, previously grown in nutrient broth up to the late log phase (12 h), and containing approximately 10^7 cfu/mL, was used in the experiments. Each culture flask was incubated for 36 h at the respective temperature for each design point in the experiments. Previous experiments had shown that maximal endoglucanase production occurred at 36 h. At the end of the fermentations, culture samples were collected and were centrifuged at 6000 rpm at 4 °C for 10 minutes. The cell-free supernatant obtained was used as crude enzyme preparation in the endoglucanase assay.

7.2.4 Endoglucanase assay

Endoglucanase activity was determined by measuring the reducing sugars released after the reaction of 200 µL of the enzyme preparation with 200 µL of 2 % CMC in 0.05 M phosphate buffer (pH 7.0) (Y. H. P. Zhang et al., 2009). The mixture was incubated for 30 minutes at 50 °C and the reaction was stopped by the addition of 800 µL of DNS reagent followed by immersing the reaction tubes in boiling water for 5 minutes. Released sugars were measured as glucose equivalent using the DNS reagent (Miller, 1959). One unit (U) of enzyme activity was defined as the amount of enzyme that liberated 1 µmol of reducing sugar per mL per minute from the substrate.

7.2.5 Screening and optimization of process variables for endoglucanase production

7.2.5.1 Screening of factors (process variables)

Three process variables (pH, temperature, and agitation speed) were screened to determine which of them significantly influenced endoglucanase production. This was done using a two-level fractional factorial design (2^{3-1}) with triplicate runs at each design point, resulting in a total of 12 runs (Table 7.1). The factor settings used for temperature, pH, and agitation were as follows: low (-1): 25 °C, 5.0, and 100 rpm; and high (+1): 37 °C, 8.0, and 200 rpm respectively. Endoglucanase activity (U/mL) was taken as the response variable. Experimental design and data analysis were done using Minitab software (version 17.1.0, Minitab Inc.). The Pareto chart and the normal plot of standardized effects were used in determining the significant factors at α = 0.05.

Table 7.1: Fractional factorial design used in this study.

Runs	Temperature (°C)	pH	Agitation (rpm)	Enzyme activity (U/mL)
1	37 (+1)	5.0 (-1)	100 (-1)	0.767
2	25 (-1)	8.0 (+1)	100 (-1)	0.604
3	37 (+1)	8.0 (+1)	200 (+1)	0.802
4	37 (+1)	5.0 (-1)	100 (-1)	0.771
5	37 (+1)	5.0 (-1)	100 (-1)	0.672
6	25 (-1)	5.0 (-1)	200 (+1)	0.390
7	37 (+1)	8.0 (+1)	200 (+1)	0.828
8	25 (-1)	5.0 (-1)	200 (+1)	0.366
9	25 (-1)	8.0 (+1)	100 (-1)	0.580
10	25 (-1)	5.0 (-1)	200 (+1)	0.390
11	25 (-1)	8.0 (+1)	100 (-1)	0.602
12	37 (+1)	8.0 (+1)	200 (+1)	0.810

7.2.5.2 Optimization of process variables by RSM

After identifying the important process variables in the screening experiments, Box-Behnken design (Box & Behnken, 1960) was then employed in determining the optimal settings of these factors (pH, temperature, and agitation) for endoglucanase production. The same factor levels as used in the screening experiment were used in the optimization

experiments but with additional runs at the centre point (31 °C, pH 6.5, and 150 rpm) designated as (0). In this case, a total of 45 runs (Table 7.2) were conducted (15 triplicate base runs) which included nine replicates at the centre point. A second-order response surface model (Montgomery, 2013) was fitted to the experimental data in order to examine the relationship between the response variable (endoglucanase activity) and the factors:

$$y = \beta_o + \beta_1 x_1 + \beta_2 x_2 + \beta_3 x_3 + \beta_{11} x_1^2 + \beta_{22} x_2^2 + \beta_{33} x_3^2 + \beta_{12} x_1 x_2 \qquad \text{Eq. 7.1}$$
$$+ \beta_{13} x_1 x_3 + \beta_{23} x_2 x_3$$

In this model, y is the endoglucanase activity; x_1, x_2, and x_3 are the process variables (temperature, pH, and agitation respectively); β_o is the model constant; β_1, β_2, and β_3 are linear coefficients; β_{11}, β_{22}, and β_{33} are quadratic coefficients; and β_{12}, β_{13}, and β_{23} are the interaction coefficients.

Minitab software was used for the experimental design, regression analysis, and response surface plots. The response optimizer function of the software was used for determining the optimal levels of the factors for endoglucanase production. Verification of the predicted optimal conditions for endoglucanase production was done by conducting replicate experiments at the optimum levels of the factors.

Table 7.2: Box-Behnken experimental design for optimization of process variables for endoglucanase production by *B. aerius* S5.2.

Runs	Temperature (x_1) (°C)	pH (x_2)	Agitation (x_3) (rpm)	Endoglucanase activity (y) (U/mL)	
				Experimental	Predicted
1	31 (0)	6.5 (0)	150 (0)	0.726	0.705
2	37 (+1)	6.5 (0)	100 (-1)	0.707	0.768
3	31 (0)	8.0 (+1)	100 (-1)	0.670	0.659
4	31 (0)	8.0 (+1)	100 (-1)	0.680	0.659
5	31 (0)	6.5 (0)	150 (0)	0.709	0.705
6	25 (-1)	8.0 (+1)	150 (0)	0.456	0.535
7	31 (0)	6.5 (0)	150 (0)	0.740	0.705
8	25 (-1)	6.5 (0)	100 (-1)	0.430	0.435
9	31 (0)	5.0 (-1)	200 (+1)	0.512	0.632
10	31 (0)	5.0 (-1)	200 (+1)	0.652	0.632
11	31 (0)	5.0 (-1)	100 (-1)	0.522	0.588
12	25 (-1)	6.5 (0)	100 (-1)	0.413	0.435
13	37 (+1)	8.0 (+1)	150 (0)	0.828	0.824
14	25 (-1)	5.0 (-1)	150 (0)	0.358	0.404
15	25 (-1)	6.5 (0)	200 (+1)	0.514	0.502
16	31 (0)	6.5 (0)	150 (0)	0.633	0.705
17	25 (-1)	5.0 (-1)	150 (0)	0.415	0.404
18	37 (+1)	6.5 (0)	200 (+1)	0.820	0.827
19	37 (+1)	5.0 (-1)	150 (0)	0.785	0.774
20	25 (-1)	6.5 (0)	200 (+1)	0.543	0.502

Table 7.2 (continued): Box-Behnken experimental design for optimization of process variables for endoglucanase production by _B. aerius_ S5.2.

Runs	Temperature (x_1) (°C)	pH (x_2)	Agitation (x_3) (rpm)	Endoglucanase activity (y) (U/mL)	
				Experimental	Predicted
21	31 (0)	5.0 (-1)	100 (-1)	0.633	0.588
22	25 (-1)	8.0 (+1)	150 (0)	0.567	0.535
23	37 (+1)	6.5 (0)	100 (-1)	0.756	0.768
24	37 (+1)	8.0 (+1)	150 (0)	0.843	0.824
25	31 (0)	8.0 (+1)	100 (-1)	0.686	0.659
26	31 (0)	6.5 (0)	150 (0)	0.668	0.705
27	31 (0)	6.5 (0)	150 (0)	0.713	0.705
28	25 (-1)	5.0 (-1)	150 (0)	0.409	0.404
29	37 (+1)	8.0 (+1)	150 (0)	0.832	0.824
30	31 (0)	6.5 (0)	150 (0)	0.711	0.705
31	25 (-1)	8.0 (+1)	150 (0)	0.541	0.535
32	31 (0)	6.5 (0)	150 (0)	0.721	0.705
33	25 (-1)	6.5 (0)	100 (-1)	0.444	0.435
34	31 (0)	8.0 (+1)	200 (+1)	0.721	0.741
35	37 (+1)	6.5 (0)	100 (-1)	0.750	0.768
36	37 (+1)	5.0 (-1)	150 (0)	0.791	0.774
37	37 (+1)	5.0 (-1)	150 (0)	0.785	0.774
38	31 (0)	8.0 (+1)	200 (+1)	0.728	0.741
39	31 (0)	8.0 (+1)	200 (+1)	0.723	0.741
40	31 (0)	6.5 (0)	150 (0)	0.723	0.705
41	31 (0)	5.0 (-1)	200 (+1)	0.672	0.632
42	25 (-1)	6.5 (0)	200 (+1)	0.541	0.502

Table 7.2 (continued): Box-Behnken experimental design for optimization of process variables for endoglucanase production by *B. aerius* S5.2.

Runs	Temperature (x_1) (°C)	pH (x_2)	Agitation (x_3) (rpm)	Endoglucanase activity (y) (U/mL)	
				Experimental	Predicted
43	31 (0)	5.0 (-1)	100 (-1)	0.658	0.588
44	37 (+1)	6.5 (0)	200 (+1)	0.847	0.827
45	37 (+1)	6.5 (0)	200 (+1)	0.834	0.827

7.2.6 Optimization of substrate component proportions for endoglucanase production by mixture design

Optimization of substrate component proportions for endoglucanase production was carried out using mixture designs under the assumption of two feedstock supply scenarios that could be encountered in a biorefinery.

7.2.6.1 Optimization using simplex centroid design

Simplex centroid design is suitable for mixtures having zero amount of one or more of the components (Stoyanov & Walmsley, 2006). This design would be applicable in a situation where a biorefinery has abundant supply of different feedstocks and either of the single or mixed feedstocks may be utilized for enzyme production. Here, there are no constraints on component proportions since it is not compulsory that all the single substrates are present. In this study, an augmented simplex centroid design was employed for modelling the relationship between substrate component proportions and endoglucanase production. The experimental design comprised two replicates each of the pure blends (vertices) and binary blends, and three replicates each of the centroid (complete mixture) and axial blends. Triplicate runs were used at the axial and centroid points in order to make better predictions about complete mixtures (Montgomery, 2013).The design resulted in a total of 24 design points (Table 7.3). The total amount of the substrate (MS) in all the experimental runs was kept at 0.60 g in 30 mL of media (i.e. 2 % (w/v) substrate concentration). Relative proportions of each of the substrate components were varied according to the design points. The optimal process variables obtained in earlier optimization experiments were applied i.e. 37 °C, pH 7.45, and 180 rpm agitation speed. The actual speed recommended by the model was 184.8 rpm but 180 rpm was used due to instrumental limitation. Four mixture models (linear, quadratic, special cubic, and full cubic) (Cornell, 2002; Montgomery, 2013) were tested in order to

fit the experimental data (Equations 7.2 – 7.5). Experimental design, model fitting, and response optimization were done using Minitab software. The predicted endoglucanase activity at the optimal component proportions was verified in a set of replicated experiments.

Linear:

$$E(y) = \sum_{i=1}^{p} \beta_i x_i$$

<div align="right">Eq. 7.2</div>

Quadratic:

$$E(y) = \sum_{i=1}^{p} \beta_i x_i + \sum \sum_{i<j} \beta_{ij} \, x_i x_j$$

<div align="right">Eq. 7.3</div>

Special cubic:

$$E(y) = \sum_{i=1}^{p} \beta_i x_i + \sum \sum_{i<j} \beta_{ij} \, x_i x_j + \sum \sum \sum_{i<j<k} \beta_{ijk} \, x_i x_j x_k$$

<div align="right">Eq. 7.4</div>

Full cubic:

$$E(y) = \sum_{i=1}^{p} \beta_i x_i + \sum \sum_{i<j} \beta_{ij} \, x_i x_j + \sum \sum_{i<j} \delta_{ij} \, x_i x_j \left(x_i - x_j \right)$$
$$+ \sum \sum \sum_{i<j<k} \beta_{ijk} \, x_i x_j x_k$$

<div align="right">Eq. 7.5</div>

In equations (7.2) through (7.5), β_i and β_{ij} are the linear and quadratic coefficients, with β_{ij} indicating synergistic (or antagonistic) blending. The parameter δ_{ij} is a cubic

coefficient indicating binary synergistic (or antagonistic) blending between the components i and j; while β_{ijk} is a cubic coefficient indicating ternary synergistic (or antagonistic) blending between the components i, j, and k (Cornell, 2002).

Table 7.3: Augmented simplex-centroid design for optimization of component proportions for *B. aerius* S5.2 endoglucanase production on MS.

Runs	Proportion			Actual amount (g)			Endoglucanase activity (U/mL)	
	EFB	OPF	RH	EFB	OPF	RH	*Experimental*	*Predicted*
1	0.1667	0.1667	0.6667	0.10	0.10	0.40	0.701	0.646
2	1.0000	0.0000	0.0000	0.60	0.00	0.00	0.765	0.756
3	1.0000	0.0000	0.0000	0.60	0.00	0.00	0.740	0.756
4	0.6667	0.1667	0.1667	0.40	0.10	0.10	0.779	0.762
5	0.0000	0.5000	0.5000	0.00	0.30	0.30	0.621	0.644
6	0.3333	0.3333	0.3333	0.20	0.20	0.20	0.608	0.696
7	0.0000	0.0000	1.0000	0.00	0.00	0.60	0.575	0.571
8	0.6667	0.1667	0.1667	0.40	0.10	0.10	0.767	0.762
9	0.5000	0.5000	0.0000	0.30	0.30	0.00	0.802	0.809
10	0.0000	1.0000	0.0000	0.00	0.60	0.00	0.623	0.629
11	0.1667	0.6667	0.1667	0.10	0.40	0.10	0.742	0.690
12	0.3333	0.3333	0.3333	0.20	0.20	0.20	0.674	0.696
13	0.0000	0.5000	0.5000	0.00	0.30	0.30	0.637	0.644
14	0.1667	0.6667	0.1667	0.10	0.40	0.10	0.682	0.690
15	0.0000	1.0000	0.0000	0.00	0.60	0.00	0.623	0.629
16	0.1667	0.1667	0.6667	0.10	0.10	0.40	0.652	0.646
17	0.0000	0.0000	1.0000	0.00	0.00	0.60	0.549	0.571
18	0.6667	0.1667	0.1667	0.40	0.10	0.10	0.802	0.762
19	0.5000	0.0000	0.5000	0.30	0.00	0.30	0.719	0.734
20	0.5000	0.5000	0.0000	0.30	0.30	0.00	0.795	0.809
21	0.5000	0.0000	0.5000	0.30	0.00	0.30	0.723	0.734

Table 7.3 (continued): Augmented simplex-centroid design for optimization of component proportions for *B. aerius* S5.2 endoglucanase production on MS.

Runs	Proportion			Actual amount (g)			Endoglucanase activity (U/mL)	
	EFB	OPF	RH	EFB	OPF	RH	*Experimental*	*Predicted*
22	0.3333	0.3333	0.3333	0.20	0.20	0.20	0.691	0.696
23	0.1667	0.6667	0.1667	0.10	0.40	0.10	0.723	0.690
24	0.1667	0.1667	0.6667	0.10	0.10	0.40	0.676	0.646

7.2.6.2 Optimization using extreme vertices design

Extreme vertices design is used when there are constraints on the proportions of some or all of the components in a mixture design experiment i.e. none of the components has zero amount (Cornell, 2002). The constraints on the component proportions make the factor space become a hyper-polyhedron rather than a simplex as seen in the unconstrained situation (McLean & Anderson, 1966). This design would be applicable in a biorefinery with limited supply of feedstocks. In this case, all the available feedstock types must be utilized i.e. all the substrate components must be present. This design was used in the present study to determine the relationship between the component proportions and endoglucanase production. The experimental design consisted of two replicates each at the vertices and edge centroids, and three replicates each at the overall centroid and axial points. This resulted in a total of 24 design points representing different combinations of EFB, OPF, RH (Table 7.4). Constraints on the component amounts (in a mixture of 0.60 g total amount) were applied as follows:

$$0.10 \text{ g} \leq x \leq 0.40 \text{ g}$$

where 0.10 g was the lower bound for each component and 0.40 g was the upper bound; and x represents EFB, OPF, or RH.

The same amount of substrate and fermentation conditions (process variables) as used in the simplex centroid design were applied. Model testing was done as reported for the simplex centroid design. Experimental design, model fitting, and response optimization were done using Minitab software. The predicted endoglucanase activity at the optimal component proportions was verified in a set of replicated experiments.

140

Table 7.4: Extreme vertices design for optimization of component proportions for *B. aerius* S5.2 endoglucanase production on MS.

Runs	Proportion			Actual amount (g)			Endoglucanase activity (U/mL)	
	EFB	OPF	RH	EFB	OPF	RH	*Experimental*	*Predicted*
1	0.5000	0.2500	0.2500	0.30	0.15	0.15	0.615	0.615
2	0.2500	0.5000	0.2500	0.15	0.30	0.15	0.609	0.617
3	0.6667	0.1667	0.1667	0.40	0.10	0.10	0.662	0.664
4	0.2500	0.2500	0.5000	0.15	0.15	0.30	0.572	0.590
5	0.4167	0.1667	0.4167	0.25	0.10	0.25	0.633	0.631
6	0.5000	0.2500	0.2500	0.30	0.15	0.15	0.619	0.615
7	0.4167	0.4167	0.1667	0.25	0.25	0.10	0.619	0.623
8	0.6667	0.1667	0.1667	0.40	0.10	0.10	0.662	0.664
9	0.1667	0.1667	0.6667	0.10	0.10	0.40	0.537	0.550
10	0.1667	0.6667	0.1667	0.10	0.40	0.10	0.413	0.412
11	0.3333	0.3333	0.3333	0.20	0.20	0.20	0.574	0.624
12	0.2500	0.5000	0.2500	0.15	0.30	0.15	0.625	0.617
13	0.3333	0.3333	0.3333	0.20	0.20	0.20	0.635	0.624
14	0.1667	0.4167	0.4167	0.10	0.25	0.25	0.588	0.592
15	0.1667	0.6667	0.1667	0.10	0.40	0.10	0.407	0.412
16	0.1667	0.4167	0.4167	0.10	0.25	0.25	0.590	0.592
17	0.4167	0.4167	0.1667	0.25	0.25	0.10	0.619	0.623
18	0.2500	0.5000	0.2500	0.15	0.30	0.15	0.639	0.617
19	0.4167	0.1667	0.4167	0.25	0.10	0.25	0.621	0.631
20	0.3333	0.3333	0.3333	0.20	0.20	0.20	0.629	0.624
21	0.1667	0.1667	0.6667	0.10	0.10	0.40	0.560	0.550

Table 7.4 (continued): Extreme vertices design for optimization of component proportions for *B. aerius* S5.2 endoglucanase production on MS.

Runs	Proportion			Actual amount (g)			Endoglucanase activity (U/mL)	
	EFB	OPF	RH	EFB	OPF	RH	*Experimental*	*Predicted*
22	0.5000	0.2500	0.2500	0.30	0.15	0.15	0.635	0.615
23	0.2500	0.2500	0.5000	0.15	0.15	0.30	0.590	0.590
24	0.2500	0.2500	0.5000	0.15	0.15	0.30	0.631	0.590

7.3 Results and discussion

7.3.1 Screening and optimization of process variables for endoglucanase production

7.3.1.1 Screening of process variables

Screening for the influential factors among the three selected factors (temperature, pH, and agitation) was carried out using the fractional factorial design. Endoglucanase activity obtained at different combinations of the factor levels are presented in Table 7.1. The Pareto chart of the standardized effects of the factors screened in this study is presented in Figure 7.1. The lengths of the bars in the chart correspond to the relative magnitude of the effects of the factors. Factors whose bars cross the reference line have significant effects (Antony, 2003). It can be seen from the figure that all the factors studied had significant effects on endoglucanase production. Temperature had the most significant effect followed by pH; agitation had the least effect.
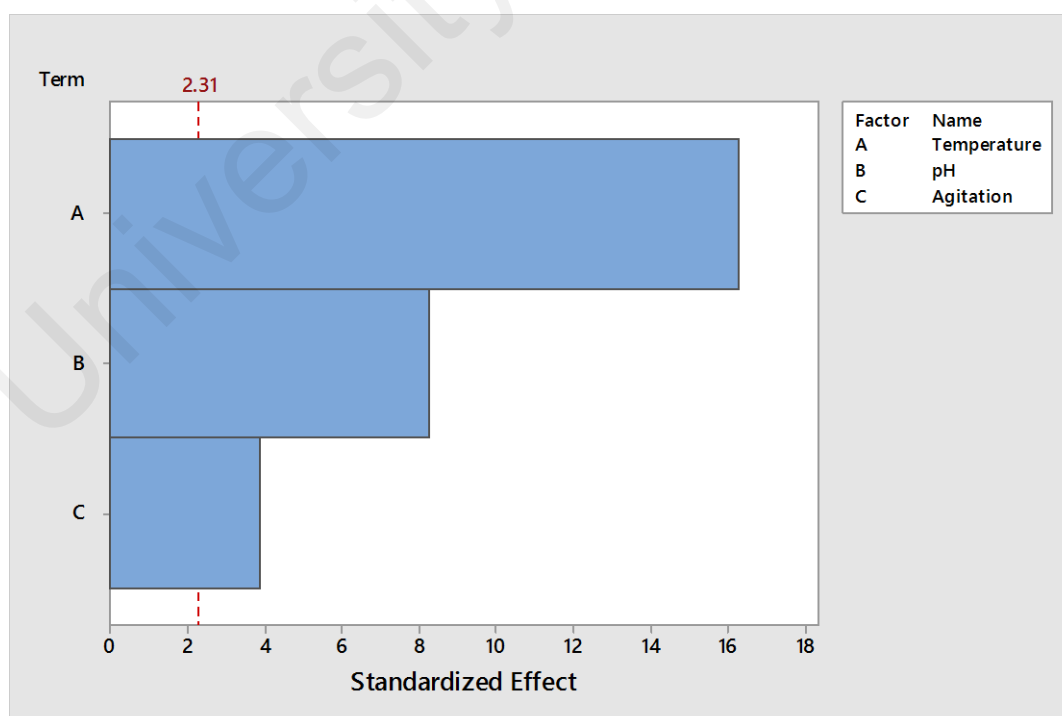
Figure 7.1: Pareto chart of the effects of temperature, pH, and agitation on endoglucanase production by *B. aerius* S5.2 (α = 0.05).

Besides the Pareto chart, another graph that provides further information about the effects of factors in a screening experiment is the normal effects plot. This plot provides information on the direction of the effect of each factor in addition to information about the significance of each effect (Antony, 2003). Figure 7.2 shows the normal effect plot for the factors screened in this study. As seen in Figure 7.2, all the factors had significant effects. However, Figure 7.2 further showed that agitation had negative effect, meaning that increase in the levels of this factor within the range tested would lead to a decrease in endoglucanase production. Conversely, for temperature and pH, which both showed positive standardized effects, an increase in factor level would result in increased endoglucanase production. These observations are in agreement with Khan and Husaini (2006) who reported that temperature, pH, agitation were the most significant factors affecting cellulase production by *Bacillus amyloliquefaciens* UMAS 1002. The current study is the first report of investigation into the effects of fermentation conditions on cellulase production by *B. aerius*.
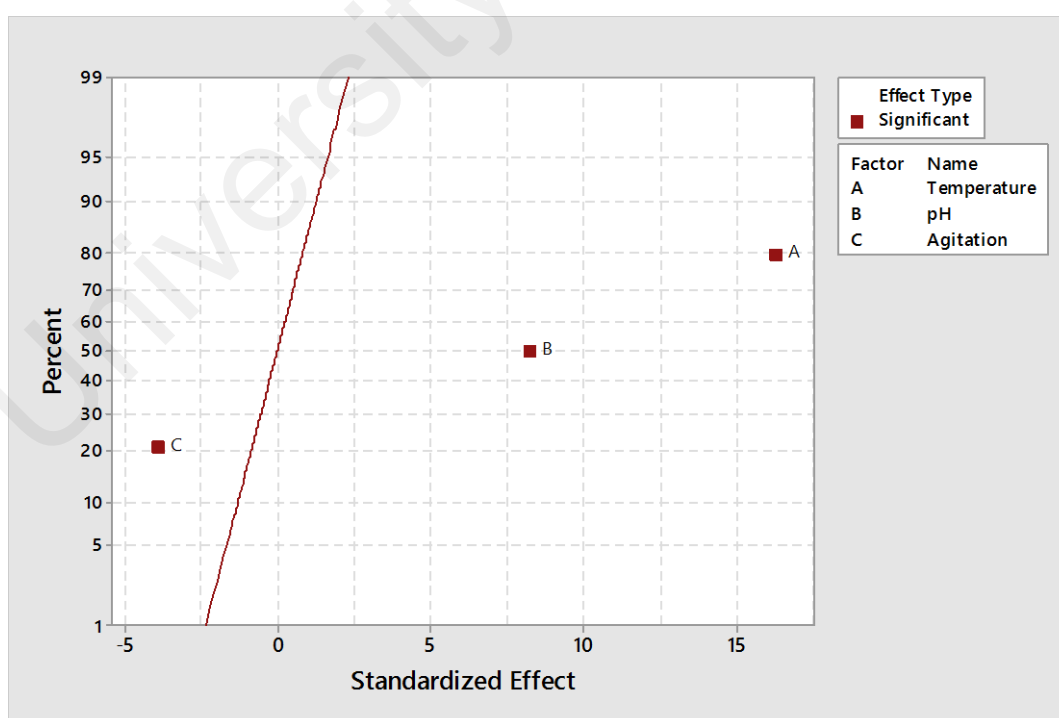
Figure 7.2: Normal plot of effects of temperature, pH, and agitation on endoglucanase production by *B. aerius* S5.2 (α = 0.05).

7.3.1.2 Optimization of process variables for endoglucanase production

Since all the factors screened were found to be significant, the relationship between these factors and endoglucanase production was further studied using response surface methods with the Box-Behnken design. Results obtained from the experiments according to this design are presented in Table 7.2. The quadratic model presented in Equation (7.6) was fitted to the experimental data using multiple regression. The predicted values of endoglucanase activity according to this model are also presented in Table 7.2.

$$y = -2.715 + 0.1223x_1 + 0.223x_2 + 0.00308x_3 - 0.001282x_1^2 \qquad \text{Eq. 7.6}$$
$$- 0.01092x_2^2 - 0.000010x_3^2 - 0.00223x_1x_2$$
$$- 0.000006x_1x_3 + 0.000126x_2x_3$$

The significance of the terms in the model (at $\alpha = 0.05$) were determined using analysis of variance (ANOVA) (Table 7.5). The ANOVA table showed that the linear and square (quadratic) effects were significant ($P < 0.05$) while the interaction effects were not significant ($p > 0.05$). The lack-of-fit for the model was also not significant and this showed that the model adequately explained the variation in endoglucanase production. Furthermore, the R^2 value of the fitting was 92.94 %, which means that only 7.06 % of the variation was not accounted for by the model. The adjusted R^2 and the predicted R^2 were 91.12 % and 88.00 % respectively.

Table 7.5: ANOVA of quadratic model for endoglucanase production.

Source	DF	Adjusted Sum of Squares	Adjusted Mean Squares	*F*-Value	*P*-value
Model	9	0.7623	0.0847	51.16	0.000
Linear effects	3	0.7231	0.2410	145.59	0.000
X_1	1	0.6502	0.6502	392.74	0.000
X_2	1	0.0491	0.0491	29.64	0.000
X_3	1	0.0238	0.0238	14.40	0.001
Square effects	3	0.0333	0.0111	6.70	0.001
X_1^2	1	0.0236	0.0236	14.26	0.001
X_2^2	1	0.0067	0.0067	4.04	0.052
X_3^2	1	0.0073	0.0073	4.39	0.044
Interaction effects	3	0.0059	0.0020	1.19	0.327
X_1X_2	1	0.0048	0.0048	2.91	0.097
X_1X_3	1	0.0000	0.0000	0.03	0.873
X_2X_3	1	0.0011	0.0011	0.64	0.428
Lack-of-fit	3	0.0116	0.0039	2.66	0.065

Based on the model, 3D response surface plots (Figs. 7.3 – 7.5) were generated in order to analyse the relationship between endoglucanase activity and the process variables. The hold values of the factors used in the surface plots were 31 °C, pH 6.5, and 150 rpm. Figure 7.3 shows the changes in endoglucanase production when agitation was held constant. It could be seen that the highest endoglucanase activity was obtained at temperatures above 35 °C and above pH 5.5. The least endoglucanase production was recorded at low values of both factors. A similar trend can be seen in Figure 7.4 when pH was held constant. Maximum endoglucanase production was favoured by agitation speeds above 120 rpm and at temperatures above 35 °C. Endoglucanase activity dropped rapidly when both factors were reduced to their minimum levels. In Figure 7.5 where temperature was held constant, it could be seen that maximum endoglucanase activity was recorded at agitation speeds above 150 rpm and at pH values above 7.0. Simultaneous reduction of medium pH and agitation decreased the endoglucanase activity drastically.
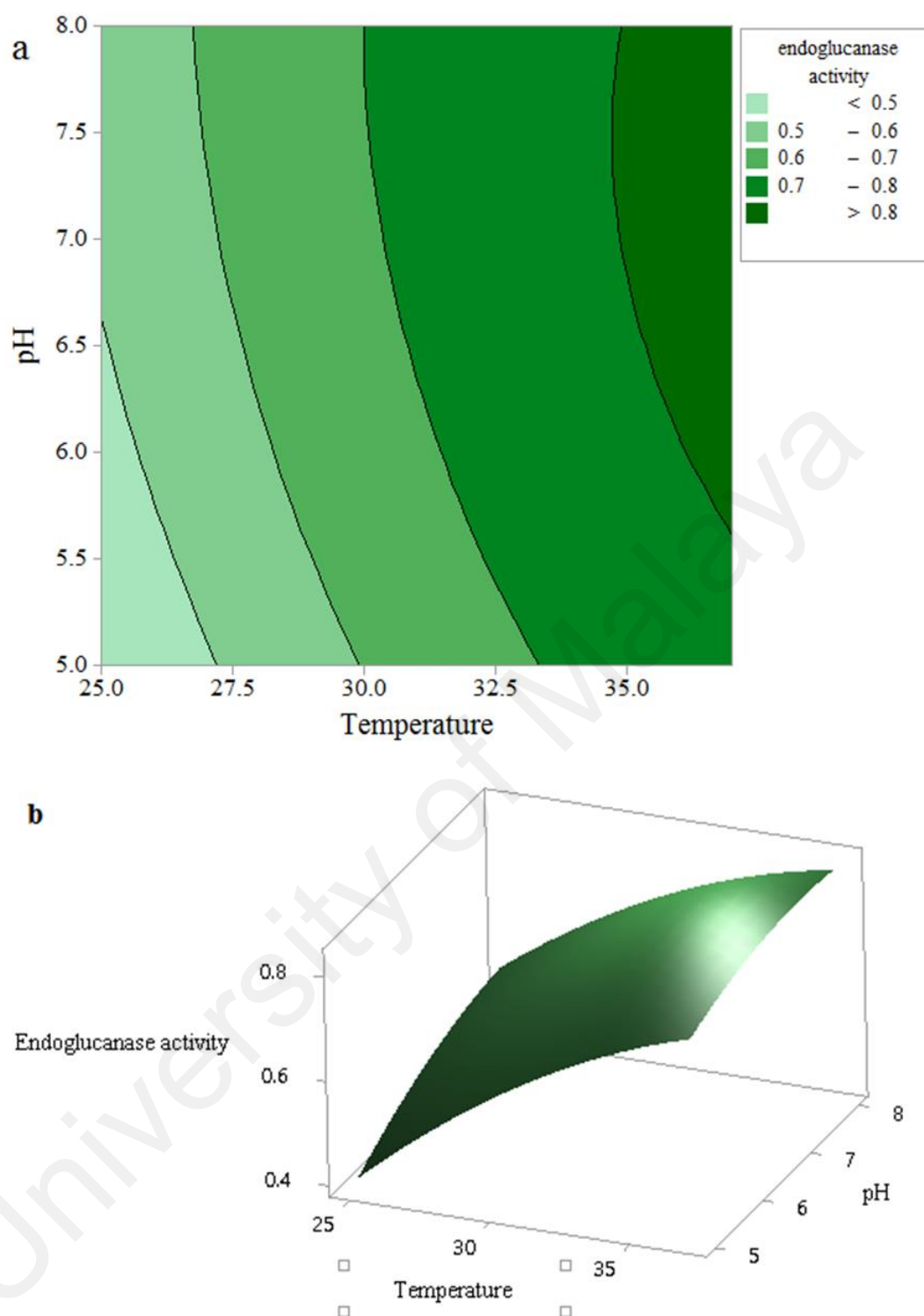
Figure 7.3: Response surface plots of endoglucanase activity *vs.* temperature and pH; (a) contour plot, (b) 3D surface plot.
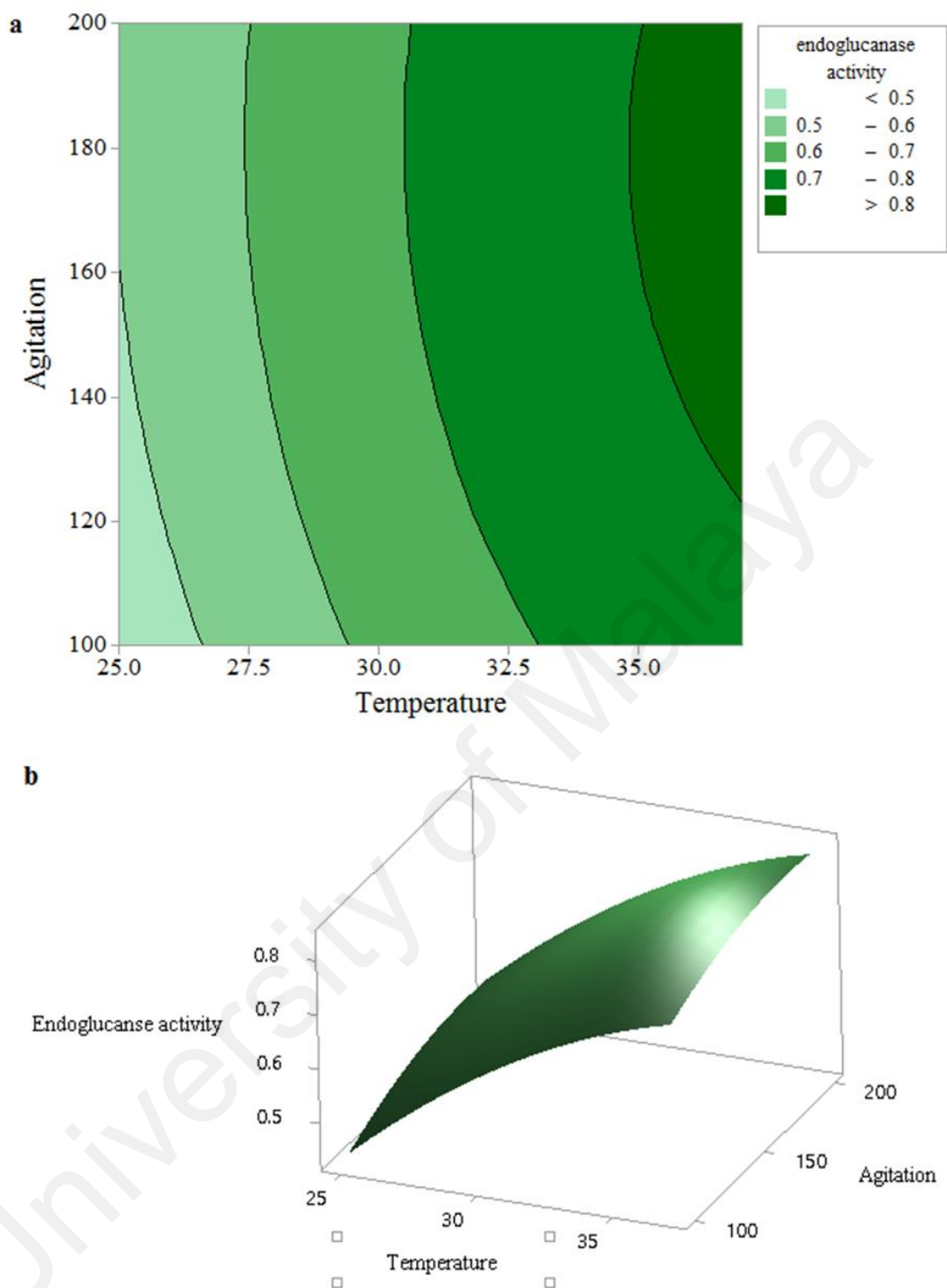
Figure 7.4: Response surface plots of endoglucanase activity *vs.* temperature and agitation: (a) contour plot, (b) 3D surface plot.
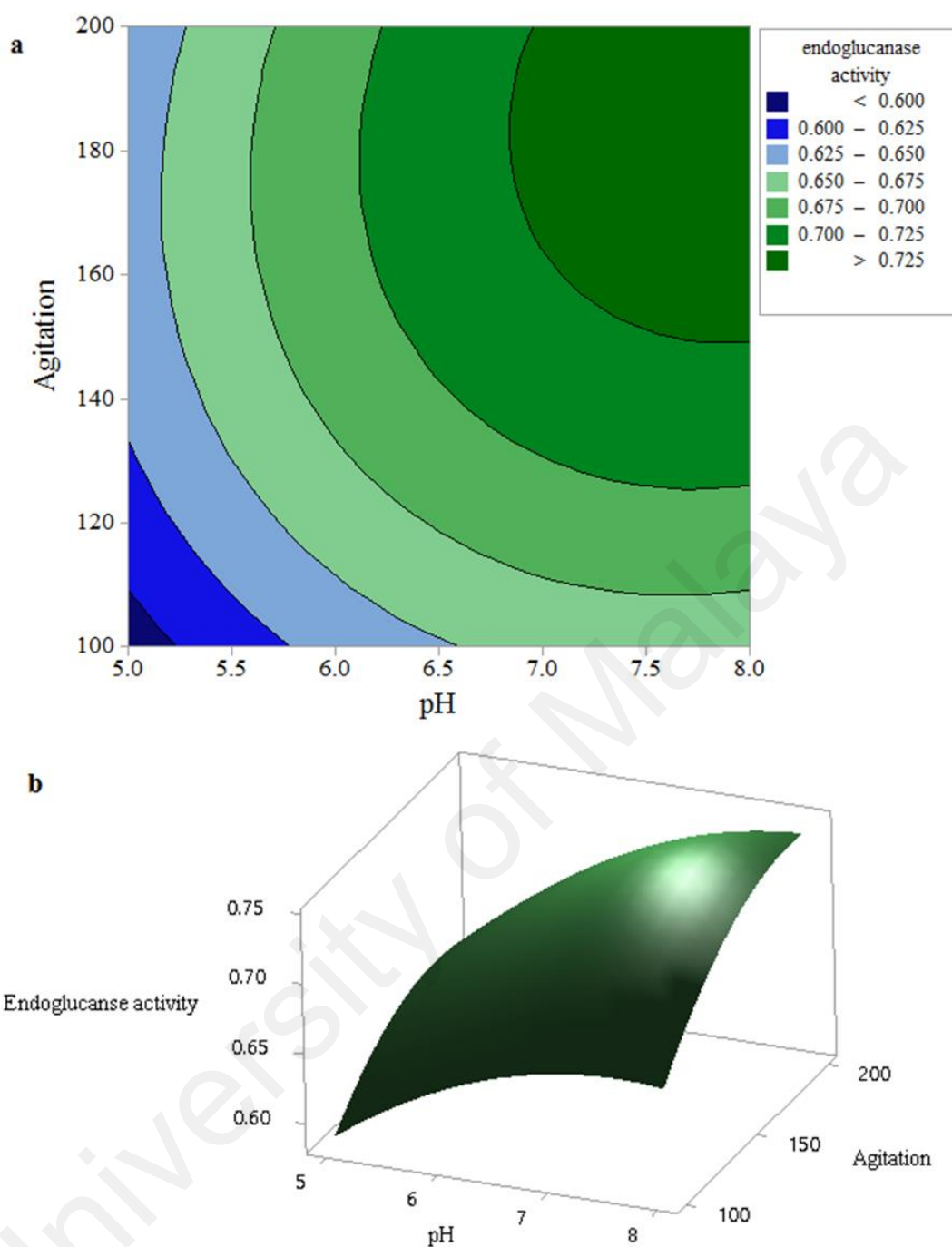
Figure 7.5: Response surface plots of endoglucanase activity *vs*. pH and agitation: (a) contour plot, (b) 3D surface plot.

Using this model, the optimum conditions for maximal endoglucanase production were predicted as: temperature of 37 °C, pH 7.45, and agitation speed of 184.8 rpm. At these settings, the endoglucanase activity was predicted at 0.842 U/mL with confidence interval of 0.804 – 0.880 U/mL and prediction interval of 0.751 – 0.933 U/mL. Additional replicated experiments were conducted at these settings in order to confirm the validity

of the model prediction. The agitation speed used in the verification experiments was 180 rpm due to the limitation of the shaker incubator used. The level of agreement between the model prediction and experimental values obtained was determined by calculating the average absolute relative deviation (% AARD) using Equation (7.7) (Y.-S. Lin & Lee, 2011).

$$\% \, AARD = \frac{100}{N} \sum_{i=1}^{N} \frac{\left| Y_{predicted} - Y_{experimental} \right|}{Y_{experimental}} \qquad \text{Eq. 7.7}$$

From the results in Table 7.6, it was clear that the model prediction was in good agreement with the experimental observations, with AARD of 6.80 %. The mean endoglucanase activity obtained from the verification experiments was 0.789 U/mL, which falls within the confidence and prediction intervals of the model prediction. This value represents about 116 % increase over enzyme titer obtained on untreated MS under un-optimized conditions (0.677 U/mL) recorded in previous studies (30 °C, pH 7.0, and 170 rpm; see Chapter 6).

Table 7.6: Endoglucanase production at model-predicted optimal process variable settings.

Experiment	Temperature (°C)	pH	Agitation (rpm)	Endoglucanase activity (U/mL)
Predicted	37	7.45	184.80	0.842
Verification 1	37	7.45	180.00	0.773
Verification 2	37	7.45	180.00	0.793
Verification 3	37	7.45	180.00	0.799

7.3.2 Optimization of substrate component proportions by mixture designs

7.3.2.1 Simplex centroid design

The simplex centroid design was used to determine the relationship between the substrate component proportions and endoglucanase production by *B. aerius* S5.2. This part also simulated a scenario of relative abundance of feedstock in a biorefinery that utilizes mixed feedstocks for enzyme production. Results of the experiments conducted according to this design are presented in Table 7.3. The wide variation in endoglucanase activity (0.549 – 0.802 U/mL) recorded with different substrate combinations, despite the fact that same fermentation conditions were used, highlighted the importance of the effects of component proportions on enzyme production. A similar observation was made by Prakasham et al. (2009) with respect to biohydrogen production from mixed substrates.

Table 7.7 shows the comparison of the various models tested to fit the experimental data. All the tested models, with the exception of full cubic model, had significant *p*-values ($p < 0.05$). However, of these three, the special cubic model showed the highest R^2 of 83.69 %, which suggested that this model was able to explain the variability in endoglucanase production to the substrate components. Although the full cubic model showed slightly higher R^2 value (83.86 %), its *p*-value was not significant. Based on this premise, the special cubic model was adopted for the study.

Table 7.7: Analysis of mixture models tested for fitting experimental data for simplex centroid design.

Model	R^2 (%)	Adjusted R^2 (%)	Predicted R^2 (%)	Model *P*-value
Linear	65.18	61.86	51.40	0.000
Quadratic	78.94	73.09	70.80	0.026
Special cubic	83.69	77.93	73.78	0.040
Full cubic	83.86	75.25	68.10	0.923

The equation derived (Eq. 7.8) based on the special cubic model was fitted to the experimental data and used to obtained the predicted values in Table 7.3.

$$y = 1.26 * EFB + 1.05 * OPF + 0.95 * RH + 1.29 * EFB * OPF + 0.78 \quad \text{Eq. 7.8}$$
$$* EFB * RH + 0.49 * OPF * RH - 7.36 * EFB * OPF * RH$$

In Equation (7.8), y represented endoglucanase activity (U/mL) and the regression coefficients are based on component amounts.

The significance of the effects in this model as well as the individual terms of the model were determined by ANOVA (Table 7.8). Terms of the model were evaluated at $\alpha = 0.05$. The linear, quadratic, and special cubic effects were all significant with p-values less than 0.05. This showed that at least one of the component terms of these models had significant effect on endoglucanase production. However, the quadratic effect of the OPF*RH interaction term was not significant, indicating that interaction between these two components did not affect endoglucanase production significantly. The positive coefficient values of the EFB*OPF and the EFB*RH terms indicated that these substrate components had synergistic effects on endoglucanase production. Furthermore, this means that the mean endoglucanase activity from these blends is greater than the mean activity that would be obtained from the individual substrates (Cornell, 2002). The negative coefficient of the special cubic term showed that the components had antagonistic interaction when combined in ternary blends. Consequently, the mean endoglucanase activity from the ternary blend would be less than the mean activity obtained from the individual components (Cornell, 2002). Among the three components, RH showed the least contribution towards endoglucanase production as the magnitude of its linear term coefficient was the least (Eq. 7.8). This observation is also supported by the fact that binary blends that contained RH showed lower magnitude of the coefficients

153

than those without it. EFB exhibited the greatest impact on endoglucanase production followed by OPF, based on the magnitude of their coefficients.

Table 7.8: ANOVA table of special cubic model for endoglucanase production by simplex centroid design (component proportions).

Source	DF	Adjusted Sum of Squares	Adjusted Mean Squares	*F*-Value	*P*-value
Model	6	0.1018	0.0170	14.54	0.000
Linear	2	0.0409	0.0204	17.52	0.000
Quadratic	3	0.0223	0.0074	6.36	0.004
EFB*OPF	1	0.0183	0.0183	15.69	0.001
EFB*RH	1	0.0067	0.0067	5.78	0.028
OPF*RH	1	0.0026	0.0026	2.27	0.150
Special cubic	1	0.0058	0.0058	4.95	0.040
EFB*OPF*RH	1	0.0058	0.0058	4.95	0.040
Lack-of-fit	3	0.0115	0.0038	6.47	0.006

The mixture response plots in Figure 7.6 show the relationship between endoglucanase production and the three substrate components. The plots showed that maximum endoglucanase activity was obtained with blends containing about 60 % EFB, 30 % OPF, and with almost zero amount of RH. Among the pure blends, EFB gave the highest activity followed by OPF, while endoglucanase activity declined drastically with pure blends of RH. These observations are in general agreement with the analysis of the model coefficients explained earlier.
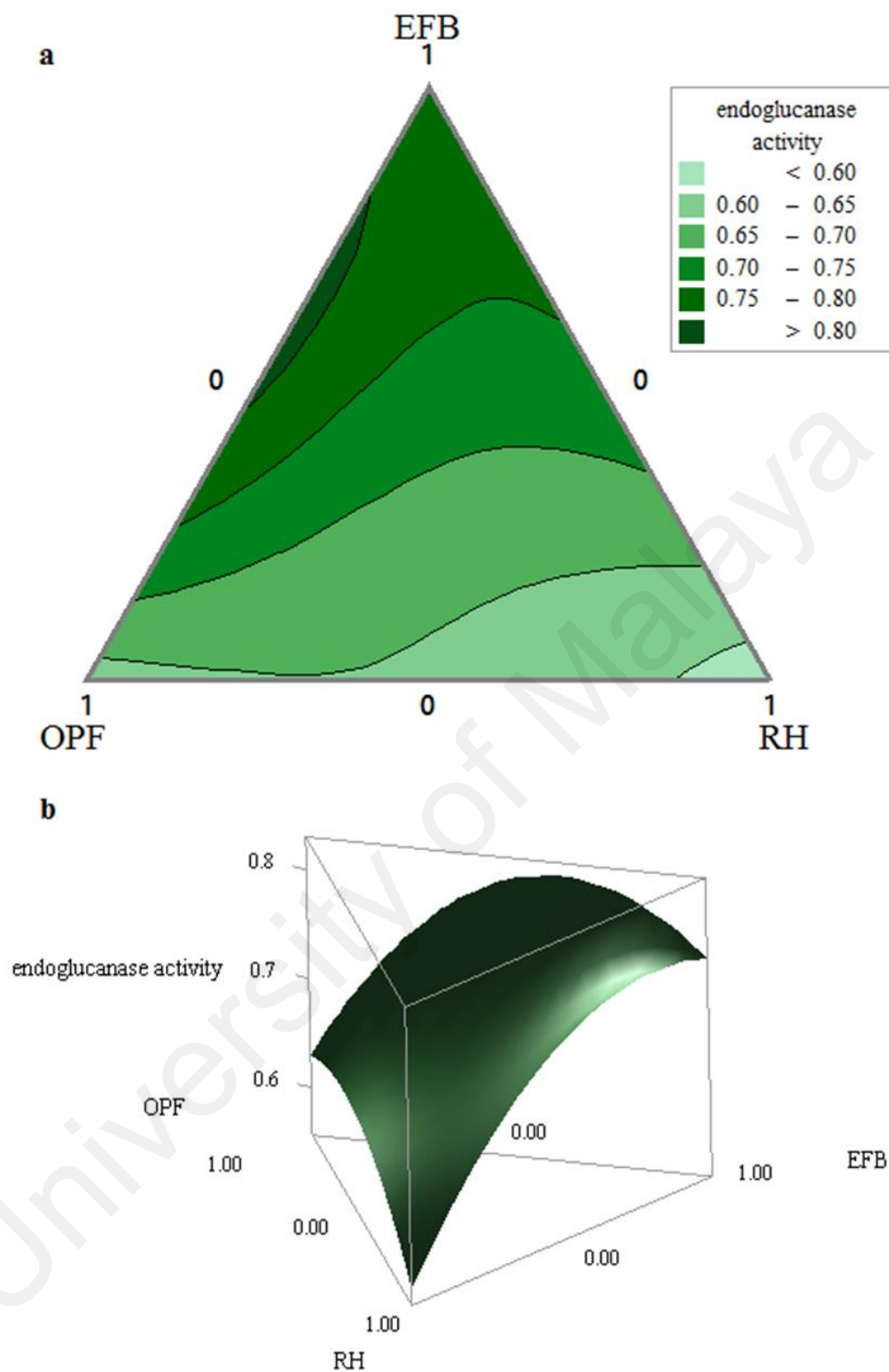
Figure 7.6: Mixture response plots of endoglucanase activity for simplex centroid design (based on component proportions; 0.00 – 1.00 represents 0 – 100 %): (a) mixture contour plot, (b) mixture 3D surface plot.

Observations from the experiments suggested that RH was unfavourable for endoglucanase production by *B. aerius* S5.2. This can be related to the physicochemical characteristics of the RH used in this study. As it was reported in Chapter six, RH contained a lot of silica bodies compared to the other single substrates (Figure 6.1). To corroborate this, comparison of the FTIR spectra (*Appendix F*) of the three substrates showed that RH had higher amounts of silica as it had the most prominent band around 800 cm^{-1}, a band that has been attributed to the bending vibrations of Si-O bonds (Shokri, Firouzjah, & Hosseini, 2009; Swann & Patwardhan, 2011). The presence of silica bodies in biomass has been shown to prevent bacterial attachment and is also inhibitory to cellulolytic microorganisms (Bae et al., 1997). Furthermore, the RH used in this study had higher composition of lignin (24.6 %) than EFB and OPF (20 % each) (*Appendix G*). The inhibitory effects of lignin on cellulase production and cellulolytic microorganisms have been demonstrated (Bigelow & Wyman, 2002; Camp et al., 1988; Han & Callihan, 1974). RH also contained significantly higher amount of ash (10.5 %) compared to EFB and OPF (approximately 1 %) (*Appendix G*). This was probably responsible for the high amount of silica in the RH as silica is one of the most abundant components of biomass ash. The removal of ash during biomass pretreatments has been associated with increased cellulase production (X. Zhao, Zhou, Zheng, & Liu, 2010).

Model-predicted optimum component combination for endoglucanase production was 0.38 g of EFB, 0.22 g of OPF, and 0 g of RH, which in terms of proportions is 63.3 % EFB, 36.7 % OPF, and 0 % RH. At this combination, the predicted endoglucanase activity was 0.817 U/mL. However, the mean endoglucanase activity obtained at the optimum substrate proportions in the verification experiments was 0.679 U/mL (Table 7.9), with AARD of 20.38 %. This showed that there was a modest agreement between the model prediction and the experimental values. A similar approach was adopted by Sathish, Lakshmi, Rao, Brahmaiah, and Prakasham (2008) in the production of glutaminase from

mixed substrate of wheat bran, Bengal gram husk, and palm seed fibre in solid-state fermentation by a *Bacillus* sp. The authors found that substrate component ratio of 66:34:0 was optimal for glutaminase production.

Table 7.9: Endoglucanase production at model-predicted optimal substrate component combination for simplex centroid design.

Experiment	Component amounts (g)			Endoglucanase activity (U/mL)
	EFB	OPF	RH	
Predicted	0.38	0.22	0.00	0.817
Verification 1	0.38	0.22	0.00	0.689
Verification 2	0.38	0.22	0.00	0.668
Verification 3	0.38	0.22	0.00	0.680

7.3.2.2 Extreme vertices design

Extreme vertices design was used in a separate study to further study the relationship between the substrate components and endoglucanase production by *B. aerius* S5.2. In this case, a condition was imposed such that each of the components must be present in the mixture. Lower and upper bound constraints of 0.10 g and 0.40 g corresponding to about 16.7 % and 66.7 % respectively for all the components were adopted. This was to simulate a biorefinery scenario where there is a limited supply of biomass feedstocks for enzyme production and the biorefinery must use the available ones in the best possible way for maximum product yield.

Results obtained from experiments conducted using this design are presented in Table 7.4. The endoglucanase activity ranged from 0.407 – 0.662 U/mL. This wide variation was an indication that endoglucanase production on the mixed substrate was sensitive to the substrate component proportions since the fermentation conditions applied in all the experimental runs were very similar. Similar observations were made with respect to the use of mixed substrates for the production of biohydrogen (Prakasham et al., 2009) and glutaminase enzyme (Sathish et al., 2008).

Various mixture models were tested for the fitting of the experimental data (Table 7.10). Based on the high R^2 value and the p-value significance (at $\alpha = 0.05$), the full cubic model was selected for use in the study. The R^2 value of this model showed that 93.65 % of variation in the endoglucanase activity recorded with the different substrate combinations could be explained by the model.

Table 7.10: Analysis of mixture models tested to fit experimental data for extreme vertices design.

Model	R^2 (%)	Adjusted R^2 (%)	Predicted R^2 (%)	Model P-value
Linear	46.98	41.93	21.00	0.001
Quadratic	80.84	75.51	70.40	0.000
Special cubic	81.27	74.67	68.16	0.537
Full cubic	93.65	90.27	87.79	0.000

The equation (Eq. 7.9) derived based on this model was used to obtain the predicted values of endoglucanase production at the various substrate combinations presented in Table 7.4.

$$y = 2.20 * EFB - 2.30 * OPF + 0.95 * RH + 6.52 * EFB * OPF - 1.23 \quad \text{Eq. 7.9}$$
$$* EFB * RH + 9.20 * OPF * RH - 15.84 * EFB * OPF$$
$$* RH - 38.52 * EFB * OPF * (EFB - OPF) + 11.75 * EFB$$
$$* RH * (EFB - RH)$$

In Equation (7.9), y represented endoglucanase activity (U/mL) and the regression coefficients are based on component amounts.

The significance of the effects in this model as well as the individual terms of the model were evaluated using ANOVA (Table 7.11). Terms of the model were evaluated at $\alpha = 0.05$. The quadratic term, EFB*RH was not significant ($p > 0.05$); this means that pure binary blends of EFB and RH did not significantly affect endoglucanase production.

Similarly, the full cubic term EFB*RH*(EFB – RH) was also not significant and this means that binary interaction between EFB and RH within ternary mixtures did not significantly affect endoglucanase production. Ternary interaction between complete blends of the three substrate components did not affect endoglucanase production as well, as the special cubic term (EFB*OPF*RH) was not significant. Examination of the coefficients of terms in the model showed that EFB and RH positively affected endoglucanase production when they constituted major proportion of the MS since their linear terms showed positive coefficients, although that of RH was of lower magnitude. However, OPF negatively influenced the response as it showed a negative coefficient. For the binary blends, EFB*OPF and OPF*RH both positively affected the response due to their positive coefficients. However, the latter was of a higher magnitude. Binary interaction between EFB and OPF within ternary mixtures influenced endoglucanase production negatively, and this was of high magnitude.

Table 7.11: ANOVA of full cubic model for endoglucanase production by extreme vertices design (component proportions).

Source	DF	Adjusted Sum of Squares	Adjusted Mean Squares	*F*-Value	*P*-value
Model	8	0.0896	0.0112	27.66	0.000
Linear	2	0.0202	0.0101	25.00	0.000
Quadratic	3	0.0241	0.0080	19.85	0.000
EFB*OPF	1	0.0038	0.0038	9.50	0.008
EFB*RH	1	0.0001	0.0001	0.34	0.571
OPF*RH	1	0.0077	0.0077	18.91	0.001
Special cubic	1	0.0004	0.0004	1.03	0.326
EFB*OPF*RH	1	0.0004	0.0004	1.03	0.326
Full cubic	2	0.0118	0.0059	14.62	0.000
EFB*OPF*(EFB – OPF)	1	0.0113	0.0113	27.84	0.000
EFB*RH*(EFB – RH)	1	0.0010	0.0010	2.59	0.128
Lack-of-fit	1	0.0001	0.0001	2.76	0.119

** The term OPF*RH*(OPF – RH) could not be estimated by the software and was removed.

Mixture response plots of endoglucanase production in response to the various substrate component combinations of the extreme vertices design are presented in Figure 7.7. The plots showed that the highest endoglucanase activity was obtained with mixture blends containing EFB in the range close to 67 % and with OPF and RH being slightly above 17 %. The response showed a characteristic cubic appearance with crest and trough at different substrate combinations. Blends with higher proportions of EFB and RH produced higher endoglucanase activity than those with higher proportion of OPF. These observations also showed that the nature of the individual substrates strongly affected enzyme production.
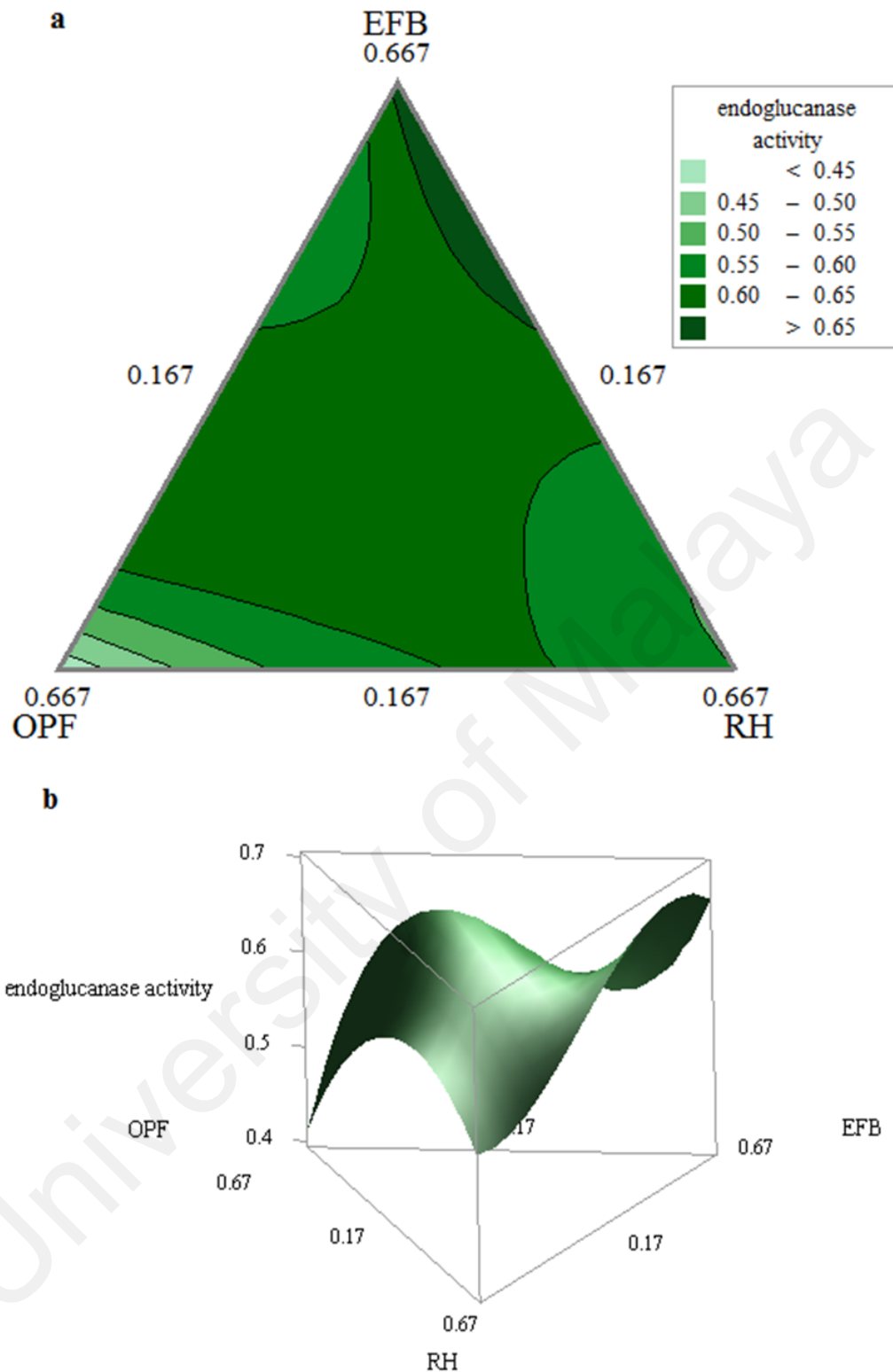
Figure: 7.7: Mixture response plots of endoglucanase activity for extreme vertices design (based on substrate proportions; 0.17 – 0.67 represents 17.0 – 67.0 %): (a) mixture contour plot, (b) mixture 3D surface plot.

The predicted optimal combination of the substrate components was 0.35 g of EFB, 0.10 g of OPF, and 0.15 g of RH, which in terms of proportions, is equivalent to 58 % EFB, 17 % OPF, and 25 % RH. At this combination, the expected endoglucanase production was 0.687 U/mL. Results from the verification experiments (Table 7.12) conducted at the predicted optimum substrate proportion produced mean endoglucanase activity of 0.679 U/mL. The AARD from these experiments was 6.25 %, indicating good agreement with the model prediction.

Table 7.12: Endoglucanase production at model-predicted optimal substrate component combination for extreme vertices design.

Experiment	Component amounts (g)			Endoglucanase activity (U/mL)
	EFB	OPF	RH	
Predicted	0.35	0.10	0.15	0.687
Verification 1	0.35	0.10	0.15	0.664
Verification 2	0.35	0.10	0.15	0.635
Verification 3	0.35	0.10	0.15	0.738

By comparing the two mixture designs used in this study, it was obvious that the extreme vertices design gave a better prediction of endoglucanase production by *B. aerius* S5.2 since there was better agreement (6.25 % AARD) between experimental- and the predicted enzyme yield than was observed with the simplex centroid design (20.38 % AARD). Hence, extreme vertices design provided a better understanding of the dependence of endoglucanase production on the substrate components.

It was observed that higher enzyme activities were obtained in the simplex centroid experiments (0.549 – 0.802 U/mL) than in the extreme vertices experiments (0.407 – 0.662 U/mL). Although all biomass samples used were of the same particle size range, the observed differences in endoglucanase titers might be due to the fact that RH used in both experiments were from different sources. Intra-species differences between the two RH samples could result in significant differences in chemical composition (silica content, lignin, etc.) and physicochemical characteristics (crystallinity, porosity, moisture

content, etc.) which could impact significantly on endoglucanase production (Williams, Westover, Emerson, Tumuluru, & Li, 2015). Also, variation in pre-processing activities and storage conditions could significantly alter the characteristics of the same biomass type from different sources (Rigdon, Jumpponen, Vadlani, & Maier, 2013).

7.4 Conclusions

This study sought to identify some influential process variables affecting endoglucanase production by *B. aerius* S5.2 on a mixed substrate comprising EFB, OPF, and RH. The process variables and substrate proportions were optimized for maximum enzyme production using principles of design of experiments. Temperature, pH, and agitation significantly influenced endoglucanase production by the bacterial culture. Optimization of these process variables using response surface methods (Box-Behnken design) resulted in 116 % increase in endoglucanase production compared to un-optimized conditions. Use of mixture designs allowed for identification of optimal substrate component combinations for maximal enzyme production. The nature of the individual substrates strongly affected enzyme production and EFB was the most suitable single substrate for endoglucanase production. Extreme vertices design gave better prediction of optimal substrate component proportions than simplex centroid design. However, the choice of design to use in a practical situation would depend on the prevailing technical, economic, and feedstock availability situations of the biorefinery. Mixture designs used in this study provided an efficient and cost-effective way of optimizing enzyme yields using the same process conditions and microorganism.

CHAPTER 8: CONCLUSIONS AND RECOMMENDATIONS

8.1 Conclusions

The main goal of this study was to demonstrate the feasibility of utilizing mixed lignocellulosic biomass as substrate for the production of bacterial endoglucanase. In this chapter, the general conclusions from the study are presented. Implications of the findings and their impact on future work and other research areas are summarized. The chapter concludes by presenting an agenda for future direction of the research.

The following conclusions can be drawn from the study:

1. The sequential targeted screening approach used in the study led to the isolation of *B. aerius* with relatively high titers of endoglucanase, FPase, and exoglucanase on MS compared to the other isolated bacterial strains. The screening approach employed underscored the limitation of applying plate screening alone for isolating cellulolytic microbes. This study is the first report on cellulase production by *B. aerius*;

2. *B. aerius* S5.2 produced higher amounts of extracellular endoglucanase on mixed substrate compared to the single substrates and the pure soluble and insoluble cellulosic substrates. This finding showed that use of MS for endoglucanase production by this strain would be advantageous for enzyme productivity with the implication of reduced production cost. The strain can also be used in CBP applications for bioethanol and/or other bioproducts;

3. Decanter cake and *Leucaena leucocephala* (LL) seeds could be alternative abundant substitutes for yeast extract and $NaNO_3$ as nitrogen sources in endoglucanase production. LL as a carbon source adjunct improved endoglucanase production significantly. This showed that the use of carefully selected agro-industrial residues as medium supplements in mixed substrate system could be an economical approach towards cellulase production;

4. Thermochemical pretreatments did not improve enzyme yields, and were thus not necessary for endoglucanase production. This could translate into the reduction of costs due to pretreatments in a practical situation. Combining the SS for pretreatment and fermentation enhanced endoglucanase production.

5. Optimization of process variables (at 37 °C, pH 7.45, and 180 rpm) using response surface methodology resulted in 116 % increase in endoglucanase production. Mixture blends with higher proportions of EFB were most favourable for higher enzyme yields. Optimal mixture proportions for tentative scenarios of abundant and limited feedstock supplies were (EFB:OPF:RH) 63.3:36.7:0 and 58:17:25 respectively. Optimization of enzyme production at different component proportions while using the same process conditions and microorganism in this study showed that mixture design is a robust tool for improving cellulase production with mixed lignocellulosic substrates;

6. The study demonstrated that mixed feedstock strategy could bring about improved and efficient production of *B. aerius* endoglucanase. New insights into some aspects of *B. aerius* cellulolytic system previously unknown were also presented.

8.2 Recommendations for future work

1. It would be interesting to investigate xylanase production by this strain. Since it has been demonstrated to produce FPase and exoglucanase, it is possible that it also possesses a complete repertoire of lignocellulosic enzymes, including xylanase, another important industrial biocatalyst;

2. Since the strain produced relatively high titers of other cellulases, future studies with MS should attempt to utilize mixture design with multiple responses option to optimize the production of complete cellulolytic complex in this strain in addition to endoglucanase;

3. Whole genome sequencing of this strain should be done in order to understand the genetic basis of its cellulase system. This would provide basic information needed to improve its cellulase production using molecular techniques;

4. Future studies should investigate a wider range of carbon sources (e.g. cellobiose, lactose, sophorose, sorbose, etc.) for their effects on cellulase production. This would provide more information regarding the regulation (induction and repression) of cellulase production in this particular strain;

5. Proteomic and metabolomic studies should be carried out in order to gain more insights into the response of this strain to the presence of mixed and single lignocellulosic substrates for endoglucanase production;

6. Endoglucanase production with this strain should be investigated at the automated, controlled bench-scale bioreactor level to determine its prospect for industrial application. In an automated, controlled bioreactor, optimization of cultivation time as one of the process variables is necessary for an economical fermentation process;

7. Properties of the produced endoglucanase on SS and MS should be studied in order to understand the effects of substrate combination on the quality of the enzyme. Purification and characterization of the endoglucanase would therefore be necessary.

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LIST OF PUBLICATIONS AND PAPERS PRESENTED

A. Publications:

1. **Oke, M. A.**, Annuar, M. S. M., & Simarani, K. (2016a). Enhanced endoglucanase production by *Bacillus aerius* on mixed lignocellulosic substrates. *BioResources, 11*(3), 5854-5869. doi:10.15376/biores.11.3.5854-5869

2. **Oke, M. A.**, Annuar, M. S. M., & Simarani, K. (2016b). Mixed feedstock approach to lignocellulosic ethanol production—prospects and limitations. *BioEnergy Research*, 1-15. doi:10.1007/s12155-016-9765-8

3. **Oke, M. A.**, Annuar, M. S. M., & Simarani, K. (2016c). Mixed lignocellulosic biomass degradation and utilization for bacterial cellulase production. *Waste and Biomass Valorization*, 1-11. doi:10.1007/s12649-016-9595-0

4. **Oke, M. A.**, Ishola, M. M., Taherzadeh, M. J., Mohamad Annuar, M. S., & Simarani, K. (2016). Effects of pretreatment of single and mixed lignocellulosic substrates on production of endoglucanase by *Bacillus aerius* S5.2. *BioResources, 11*(3), 6708-6726. doi:10.15376/biores.11.3.6708-6726

B. Conference presentations

1. Oke, M. A., Philip, K., & Ajam, N. (2013). *Bioethanol production from mixed lignocellulosic substrates using cellulolytic termite gut bacteria and yeast.* Paper presented at the 20th MSMBB Scientific Meeting and MSMBB 25th Anniversary, Research Management and Innovation Complex (RMIC), University of Malaya, Malaysia.

2. Oke, M. A., Annuar, M. S. M., & Simarani, K. (2014a). *Cellulase production and degradation of pretreated oil palm and rice mixed lignocellulosic residues by Pseudomonas cellulosa ATCC 55703*. Paper presented at the The 4th ASEAN Science Congress and Sub Committee Conferences (The ASEAN Conference on Science and Technology), IPB International Conference Center, Bogor, Indonesia.

3. Oke, M. A., Annuar, M. S. M., & Simarani, K. (2014b). *Mixed biomass approach to lignocellulosic biorefinery: Enhancement of endoglucanase production by Bacillus aerius S5.2 on mixed lignocellulosics*. Paper presented at the International Postgraduate Research Awards Seminar (INPRAS), University of Malaya, Kuala Lumpur, Malaysia.

4. Oke, M. A., Annuar, M. S. M., & Simarani, K. (2014c). *Mixed lignocellulosic biomass as viable feedstocks for the production of biofuels and other bio-commodities*. Paper presented at the 16th International Biotechnology Symposium and Exhibition (IBS2014), The Events Center of Ceara, Fortaleza, Brazil.

5. Oke, M. A., Annuar, M. S. M., & Simarani, K. (2014d). *Sequential screening of bacteria for cellulolytic ability and production of endoglucanase on NaOH-heat*

pretreated single and mixed lignocellulosic residues by Bacillus subtilis S8.2. Paper presented at the 19th Biological Sciences Graduate Congress (BSGC), National University of Singapore, Singapore.

6. Oke, M. A., Annuar, M. S. M., & Simarani, K. (2015). *Optimization of bacterial endoglucanase production on unwashed, untreated mixed lignocellulosic substrate using factorial and response surface designs*. Paper presented at the 12th Asian Congress on Biotechnology (ACB 2015), Istana Hotel, Kuala Lumpur, Malaysia.
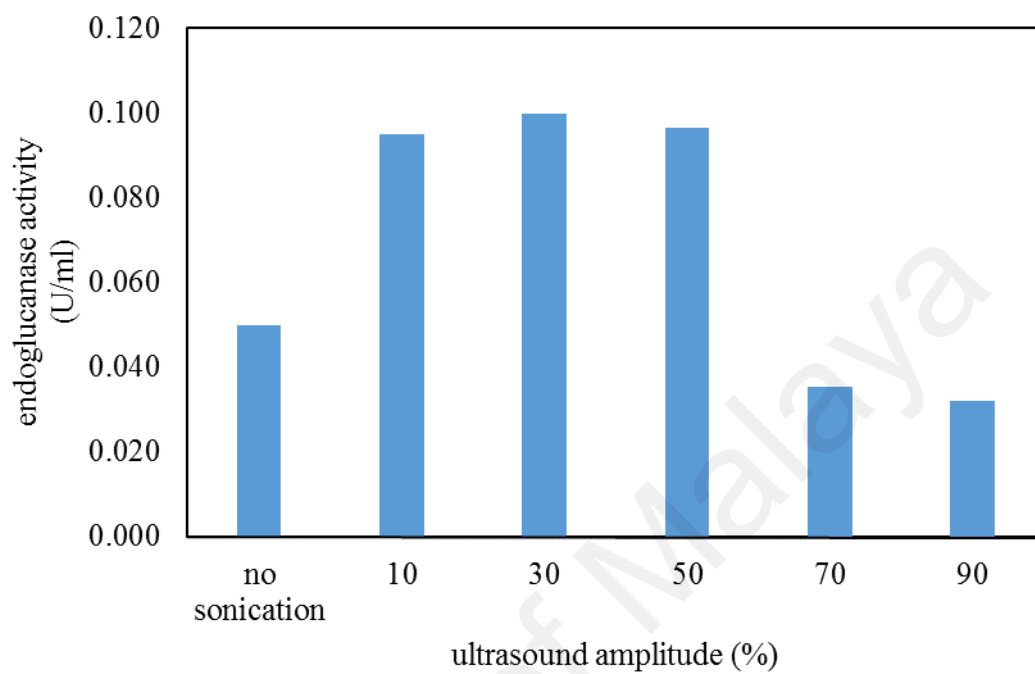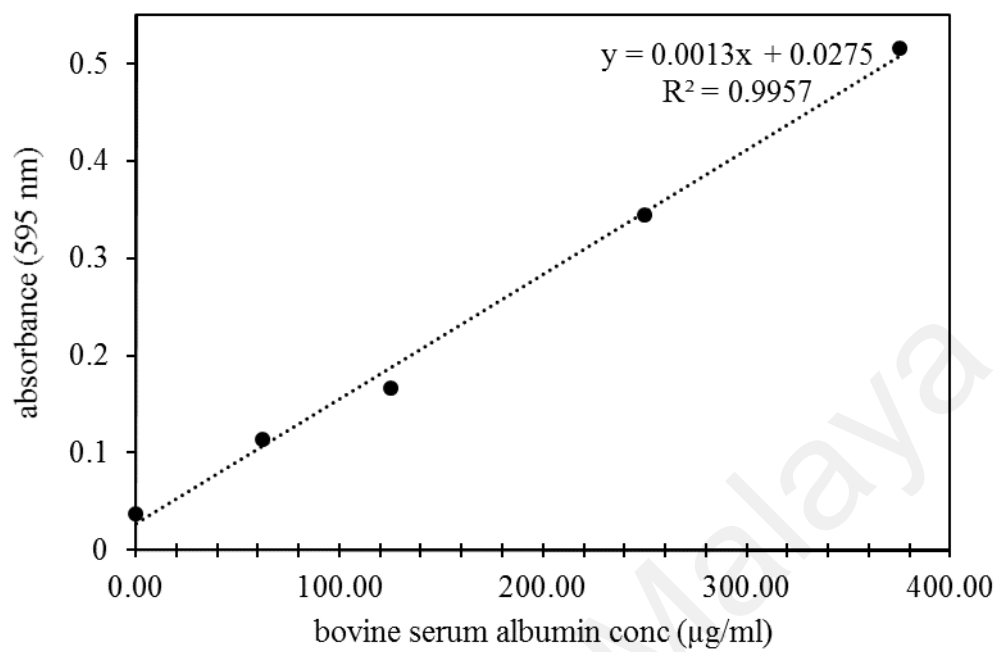
Appendix A: Determination of optimal sonication amplitude for extraction of intracellular enzyme fractions from *B. aerius* S5.2.

Appendix B: Protein standard curve.

Appendix C: Determination of MS dry weight loss as a result of *B. aerius* S5.2 activity.

Sample	Filter paper weight (g)		Weight of hydrolyzed MS (W_1-W_0) (g)	Weight loss $[1-(W_1-W_0)]$ (g)	Percentage loss (%)	Average weight loss (%)
	Initial (W_0)	final (W_1)				
Inoculated						
I	2.39	3.11	0.72	0.28	28.00	25.33 ± 2.52
II	1.54	2.31	0.77	0.23	23.00	
III	1.55	2.30	0.75	0.25	25.00	
Control	1.55	2.36	0.81	0.19	19.00	19.00

204

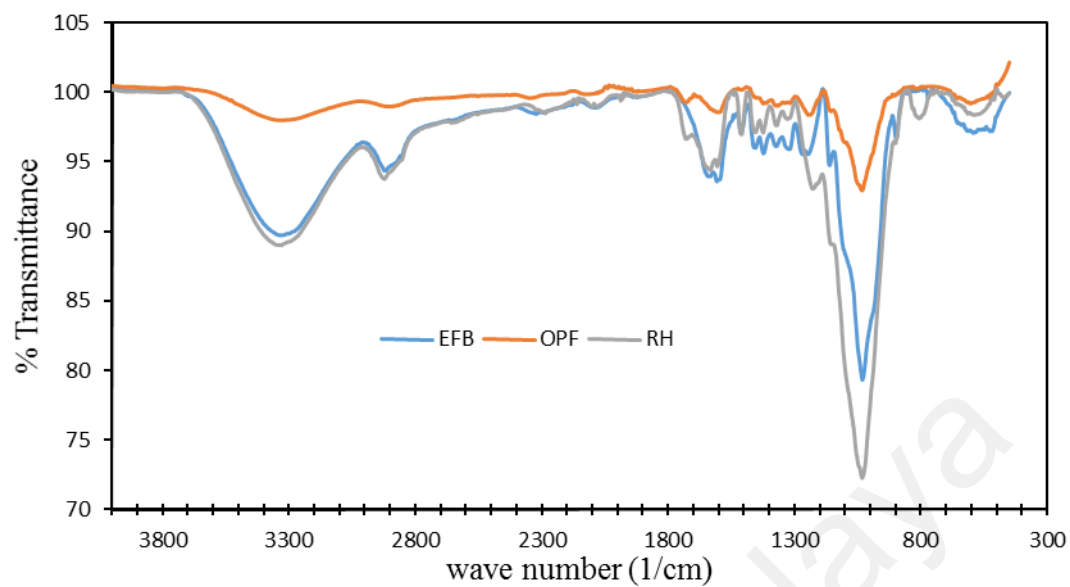**Appendix D:** *Leucaena leucocephala* **pods.**

Appendix E: *Leucaena leucocephala* **seeds.**

Appendix F: FTIR spectra of untreated EFB, OPF, and RH.

Appendix G: Chemical composition of untreated single substrates used in the study.

Substrate	Chemical composition (%)				
	Cellulose	Hemicellulose	Lignin	Ash	Others
EFB	34.98 ± 1.20^a	19.11 ± 4.17^a	20.40 ± 0.26^b	1.08 ± 0.60^b	24.44 ± 5.02^a
OPF	33.54 ± 0.81^a	16.68 ± 0.44^a	20.09 ± 0.22^b	1.11 ± 0.37^b	28.58 ± 1.00^a
RH	30.70 ± 1.20^a	14.11 ± 0.50^a	24.55 ± 0.01^a	10.48 ± 0.53^a	20.17 ± 2.21^a

Appendix H: Composition of major components of MS samples before and after 48 h of endoglucanase production by *B. aerius* S5.2.

MS	Chemical composition (%)					
	Cellulose		Hemicellulose		Lignin	
	Before fermentation	After fermentation	Before fermentation	After fermentation	Before fermentation	After fermentation
Untreated	31.97 ± 1.10^b	38.43 ± 2.02^a	15.07 ± 0.24^a	15.40 ± 1.05^a	20.88 ± 0.10^b	24.88 ± 0.07^a
Acid-pretreated	48.65 ± 0.39^b	54.86 ± 0.29^a	6.88 ± 0.97^a	5.17 ± 0.11^b	30.87 ± 1.45^a	29.15 ± 0.52^a
Alkali-pretreated	37.84 ± 1.62^b	42.60 ± 0.05^a	19.04 ± 0.87^a	17.88 ± 0.17^a	23.36 ± 0.52^a	23.23 ± 0.46^a
Water-pretreated	38.31 ± 0.56^b	42.04 ± 0.81^a	19.80 ± 0.47^a	17.51 ± 0.84^b	23.73 ± 1.86^a	24.29 ± 0.65^a
NMMO-pretreated	40.20 ± 1.35^b	44.57 ± 1.39^a	20.07 ± 0.57^a	13.26 ± 0.54^b	22.29 ± 0.27^b	25.06 ± 0.41^a

Data were expressed on a dry weight basis. [a-b] Values represented means of three replicates ± standard deviation (SD). Values within the same row for each structural component and having same superscript letters are not significantly different ($p > 0.05$).