THE DEVELOPMENT OF EFFICIENT FUNGAL BIOMASS-DEGRADING ENZYME MIXTURES FOR SACCHARIFICATION OF LOCAL LIGNOCELLULOSIC FEEDSTOCK

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A THESIS SUBMITTED AS A PART OF THE REQUIREMENTS FOR THE DEGREE OF MASTER OF ENGINEERING IN ENERY TECHNOLOGY AND MANAGEMENT

THE JOINT GRADUATE SCHOOL OF ENERGY AND ENVIRONMENT AT KING MONGKUT'S UNIVERSITY OF TECHNOLOGY THONBURI

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ABSTRACT

Conversion of agricultural biomass to biofuels and value-added chemicals is the basis of the sustainable and environmentally-friendly biorefinery industry. The development of effective lignocellulolytic microbial and enzyme systems is a challenge for the successful establishment of cost-effective biomass conversion processes. The culture media parameters such as carbon, nitrogen sources and types of medium were investigated using one factor at a time (OFAT) analysis. The optimization of fermentation conditions including concentration of carbon source, inducer and initial pH were further studied using multivariate analysis; besides, addition of oil palm empty fruit bunch (OPEFB) was observed as co-substrate for enhancing yield of cellulases. Among various complex biomass and synthetic substrates for carbon and nitrogen sources contained in fermentation media, 1% (w/v) OPEFB, 2% microcrystalline cellulose (AVICEL®) and 1% soybean meal (SBM) in distilled water were found to be potent sources for the production of cellulase by *Chaetomium globosum* BCC5776. The incubation temperature, time and initial pH were 30 ºC, 6 days and 5.8, respectively. The maximum volumetric productivity of cellulase was 0.40 FPU/ml with specific activity of 0.79 U/mg proteins from submerged fermentation. Further optimized culture fermentation condition, the BCC5776 fungal enzyme extract was produced in 1-L and 5-L bioreactor. Comparable yield of cellulase activity (0.359 FPU/ml, 0.77 U/mg) was obtained when the enzyme production was up-scaled in a 5-L bioreactor. The crude cellulose enzyme from BCC5776 worked optimally in temperature range of 50-60ºC with pH range of 5.0-6.0. A synergistic enzyme system for the hydrolysis of alkaline pretreated rice straw was then optimized based on mixture design approach based on the synergy of the BCC5776 crude fungal enzyme extracts with commercial enzymes β-glucosidase Novozyme® 188 and hemicellulase Accellerase® XY. Using the full cubic model, the optimal formulation of the enzyme mixture was predicted to the percentage of BCC5776:Novozyme® 188: Accellerase® XY = 44.4:20.6:35.0, which

produced 572.68 mg reducing sugar/g pretreated rice straw enzymes. The highest total reducing sugar was approximately 764.69 mg reducing sugar/g pretreated rice straw using enzyme loading equivalent to 15.53 FPU/g when amount of enzyme dosage was increased four folds. The work showed potential of cellulase from *Chaetomium globosum* BCC5776 for lignocellulose hydrolysis for further conversion to fuels and chemical in sugar-platform biorefineries.

Keyword: Biomass, cellulase, *Chaetomium globosum*, saccharification

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CHAPTER 1

INTRODUCTION

1.1 Rationale

Nowadays, the crisis of rising prices of crude oil, particularly for these industrial and transportation sectors, due to rapidly increasing fuel demands and decreasing oil supplies leads to the insufficiency of fundamental feedstock (coal, oil and gas). In addition, consumption of petroleum also emits excessive CO_2 into the atmosphere, resulting in environment concerns such as global climate change because of warmer temperatures and air pollution [1].

Many countries are concerned about energy and environment issues, and thus are encouraged to introduce clean energy technology. Study on the natural bioconversion process on lignocellulosic plant biomass not only gives insights on the roles of biological processes in maintaining the carbon balance of the eco-system but also of great interest for biotechnology application. Recently, one of the most attractive approaches is to utilize abundant lignocellulosic materials instead of conventional fuels since lignocellulose is renewable, clean, inexpensive and no harmful to environment; besides, it does not negatively affect to human food supply. There are two different technical platforms: thermo-chemical platform and biotechnological platform that are constructed to produce lignocellulosic feedstock biorefinery. In the context, production of fuels, chemicals and materials based on biomass feedstock is an interesting alternative. Several processes such as gasification, pyrolysis and hydrolysis relating to biomass conversion into some chemicals and energy production are shown in Fig. 1.1. Some processes have been mainly used in industry while some still need further development to meet technical and economic criteria for commercialization.

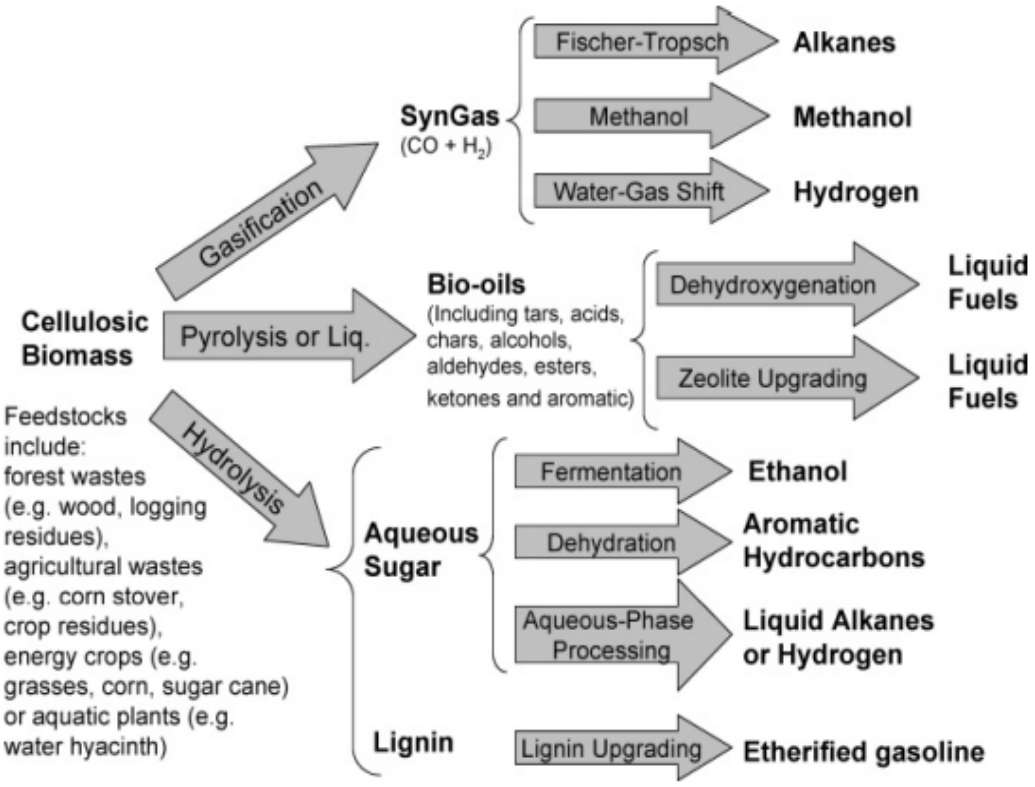


Figure 1.1 Strategies for production of fuels and intermediate chemicals from lignocellulosic materials [2].

Lignocellulosic biomass is a biological material derived from organismsliving in agricultural wastes, wood wastes, forestry residues, animal dung, sewage sludge, etc. They are considered to be largest potential carbon source on earth. It is consisted of cellulose, hemicellulose, lignin as primary constituents and others such as extractives, ash and protein. These compositions vary in different types of lignocelluloses, as shown in Table 1.1. Bioconversion of cellulosic and hemicellulosic materials into fermentable sugars is necessary because these sugars are primary source of bioenergy and biorefinery feedstock products e.g. bioethanol, biochemicals and bioplastic, etc. The main process that used to deliver sugar feedstock is hydrolysis of lignocelluloses to sugar. Development of a practical biomass hydrolysis technology for efficient biomass conversion is thus a great challenge for the development of biorefinery industry. Normally, hydrolysis process can be classified into two groups depending on the applied method; (1) chemical hydrolysis and (2) enzymatic hydrolysis. However, applying chemical method has some disadvantages compared to hydrolysis using enzymes in term of cost at downstream processing and sugar impurity. Currently, enzymatic saccharification would be a promising way to degrade plant biomass into sugar monomers for production in biorefineries.

Table 1.1 The contents of cellulose, hemicelluloses, lignin and other compounds in various terrestrial biomass species[a][2].

Biomass components (dry wt %)	corn stover	switch grass	sugarcane	sweet sorghum	eucalyptus
Celluloses	36	40-45	22	35	48
Hemicelluloses	23	31-35	15	17	14
Extractives (starches, terpenes)	6	0	43	23	2
Lignin	17	6-12	11	17	29
Uronic acid		0	0	1	4
Proteins		5-11			
Ash	10	5-6	9	5	1

[a]Adapted from Towler *et al.*,2004

A variety of cellulolytic, hemicellulolytic, and lignolytic enzymes produced by various microbes, including bacteria and fungi, are used in the hydrolysis process. Among them, fungi are the major plant biomass degrading enzyme producers, which play a major role in plant biomass decomposition in nature. Lignocellulolytic enzyme from fungi are divided largely into three groups which are cellulases, hemicellulase and ligninases. Table 1.2 summarizes a few different fungi producing different lignocellulolytic enzymes. Cellulases, which include endoglucanases, exoglucanases and β-glucosidase, from various fungal genera e.g. *Trichoderma* sp., *Aspergillus* sp., *Penicillium* sp., *Chaetomium* sp., etc. are highly efficient in degradation of cellulose into glucose monomer. These enzymes act synergistically to catalyze cellulosic materials.

There are some reports on fungal strains producing cellulases, for examples, *Aspergillus* is one of the best well-known that normally has high β-glucosidase activity but lower amount of endoglucanase [3]. In contrast, *Trichoderma viride* has high endoglucanase activity but lower β-glucosidase levels and hence limits to degrade efficient celluloses. Apart from cellulases, hemicellulases are also important enzymes for decomposition of hemicelluloses into C_5 sugars (i.e. pentoses). Different techniques including mutagenesis, co-culturing and heterologous gene expression are applied to increase fungal enzymes production. Nevertheless, optimization method is also used to enhance the production of enzymes. Many physical and chemical parameters affect enzyme production such as pH, temperature, nitrogen sources, and phosphorous sources, etc. These factors are needed to be optimized for improvement of enzymes production/activity from fungi.

Table 1.2 Examples of different fungi producing lignocellulolytic enzymes [4].

	Group	**Fungal strain**	**Enzymes**
Aerobic fungi (Extracellular lignocellulolytic enzymes)	Ascomycetes	*T. reesei*	Cellulases (CMCase, CBH, BGL), Hemicellulase (xylanase)
		A. niger	Cellulases, Xylanases
	Basidiomycetes	*P. chrysosporium*	Cellulases (CMCase, CBH, BGL), CDH, LiP, MnP, Hemicellulase (xylanases)
		F. palustris	Cellulases (CMCase, CBH, BGL)
Anaerobic rumen fungi (Chytridiomycetes) (Cell-wall associated lignocellulolytic enzymes, "cellulosome")	*Anacromyces*	*Anacromyces Mucronatus* 543	Cellulases (CMCase), Hemicellulase (xylanase)
	Caccomyces	*Caccomycescommunis*	Cellulases, Hemicellulase (xylanase, β-D-xylosidase)
	Neocallimastix	*Neocallimastixfrontalis*	Cellulases, Hemicellulases (xylanase, β-galactosidase)
	Orpinomyces	*Orpinomyces sp.*	Cellulases (CMCase, CBH, β-glucosidase), Hemicellulases (xylanase, mannannase)
	Piromyces	*Piromyces sp.*	Cellulases (CMCase, CBH, β-glucosidase), Hemicellulases (xylanase, mannannase)

CMCase: Carboxymethylcellulases (endoglucanase), CBH: Cellobiohydrolases, BGL: β-glucosidases, CDH: Cellobiose dehydrogenase, MnP: Manganese peroxidises, LiP: Lignin peroxidises.

In this research, an efficient biomass degrading enzyme was developed based on the crude multi-activity cellulolytic and hemicellulolytic enzymes from a soft-rot fungus *Chaetomium globosum* BCC5776. Production media and fermentation conditions were studied to increase enzyme production capability of the wild-type fungal strain, along with trials on development of hyper-producing mutant strains using mutagenesis techniques. The crude enzyme were used for formulation of efficient biomass-degrading enzyme mixture by experimental mixture design approach and then used for hydrolysis of alkali-pretreated rice straw to production of fermentable sugars. The work provides an alternative lignocellulose-degrading enzyme potent for on-site enzyme production in the development of a feasible biorefinery industry.

1.2 Literature Review

As described above, the main objective of this research is to study fungal biomass-degrading enzyme mixtures based on crude multi-activity cellulolytic and hemicellulolytic enzymes for the saccharification of local lignocellulosic feedstock. The work aims to study cellulase production from *C. globosum* BCC 5776 by submerged fermentation and formulate crude enzyme mixtures comprising BCC 5776 and commercial enzymes or crude/recombinant enzymes in ENZ database for biomass saccharification. In this work, enzyme production by the fungal strain and enzymatic hydrolysis were investigated. Details of the previous researches are presented below:

1.2.1 Enzyme production

Cellulolytic enzymes, including endoglucanases, exoglucanases and β-glucosidases, are required in order to completely hydrolyze cellulose. These hydrolytic enzymes act synergistically on conversion of amorphous and crystalline regions of cellulose into glucose. In order to improve cellulase production, many factors (physical and chemical) need to be optimized. Several methods, particularly fermentation media optimization and mutagenesis, have been applied to produce higher yield of cellulases. Therefore, the previous researches are reviewed as follows:

1.2.1.1 Optimization of fermentation media for cellulase production

Ojumu *et al.* (2003) [5] studied cellulase production by *Aspergillus flavus* Linn isolate NSPR 101 fermented on different lignocelluloses. The substrates used in this work were bagasse, sawdust and corncob. The fermentation medium for production of cellulase (FPase) with various lignocelluloses as carbon source was optimized to the following condition: 200 rpm agitation speed at 35ºC for 52 h. They found that the maximum cellulase production (FPase) was 0.0743 IU/ml with sawdust while bagasse and corncob were 0.0573 IU/ml and 0.0502 IU/ml, respectively at about 12 h of production for all the lignocellulosic materials used (Fig. 1.2). The highest cellulase productivity with sawdust may be due to its very high percentage of cellulose which is the major component of cell walls of wood. The results were achieved when the lignocellulosic residues (bagasse, sawdust and corncob) were pretreated with ball milling (particle size 0.5 mm) for 48 h and then soaked in 1% (w/v) NaOH solution at a ratio of 1:10 (substrate:solution) for 2 h at

room temperature. The treated substrates were neutralized before inoculation into liquid fermentation medium for cellulolytic enzyme production.

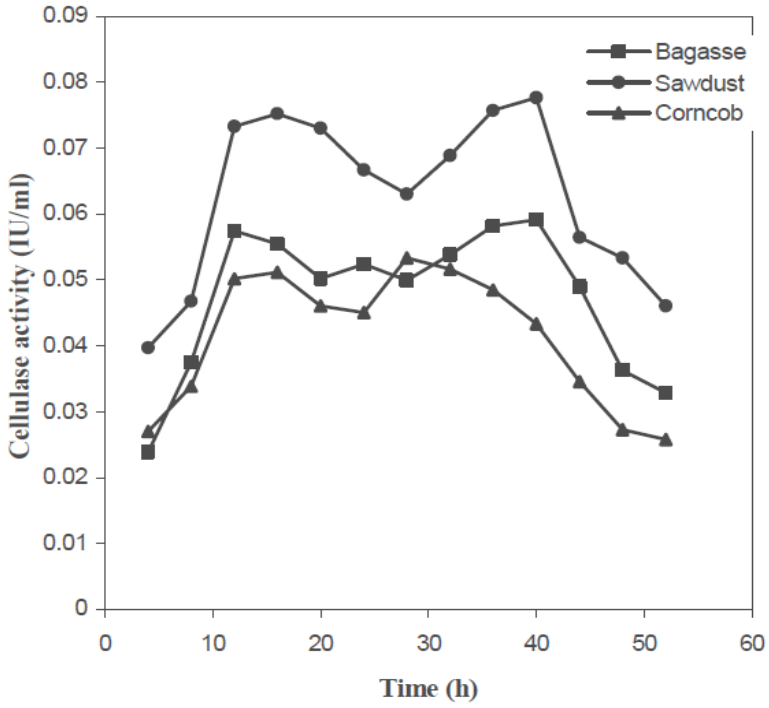


Figure 1.2 Substrate effects on *A. flavus* cellulase activity grown in optimized medium at 200 rpm agitation and 1.00 vvm aeration [5].

Narasimha *et al*. (2006) [6] optimized the production conditions for cellulolytic enzymes from *Aspergillus niger* in liquid shake cultures by investigating the best cellulosic substrate used as the carbon source. Primarily, enzyme production in three different media (minimal, basal and Czapek-Dox) containing 1% cellulose as the carbon source were observed over a period of 3 weeks at 28ºC on a rotary shaker 180 rpm. Total activity of cellulase complex and/or individual component enzyme activity reached the maximum of 1.70 FPU/ml on the Czapek-Dox medium at 7 days followed by 1.59 FPU/ml and 1.01 FPU/ml in basal medium and minimal medium, respectively. Dose response cellulose supplementation within a range of 0.5 – 2.0% were optimized to enhance the yield of cellulase production. The optimal concentration of cellulose was 1.5% for cellulase production. The values of FPase, CMCase and β-glucosidase were 1.75 FPU/ml, 0.650 U/ml, and 1.12 U/ml, respectively. Further increases in cellulose concentrations beyond 1.0% level did not result in proportionate increases in yields of fungal biomass and protein content.

Sathyavrathan and Krithika (2013) [7] compared cellulase production levels of *Aspergillus niger* (NRRL-322) and *Trichoderma reesei* (NCIM-1052). In the first part of this research, they studied the production of cellulase from both *A. niger* and *T. reesei* in 1-L flask containing 300 mL of commercial media (potato dextrose) for 120 h, 30ºC with shaking at 150 rpm. The result of enzyme activities from *A. niger* and *T. reesei* was shown in Fig. 1.3. They obtained cellulase production in *Aspergillus niger* (NRRL-322) equivalent to 23.3 U/ml whereas the activity from *Trichoderma reesei* (NCIM-1052) was 38.75 U/ml. In media optimization for production of cellulase in *T. reesei*, four different media containing different carbon sources and concentration were used: 1% wheat bran, 1% cellulose, 0.6% dextrose, and 2% groundnut. The enzyme activity as shown in Fig. 1.4 was 43.067 U/ml for the medium containing 2% groundnut.

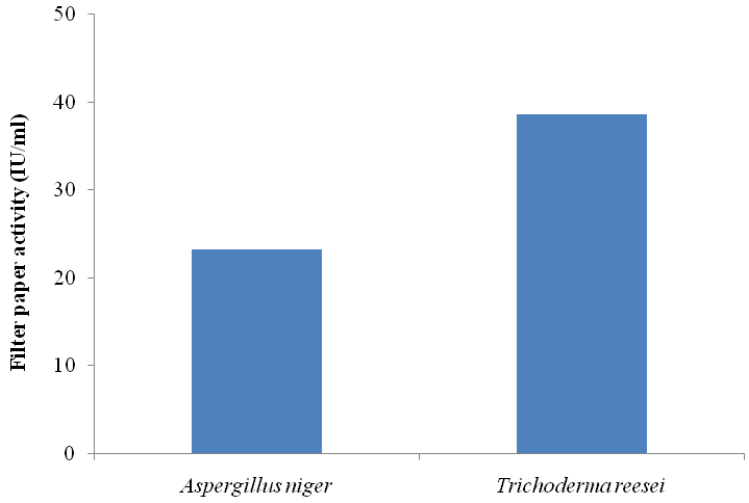


Figure 1.3 Cellulase production from *Aspergillus niger* vs *Trichoderma reesei* [7]. One unit of enzyme refers to the amount of enzyme that produces 1 µg of reducing sugar released in terms of glucose per minute.

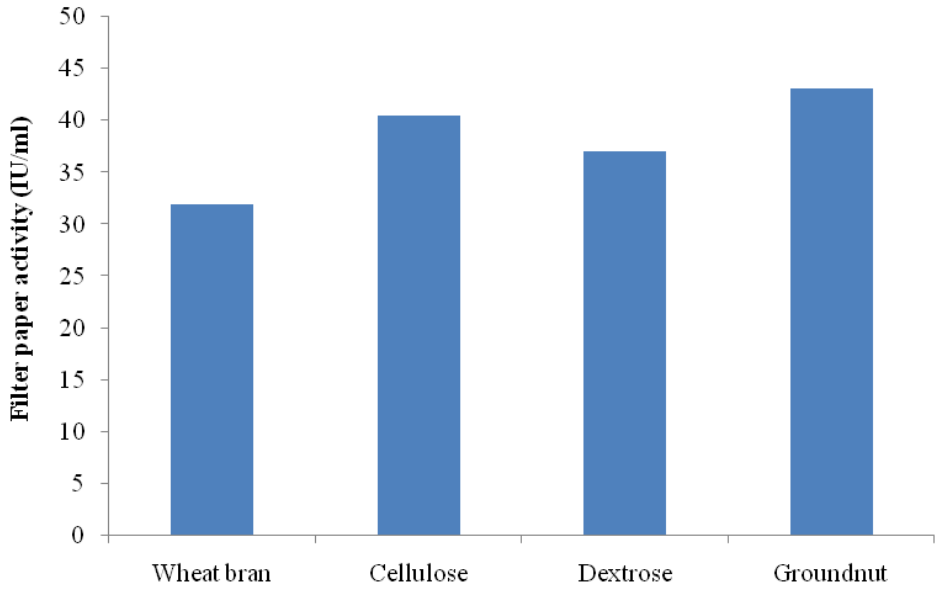


Figure 1.4 Cellulase activity of *Trichoderma reesei* in media containing various substrates [7]. One unit of enzyme refers to the amount of enzyme that produces 1 µg of reducing sugar released in terms of glucose per minute.

Moosavi-Nasab and Majdi-Nasab (2008) [8] studied cellulase production by *Trichoderma reesei* using sugar beet pulp (SBP) as a substrate. They obtained 0.46 IU/ml of filter paper activity under the optimal conditions when *T. reesei* was fermented in 100 ml of amedium containing the mixture of chemical salts and SBP substrate. The optimal condition described in this study was at 30ºC, initial pH 4.8, and agitation speed 180 rpm for 5 days.

Later on, Rashid *et al*. (2009) [9] studied optimization of nutrient supplients for cellulase production. *Trichoderma reesei* RUT C-30 (ATCC 56765) was employed to optimize media containing palm oil mill effluent (POME) as basal media. The Plackett-Burman experimental design was used for optimization in order to identify significant ingredients in the media for cellulolytic enzyme. In total 12 experimental runs (combinations) with 11 independent variables: total suspend of solid (TSS) of POME, cassava powder, wheat flour, sugar, cellulose, peptone, $(NH_4)_2SO_4$, KH_2PO_4, Tween-80, $MnSO_4.H_2O$ and $MgSO_4.7H_2O$ were screened in this work (Table 1.3). The main effect of each variable was evaluated for the significant effect of the eleven different co-substrates and mineral elements on the production of cellulase using POME as a basal medium. The results shown in Fig. 1.5 showed that sugar, cellulose, peptone, KH_2PO_4, Tween-80 and $MnSO_4.H_2O$ had positive effect on FPase and CMCase activity. Run 12 provided the

maximum filter paper activity (FPA) of 2.907 U/ml. Then, the experimental design of one factor at a time (OFAT) was also manipulated to observe the optimum levels of the parameters which are TSS of POME, sugar, cellulose, peptone, KH_2PO_4,Tween-80, and $MnSO_4.H_2O$. Each variable was investigated independently at different levels.

Table 1.3 The output of 12 experimental runs with 11 independent variables in Plackett-Burman experimental design for optimization of cellulase production [9].

Run	A	B	C	D	E	F	G	H	J	K	L	FPA	CMC
	(%, w/v)											(U/ml)	
1	0.5	0	0	0	0	0	0	0	0	0	0	0.166	0.506
2	2	0	2	0	0	0	0.5	0.25	0.2	0	0.02	0.004	0.060
3	2	0	2	2	0	0.5	0	0	0	0.05	0.02	0.020	0
4	2	0	0	0	2	0.5	0.5	0	0.2	0.05	0	0.037	0.062
5	2	2	0	2	2	0	0.5	0	0	0	0.02	0.018	0.012
6	0.5	0	2	2	2	0	0.5	0.25	0	0.05	0	0.915	5.718
7	0.5	2	0	0	0	0.5	0.5	0.25	0	0.05	0.02	0.390	0.896
8	2	2	0	2	0	0	0	0.25	0.2	0.05	0	0.069	0.081
9	0.5	2	2	0	2	0	0	0	0.2	0.05	0.02	1.884	6.954
10	0.5	2	2	2	0	0.5	0.5	0	0.2	0	0	0.641	1.257
11	2	2	2	0	2	0.5	0	0.25	0	0	0	0.017	0.084
12	0.5	0	0	2	2	0.5	0	0.25	0.2	0	0.02	2.907	6.5

A, TSS of POME; B, Cassava powder; C, wheat flower; D, sugar; E, cellulose; F, peptone; G, $(NH_4)_2SO_4$; H,KH_2PO_4; J, tween 80; K, $MnSO_4.H_2O$; L, $MgSO_4.7H_2O$; The (+) indicates the high and (-) indicates the low level.

However, carboxymethylcellulase (CMC) was also investigated in Plackett-Burman experimental design. In order to optimize two independent variables, including peptone and cellulose, the face centered central composite design (FCCD) program under response surface method (RSM) was used. The maximum cellulase activity (CMC) was predicted using FCCD was equivalent to 17.84 U/ml on the center point with 0.5% concentration of peptone and cellulose as showed in Fig. 1.6.Furthermore, a set of experiment (5 runs) was performed to verify the optimization result to validate the developed model (Table 1.4). The highest cellulase activity of 18.53 U/ml was obtained under the optimum conditions, which was slightly higher than the predicted value of 17.84 U/ml. The medium constituent concentration of peptone and cellulose was also 0.5% (w/v).

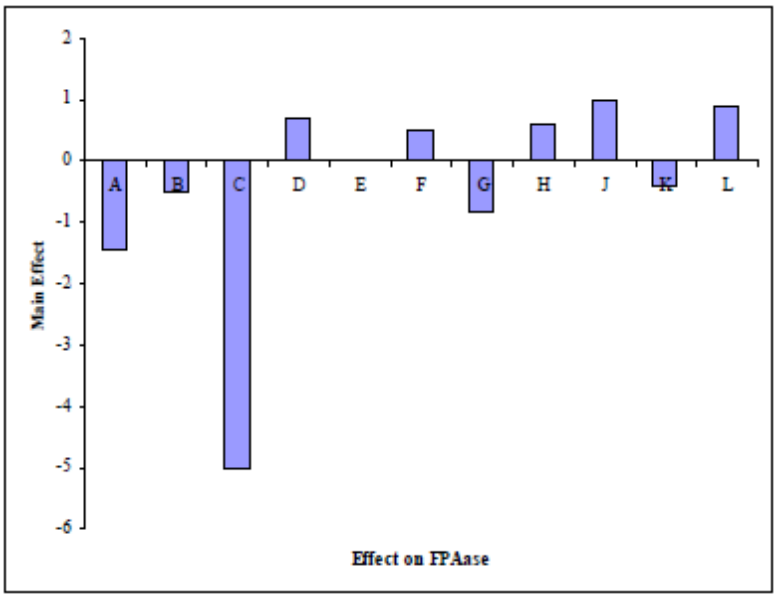
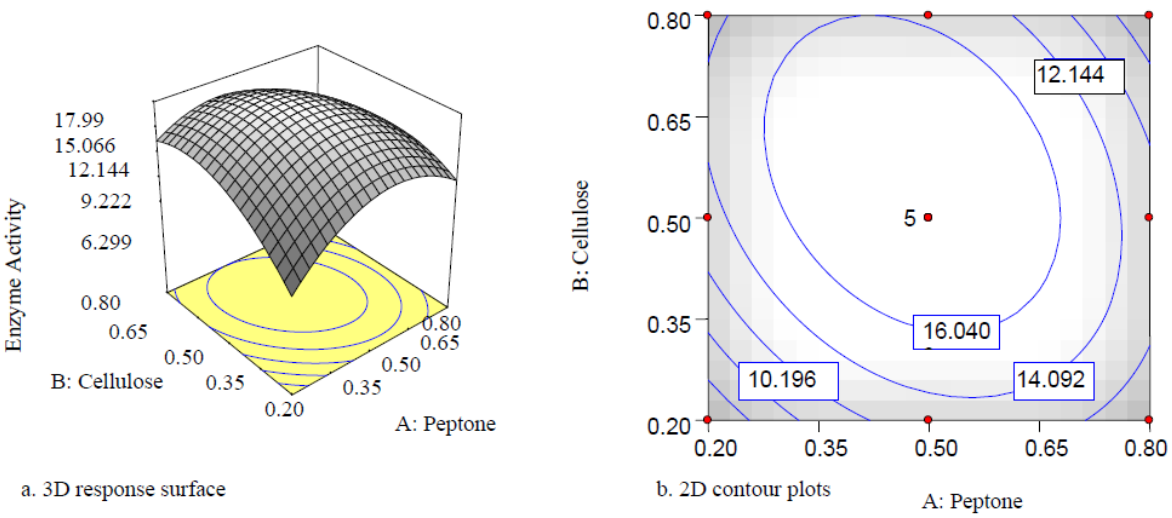


Figure 1.5 Main effects of the medium constituents on cellulase production (FPase) from Plackett-Burman experimental results (A: TSS(POME), B: cassava, C: wheat, D: sugar, E: cellulose, F: peptone, G: $(NH_4)_2SO_4$, H: KH_2PO_4, J: Tween-80, K: $MnSO_4.H_2O$, L:$MgSO_4.7H_2O$) [9].



a. 3D response surface

b. 2D contour plots

Figure 1.6 Effects of peptone and cellulose concentrations (%, w/v) on the production of cellulase [9].

Table 1.4 Validation of the developed polynomial equation for optimum medium constituent [9].

Run	A:Peptone (%w/v)	B:Cellulose (%w/v)	Cellulase (U/ml)	
			Predicted	Experimental
1	0.3	0.6	16.47	16.3
2	0.4	0.6	17.71	17.42
3	0.5	0.5	17.84	18.53
4	0.6	0.4	16.67	17.55
5	0.7	0.3	14.19	13.37

Gautum *et al.* (2010) [10] investigated the optimization of medium and cellulase production in submerged fermentation by *Trichoderm viride*. The objective of this study was to reduce the production cost of cellulase. Therefore, municipal solid waste (MSW) residue was introduced to use as the alternative carbon source. *T. viride* was grown in media containing 50 ml of basal medium and was incubated in stationary condition at 40 ± 2ºC up to 7 days. Many factors influencing cellulase production were studied including incubation time, pH, temperature, carbon source (both commercial and alternative carbon sources), nitrogen source, and cations. The optimal condition for maximum production of cellulolytic enzymes was determined. Five different carbon sources and concentrations were observed in this work: glucose, maltose, cellulose, sucrose, and carboxymethylcellulose (CMC). They found that sucrose led to the maximum cellulase activities; 2.68 U/ml, 2.17 U/ml and 2.06 U/ml for FPase, CMCase and β-glucosidase respectively. The effect of the best carbon source with different concentrations was showed in Table 1.5.

Table 1.5 Effects of concentration of carbon sources (%) on cellulase production (U/ml) by *Trichoderma viride* [10].

Conc.	Sucrose		
(%)	FPase	CMCase	β-glucosidase
0.5	0.67±0.05	0.50±0.06	0.42±0.04
1.0	2.68±0.07	2.17±0.07	2.06±0.08
1.5	2.21±0.08	1.51±0.06	1.51±0.08
2.0	1.55±0.05	0.86±0.05	0.90±0.06
2.5	0.84±0.04	0.64±0.04	0.74±0.05
3.0	0.44±0.03	0.31±0.03	0.31±0.04

The highest cellulase activity was obtained with 1.0% yeast extract as the nitrogen source (Table 1.6),which was used to replace the nitrogen source in basal medium. The cellulase activities of FPase, CMCase and β-glucosidase were 2.21 U/ml, 1.93 U/ml, and 1.94 U/ml, respectively with 1.0% concentration of the nitrogen source. Some kinds of cation such as Na^+ and K^+ did not significantly affect cellulase production; in contrast, addition of 10 mM concentration of Ca^{2+} could enhance cellulase activities.

Table 1.6 Effects of concentration of nitrogen sources (%) on cellulase production (U/ml) by *Trichoderma viride* [10].

Conc.	Yeast extract		
(%)	FPase	CMCase	β-glucosidase
0.5	0.31±0.04	0.32±0.05	0.37±0.03
1.0	2.21±0.08	1.93±0.07	1.94±0.06
1.5	1.89±0.07	1.62±0.06	1.71±0.08
2.0	1.52±0.07	0.84±0.06	0.91±0.06
2.5	0.91±0.06	0.63±0.05	0.46±0.05
3.0	0.53±0.04	0.48±0.04	0.39±0.05

Other parameters, such as pH, temperature, incubation period, and alternative carbon source (MSW), also presented in Figs.1.7A-D. The results that in Fig. 1.7D reveal MSW residue was the best carbon substrate for FPase, CMCase and β-glucosidase production by *Trichoderma* sp. and 4.0% MSW residue led to the highest cellulase production of FPase 1.77 U/ml, CMCase 1.95 U/ml, and β-glucosidase1.66 U/ml.

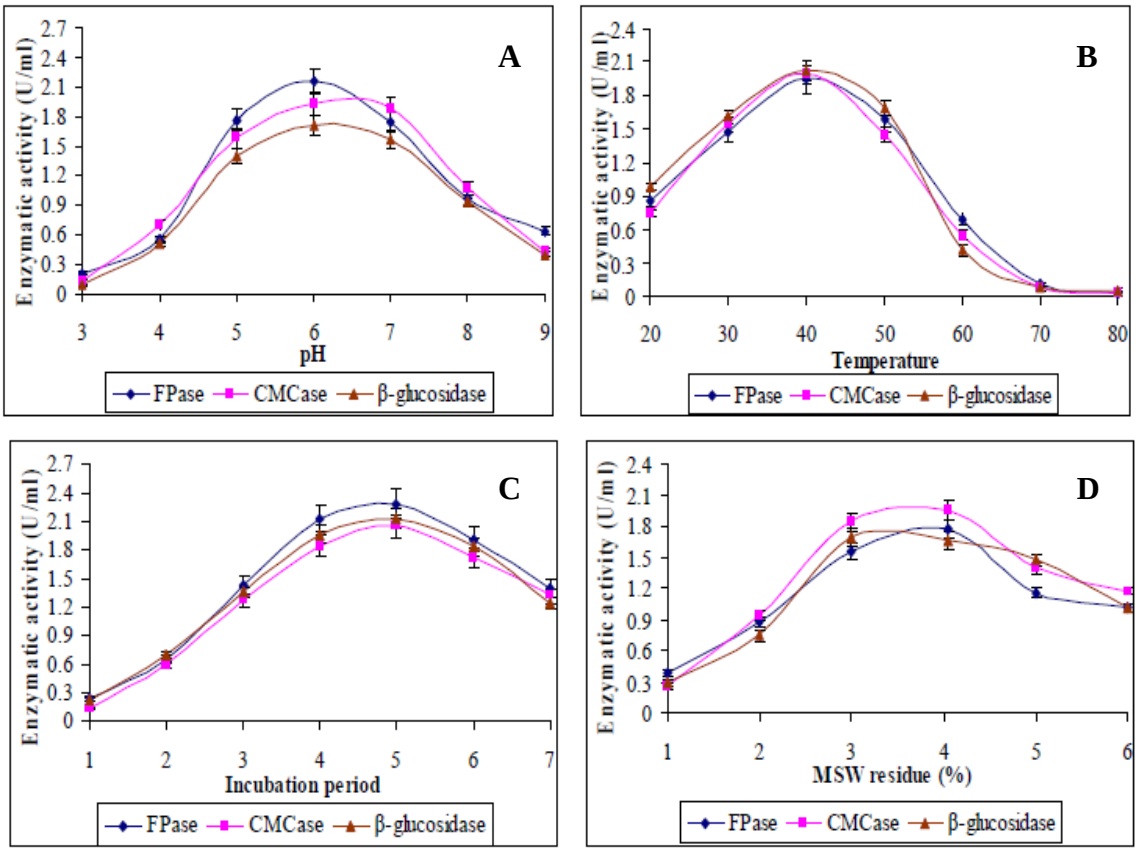


Figure 1.7 Effect of cellulase production (U/ml) by *Trichoderma viride*: (A) pH, (B) temperature, (C) incubation time, (D) concentration of MSW used as alternative carbon source [10].

Goldbeck et *al.* (2012) [11], studied cellulolytic enzymes production by *Acremonium strictum* isolated from Brazilian Biome using different substrates. Submerged fermentation was initially carried out using commercial substrates including AVICEL®, SERVACEL® (microcrystalline cellulose), and carboxymethyl cellulose (CMC) as the carbon sources. The culture medium contained 20 g/l of the substrates and incubated at 30ºC with 150 rpm shaking for 240 h. The samples were collected at 48 h intervals and then centrifuged to separate the supernatant (crude enzyme) and precipitate (microbial cells) for further analysis of enzyme activities. The activities of CMCase, FPase, cellobiase, and β-glucosidase were shown in Fig. 1.8a-d. They found that the greatest cellulase production was achieved in AVICEL®.

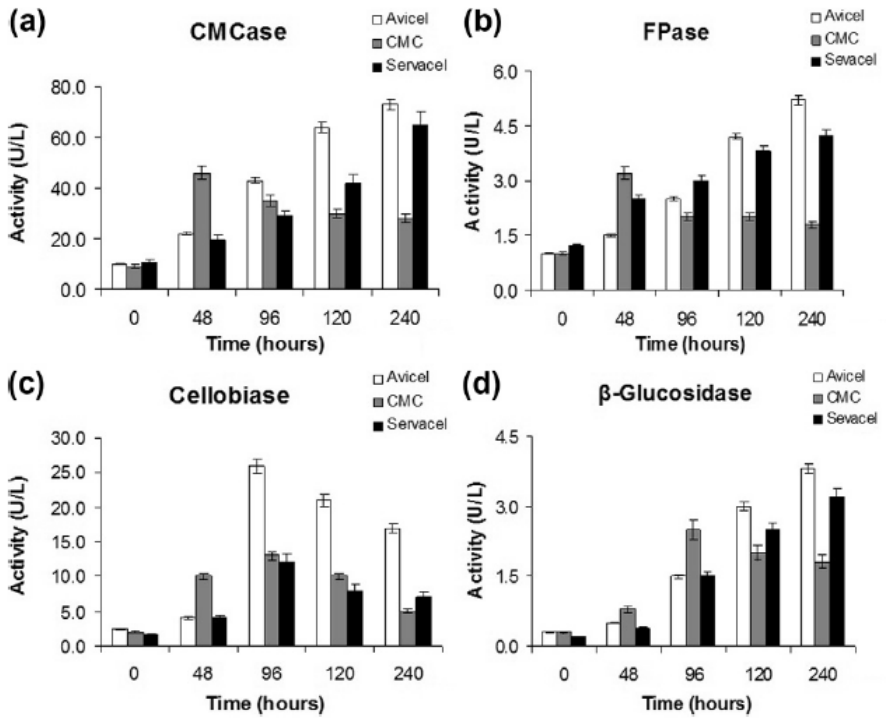


Figure 1.8 Comparison of enzyme activities from *A. strictum* using different commercial substrates(microcrystalline cellulose AVICEL®, SERVACEL® and carboxymethylcellulose, CMC) during 240 h of fermentation: (a) CMCase activity, (b) FPase activity, (c) cellobiase acitivty, (d) β-glucosidase activity [11].

In the next step, lignocellulosic biomass was used to compare with the best commercial substrate i.e. AVICEL® in liquid fermentation. Four different conditions of bagasse as the substrate were used to study cellulolytic enzyme production by *A. strictum*: raw sugarcane bagasse (without pretreatment) and bagasse subjected to mild (12 kgf/cm2; 188.5ºC), average (15 kgf/cm2; 198ºC), and strong (19 kgf/cm2; 210.4ºC) pretreatment along with microcrystalline cellulose AVICEL®. The results of four different enzymatic activities were shown in Fig. 1.9a-d. The maximum cellulase activities were 139.42 U/ml (at 240 h of fermentation), 10.82 U/ml (at 192 h of fermentation), 27.72 U/ml (at 92 h of fermentation), and 3.48 U/ml (at 240 h of fermentation) for CMCase, FPase, cellobiase and β-glucosidase, respectively with mild pretreated sugarcane bagasse.

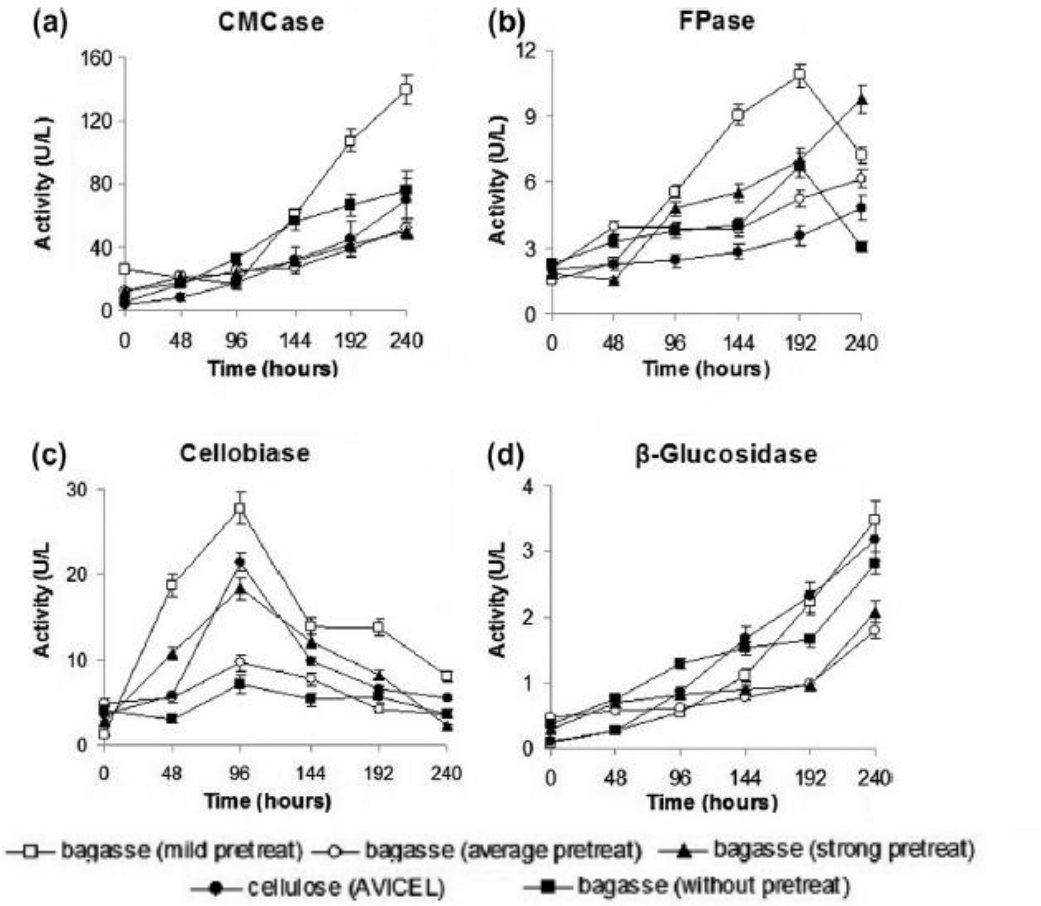


Figure 1.9 Relationship of CMCase activity (a), FPase activity (b), cellobiase activity (c), and β-glucosidase activity (d) to fermentation time using sugarcane bagasse pretreated with different intensities and microcrystalline cellulose (AVICEL®) [11].

1.2.1.2 Improvement of fungal strains by mutation for cellulase production

Kovácset *et al.* (2008) [12] developed *Trichoderma atroviride* mutants for enhancing the production of cellulase and β-glucosidase on pretreated willow. Wild-type strain F-1505 was employed as the parent strain. Firstly, 150 wild-type *Trichoderma* strains coming from 30 countries over the world were screened to identify the best cellulase producer strains in shake flask fermentation on two different media containing pretreated willow and compared to *Trichoderma reesei* (RUT-30). The strains *Trichoderma atroviride* (TUB F-1505) was proved to be the best cellulase producer (filter paper activity, FPA) ~0.32 FPU/ml.

Both chemical and physical methods of mutagenesis were introduced to improve cellulase production. The origin of the mutant strains is summarized in Fig. 1.10. The best

10 mutants from more than 30,000 mutant colonies were cultured for 3 day shake flask fermentation on pretreated willow as the carbon source, containing 150 ml of medium at 30°C on a rotary shaker 220 rpm and after that they were analyzed the activities of cellulase including FPA, endoglucanase (EG) and β-glucosidase compared to TUB F-1505 and RUT-30 as shown in Table 1.7. It was found that cellulase activity from all mutant strains were in the range of 0.48-0.68 FPU/ml of filter paper activity (25-45% FPA improvement compared to wild-type strain TUB F-1505). All mutant strains found to be good producers for production of β-glucosidase with the activity of more than 4.7 IU/ml when compared to RUT-30 (<0.1 IU/ml) while endoglucanase activity in almost all mutant strains was similar to RUT-30 which was in the range of 100-160 EG/ml. Three selected mutant strains (TUB F-1663, TUB F-1740 and TUB F-1753) that produced high levels of cellulase were then cultivated in submerged fermentation using different types of pure cellulose as carbon source. The maximum FPase and β-glucosidase activity of the mutant strains reached in 2-3 days equivalent to 0.45-0.60 FPU/ml and 4.5-8.0 IU/ml, respectively as shown in Fig. 1.11a-b when compared to the wild-type strain F-1505 and *T. reesei*RUT-30.

The produced enzymes are introduced to hydrolyze the pretreated lignocellulosic biomass. One hundred milliliters of crude enzymes solution that obtained from *T. reesei* RUT-30, wild-type strain F-1505 and its mutants were used and compared to the commercial enzyme mixtures (Celluclast 1.5L and Celluclast 1.5L + Novozyme 188) with 100 h of incubation time. It was found that enzyme from mutant TUB F-1753 strain produced high glucose concentration of 3-6 mg/ml which was slightly lower than Celluclast 1.5L + Novozyme 188 (4-7 mg/ml).

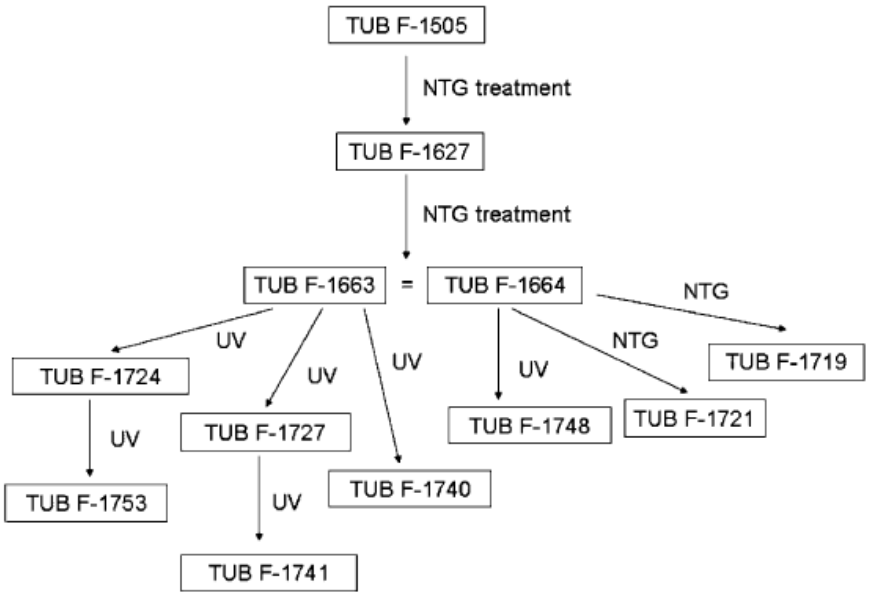


Figure 1.10 The parent wild-type strain *Trichoderma atroviride* TUB F-1505, and the developmentof high cellulase producing mutants (mutation tree) with UV-light and *N*-methyl-*N*-nitro-*N*-nitrosoguanidine (NTG) treatment [12].

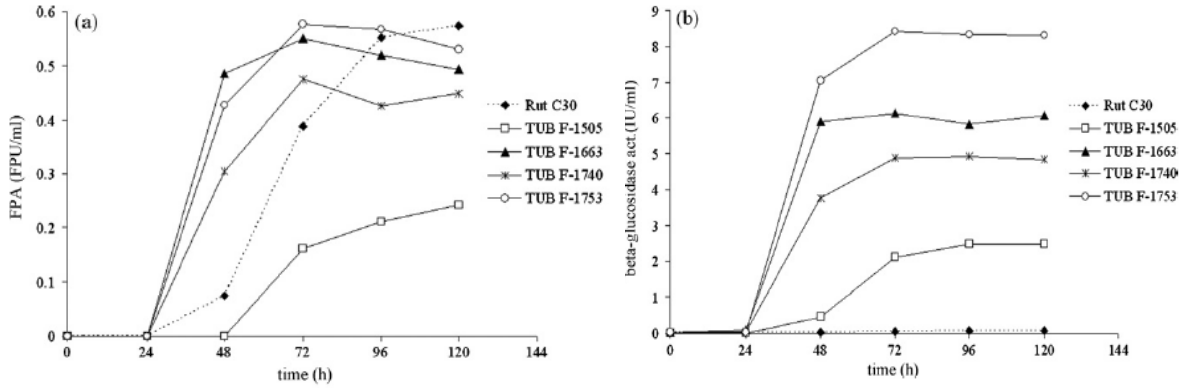


Figure 1.11 Time course of cellulase (FPA) (a) and β-glucosidase (b) production of the *Trichoderma atroviride* strains compared to *Trichoderma reesei* RUT C30 on pretreated willow (medium 2W) in lab scale agitated fermenters [12].

Table 1.7 Shake flask fermentation on pretreated willow with *Trichoderma reesei* RUT C30, the wild-type strain *Trichoderma atroviride* TUB F-1505 and the 10 best *T. atroviride* mutants[12].

	Strain	Medium	Final pH	FPA (FPU/ml)	β-Glucosidase activity (IU/ml)	Endoglucanase activity (EG/ml)
	Rut C30	1W	6.0	0.62	0.07	132.3
		2W	5.8	0.63	0.05	122.1
Wild strain	TUB F-1505	1W	6.2	0.41	5.30	106.0
		2W	5.7	0.42	4.67	103.0
1	TUB F-1663	1W	5.9	0.65	9.01	137.8
		2W	5.6	0.64	6.30	104.4
2	TUB F-1721	1W	6.2	0.65	9.95	155.5
		2W	6.0	0.66	6.70	153.7
3	TUB F-1724	1W	6.2	0.61	11.70	160.6
		2W	6.0	0.61	8.73	143.6
4	TUB F-1727	1W	5.9	0.61	9.67	146.4
		2W	5.6	0.64	7.01	115.2
5	TUB F-1740	1W	6.0	0.63	9.99	157.5
		2W	6.0	0.66	7.26	145.8
6	TUB F-1741	1W	5.9	0.68	9.83	143.5
		2W	5.6	0.58	6.92	124.7
7	TUB F-1746	1W	6.0	0.56	8.96	158.8
		2W	6.0	0.64	7.86	143.3
8	TUB F-1748	1W	5.9	0.50	9.59	121.2
		2W	5.7	0.58	6.98	100.3
9	TUB F-1752	1W	5.9	0.62	8.81	116.0
		2W	5.7	0.54	6.04	97.9
10	TUB F-1753	1W	6.0	0.57	10.28	155.5
		2W	6.0	0.60	8.99	145.2

Vu *et al.* (2009) [13] researched cellulase production by isolated *Aspergillus* sp. using repeated and sequential mutagenesis. Initially, 320 fungal isolates were screened and identified as *Aspergillus* sp. The fungal strain namely K-10 was found to be the best potent strain for production of cellulase; therefore, this strain was further mended by mutation for higher cellulase production. Two methods of mutation were manipulated for strain improvement. In method I, a number of mutant strains was screened and tested for cellulase activities (CMCase, FPase and β-glucosidase). It was found that the best mutant strain NTG-4 was obtained from 4th NTG treatment which could produce higher cellulase yield. In the other hand, the mutant strain R20-2 was initially obtained after the first mutation treatment of method I and further mutated by method II. The best mutant strain namely GV-3 was further improved by 3rd simultaneous treatment with NTG and UV. Both mutant strains NTG-4 and GV-3 were observed for enhancing capability of cellulolytic enzyme. The result, as shown in Table 1.8, clearly showed that cellulase producing mutant strains could be obtained from both methods.

Table 1.8 Improvement of the production of CMCase by treatment with various mutagenic agents [13].

Method I			
Mutagenic treatment	Selected mutant	Enzyme production improved (%)	CMCase activity (U/ml)
None	K-10	100[b]	18.73
1st γ-ray	R-20	127.5	23.88
2nd γ-ray	R20-2	138.0	25.85
Uvc	RV-10	142.5	26.69
1st NTG	NTG-1	158.4	29.67
2nd NTG	NTG-2	165.3	30.96
3rd NTG	NTG-3	185.3	34.71
4th NTG	NTG-4	202.7[c]	37.86

Method II			
Mutagenic treatement	Selected mutant	Enzyme production improved (%)	CMCase activity (U/ml)
None	K-10	100[b]	18.73
1st γ-ray	R20	127.5	23.88
2nd γ-ray	R20-2	138.0	25.85
1st simultaneous treatment with NTG[d] + UV	GV-1	141.6	26.52
2nd simultaneous treatment with NTG + UV	GV-2	135.3	25.34
3rd simultaneous treatment with NTG + UV	GV-3	128.2	24.01

[a]CMCase, carboxymethyl cellulose.
[b]The 100% CMCase activity was 18.73 U/ml.
[c]UV, ultraviolet.
[d]NTG, N-methyl-N'-nitro-N-nitrosoguanidine.
The 202.7% CMCase activity was 37.86 U/ml.

Moreover, the stability of the mutant Aspergillus *sp*. NTG-4 for cellulase production was studied. The mutant strain NTG-4 was subcultured onto PDA plates for over seven months. Each subculture (total 19 times) was tested for cellulase activities– CMCase, FPase and β-glucosidase. The production of cellulase was achieved by solid-state fermentation (SSF) using wheat bran as a substrate. It was found that the mutant strain could maintain the same production yield of cellulase consistently (Fig. 1.12). The higher

activities of CMCase, FPase and β-glucosidase were 37.84 U/ml, 35.18 U/ml and 27.12 U/ml over the study period.

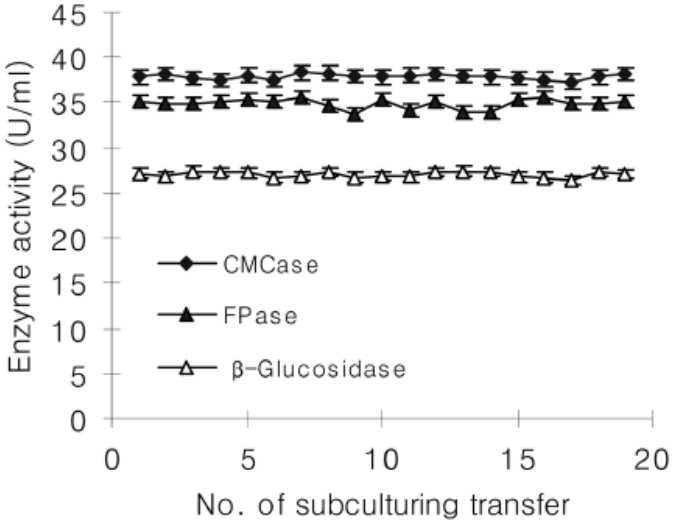


Figure1.12 Stability of the production of CMCase, FPase, and β-glucosidase by mutant *Aspergillus* sp. NTG-4. CMCase, carboxymethylcellulase; FPase, filter paper cellulase [13].

1.2.2 Hydrolysis

Enzymatic saccharification is the second step of the biorefinery process to produce sugar. Cellulases and hemicellulases are used in this step for cleaving cellulose and hemicelluloses, respectively into soluble monomeric sugars including hexoses (6-carbons) and pentoses (5-carbons). Improvement of catalytic efficiency of enzymes is very important because it will reduce the cost of hydrolysis by enabling lower enzymes dosage [4] and increase the sugar yields. Many factors e.g. temperature, pH, ionic strength and surface area are in hydrolysis process using enzymes as they are related to enzyme catalytic performance and stability as well as adsorption of cellulases on microcrystralline cellulose. Relatively high temperature and low pH cause leads to lower enzyme catalytic efficiency and stability. The surface area of lignocellulosic substrates plays an important role in the interaction between the cellulases and cellulose. The adsorption is proportional to the initial cellulose concentration. Consequently, the pretreatments of cellulosic substrates like ball milling, steam treatment and chemical pretreatment are performed to increase the available surface area for enzymatic attack. Moreover, high temperature and low pH tolerant enzymes are preferred in hydrolysis of pretreated lignocellulosic materials

at industrial scales. In most cases, the optimum temperature for enzymatic hydrolysis is in the range of 30 to 55ºC which is suitable for enzyme activity [14].

Aderemi *et al.* (2008) [15] described the kinetics of glucose production from rice straw by *Aspergillus niger*. In addition, they observed the effects of particle size varying from 425 to 75 µm on glucose yield. They found that decreasing the particle size of rice straw would increase glucose yield from 43% to 87% (Fig. 1.13) whereas the optimal temperature and pH were investigated in the range of 45-50 ºC and 4.5-5.0 respectively. The study also presented that the rate of glucose production was depended on the effect of ammonia pretreatment of rice straw, substrate concentration and cell loading

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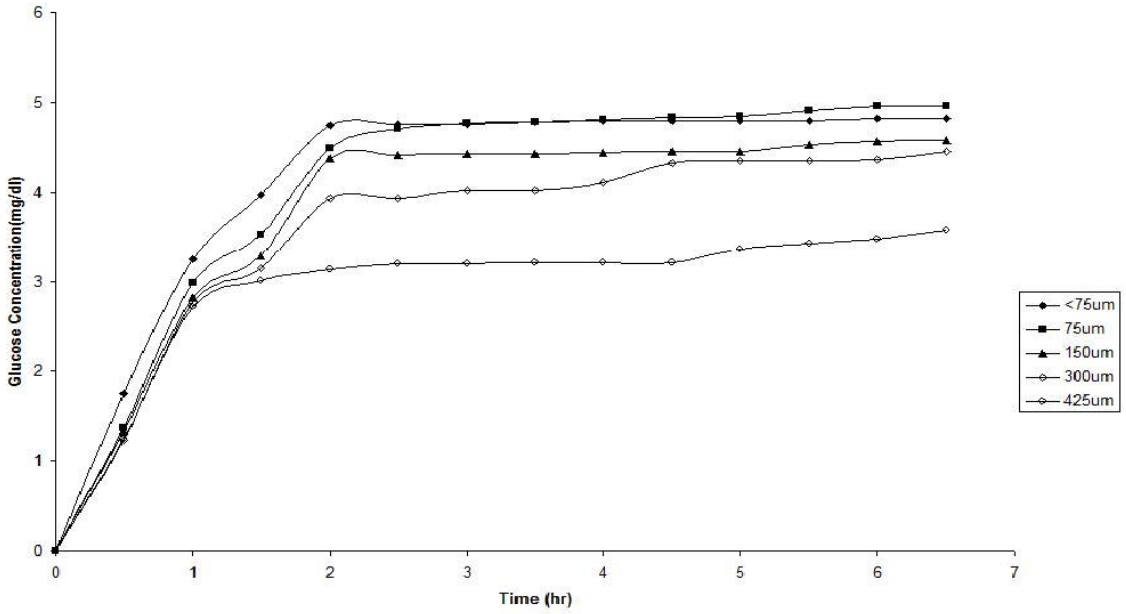

Figure 1.13 Effect of particle size on glucose yield [15].

Buaban *et al.* (2010) [16] studied bioethanol production from ball milled bagasse using on-site produced fungal enzyme mixtures and fermentation of xylose by *Pichia stipitis*. Crude enzymes prepared from *Penicillium chrysogenum* BCC 4504 (5 FPU/g substrate) and *Aspergillus flavus* BCC 179 (10 U/g substrate based on β-glucosidase activity) were used and compared to commercial enzymes which are Acremonium cellulase (5 FPU/g substrate) and Optimash BG (20 U/g substrate of xylanase) in the hydrolysis reaction. The bagasse hydrolysis reaction contained 5% (w/v) of alkaline pretreated bagasse in 50 mM sodium acetate buffer, pH 5.0 with 1 ml of enzyme mixtures. The reaction mixture was incubated at 45 ºC for 72 h and mixed with a horizontal rotator.

The monosaccharide sugars were determined using HPLC. The sugar yield (%) from enzymatic hydrolysis with different enzyme preparations was investigated as shown in Fig. 1.14. It was found that the maximum yield of sugars of 84 ± 0.7% glucose and 70.4 ± 1.1% xylose were obtained from combination of the *P. chrysogenum* BCC 4504 cellulase with *A. flavus* BCC 179 enzyme preparation containing high β-glucosidase and xylanase activities.

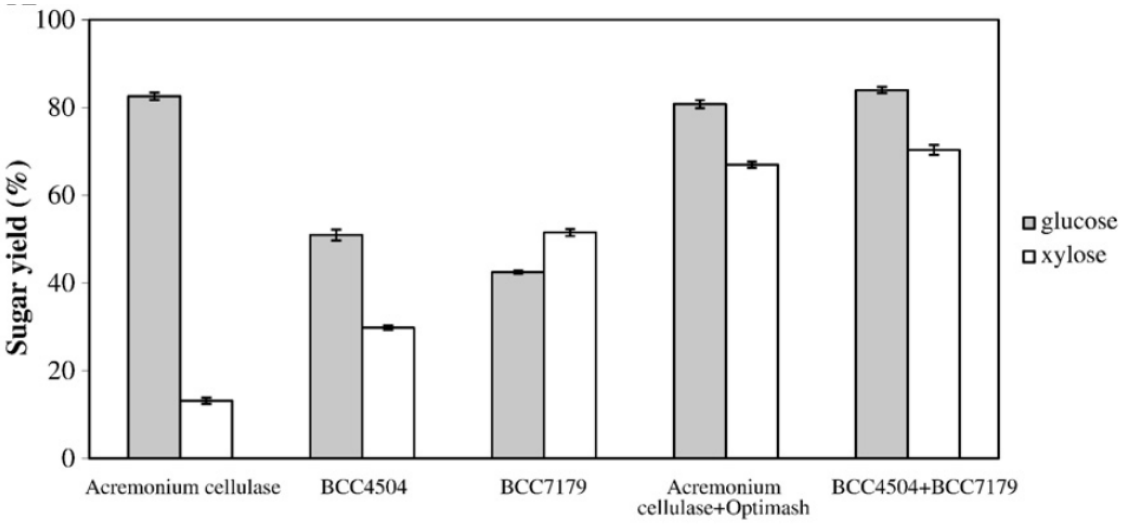

Figure 1.14 Effect of enzyme mixtures hydrolysis of 5% (w/v) pretreated bagasse in 50 mM sodium acetate buffer, pH 5.0, for 72 h at 45 °C. Enzymes used included 5FPU/g substrate of Acremonium cellulase, 20 U (as xylanase activity)/g substrate of Optimash BG, 5 FPU/g substrate of cellulase preparation from *P. chrysogenum* BCC4504, and 10 U (as β-glucosidase activity)/g substrate of enzyme preparation from *A. flavus* BCC7179 [16].

Zhong *et al.* (2009) [17] studied the optimization of enzymatic hydrolysis and ethanol fermentation from ammonia fiber expansion (AFEX)-treated rice straw. They started that the hydrolysis efficiency of lignocellulosic biomass increased when combining of enzymes such as cellulase, xylanases and pectinases. It was found that the addition of Multifact® xylanase at 2.67 mg protein/g glucan and Multifect® pectinase at 3.65 mg protein/g glucan was optimized to greatly increase sugar conversion of AFEX-treated rice straw. Glucan and xylan conversion into monomeric sugars, as shown in Fig. 1.15, reached 81.7% and 75.8% whereas cellulose usage was only one-fifth of that (15 FPU/ g glucan loading) about 80.6% of glucan conversion and 89.6% of xylan conversion.

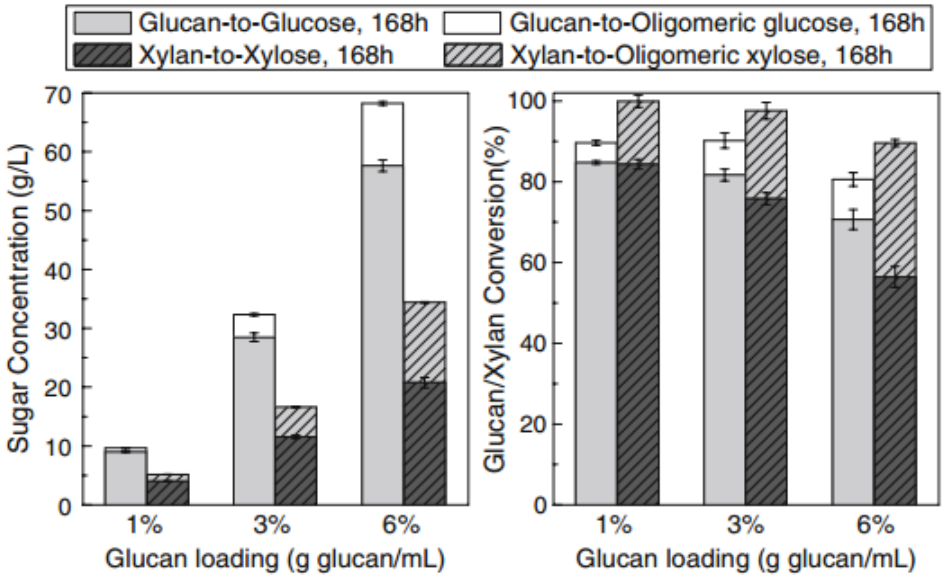

Figure 1.15 Monomeric and oligomeric sugar analysis of hydrolysate at different glucan loadings (1%, 3%, and 6%). Enzyme complex: Spezyme® CP (15 FPU/g glucan), Novozyme™ 188 (64 pNPGU/g glucan), Multifect® xylanase (2.67 g protein/g glucan), Multifect® pectinase (3.65 g protein/g glucan). Enzyme loading, 6% glucan loading; temperature, 50 °C; shaking speed, 150 rpm (1% glucan loading), 250 rpm (3% and 6% glucan loading). Values are means of duplicate experiments [17].

Zhao *et al.* (2011) [18] studied enzymatic saccharification of alkali/peracetic acid (PAA)-pretreated bagasse. The effects of initial solid consistency (2.5%–10%), cellulase loading (0–20 FPU/ml) and addition of supplemental β-glucosidase (0–40 CBU/g solid of supplemental β-glucosidase) on the enzymatic conversion of glycan were studied. The pulp was digested at 50 ± 0.5ºC and pH 4.8 in 0.1M sodium acetate buffer on 130 rpm for 5 days. It was found that conversion of enzymatic glycan reached 80% after 24 h incubation period when enzyme loading was 10 FPU/g solid.

Suwannarangsee *et al.* (2012) [19] demonstrated the use of a systematic approach for the design and optimization of a synergistic enzyme mixture of fungal enzymes and expansin (nonenzymatic proteins) for lignocellulosic degradation. The enzyme mixture for hydrolysis of alkali-pretreated rice straw was optimized based on the synergy of crude enzymes from a local fungal strain, a bacterial expansin and a commercial *T. reesei* commercial cellulase (Celluclast™). In order to screen fungal enzymes with synergy to Celluclast™ 1.5L, thirteen fungal strains were selected for crude enzyme preparations and screened for their synergistic actions with the commercial cellulase (Fig. 1.16). Only three

strains (BCC 309, BCC 199, and BCC 4441) had high capability to hydrolyze alkali-pretreated rice straw. As the second screening, the three fungal enzyme extracts were produced in scaled-up amounts, and the concentrated extracts were used for synergistic activity studied at higher level of enzyme loading (2.5 FPU/g each). The BCC crude fungal enzymes were mixed with the commercial cellulase Celluclast™ 1.5L at the ratio of 50:50. The yield of reducing sugar (mg/g) from the mixture of BCC enzyme and commercial enzyme was 70% greater than Celluclast™1.5L alone, showing their synergistic action. The supplementation of the Celluclast™/BCC 199 enzyme extract were then mixed with recombinant *B. subtilis* BCC 23086 expansin (BsEXLX1) at different concentrations in order to increase reducing sugar yield from the rice straw hydrolysis. The result showed that the reducing sugar yield significantly increased in range of 5.5% to 17.8% (Fig. 1.17). The increase in expansin concentration resulted in increasing reducing sugar yields when low concentrations of expansin were applied.

However, further increases in the expansin dosage led to a decrease in the reducing sugar yields. The result clearly demonstrated the enhancement effect of expansin on the hydrolysis of lignocellulosic biomass, which possesses more complexity than a pure cellulose fibre-like filter paper, with a high cellulase dosage similar to that used in biomass hydrolysis. The hydrolysis reaction were performed in 1.5-ml tubes with a total reaction volume of 1 ml containing 1% (w/v) pretreated-rice straw, 50 mM sodium acetate buffer (pH 5.0),1 mM sodium azide, and the indicated amount of enzyme. The reactions were incubated at 50 ºC for 48 h with shaking at 200 rpm. The reducing sugar yields were determined. A systematic mixture design was used to optimize the optimal ratio of enzyme mixtures for rice straw hydrolysis. The optimal formulation of enzyme mixtures containing Celluclast™: BCC 199: expansin was 41.4:37.0:21.6, which produced 769 mg reducing sugar/g biomass using 2.82 FPU/g of pretreated rice straw (Fig. 1.18).

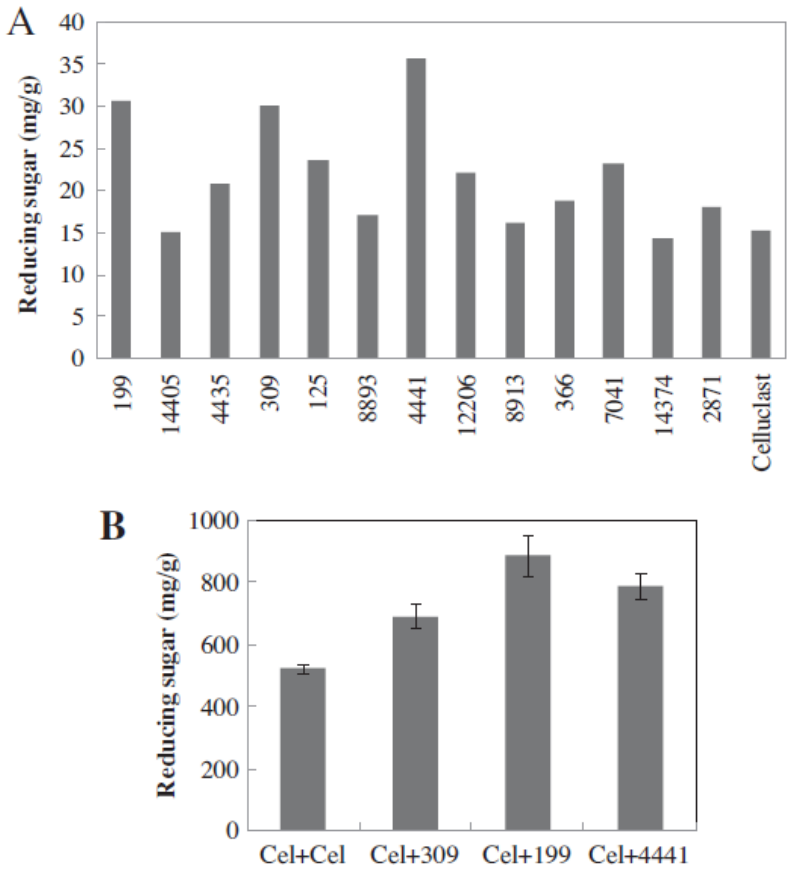

Figure 1.16 (A) The primary screen of different combinations of Celluclast™ and crude fungal enzyme extracts. A total of 0.01 FPU of Celluclast™ and 0.01 FPU of the BCC enzyme extracts were mixed for a total enzyme dosage of 0.4 FPU/g of pretreated rice straw. The Celluclast™ control reaction contained 0.02 FPU of Celluclast™ only. (B) The secondary screen of Celluclast™ and selected BCC enzymes. A total of 0.125 FPU of Celluclast™ and BCC enzyme extract was mixed for a total enzyme dosage of 5 FPU/g pretreated rice straw [19].

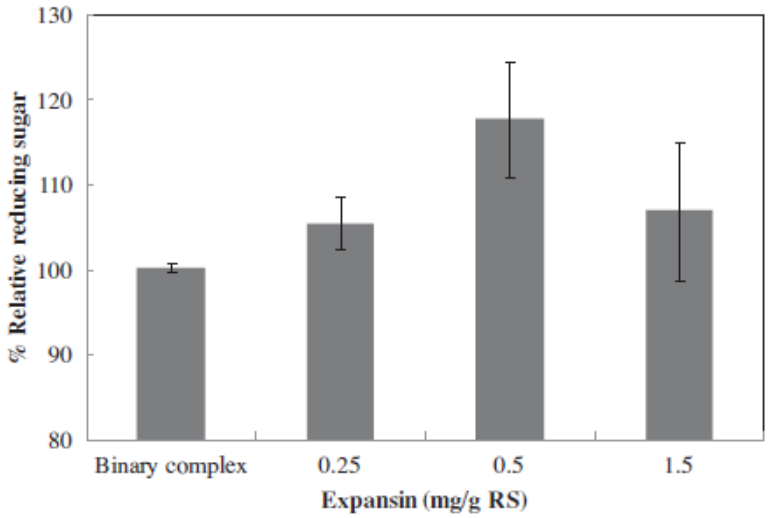

Figure 1.17 The synergistic effect of expansin on the binary Celluclast™-BCC 199 enzyme extract mixture on rice straw hydrolysis. The reactions contained 5 FPU/g of a 50:50 Celluclast™:BCC enzyme extract blend with the indicated amount of expansin. The reactions were incubated at 50ºC for 48 h [19].

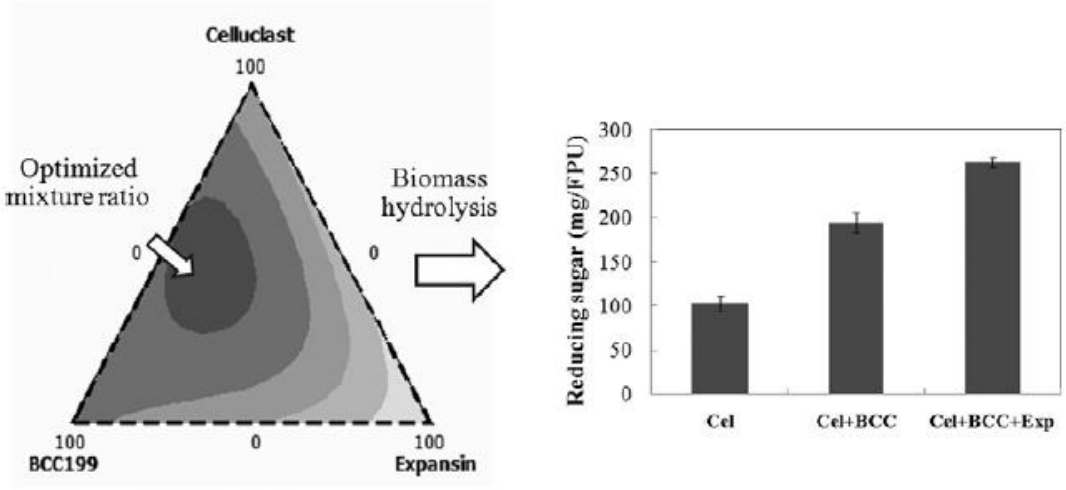

Figure 1.18 Optimization of the ternary enzyme mixture using a systematic mixture [19].

1.3 Research objectives

In order to screen for high-level cellulase producers for biomass saccharification process, a number of fungal strains in the BIOTEC Culture Collection (BCC) were screened for cellulase production. Among the fungal strains, *Chaetomium globusum* BCC 5776 was found to be a potent candidate with cellulase productivity of 0.20 FPU/ml from submerged fermentation. The work in this study thus aims to further develop the capability of this strain for cellulase production by optimization of fermentation media and conditions

by using systematic experimental design method and scale-up in lab-scale bioreator. Improvement of the fungal capability by screening of mutant strains will also be explored. An efficient enzyme system composing BCC5776 crude enzyme and other enzyme with complement activities was then characterized and optimized using the mixture design approach. The work will provide a potent candidate strain for further on-site cellulase production for biomass conversion process.

1.4 Scope of work

- Optimization of fermentation media using complex and synthetic substrates for carbon, nitrogen sources, and trace elements and cultivation conditions, e.g. temperature and pH for efficient cellulosic biomass-degrading enzyme from BCC 5776.
- Scaled-up production of cellulolytic enzymes under optimal condition in bioreactor and downstream processing for enzyme preparation.
- Study of the development of hyper-producing mutant strains base on BCC 5776 using mutagenesis technique i.e. UV-irradiation.
- Biochemical characterization of crude enzyme preparation, e.g. optimal working temperature, pH and stability and composite enzyme activities.
- Formulation of efficient enzyme mixtures based on BCC 5776 crude enzyme with commercial enzymes or crude/recombinant enzymes in ENZ database using experimental mixture design approach.

CHAPTER 2

THEORIES

2.1 Biorefinery

Biorefinery is a facility that integrates lignocellulosic biomass conversion processes for the sustainable production of bio-based products: bioenergy (heat and power), biofuels (e.g. ethanol, gasoline) and bio-chemicals (e.g. xylitol, furfural) for an agriculture-based country such as Thailand. The lignocellulosic materials biorefinery feedstock can be built on two different technical platforms which are biological platform and thermochemical platforms. The concept of biorefinery, as shown in Fig. 2.1, is hence analogous to today's petroleum refineries that produce not only gasoline but also petrochemical refinery products. Development of an efficient bioprocess results in high efficient utilization of surplus agricultural wastes, leading to overall improvement on the economics of the agricultural sector and providing an alternative technology to the current petroleum-based industry.

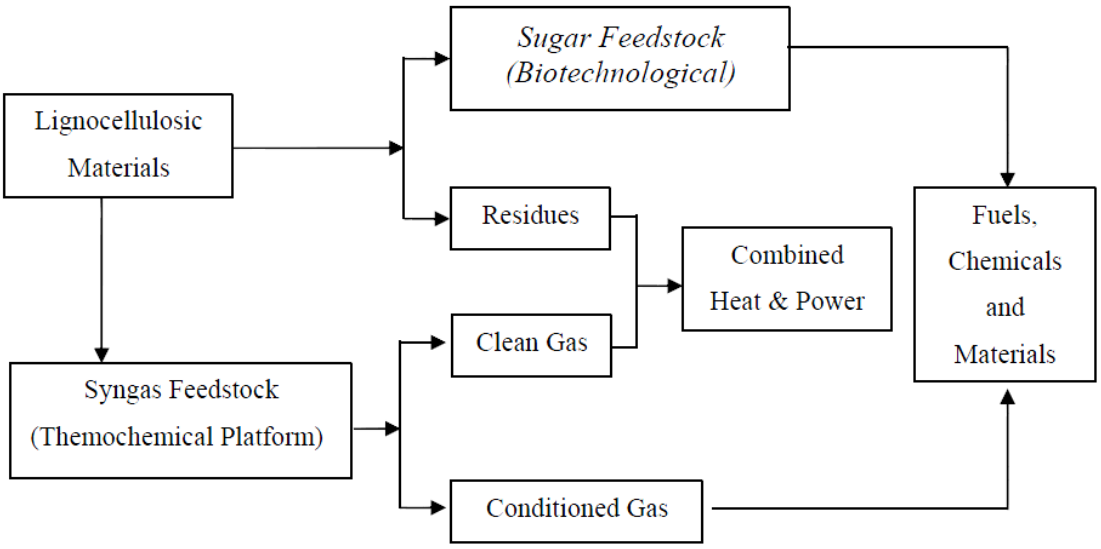

Figure 2.1 Conceptual lignocellulosic feedstock biorefinery [20].

2.2 Structure and chemistry of lignocellulosic biomass

Lignocellulosic plant biomass represents the most abundant renewable carbon source on earth. Generally, lignocelluloses are composed of three major components: cellulose, hemicelluloses and lignin. In addition, some amounts of ash, proteins and pectin are also found in biomass. These compositions are formed into a complex structure, as shown in Fig. 2.2. The details of each component are described below**.**

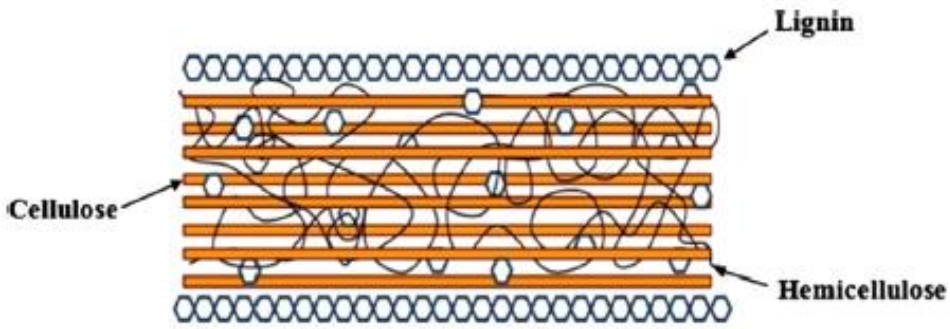

Figure 2.2 General structure of lignocellulosic biomass feedstock (cellulose, hemicelluloses and lignin) [21].

2.2.1 Cellulose

Cellulose is a linear homopolysaccharide that is built up of D-glucopyranose monomers ($C_6H_{10}O_5$) and linked together by β-1,4-glycosidic bonds, unlike starch-based plants held by α-1,4-glycoside linkages. The smallest repetitive unit of cellulose is cellobiose. The structure of cellulose is composed of two regions: the highly order crystalline region and the loosely packed amorphous area. Each cellulose chain is coupled by hydrogen bond, resulting in parallel arrangement of crystalline structures known as "microfibril". The result of its highly ordered crystalline structure makes the cellulose more difficult to degrade than hemicellulose [22]. Bundles of microfibril form fibres that are embedded in a matrix of hemicelluloses and lignin.

2.2.2 Hemicellulose

Hemicellulose, the second most abandant component of biomass, is a more complex structure than cellulose. It is a heteropolymer which comprise pentoses (including arabinose and xylose), hexoses (mainly mannose, less glucose and galactose) and sugar acids (often acetylated or methylated). The composition of hemicellulose varies substantially in nature and depends on the species. There are usually three basic forms of

hemicellulose: 1,4-β-D-xylans, 1,4-β-D-mannans and 1,3- and 1,4-β-D-galactans. In hardwood, glucuronoxylans and glucomannans are dominant components while galactoglucomannans and arabinoglucuroxylans are main composition of softwood. Xylans refer to as the glucuronoxylans and arabinoglucuroxylans. In contrast to cellulose, hemicellulose contained relatively shorter chains and highly branched structure, hence it is weaker and can be hydrolyzed easily.

2.2.3 Lignin

Lignin is the third main component of lignocellulosic residues and connects both cellulose and hemicelluloses, forming a unique structure under its physical seal. Lignin is highly recalcitrant to physical, chemical, and microbial attacks, resulting to rigidity and strength of the plants. Lignin is an amorphous heterogeneous complex and non-linear polymer. Normally, it consists of three basic aromatic alcohols including p-coumaryl, coniferyl and sinapyl linked through carbon-carbon and ether bonds. In addition, some kinds of plant, mainly grass and dicotyledon contain large numbers of phenolic acids such as ferulic acid and p-coumaric which are esterified to alcohol groups of each other and to other alcohols such asp-coumaryl and sinapyl alcohols [23]. The solubility of lignin in water is around 180ºC under neutral conditions; however, it also dissolves in alkaline, neutral or acid solvent depended on the precursor of the lignin (p-coumaryl, coniferyl and sinapyl alcohol) [24].

2.3 Biomass saccharification

Saccharification of lignocellulosic agricultural biomass is the basic process in a biorefinery. The goal of hydrolysis is to cleave cellulosic and hemi-cellulosic polysaccharide into their monomers. Complete hydrolysis of cellulose results in glucose whereas the hemicullulose provides pentoses and hexoses. Hydrolysis of lignocellulosic biomass into sugars can be performed by either chemical or enzymatic. Chemical hydrolysis relates to expose the lignocellulosic biomass with acids or alkaline for a period of time at a specific temperature, and hence results in release of sugars from both of cellulose and hemicellulose. Furthermore, chemical hydrolysis can be classified into two methods depending on types of chemical: (1) acid hydrolysis (e.g. H_2SO_4 and HCl) and (2) alkaline hydrolysis (e.g. NaOH and NH_3). However, this method gives higher yield of fermentable sugars (e.g. 90% of theoretical glucose yield) than enzymatic saccharification [25]. Although, the chemicals process lead to generation of inhibitory by product while

need detoxification step. Enzymatic hydrolysis of cellulose, the main component in lignocellulosic biomass is carried out by cellulolytic enzymes which are highly specific.

The hydrolysis product is mainly glucose which can be further converted into bioethanol and other chemicals. Utility cost of enzymatic hydrolysis is low compared to acid or alkali hydrolysis because enzyme hydrolysis is usually conducted at mild conditions (pH 4.8 and temperature 45-50^{o}C) and does not have corrosion problem as chemical hydrolysis. The process is also more environmentally friendly and has been recognised as a potent method for glucose production from biomass. As the chemical composition and physical properties of different agricultural biomass varies, the optimization of hydrolysis process, particularly the pretreatment step and active enzyme formulations for specified biomass, is thus necessary for their efficient conversion and production of the target products.

2.4 Lignocellulolytic enzyme-producing fungi

Lignocellulolytic enzymes-producing fungi are widespread, and include species from the ascomycetes (e.g. *T. reesei*), basidiomycetes including white-rot fungi (e.g. *P. chrysosporium),* brown-rot fungi (e.g. *Fomitopsis palustris*), and finally, a few anaerobic species (e.g. *Orpinomyces sp.*) that degrade cellulose in gastrointestinal tracts of ruminant animals [26]. Biomass degradation by these fungi is performed by complex mixtures of cellulases, hemicellulases and ligninases, reflecting the complexity of the materials. The ability of rot fungi to degrade wood varies among fungal species and depends on the chemical properties of wood and on its structural features [27]. Interestingly, soft rot fungi cause substantial losses in lignocellulose properties in early stages of attack and in various environments. Previous papers of the fungal degraded lignocellulosic biomass, having established that *C. globosum* is able to reduce wood strength by acting preferentially upon the wood constituents. Several researchers reported that *C. globosum* is a strong cellulose decomposer and expressed a very effective antagonist of various soil microorganisms [28]. The details of biological properties of *C. globosum* are described as follows:

2.4.1 *Chaetomium globosum*

Chaetomium is a fungus belonging to Ascomycota of the family Chaetomiaceae, which was established by Kunze in 1817. *Chaetomium* Kunze is one of the largest genera of saprophytic ascomycetes which comprise more than 300 species worldwide [29]. Members of this genus typically have superficial, ostiolar perithecia, covered in hairs. Asci are often clavate and evanescent, bearing eight spores. Ascospores are usually lemon-shaped, commonly colored olive-brown (Fig. 2.3). Mycelia often grows in conglomerate masses that resemble rope [30]. *C. globosum* are well known as coprophilous, seed and soil fungi and also found in organic compost [31]. They degrade cellulose and other organic material. Laccase, a kind of polyphenol oxidase, can oxidize an array of organic and inorganic substrates. Laccase is considered to be a potentially important enzyme for industries.

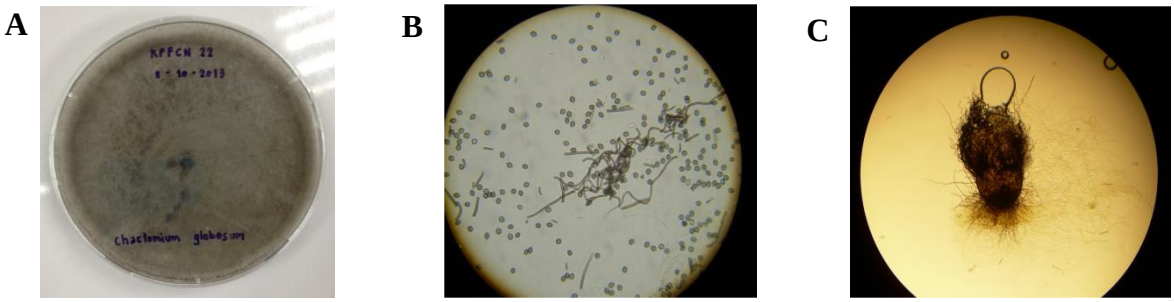

Figure 2.3 *C. globosum* (A) 7-days-old culture on PDA, (B) *Chaetomium* ascospores with their characteristic football shape and (C) *Chaetomium* ascoma.

C. globosum is one of the most well-known strains that produce laccase [32]. Moreover, there are several reports indicating that *C. globosum* is effective in reducing damage caused by seed rot and damping off, of several seed borne and soil borne plant pathogens like *Pythium ultimum*, *Alternaria raphani*, *A. brassicola*, *Fusarium* spp. [33] Studies in laboratory have indicated the potentiality of *C. globosum* for the biological control of spot blotch disease of wheat caused by *Bipolaris sorokiniana* and Ascochyta blight of chick pea [34].Apple scab disease caused by *Venturia inaequalis* has been significantly controlled by foliar spray of *C. globosum* ascospore suspension [35]. In addition,several types of compounds have been investigated from *C. globosum* e.g. chaetoglobosins A and C when cultured on building material. The presence of these mycotoxins can be lethal to mammalian cells which act by binding to actin leading to inhibition of cell division, locomotion, and formation of cell surface projections [36].

2.5 Lignocellulose degrading enzyme

Enzymes are catalysts bearing some excellent properties (high activity, selectivity and specificity) that permit to the most complex chemical processes to execute under the most benign experimental and environmental conditions [37]. In nature, lignocellulosic biomass is degraded with the cooperation of cellulolytic and hemicellulolytic enzymes from many microorganisms including aerobic and anaerobic bacteria and fungi. These lignocellulose degrading enzymes act cooperatively in plant biomass degradation in nature and play important roles in a range of industries, including animal feed, pulp & paper and the promising biomass conversion and biorefinery industry. The details of cellulases, hemicellulases and lignases are described below.

2.5.1 Cellulases

Cellulases are usually a mixture of several enzymes (Fig. 2.4). At least three major groups of cellulases are involved in the hydrolysis process: (1) endoglucanase (EG, endo-1,4-D-glucanohydrolase, or EC 3.2.1.4), which attacks regions of low crystallinity in the cellulose fiber, creating free chain-ends; (2) exo-glucanase or cellobiohydrolase (CBH, 1,4-β-D-glucan cellobiohydrolase, or EC 3.2.1.91), which degrades the molecule further by removing cellobiose units from the free-chain ends. In addition, cellobiohydrolase also slowly degrades crystalline cellulose. Both endoglucanases and cellobiohydrolases are active on amorphous (non-crystalline) cellulose (3) β-glucosidase (EC 3.2.1.21) which hydrolyses cellobiose to produce glucose [23].

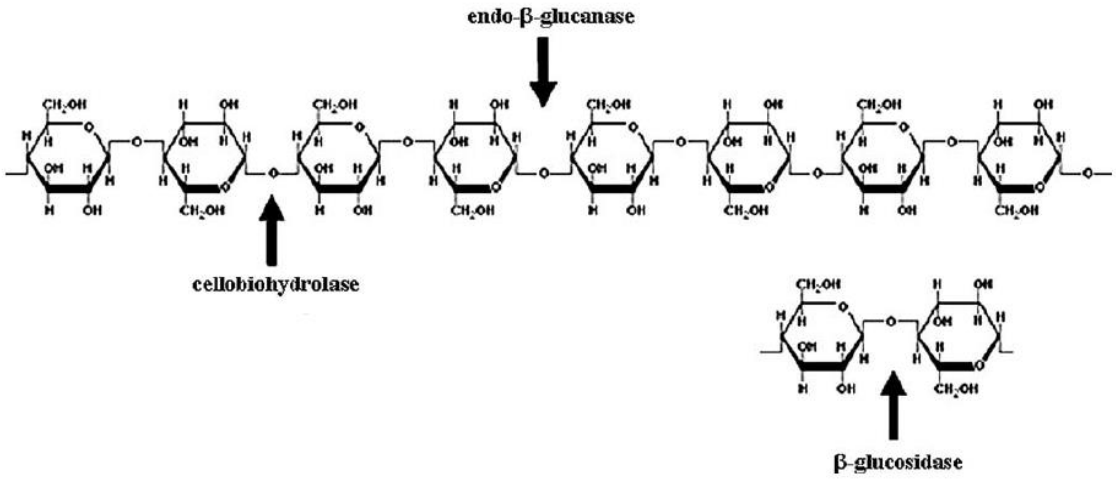

Figure 2.4 Molecular structure of cellulose and site of action of endoglucanase, cellobiohydrolase and β-glucosidase [3].

2.5.2 Hemicellulases

To complete the hydrolysis of hemicellulose into monosaccharide sugars, i.e. pentoses and hexoses, a number of accessory enzymes are required, as shown in Fig. 2.5. In xylan degradation, for instance, endo-1,4-β-xylanase, β-xylosidase, β-glucuronidase, α-L-arabinofuranosidase and acetylxylan esterase all act on the different heteropolymers available in nature. In glucomannan degradation, β-mannanase, and β-mannosidase cleave the polymer backbone.

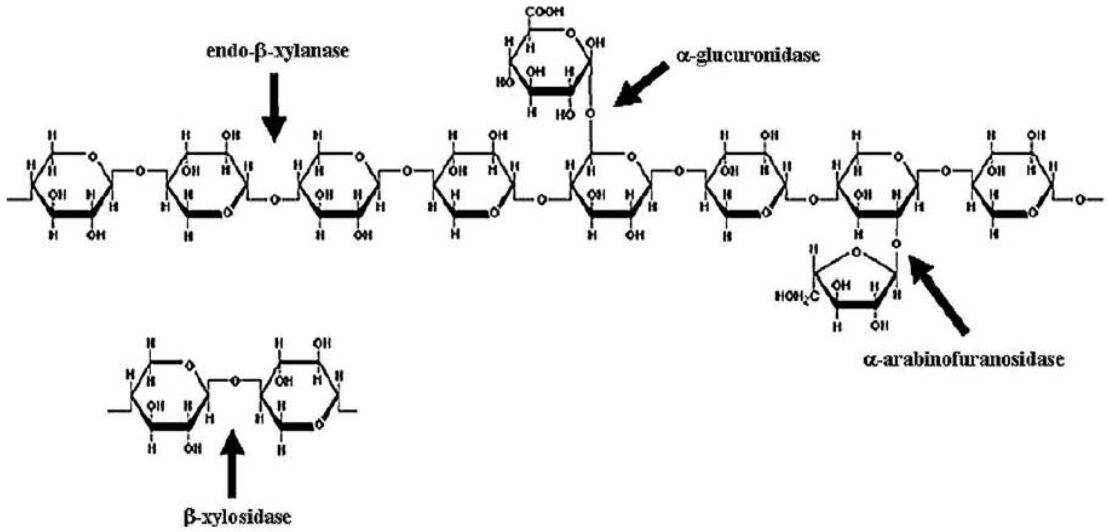

Figure 2.5 Polymeric chemical structure of hemicellulose and targets of hydrolytic enzymes involved in hemicellulosic polymer degradation [3].

2.5.3 Ligninases

Unlike cellulose and henicellulose, lignin is the most abundant of renewable aromatic compounds, including non-phenolic (80-90%) and phenolic structures [38]. The degradation of lignin consists of two ligninolytic families; (1) phenol oxidase (laccase) and (2) peroxidases [lignin peroxidase (LiP) and manganese peroxidase (MnP)] [39].In part of non-phenolic structure, LiP is able to oxidize but it was not detected in many lignin degrading fungi. Moreover, it has been widely accepted that the oxidative ligninolytic enzymes are not able to penetrate the cell walls due to their size. Thus, it has been suggested that prior to the enzymatic attack, low-molecular weight diffusible reactive oxidative compounds have to initiate changes to the lignin structure.

2.6 Enzyme production

Several cellulolytic and hemicellulolytic enzymes have been employed for many applications and have biotechnological potential for various industries, including fuels, chemicals, food, textile, pulp, paper, animal feed, and breweries[40, 41]. In general, the production of a wide variety of enzymes that are highly beneficial to individuals and industry is obtained from fermentation. The basic bioprocess technologies that employed for production of enzymes can be divided broadly into submerged fermentation (SmF) and solid-state fermentation (SSF) depending on the type of substrates used during fermentation. Some researches of cellulase production using different types of bioprocesses (submerged fermentation and solid-state fermentation) are presented in Table 2.1. These techniques have been further refined based on various parameters such as the substrates used, environmental parameters and the organisms used for fermentation which are shown in Table 2.2 [42]. In addition, over the past few decades, many scientists have been trying to improve the production titers by adopting new approaches, which include the use of better bioprocess technologies, using cheaper or crude raw materials as substrates for enzyme production, bioengineering the microorganisms, etc. [43, 44]. More details descriptions of current technologies for enzyme production are reported as follows:

2.6.1 Submerged Fermentation (SmF) or Liquid Fermentation (LF)

Submerged fermentation has been defined as fermentation in the presence of excess water. Free flowing liquid substrates such as molasses and broths are utilized in SmF. This fermentation technique is suitable for various microorganisms, especially bacteria and filamentous fungi [45], which prefer high moisture content. Almost large-scale enzyme producing facilities are using the proven technology of SmF due to better monitoring and ease of handling. Moreover, the purification of products obtain easier than solid-state fermentation [46].

2.6.2 Solid-state Fermentation (SSF)

Solid-state fermentation (SSF) is defined as fermentation in the absence or near absence of free water [47]. The substrates utilized in SSF as solid forms such as wheat bran, baggasse and paper. In this fermentation technique, it is proper for fungi and other microorganisms that require low moisture content. In contrast, it cannot be used in

fermentation processes involving organisms that require high a_w (water activity), such as bacteria [48].

2.6.3 Strain improvement of existing industrial microorganisms for enzyme production

The improvement of lignocellulolytic enzymes producing microbes is referred to as modern biological approaches that are opportunities for increasing cellulases and hemicellulases with greater specific activities, resulting in high degradation of lignocelluloses into monomeric sugars [23]. Optimizing enzyme mixtures is interesting bioprocess technology that can increase enzyme specific activity by combining purified individual proteins or individual domains either from the producing organisms, or from recombinant organisms expressing a single cloned lignase, hemicellulase or cellulase encoding gene [23].

For more than fifty years in biotechnology research, mutagenesis is a process by which the genetic information of an organism is changed in a stable manner, resulting in a mutation. Hyper-cellulolytic enzymes can be obtained by physical/chemical treatment. Mostly ultraviolet (UV) and microwave irradiation are brought into physical treatments. For chemical treatment, many chemical mutagens such as NTG, H_2O_2 are required for biological activation to become mutagenic. Apart from mutagenesis approach, co-cultivation of the cellulolytic organisms complementing the desired cellulolytic component has been recently attempted for achieving an increased rate of lignocellulosic bioconversion. This technique is defined as a simultaneous cultivation of two or more species of microorganism in the same medium while genetic manipulation techniques including recombinant DNA technology and protein engineering are also being used as a powerful modern approach for efficient lignocellulosic bioconversion. Recombinant DNA technology offers significant potential for improving various aspects of lignocellulolytic enzymes such as production, specific activity, pH and temperature stability as well as creating “synthetic” designer enzymes for specific applications [49, 50].

Table 2.1 Types of bioprocesses and substrates used in cellulase production [46].

No.	Factor	Submerged fermentation	Solid state fermentation
1	Substrate	Soluble substrates (usually sugars)	Insoluble polymeric substrates (starch, cellulose, pectin, lignin)
2	Aseptic condition	Heat sterilization and aseptic control	Vapor treatment, even non-sterile conditions can be used
3	Water	High volumes of water consumed and effluents discarded	Limited consumption of water; low amount or, no effluent produced and discarded
4	Metabolic heating	Easy control of temperature	Low heat transfer capacity, difficulty in control of temperature
5	Aeration	Limitation by soluble oxygen, high level of air required	Easy aeration and high surface exchange air/substrate
6	pH control	Easy pH control	Buffered solid substrates
7	Mechanical agitation	Good homogenization	Static conditions preferred
8	Scale up	Industrial equipments available	Need for engineering and new design equipment
9	Inoculation	Easy inoculation	Spore inoculation(fungi), batch
10	Contamination	Risks of contamination for single strain bacteria	Risk of contamination for low rate growth fungi
11	Energetic considerations	High energy consuming	Low energy consuming
12	Volume of equipment	High volumes and high cost technology	Low volumes and low costs of Equipments
13	Effluent and pollution	High volumes of polluting effluents generated	No effluents, less pollution generated
14	Concentration of products	Low yield and diluted product	Highly concentrated product

Table 2.2 Comparison of some important factors between submerged and solid state fermentation (adapted and modified from [48, 51-53]).

Microorganism	Substrate	Method	Magnitude	Enzymes: activity
Neurospora crassa	Wheat straw	SmF	Shake flask	FPAse 1.33 U/ml, CMCase 19.7 U/ml, BGL 0.58 U/ml
Penicillium janthinellum	Sugar cane bagasse	SmF	Shake flask	FPAse 0.55 U/ml, CMCase 21.5 U/ml, BGL 2.3 IU/ml
Phaenerocheate chrysosporium	Cellulose (Avicell)	SmF	100-l fermentor	Cellulase 29 mg/g cellulose
Rhodothermus marinus	CM cellulose	SmF	150-l fermentor	Endoglucanase 97.7 U/ml
Streptomyces sp T3-1	Carboxymethyl cellulose	SmF	50-l fermentor	CMCase 148 IU/ml, Avicellase 45 IU/ml, BGL 137 IU/ml
Streptomyces drozdowiczii	Wheat bran	SmF	Shake flask	CMCase 595 U/l
Thermotoga maritima	Xylose	SmF	Shake flask	Cellobiase 11 mU/ml, Avicellase 0.3 mU/ml Beta-glucosidase 30 mU/ml
Trichoderma reesei	Steam treated willow	SmF	22-l fermentor	FPAse 108 U/g cellulose
Trichoderma reesei RUT C30	Cellulose (Avicell)	SmF	Microbubble dispersion bioreactor	FPAse 1.8 U/ml
Trichoderma reesei RUT C30	Corrugated cardboard	SmF	30-l fermentor	FPAse 2.27 U/ml
Trichoderma reesei ZU-02	Corn Stover Residue	SmF	30-l fermentor	Cellulase 5.48 IU/ml, FPAse 0.25 U/ml
Trichoderma viridae	Sugar cane bagasse	SmF	Shake flask	FPAse 0.88 U/ml, CMCase 33.8 U/ml, BGL 0.33 U/ml
Penicillium occitanis	Paper pulp	SmF-fed-batch	20-l fermentor	FPAse 23 IU/ml, CMCase 21 IU/ml
Trichoderma reesei	Xylose/sorbose	SmF-continuous	Bioreactor	FPAse 0.69 U/ml/h
Aspergillus niger NRRL3	Wheat Bran/Corn cob	SSF	Flask	Cellobiase 215 IU/g, cellulose
Bacillus subtilis	Banana waste	SSF	Shake flask	FPAse 2.8 IU/gds, CMCase 9.6 IU/gds, Cellobiase 4.5 IU/gds
Bacillus subtilis	Soybean industry residue	SSF	Cylindrical bioreactor	FPAse 1.08 U/mg protein
Mixed culture: Trichoderma reesei, Aspergillus niger	Rice Chaff/Wheat Bran (9:1)	SSF	Flask	FPAse 5.64 IU/g
Penicillium decumbans	Wheat straw/bran (8:2)	SSF	SSF bioreactor	Fpase 20.4 IU/g
Thermoascus auranticus	Wheat straw	SSF	Perforated drum bioreactor	FPAse 4.4 U/gds, CBH 2.8 U/gds, Endoglucanase 987 U/gds, BGL 48.8 U/gds
Trichoderma reesei ZU 02	Corn cob residue	SSF	Tray fermentor	FPAse 158 U/gds
Trichoderma reesei RUT C30	Wheat bran	SSF	Shake flask	3.8 U/gds FPU

2.7 Experimental design

In general, the design of experiments (DOEs) or experimental design is the study of planning processes to meet specified objectives. Planning an experiment suitably is very important in order to assure that the right type of data and a sufficient sample size and power are available to answer the research questions of interest as clearly and efficiently as possible. Formal planned experimentation is often used in evaluating physical objects, chemical formulations, structures, compositions, and materials. In the design of experiments, the investigators is often interested in the effect of some process or intervention (the "treatment") on some objects (the "experimental units"), which may be people, parts of people, groups of people, plants, animals, etc [54]. Design of experiments is thus a discipline that has very broad application across all the natural and social sciences and engineering. In statistical experimental design, many principles are involved e.g. comparison, randomization, replication, blocking, orthogonality, and factorial experiments. In practice, a use of factorial experiments is widespread and easy when compared to one-

factor-at-a-time (OFAT) method because this method can evaluate the effects and possible interactions of several factors (independent variables) at the same experimental period. Analysis of experiment design is built on the foundation of the analysis of variance (ANOVA), which is a collection of models that partition the observed variance into components, according to the factors that the experiment must estimate or test.

2.8 Mixture designs

Mixture designs [55] are used to vary the proportions of mixture components. They differ from the other designs that optimize intensive properties like temperature or extensive ones like the total quantity of material used in an experiment since the properties of mixtures depend on ingredient proportions, *xi*, and not on their absolute values. As such these proportions are not independent variables since:

$$\sum_{i=1}^{q} x_i = 1 \text{for i = 1, 2,….., } q. \quad (1)$$

As a consequence, mixture models have mathematical expressions that are different from those involving independent variables:

$$\hat{y} = \sum_i^q b_i x_i + \sum_{i \neq j}^q \sum_j^q b_{ij} x_i x_j + \sum_{i \neq j}^q \sum_{j \neq k}^q \sum_k^q b_{ijk} x_i x_j x_k + \ldots, (2)$$

most noticeably the absence of the constant b_0 term. Experimental designs can be made for any number of components but investigation of three-component systems is the most common. A simplex centroid design with axial points, presented in the concentration triangle shown in Fig. 2.6, is especially useful for ternary studies. The component proportions of this design are given in the middle columns of Table 2.3. Each point at a vertex of the triangle represents a pure component or a mixture of components. The points centered on each leg of the triangle represent 1:1 binary mixtures of the components or mixtures of their neighboring vertex points.

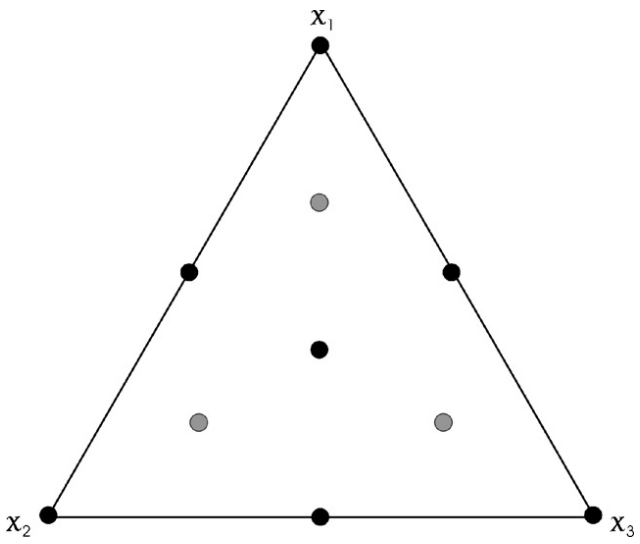

Figure 2.6 A simplex centroid design with axial points for a three-component mixture system.

The point in the center of the triangle represents a 1:1:1 ternary mixture of the three pure components or mixtures represented at the vertices. The axial points contain a 2/3 portion of one of the ingredients and 1/6 portions of the other two. This simplex centroid with axial point design is important since it permits the evaluation and validation of linear, quadratic and special cubic models. The special cubic model for a ternary system has seven terms.

$$\hat{y} = b_1x_1 + b_2x_2 + b_3x_3 + b_{12}x_1x_2 + b_{13}x_1x_3 + b_{23}x_2x_3 + b_{123}x_1x_2x_3 \qquad (3)$$

Table 2.3 Coded pseudo component proportions for mobile phases of a simplex centroid design with axial points and their resolution factor response values.

Mixture	x_{ACN}	x_{MeOH}	x_{THF}	Resolution
1	1	0	0	0.99, 1.07
2	0	1	0	5.31, 5.64
3	0	0	1	4.12, 4.34
4	0.5	0.5	0	3.79, 3.98
5	0.5	0	0.5	3.88, 4.07
6	0	0.5	0.5	5.85, 6.16
7	0.333	0.333	0.333	5.22, 5.21
8	0.667	0.167	0.167	3.42, 3.50
9	0.167	0.667	0.167	5.80, 5.81
10	0.167	0.167	0.667	4.84, 4.83

The first three represent the linear model, which is only valid in the absence of interactive effects between components, i.e. ideal solutions in physical chemistry. The next three terms represent synergic or antagonistic binary interactive effects for all possible pairs of components and, along with the linear terms, forms the quadratic model. The last term represents a ternary interactive effect and is usually important for systems having maximum or minimum values in the interior of the concentration triangle.

CHAPTER 3

METHODOLOGY

3.1 Raw material preparation

All of the biomass used in this work was obtained locally. Rice straw was obtained from a local farm in Ayutthaya, Thailand. Palm empty fruit bunches were obtained from Suksomboon Palm Oil Co., Ltd., Chonburi, Thailand. Sugarcane bagasse was obtained from Phu Kiaew Bioenergy, Chiayapoom, Thailand. Corn cob was obtained from Suwan farm, Nakorn Ratchasrima, Thailand. Sawdust was obtained from Taladthai, Pathumthani, Thailand. After collection, the biomass was physically processed using a SM2000 cutting mill (Retsch, Haan, Germany), sieved through a 0.5 mm mesh then air-dried and stored at room temperature. The chemical composition of rice straw in this study including cellulose 35.51%, hemicellulose 13.78%, lignin 25.03% and ash 22.01% were determined according to National Renewable Energy Laboratory (NREL) method [56].

3.2Alkaline pretreatment of rice straw

Milled rice straw was pretreated with 10% (w/v) sodium hydroxide (unpublished report by BIOTEC, Thailand). The pretreatment was carried out at 80 °C for 90 minutes at ratio of 1 g (rice straw) per 3 ml NaOH solution in an autoclave (Tomy autoclave SS-325, Tomy, Japan). After pretreatment, solid and liquid fractions were filtered through a filter paper (Whatman No.5). The solid fractions were washed with tap water until the pH was neutral. The pretreated substrate was dried at 60^{o}C overnight and kept in a room temperature for further experimental study. Samples were taken from solid fractions and analyzed for its composition after alkaline pretreatment according to NREL method.

3.3 Microorganism

The fungal wild-type strain *Chaetomium globosum* BCC5776 was selected as a potential producer of cellulases based on extensive screening (unpublished data). This microorganism was obtained from the BIOTEC Culture Collection, BIOTEC, Thailand (www.biotec.or.th/bcc). The fungus was maintained at room temperature on potato dextrose agar (PDA) plate consisting of potato, 200 g/L; dextrose, 20 g/L and agar, 15 g/L for 7 days before sub-cultivation.

3.4 Preparation of fungal mutants

Before mutation, the number of living spore number on the Petri plate culture of *C. globosum* BCC5776 was determined by serial dilutions, propagation on the PDA medium and colony counting. Mutants were prepared by UV-irradiation. The spores from 7-days Petri plate culture were scraped and suspended in 10 ml of sterile water containing 0.1% Tween-80. The spore suspension was then transferred to Petri dishes to a depth of 2 mm. The time of irradiation were varied from 5 to 30 min. The treated spore suspensions were spread in 0.1 ml quantities on the surface of selection medium agar (SMA) plates and incubated at room temperature for 14 days. Selection medium agar was prepared as described: 0.1% TritonX-100; 2% agar, 1% peptone, 1% AVICEL® and 10% (v/v) basal 10X. Single colonies representing different morphologies on the SMA dishes were point inoculated into AVICEL®-wheat bran-soybean meal (AWS) broth including 4% AVICEL®, 4% wheat bran and 1% soybean meal and incubated at 30ºC for 7 days at 200 rpm. A subculture of single colonies from the SMA plates was inoculated onto the PDA agar medium and later retested in shake flask fermentation.

3.5 Optimization of cellulase production

In the first step of the optimization process, the one-factor-at-a-time (OFAT) method was used to identify the significance of the ingredients of the media components. The main parameters selected for the experiment were carbon source, medium types and nitrogen source, respectively with the working volume of 50 ml in 250 Erlenmeyer flask and incubated at 30ºC with rotary shaking at 200 rpm for the optimum production of cellulase enzyme particular filter paper cellulase (FPase). Samples were taken at every 0, 72, 144, 216 and 288 hours to assay the cellulase activity using DNS method (Miller, 1959) as describe in Section 3.13.2.The OFAT method is a traditional method of designing experiments involving the testing of factors, or causes, one at a time instead of all simultaneously [57]. The range of the each parameter was investigated in different level by changing the value alone. All the experiments were performed in triplicate and the average of cellulase activity was used as the response (dependent variable). The main effect of each variable was analyzed using the statistical software package, SPSS 16.0 (Statsoft, USA).

In the second step, cellulase production optimization using multivariate analysis was used to investigate by designing an experiment for the optimization of these three parameters: concentration of AVICEL®, concentration of lactose and initial pH. Table 3.1

shows a total of 9 experiments run with different combinations of three factors and 3 replicates. Data analyze by SPSS 16.0 (Statsoft, USA) for all statistical experiment designs. The model used to find the significant interaction effect of these of cellulase production.

Table 3.1 Combinations of three parameters based on multivariate analysis for enhancing cellulase yields under submerged fermentation after 144 h of fermentation.

Run No.	AVICEL® concentration (%)	Lactose concentration (%)	pH
1	2	0.1	5.8
2	2	0.05	5.8
3	6	0.1	5.8
4	6	0.05	5.8
5	2	0.1	7.0
6	2	0.05	7.0
7	6	0.1	7.0
8	6	0.05	7.0
9	4	0.00	5.5

3.6 Enzyme production

3.6.1 Inoculum preparation and cultivation

The fungal culture was prepared from fungus grown on PDA by excising five agar pieces covered with a profuse mycelial mat using a No. 2 cork borer (Ø = 0.5 cm) and inoculated in the optimized cellulase production medium (results from Section 3.5). The medium was maintained at 30 ºC for 6 days with rotary shaking at 200 rpm. The cells again activated in same way and were ready for the further scale-up production of cellulase in 1-L flask and 5-L bioreactor. The initial pH of medium was adjusted to pH 5.8 based on preliminary studies carried out in our laboratory to determine optimal cellulase production of *C. globosum* BCC5776.

3.6.2 Batch fermentation experiment

A 10% (v/v) inoculum of a 1-L flask was inoculated into a 5-L bioreactor, batch fermenter, (Satorius, BIOSTAT B-DCU, Germany) containing a 3-L working volume. The optimized production medium contained 2% AVICEL® (60 g), 1% OPEFB (30 g), 1% soybean meal (30 g), 1% (v/v) lactose (300 ml), 1M potassium phosphate buffer pH 5.8

(150 ml) and distilled water. The cultures were also incubated under identical conditions, as mentioned above, for 6 days.

3.7 Crude fungal enzyme extract preparation

For the preparation of crude enzyme extracts, the cells were separated by centrifugation at 10,000 rpm for 10 min and the supernatants were filter–sterilized through a 0.2 µm Supor®-200 membrane (Pall Corp, Ann Arbor, MI). The selected crude enzyme extracts were concentrated by ultra filtration on a Minimate™ tangential flow filtration (TFF) system using a Minimate ™ TFF capsule with a 10-kDa MWCO membrane (Pall Corp, Ann Arbor, MI). The enzyme solutions were kept at 4ºC until used.

3.8 Enzymatic hydrolysis of biomass

The hydrolysis reactions were performed in 1.5-ml tubes with a total reaction volume of 1 ml, which contained 5% (w/v) pretreated rice straw, 50 mM sodium acetate buffer (pH 5.5) and the indicated amount of the enzyme tested (BCC5776 enzyme extract, Accellerase® XY, Accellerase® 1500, Optimash® BG and Novozyme® 188). The vessel was incubated at 50ºC for 48 hours with a vertical rotation of 200 rpm. Hydrolysis experiments were performed in triplicate. The released reducing sugar concentration was analyzed based on the amount of liberated reducing sugars using the 3,5-dinitrosalisylic acid (DNS) method. The fermentable sugars profiles were analyzed according to section 3.13.3 by high performance liquid chromatography (HPLC).

3.9 Screening for synergistic commercial enzymes in hydrolysis

An equal amount of protein (0.29 mg protein/ml) from crude fungal enzyme samples and other commercial enzymes, including Accellerase® XY (hemicellulase from Dupont, Rochester, USA), Accellerase® 1500 (cellulase from Dupont, Rochester, USA), Optimash® BG (β-glucanase/xylanase from Dupont, Rochester, USA), and Novozyme® 188 (β-glucosidase from Novozyme A/S, Bagsvaerd, Denmark), were synergistic in the primary screening experiment.

3.10 Enzyme assays

The crude enzyme activities were analyzed based on the method of Wood and Bhat.(1988)[58], according to the standard procedure recommended by the Commission on Biotechnology, IUPAC, with some modifications in terms of the total reaction volume. For

the filter paper cellulase (FPase) analysis, the 3.5-ml reactions contained an appropriate dilution of the enzyme in 100 mM sodium acetate buffer (pH 5.5) and a piece of 1 × 6 cm Whatman No. 1 filter paper as a substrate. The reaction was incubated at 50ºC for 60 min. The carboxymethyl cellulase (CMCase) and xylanase activities were assayed using 1% carboxymethyl cellulose and 1% birchwood xylan as the substrates, respectively. The 3-ml reactionswere incubated at 50º C for 30 min. The mannanase activity was assayed using 0.5% locust beangum as the substrate, the total reaction volume of 3-ml was performed in 100 mM sodium acetate buffer (pH 5.5) and incubated at 50ºC for 30 min.

The amylase and pectinase activities were also assayed using 1% soluble starch and 0.5% pectin from citrus peel as the substrates, respectively, according to the above method and incubated at 50ºC for 30 min. The released reducing sugars were measured by the DNS method (Miller, 1959), as described in Section 3.13.2. One unit (U) of enzyme activity is defined as the amount of enzyme required to liberate 1 µmol of glucose or xylose or mannose per min under the assay conditions. To measure β-glucosidase and β-xylosidase activities, the reaction mixture contained an appropriate amount of enzyme solution, 100 mM sodium acetate buffer (pH 5.5), and 0.1% (w/v) *p*-nitrophenyl-b-D-glucopyranoside (PNPG; Sigma) or *p*-nitrophenyl-b-D-xylopyranoside (PNPX; Sigma) as a substrate, respectively. After incubation at 50ºC for 30 min, the reaction was terminated by the addition of 2 ml of 1 M Na_2CO_3.

The quantity of *p*-nitrophenolate was measured spectrophoto metrically at 405 nm. One unit (U) of enzyme activity is defined as amount of enzyme that produces 1 µmole p-nitrophenol per minute under the assay conditions. The total protein concentrations of the crude enzyme extracts were determined using Bradford's method with the BIORAD protein assay reagent (BioRad, Hercules, CA) and bovine serum albumin (BSA) as the standard protein. The experiments were performed in triplicate.

3.11 Optimal working temperature, pH and stability

3.11.1 Optimum temperature and temperature stability

The thermal stability of the cellulolytic, hemicellulolytic, and other enzymes,i.e. mannanase, pectinase and amylase, were determined by incubating the crude enzyme preparations at temperatures in the range of 30-70ºC for 1 h. The filter paper cellulase (FPase), carboxymethyl cellulase (CMCase), xylanase, mannanase, pectinase and amylase

activities were analyzed using substrates according to Section 3.9 in small scale with total reaction volume of 1.2 ml and incubated at optimum temperature and pH for 10 min except FPase (60 min). For optimum temperature, the carboxymethyl cellulase (CMCase), xylanase, mannanase, pectinase and amylase activities were assayed in 100 mM sodium acetate buffer (pH 5.5) at 50ºC for 10 min. FPase activity was assayed in 100 mM sodium acetate buffer (pH 5.5) at 50ºC for 60 min. The amounts of reducing sugars were analyzed based on the DNS method as mentioned below (Section 3.13.2).

3.11.2 Optimum pH and pH stability

The pH stability of the cellulolytic, hemicellulolytic and other enzymes were evaluated by incubating the crude enzyme preparations at room temperature with different pHs in the range of 2.0-10.0 for 1 h and measuring theses activities with substrates according to Section 3.9 at optimum temperature and pH for 10 min. excepting for FPase activity, which was incubated for 60 min. The buffers were 100 mM glycine buffer (pH 2.0-4.0), 100 mM sodium acetate buffer (pH 4.0-6.0), 100 mM phosphate buffer (pH 6.0-8.0) and 100 mM Tris (pH 8.0-10.0). The amounts of reducing sugars were analyzed based on the DNS method as mentioned below (section 3.13.2).

3.12 Experimental mixture design

A {3,3}-augmented simplex lattice design was examined with Minitab 16.0 software (Minitab Inc., State College, PA) to define an optimal formulation of BCC5776, Novozyme® 188 (Novozyme A/S, Bagsvaerd, Denmark) and Accellerase® XY (Dupont, Rochester, USA). With three components and a lattice degree of 3, the design consisted of 13 experimental points, which were investigated in quadruplicate. In the mixture experiments, the sum of all the components in the blends was always 100%. The three independent variables in the mixture design consisted BCC5776 (X1), Novozyme® 188 (X2), and Accellerase® XY (X3). The amount of reducing sugar (Y1) was applied as dependent variables for the analysis and simulation of the respondent model. After the regression analysis, the full cubic model was used to simulate the optimized ratio of the mixture components. The canonical correlation of the full cubic model is shown in Eq. (1):

$$\boldsymbol{Y} = \sum_{i=1}^{3} \boldsymbol{\beta_i X_i} + \sum \sum_{i<j}^{3} \boldsymbol{\beta_{ij} X_i X_j} + \sum \sum_{i<j}^{3} \boldsymbol{\delta_{ij} X_i X_j (X_i - X_j)} + \sum \sum \sum_{i<j<k}^{3} \boldsymbol{\beta_{ijk} X_i X_j X_k} \quad (1)$$

where Y is a predicted response, β_i is a linear coefficient, β_{ij} is a quadratic coefficient, and β_{ijk} is a cubic coefficient. δ_{ij} is a parameter of the model. $\beta_i X_i$ represents the linear blending portion, and the parameter β_{ij} represents either synergistic or antagonistic blending.

3.13 Analysis

3.13.1 Chemical component analysis

Untreated and pretreated rice straw samples were analyzed according to the standard NREL method. The biomass sample was sieved through +60/+400 (250 lm/38 lm sieve openings) sieve plates on a horizontal sieve shaker. The biomass retained on this plate was used for extraction and compositional analysis. Water and ethanol extraction of biomass was carried out using an accelerated solvent extractor to remove the non-structural material base on NREL protocols. Structural carbohydrates were analyzed using high performance liquid chromatography.

3.13.2 Reducing sugar analysis by DNS method

The concentration of released reducing sugars from biomass hydrolysis was assayed using dinitrosalicylic acid (DNS) method (Miller, 1959) by measuring the absorbance at 540 nm on a micro-plate UV-Vis spectrophotometer (Thermo Scientific Multiskan Ascent). The amount of reducing sugars was calculated based on a standard curve of glucose.

3.13.3 High Performance Liquid Chromatography (HPLC)

Products from enzymatic saccharification were analyzed using a high performance liquid chromatograph (SPD-M10A DAD, Shimadzu) equipped with a refractive index detector and Aminex HPX-87H column (Bio-Rad, Hercules, CA, USA). Samples were centrifuged in 2 ml eppendorf tubes at 10,000 rpm for 10 minutes. The supernatant was filtered using 0.2 μm filters. The column temperature was set at 65 °C. The eluent was 50 mM H_2SO_4 in isocratic mode with a flow rate of 0.05 ml/min.

3.14 Protein characterization and identification

Proteomic analysis is a technique for both the separation and analysis/identification of proteins. Appropriate dilutions of the secreted enzymes of BCC5776 were applied to a 10% SDS–PAGE gel and separated using a MiniProtean II cell (Biorad) at 15 mA for 90

min. The protein bands were visualized using Coomassie blue R-250 staining and were manually excised into five fractions according to their apparent molecular weights. These protein fractions were digested by trypsin and analyzed by LC/MS/MS as previously described [59]. The tryptic peptides were resuspended in 0.1% formic acid and analyzed on a Finnigan LTQ linear ion trap mass spectrometer (Thermo Electron, San Jose, CA). MS/MS spectra were searched using the Mascot search engine (Matrix Science, Boston, MA) against the NCBI-nr database. For protein identification, redundant queries, which are apparently identical proteins that are registered in different species, were typically discarded. The candidate protein queries were mapped to the UniProt Knowledge Base [60].

CHAPTER 4

RESULTS AND DISCUSSION

4.1 The effects of medium components on cellulase production performance under submerged fermentation (SmF)

Cellulase production is influenced by a number of factors, including the strain used, the culture conditions and the substrates [61]. The relationship between these variables has an effect on production of the cellulase enzyme complex [62]. In this study, cellulolytic enzyme production from *C. globosum* BCC5776 was optimized in submerged fermentation. Effects of culture medium compositions using different carbon sources, nitrogen sources, minerals, inducers, etc. were studied in order to enhance cellulase yield with the focus on filter paper cellulase (FPase) for subsequent downstream saccharification.

4.1.1 Effects of carbon sources

In the first step, five carbon sources, including commercial substrate (AVICEL®) and lignocellulosic materials (corn cob, rice straw, sawdust and sugarcane bagasse), were compared as the sole carbon source for the production of cellulases. The fungal culture was prepared from fungus grown on PDA by excising five agar pieces covered with a profuse mycelial mat using a No. 2 cork borer (Ø = 0.5 cm) and inoculated in cellulase production medium containing 50 ml working volume in which 4% (w/v) of different carbon sources and 4% (w/v) of peptone as a nitrogen source in basal medium. The shake flasks were maintained at 30ºC for 288 hours with rotary shaking at 200 rpm. The filter paper cellulase (FPase) and carboxymethyl cellulase (CMCase) activities were assayed by the release of reducing sugars using 3,5-dinitrosalicylic acid (DNS) method [63]. The amount of reducing sugars was determined via absorbance measurements (Thermo Scientific Multiskan Ascent) at 540nm based on the standard curves prepared using the corresponding sugars. The experiment was done in triplicate.

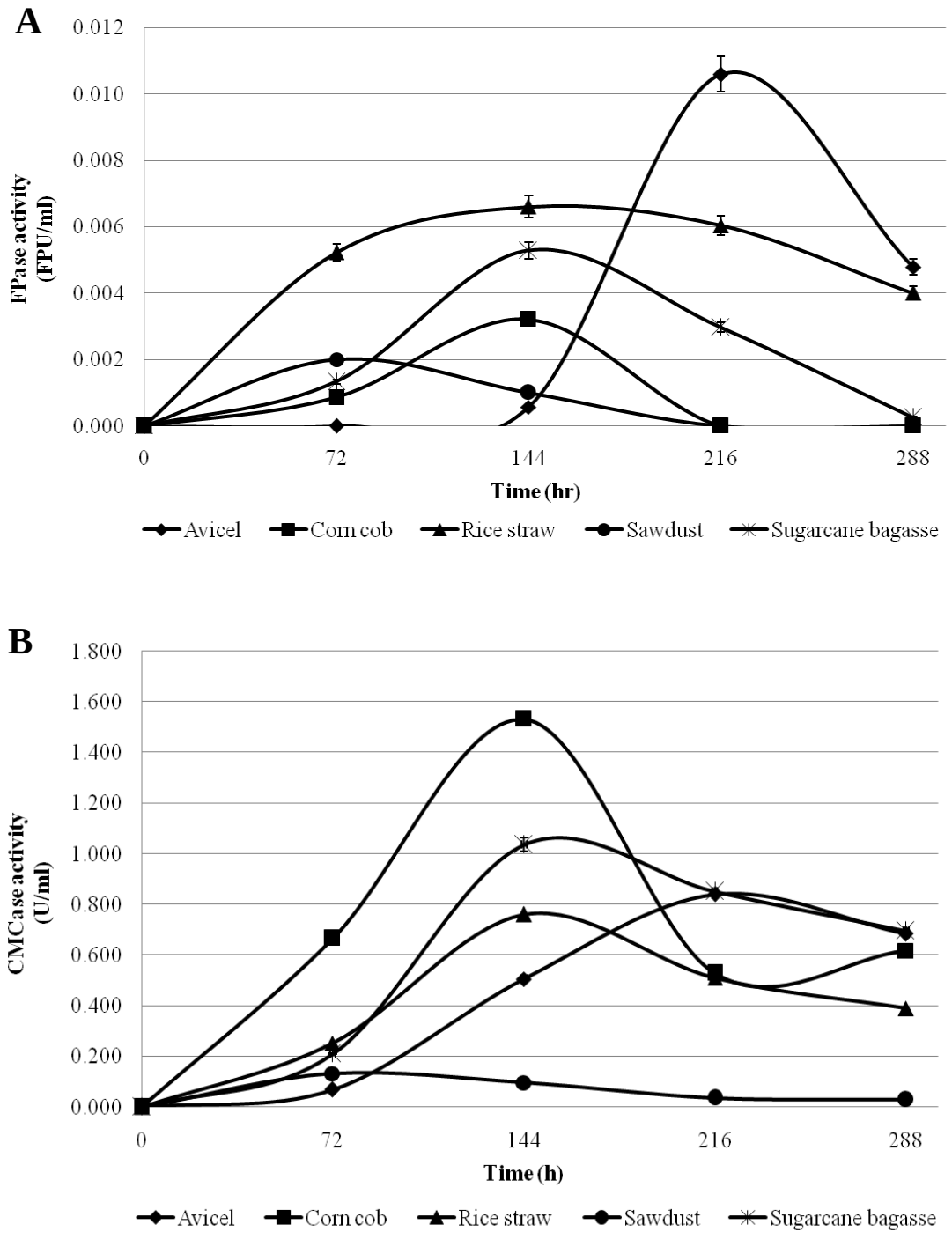

Figure 4.1 Comparison of (A) filter paper cellulase (FPase) and (B) carboxymethyl cellulase (CMCase) activities from *C. globosum* BCC5776 during 288 h of fermentation. The working volume of 50 ml in 250 ml Erlenmeyer flask contained 4% (w/v) of different carbon sources (microcrystalline cellulose AVICEL®, corn cob, rice straw, sawdust and sugarcane bagasse) and 4% (w/v) of peptone in basal medium and incubated at 30 ºC with continuous shaking at 200 rpm.

According to the results, the microcrystalline cellulose AVICEL® was found to be an effective carbon source for cellulose degrading enzymes by *C. globosum*. The highest FPase activity was 0.011 FPU/ml when cultivated using the AVICEL® as the sole carbon source after 216 h of fermentation. On the other hand, the cultivation using rice straw, corn cob, sugarcane bagasse and sawdust gave the maximum FPase activities of 0.005, 0.003, 0.005 and 0.002 FPU/ml, respectively after 144 h of fermentation, except for sawdust, where there was a small increase from 0 h to 72 h before a decrease in the activity after 144 h (Fig. 4.1A). The lower FPase activities obtained from lignocellulosic residues when compared to pure cellulose (AVICEL®) may be due to lignin which prevents enzymatic and microbiological access to cellulose [64]. Endoglucanase or CMCase activity (Fig. 4.1B) were also observed with a maximum value of 1.532 U/ml for cultivation using corn cob as carbon source after 144 h of fermentation. This was followed by sugarcane bagasse, AVICEL®, rice straw and sawdust with activities of 1.036, 0.840, 0.761 and 0.132 U/ml, respectively under the same period of fermentation corresponding to FPase activities. The highest CMCase productivity (91.920 U/ml.h) with corn cob was probably due to its cellulose structure. Azubuike and Okhamafe (2012) [65] studied microcrystalline cellulose derived from corn cob compared with AVICEL® PH 101. It was found that cellulose from corn cob has lower crystallinity which implies a more disordered structure, resulting in a more amorphous structure.

4.1.2 Effects of medium types

The culture media prepared in basal medium or distilled water were compared regarding production of cellulases by *C. globosum* BCC5776. The basal medium used in this experiment was composed of 2.5 g/L NH_4NO_3; 1.5 g/L $MgSO_4.7H_2O$; 1.2 g/L KH_2PO_4; 0.6 g/L KCl; 1 g/L peptone. Different fermentation runs were carried out in 250 ml Erlenmeyer flasks with 50 ml working volume containing 4% (w/v) of microcrystalline cellulose (AVICEL®) and 4% (w/v) of peptone in different culture media. The cultures were incubated under the same condition for 288 h. The experiment was performed in triplicate.

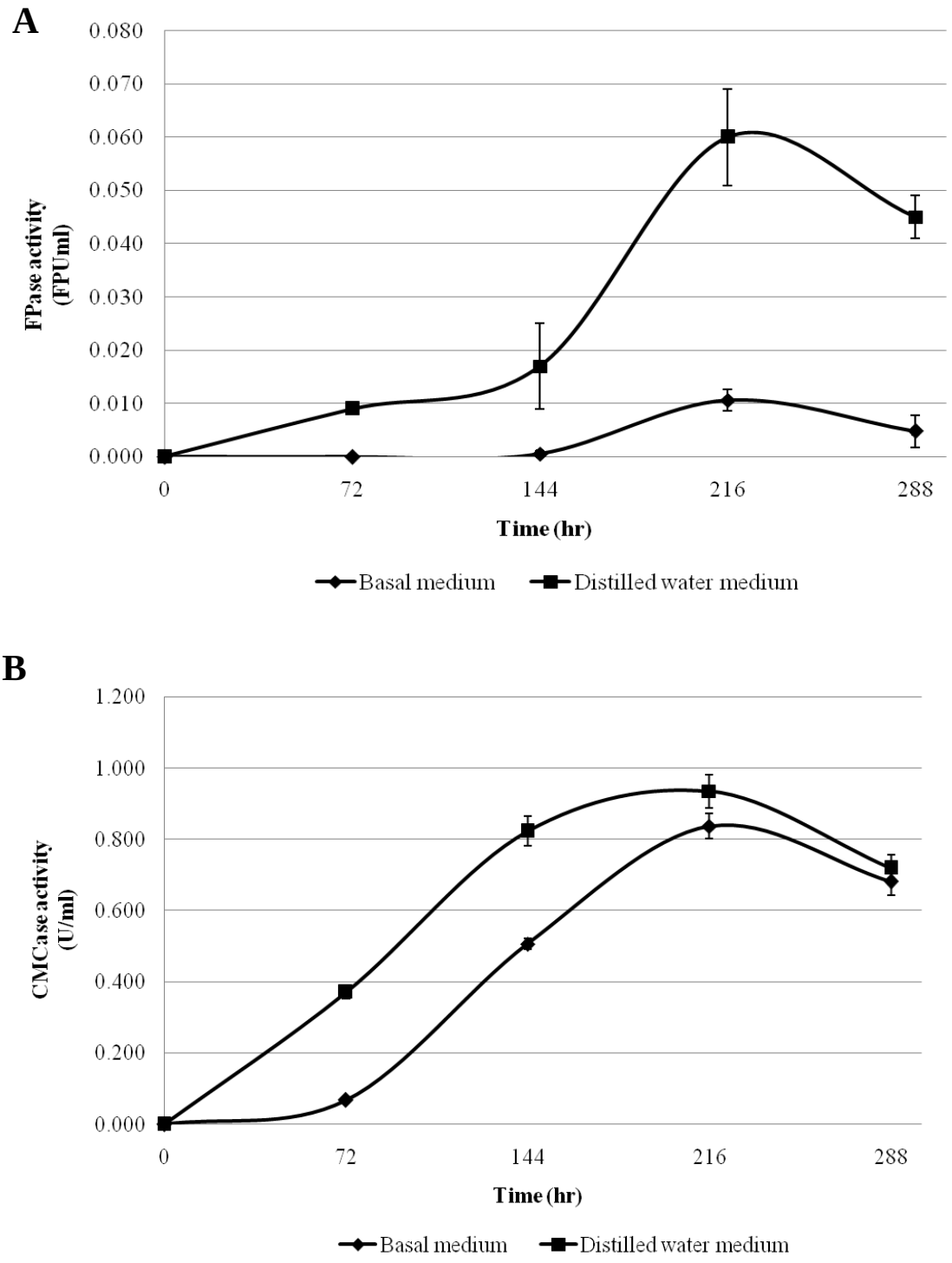

Figure 4.2 Comparison of (A) filter paper cellulase (FPase) and (B) carboxymethyl cellulase (CMCase) activities from *C. globosum* BCC5776 during 288 h of fermentation. The working volume of 50 ml in 250 ml Erlenmeyer flask contained 4% (w/v) of microcrystalline cellulose AVICEL® and 4% (w/v) of peptone in different culture media and incubated at 30 ºC with continuous shaking at 200 rpm.

Activities of FPase and CMCase were recorded (Fig. 4.2A-B) over the 288 h of fermentation of *C. globosum* BCC5776, employing the culture media prepared in basal medium or distilled water. It was observed that FPase activity led to significantly higher ($p < 0.05$) of 0.060 ± 0.01 FPU/ml when the culture media prepared in distilled water as compared to the basal medium after 216 h of fermentation. Similarly, CMCase produced at the peak enzyme activity was 0.935 ± 0.06 U/ml at 216 h in liquid culture carried out using distilled water when compared with that obtained in basal medium. Modified basal medium used in this experiment markedly affected cellulase production. This may be because the effect of metal ions e.g. Mg^{2+}, Ca^{2+}, Fe^{2+}, Co^{2+} and Zn^{2+} on the growth of fungi and enzyme production. John and Jennings (1965) [66] investigated that some concentration of ions affected the extent of the inhibition of growth of several fungi such as *Asteromyces cruciatu*s, *Dendryphiella arenari*, *Chaetomium globosum*, etc. The inhibition might be related to an unfavorable osmotic environment. In addition, the effect of balance between different metal ion concentrations could be more important than their individual effects [67]. For examples, Mg^{2+} is needed for cellulase production, but it is also inhibitory at high concentration (more than 5 mM) and inhibition is counteracted by Ca^{2+}.

4.1.3 Effects of nitrogen sources

The nitrogen source is one of the most important factors for a submerged fermentation medium to produce enzymes. Organic nitrogen sources such as peptone and yeast extract showed better results on cellulase yield and activity than inorganic nitrogen sources [68]; moreover, they were inexpensive as compared to inorganic nitrogen sources [69]. Therefore, different types of organic nitrogen sources including peptone, yeast extract and soybean meal (SBM) were independently added to distilled water at 1% (w/v) concentration in this work. The data shown in Fig. 4.3A-B revealed that among the 3 organic nitrogen sources investigated, the soybean meal (SBM) was found to be the best nitrogen source producing both FPase and CMCase ($p < 0.05$) by *C. globosum* BCC5776 with maximum enzyme activities of 0.253 ± 0.02 FPU/ml and 8.790 ± 0.10 U/ml, respectively at 144 h of cultivation. The soybean meal not only increased cellulase production but also increased mycelia growth. However, the presence of yeast extract and peptone decreased the activities of cellulolytic enzymes. Similarly, Umikalsom *et al.* (1997) [28] reported that the cellulase production by*C. globosum* was inhibited when increasing the concentration of peptone and yeast extract over 0.6% (w/v). This is contrary

to the findings of Enari *et al.*(1977)[70],who found an effective organic nitrogen source for cellulase production by *T. viride* from peptone and yeast extract.

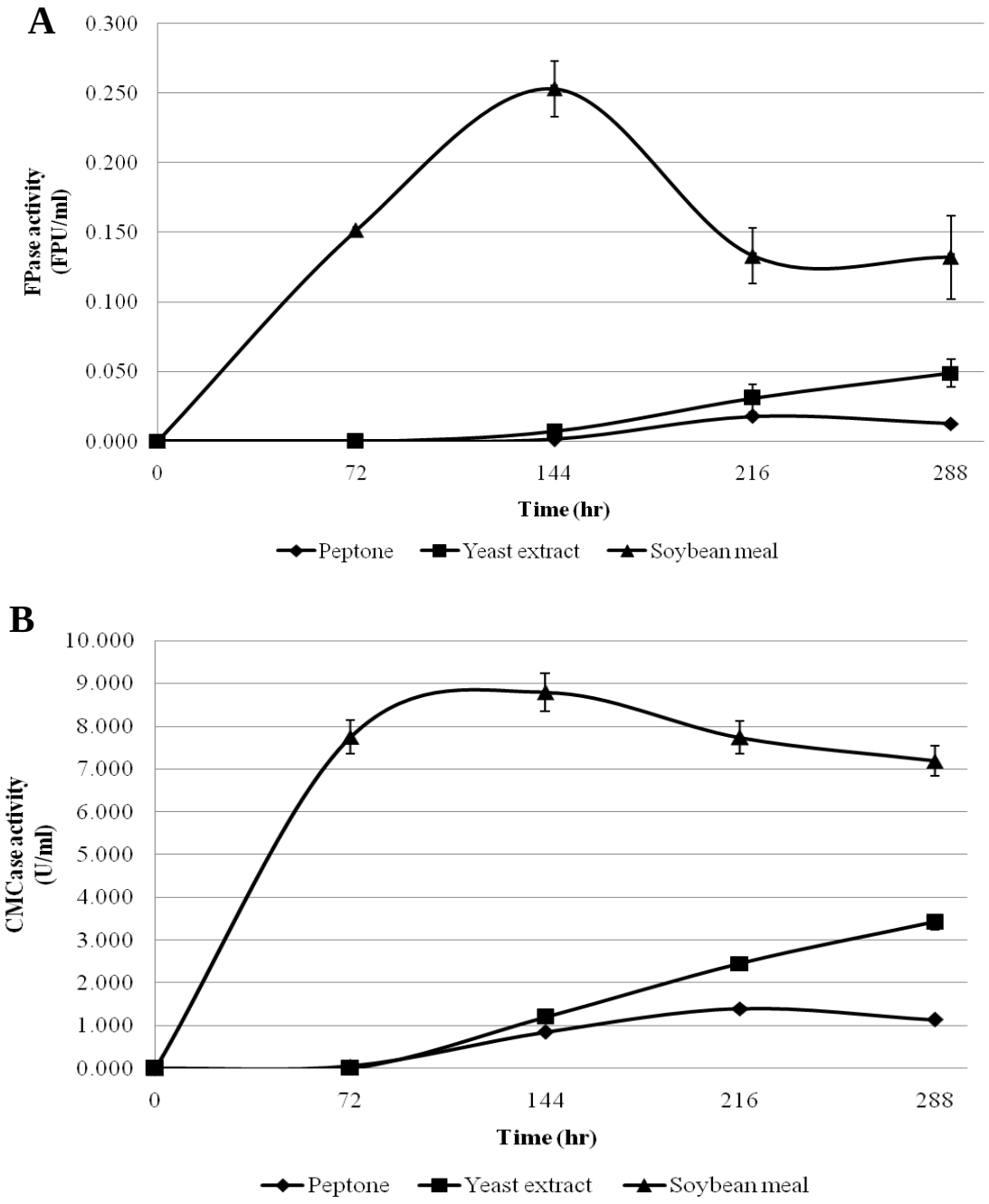

Figure 4.3 Comparison of (A) filter paper cellulase (FPase) and (B) carboxymethyl cellulase (CMCase) activities from *C. globosum* BCC5776 during 288 h of fermentation. The working volume of 50 ml in 250 ml Erlenmeyer flask contained 4% (w/v) of microcrystalline cellulose AVICEL® and 1% (w/v) of different nitrogen sources in distilled water medium and incubated at 30 ºC with continuous shaking at 200 rpm.

4.2 Optimization of cultural conditions for efficient cellulolytic enzymes production

4.2.1 Multivariate analysis of medium conditions

Traditional fermentation medium optimization by 'One factor at a time (OFAT)' approach was firstly used to identify the medium components which had significant effects on cellulolytic enzymes production. Optimization of the medium composition and conditions (multivariate analysis) for production of extracellular cellulolytic enzymes was then studied to enhance the yield of enzymes. Parameters influencing cellulase production performance focused on this study were (1) concentrations of AVICEL®; (2) concentrations of lactose and (3) initial pH. AVICEL® concentration is related to initial cellulose content as main carbon source for fungal growth in the culture medium. Thereby, this parameter is considered one of the significant factors in cellulolytic enzymes production efficiency. Addition of lactose as second carbon substrate is also optimized to induce cellulase production level [71]. Effect of initial pH is also considerable factor influencing cellulase production. Earlier preliminary studies in laboratory on the effect of pH showed that an increase in pH over 8.0 of the medium decreased cellulase activities. The optimum pH for fungal cellulases varies from species to species though in most cases. For examples, *Aspergillus terreus* significantly produce all the three main components of cellulase at initial pH ranging from pH 5.0 to 6.0. The suitable initial pH obtained from this study was in the range of those reported for cellulase production by Mahdi *et al.* (2011) [67]. On the other hand, cellulase production by *Aspergillus niger* MS82 was maximal when the initial culture pH was adjusted to pH 6.0 or pH 7.0 [72].

In the optimization, the response variables analyzed were FPase and CMCase activities at 144 h of fermentation. The experiment, as shown in Table 4.1, was applied to the optimization of the three parameters: AVICEL® concentration at 2%, 4% and 6%; lactose concentration at 0%, 0.05% and 0.1%; and initial pH of the medium at 5.5 (without pH control), 5.8 and 7.0 (potassium phosphate buffering agent). Each optimization experiment was performed in triplicates. All experiments were performed in 250 ml Erlenmeyer flasks with a working volume of 50 ml containing AVICEL®, lactose, soybean meal in distilled water medium with or without initial pH control at 30^{o}Cand incubated for 144 h with continuous shaking. The conditions for each experiment run were summarized. In this study, a total of 9 experiment run with different condition of three parameters. The FPase and CMCase were presented in FPU/ml and U/ml, respectively.

According to Table 4.1, the maximum cellulolytic enzymes activity was obtained when decreasing the concentration of AVICEL® added to the culture medium, increasing the concentration of lactose to 0.1% (v/v) and controlling initial pH at 5.8. Regarding both FPase and CMCase activities, the highest activities recorded after 144 h of fermentation were 0.322 FPU/ml and 7.192 U/ml in run no. 1 as compared to control (run no. 9).

Table 4.1 Design of fermentation parameter optimization experiment and their response variables (FPase and CMCase activities) after 144 h of fermentation.

Run No.	AVICEL® concentration (%)	[a]Lactose concentration (%)	[b]pH	FPase activity (FPU/ml)	CMCase activity (U/ml)
1	2	0.1	5.8	0.322	7.192
2	2	0.05	5.8	0.222	7.022
3	6	0.1	5.8	0.198	6.683
4	6	0.05	5.8	0.183	6.867
5	2	0.1	7.0	0.170	5.536
6	2	0.05	7.0	0.197	5.097
7	6	0.1	7.0	0.172	5.069
8	6	0.05	7.0	0.169	6.116
[c]9	4	0	5.5	0.208	6.370

[a]The initial concentration of lactose used was 1% (v/v).
[b]The final molar concentration of pH at 5.8 and 7.0 used were 50 mM.
[c]Without pH control.

The lowest FPase and CMCase activities observed were 0.169 FPU/ml in Run no. 8 and 5.067 U/ml in Run no. 7. AVICEL® supplementation at 2% (w/v) was optimal for cellulase production and after optimum level the enzyme production was reduced at higher AVICEL® concentration probably due to catabolic repression. Other reasons might be attributed either to adsorption of the cellulase to AVICEL® or limited aeration due to the high viscosity of the culture medium. Further increase the concentration of cellulose beyond 2% (w/v) did not enhance proportionally in enzymatic activities. The proper concentration of lactose for inducing cellulase production in this study was 0.1% (v/v). Shiang *et al.* (1991) [73] reported a possible regulation mechanism of cellulase biosynthesis at a particular concentration of xylose, glucose and cellobiose may induce a cellulose regulatory protein called cellulase activator molecule (CAM). The level of CAM is possibly related to substrate concentration and some unknown factors imparted by moderators. The initial pH of the culture medium on production of cellulolytic enzymes by *C. globosum* BCC5776 substantially influenced when controlling initial pH of the medium

at 5.8 (Run no. 1-4), whereas the enzyme remained accumulated in the cell at pH 7.0 (run no. 5-8) and pH 5.5 (Run no. 9). The optimum initial pH of 7.0 was described for various extracellular cellulolytic fungi such as *Neurospora crassa*, *Humicola fuscoatra* and *A. fumigates* [74-76]. Therefore, controlling initial pH at the optimal values in the fast-producing phases for cellulase production could be more advantageous for celluase production by *C. globosum* BCC5776.

4.2.2 Analysis ofaddition of lignocellulosic waste for enhancing yield of cellulases

Though the yield enhancement of cellulolytic enzymes by various commercial substrates has been mostly studied, lignocellulosic wastes or agro-industrial wastes were also well used as an alternative substrate of fermentation for industrial enzymes production, i.e. cellulases, hemicellulases and pectinase due to their lower cost. In the past few decades, many publications have been revealed that abundant lignocellulosic materials in combination with pure cellulose could produce cellulases with an increased amount. In this study, oil palm empty-fruit-bunch (OPEFB) received after stripping process generating palm oil was utilized as co-substrate at 1% (w/v) concentration under submerged fermentation in order to improve the yield and attribute of cellulases produced by *C. globosum* BCC5776. The results of effect of additional lignocellulosic materials i.e. OPEFB on filter paper cellulase (FPase) (Fig. 4.4A) and endoglucanase (CMCase) (Fig. 4.4B). The presence of OPEFB as co-substrate at 1% concentration in fermentation medium could be seen that the activities of FPase and CMCase were 0.400 FPU/ml and 10.572 U/ml, representing an improvement by 96.08% and 41.73%, respectively compared to that without pH control. In general, cellulolytic enzyme activity increased as the concentration of co-substrate increased. Alam *et al.* (2005) [77] who studied the effect of concentration of wheat flour as co-substrate in range of 1% to 5% for production of cellulases, they investigated that the maximum enzyme production attained was 0.0433 units (mmoles glucose/ml.min) at 2% wheat flour. Further increase in the co-substrate concentration resulting in no significant increased of cellulase activity.

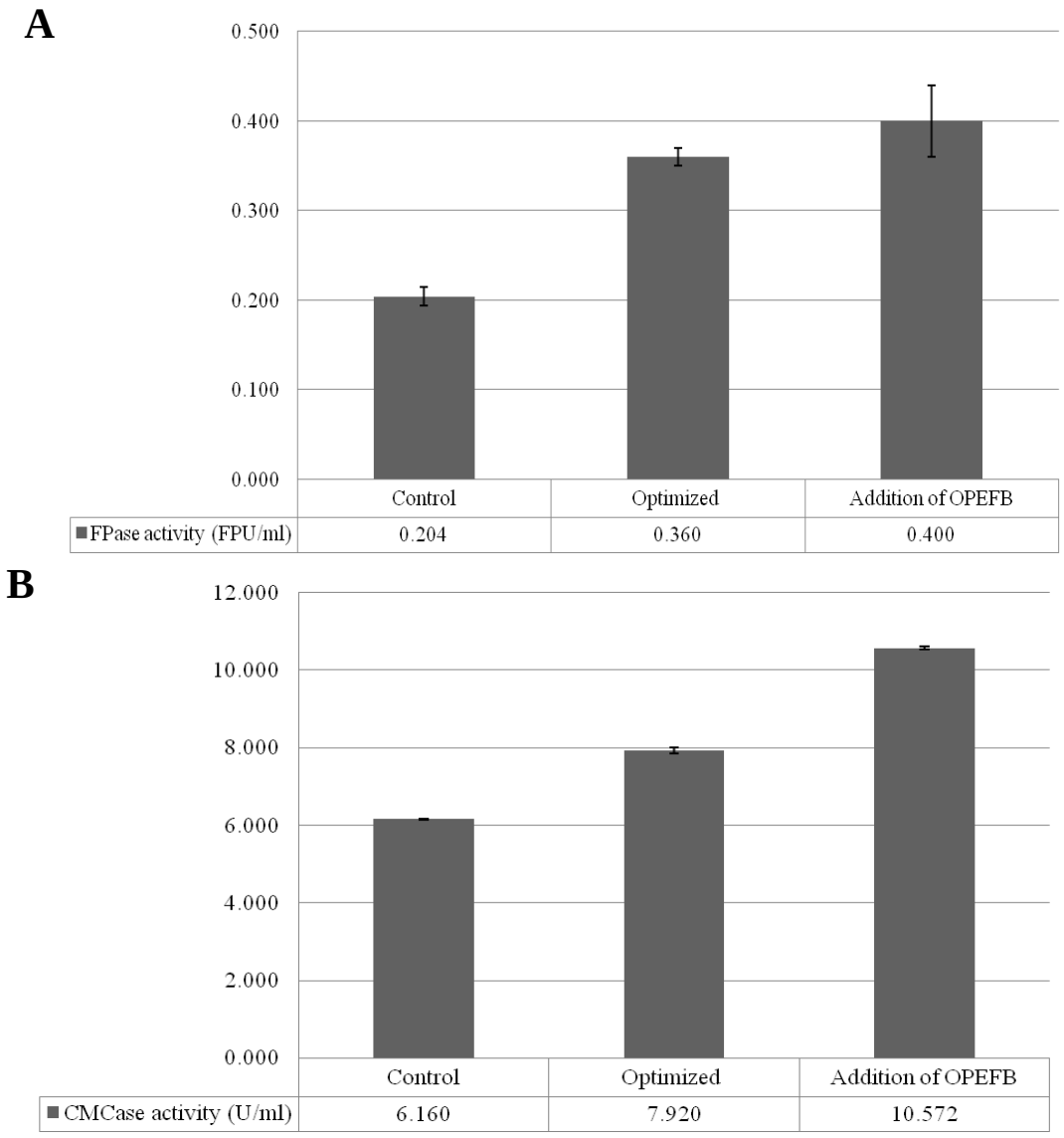

Figure 4.4 Comparison of (A) filter paper cellulase (FPase) and (B) carboxymethyl cellulase (CMCase) activities under various conditions from *C. globosum* BCC5776. 250 ml Erlenmeyer flask containing 50 ml of distilled water medium. Control: 4% (w/v) of microcrystalline cellulose AVICEL® and 1% (w/v) of soybean meal with pH uncontrolled (initial pH 5.5). Optimized: 2% (w/v) of microcrystalline cellulose AVICEL® consolidated with lactose (0.1%) and 1% (w/v) of soybean meal with initial pH of 5.8. Addition of OPEFB: 2% (w/v) of microcrystalline cellulose AVICEL® supplemented with 1% (w/v) of OPEFB and 0.1% (v/v) of lactose and 1% (w/v) of soybean meal with initial pH of 5.8. After that, the different medium conditions were incubated at 30 ºC for 144 h of fermentation with continuous shaking at 200 rpm.

4.3 Scale up production of cellulases under optimal fermentation conditions

In order to achieve the large volumetric cellulolytic enzymes, the fermentation in the 1-L Erlenmeyer flask and the 5-L batch bioreactor were carried out under the optimal fermentation conditions, which contained 2% AVICEL® supplemented with 1% OPEFB, 0.1% lactose, 1% soybean meal, 10% inoculums in liquid culture prepared in distilled water (initial pH 5.8) as described in Section 4.1.5, and then incubated at 30 ºC with rotary shaking at 200 rpm. The time profiles of filter paper activity (FPA) for total cellulase activity were shown in Fig. 4.5. The fermentation result gradually increased and reached the maximum activity at 144 h. The FPA in 1-L Erlenmeyer flask and 5-L bioreactor were 0.398 FPU/ml and 0.359 FPU/ml with its specific activities of 0.79 and 0.77 U/mg proteins, respectively. At the larger scale production of filer paper cellulase the slightly lower cellulase yield was obtained. This might be due to bulk quantities of the culture medium decreased capability of mass and heat transfer. To overcome this constraint, titer improvement by fed-batch or continuous process has been developed for improving enzyme production.

4.4 Study of fungal strain improvement of existing industrial microorganisms for cellulase production

Many investigators have attempted to study the over-production of cellulases, since high production cost and low yields of cellulases are the major obstacles for industrial applications. In the last few decades, demand of various industrial enzymes especially lignocellulolytic enzymes has been increased in both quantitative and qualitative enhancement by way of strains improvement and/or medium optimization for higher enzymatic yield [78]. A reduction in the cost of production, an improvement of fungal strains and an increase of cellulase activity in a short cultivation time are crucial to save the processing cost of cellulase production [79]. Random mutagenesis is a conventional approach to dominate genetics of lignocellulolytic microorganisms. The potential of a microorganism to mutate is an important property conferred by DNA, since it creates new variations in the gene pool[80]. The use of ultraviolet (UV) irradiation as mutagenic agent is a simple method. Therefore, the purpose of this study was to investigate hyper-production strain development for extracellular cellulase (FPase and CMCase) by mutating *C. globosum* BCC5776 using UV-irradiation approach.

The wild type *C. globosum* BCC5776 was exposed to UV radiation at different time intervals ranging from 0 to 30 minutes. This result provided 5 mutants with different productivities of FPase and CMCase (Table 4.2). The result demonstrated that the highest cellulase production was obtained from UV4,while it was obtained from UV express for 20 min which showed a 8.5-fold improved in FPase activity as well as 1.8-fold increased in the activity of CMCase compared to the wild-type BCC5776. The mutant strain UV4 was selected as hypercellulase-producing mutant; however, subcultivation for one time, the activity of cellulases could not maintain continually in further subcultivation of the strain for one generation on selection media agar (SMA) plate. This result conformed with Prabakaran *et al.* (2009) [78] who reported that UV light induced mutant with high enzyme production capability of *A. fumigates,P. chrysogenum* and *V.terrestre* when exposed to 5 minutes. Nonetheless, longer period of exposure to UV rays (> 5 minutes) was proved to be harmful to the enzyme production. With increase in exposure time, all of the enzymes production was decreased. The difference in response to UV radiation and stability of the mutant might be related to the difference in the nature of the wild type strain used in this study.

Table 4.2 Effects of time exposure to ultraviolet (UV) irradiation on FPase and CMCase production by *C. globosum* BCC5776 in SmF. n = 2, ± standard deviation.

Mutant strains	Exposed time (min)	FPase activity (FPU/ml)			CMCase activity (U/ml)		
		Day 4	Day 5	Day 7	Day 4	Day 5	Day 7
Wild-type (BCC5776)	0	0.009 ± 0.00	0.017 ± 0.01	0.020 ± 0.01	2.379 ± 0.02	2.221 ± 0.03	4.509 ± 0.09
UV1	5	0.000 ± 0.00	0.015 ± 0.00	0.030 ± 0.01	0.359 ± 0.01	0.384 ± 0.01	0.908 ± 0.04
UV2	10	0.000 ± 0.00	0.000 ± 0.00	0.000 ± 0.00	0.000 ± 0.00	0.000 ± 0.00	0.000 ± 0.00
UV3	15	0.058 ± 0.02	0.106 ± 0.03	0.160 ± 0.01	3.987 ± 0.05	3.945 ± 0.11	8.996 ± 0.06
UV4	20	0.019 ± 0.01	0.103 ± 0.04	0.170 ± 0.01	3.467 ± 0.02	3.601 ± 0.09	8.333 ± 0.05
UV5	30	0.000 ± 0.00	0.000 ± 0.00	0.000 ± 0.00	0.000 ± 0.00	0.000 ± 0.00	0.000 ± 0.00

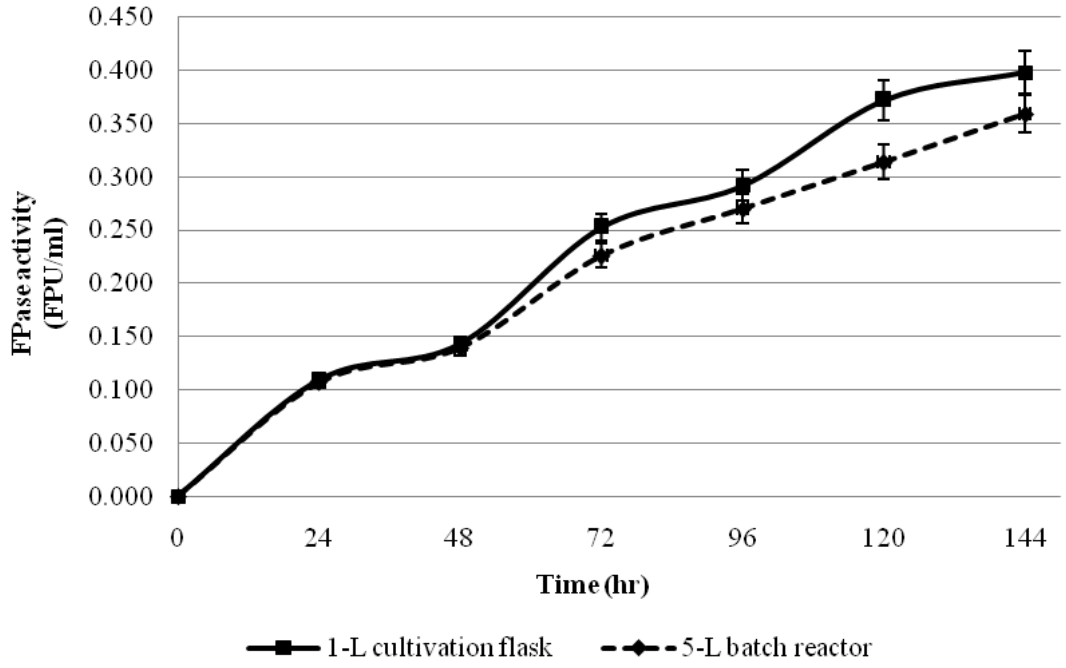

Figure 4.5 Fermentation of the substrate scaled-up in 1-L cultivation flask and 5-L bioreactor performed under optimal conditions containing 2% (w/v) microcrystalline cellulose AVICEL®, 1% (w/v) OPEFB, 0.1% (v/v) lactose and 1% (w/v) soybean meal in distilled water, initial pH 5.8 and incubated at 30°C for 144 h with continuous shaking at 200 rpm.

4.5 Biochemical characterization of crude enzyme preparation

4.5.1 Compositions of enzyme activity profiles

The *C. globosum* BCC5776 crude enzyme extracts from the 5-L fermenter were used to study the composite cellulolytic and hemicellulolytic activity profiles. The biochemical characterization from the native fungal strain BCC5776 was shown in Table 4.3. The results demonstrated substantially weak β-glucosidase (1.95 U/mg) and β-xylosidase (0.05 U/mg) activities which associated to sugar yield in saccharification process. In order to achieve monomer sugar (mainly glucose and xylose),β-glucosidase and β-xylosidase are necessary since they hydrolyze cellobiose to glucose ultimately. Regarding the high specific activities of its endo-acting glycosyl hydrolases,the crude enzyme of *C. globosum* showed CMCase as the major activity (20.39 U/mg) together with endo-activity attacking hemicelluloses including xylanase (36.62 U/mg) and mannanase activity (17.66 U/mg). It also possesses relatively high β-glucosidase activity but with low β-xylosidase activity. The results then showed the strong endo-activity for cellulase and

hemicellulase hydrolysis. Besides, some groups of glycoside hydrolases e.g. amylase, mannanase and pectinase were also slightly secreted from BCC5776.

Fig. 4.6 shows the composite enzyme activity per FPU of the BCC5776 enzyme extract. It was found that the enzymes from the native fungal strain BCC5776 exhibited relatively higher in cellulolytic enzyme i.e. CMCase (39.25 U/FPU) and hemicellulolytic enzymes i.e. xylanase (70.50 U/FPU) and mannanase (34.00 U/FPU). Other enzymes involving in biomass degradation such as pectinase (2.00 U/FPU) and amylase (1.25 U/FPU) were also found in crude enzyme extract from *C. globosum* BCC5776. However, regardless of the high specific activities of its endo-actingglycosyl hydrolases, which target cellulose and hemicellulose, BCC5776 enzyme extract exhibited substantially weak β-glucosidase (3.65 U/FPU) and β-xylosidase (0.10 U/FPU) activities. This evidence suggests that the synergism of β-glucosidase and β-xylosidase withBCC5776 crude enzyme extract for the hydrolysis would be relevant for complementing the composite activities of cellulase, particularly for its downstream lignocelluloses degrading glucosidase and xylosidase activities.

Table 4.3 The composite enzyme activity profiles of *C. globosum* BCC5776 enzyme extracts.

C. globosum		
Activity	U/mg	U/ml
CMCase	20.39	15.70
FPase	0.52	0.40
B-glucosidase	1.95	1.50
Xylanase	36.62	28.20
B-xylosidase	0.05	0.04
Amylase	0.65	0.50
Mannanase	17.66	13.60
Pectinase	1.04	0.80

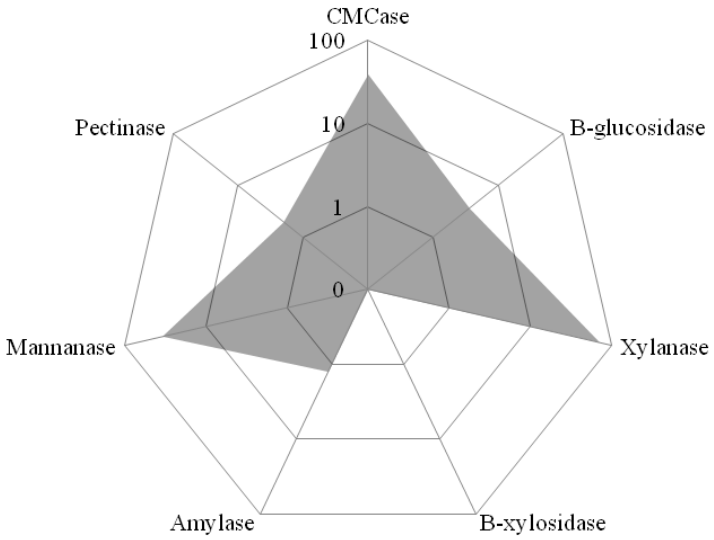

Figure 4.6 The composite enzyme activity per FPU of the BCC5776 enzyme extract. The Y-axis is in a logarithm scale (log U/FPU).

4.5.2 Optimal working temperature, pH and stability

Physical factors, including temperature and pH, play important roles in enzyme activity and stability, and have a profound effect on enzyme activity related to changes in enzyme configurations as well as catalytic performance. In general, range of temperature and pH suitable for biomass-degrading enzymes are within 50-65ºC and pH of 4.6-7.5 for a variety of fungal strains e.g. *Aspergillus niger*, *Aspergillus ornatus*, *Penicillium rubrum* and *Neurospora crassa* [3].

The effects of temperature on relative activities of BCC5776 enzyme extracts were shown in Fig. 4.7. The optimum temperature of FPase activity was 50 ºC (0.064 FPU/ml) as well as CMCase activity (0.52 U/ml). At temperature higher than 50 ºC, the FPase activity decreased dramatically to 19.68% of its optimum activity whereas CMCase activity could retain most of its optimum activity which can be retained for >74% and 60% activity at 70 and 80 ºC. For the crude xylanase activity, the optimum temperature was found to be 60ºC with its activity of 5.83 U/ml. The reaction rates remained relatively high within a temperature range of 50-70ºC. When analyzed at higher temperature i.e. 80ºC, the xylanase lost 37% activity of its optimum activity. Besides cellulolytic and hemicellulolytic enzymes, mannanase, pectinase and amylase were examined. The optimum temperature for mannanase and pectinase of fungal strain were also found between 50 and 60ºC with theirs activity of 3.61 U/ml and 0.99 U/ml respectively. For

amylase, the optimum temperature was found at 60ºC with its activity of 0.81 U/ml. Some of the literature discusses the temperature of influence on cellulase adsorption. A positive relationship between adsorption and saccharification of cellulosic substrate was observed at temperature below 60°C. The adsorption activities beyond60°C decreased possibly because of the loss of enzyme configuration leading to denaturation of the enzyme activity [81].

The effects of pH on relative activities of BCC5776 enzyme extracts was investigated at pH values ranging from 2 to 10 at 50°C. The bell curves are shown in Fig. 4.8. For cellulolytic enzymes, the FPase was found to be active in the narrow pH range of 5.0 to 6.0 whereas CMCase was working in the broad pH range of 3.0 to 8.0 with the maximum activities of FPase and CMCase at pH 5.0 (0.065 FPU/ml and 2.387 U/ml). At pH below 5.0, 49% (0.032 FPU/ml) of the maximal FPase activity was maintained. No FPase activity was observed at pH 2.0. In contrast to FPase, the relative CMCase activity was about 37% (0.024 FPU/ml) of its maximum activity at pH below 3.0. Similarly, enzyme reaction rates increased steadily from pH 5.0 to 7.0, rising at pH 5.0 with the maximum activity of 4.528 U/ml. The highest xylanase relative activity was obtained at pH 5.0 which its activity of 4.528 U/ml. At a neutral pH (7.0), most xylanase retained about 99% of its optimum activity. After increased pH above 7.0, the activity decreased sharply. The enzyme still retained 88%, 70% and 60% of its optimum activity at higher pH (7.0-10.0). Mannanase and pectinase reached to their maximum activity of 0.067 U/ml and 1.012 U/ml at pH 5.0 and pH 6.0 except amylase, the optimum pH was 7.0 with 3.093 U/ml. Studies carried out with these experiment results could conclude that the most suitable pH for enzyme activity was within mild acidic to neutral region.

By studying the crude enzyme stability (Fig. 4.9) at different temperatures (30-70°C) under the same conditions of optimum activity assay, the results of the present study revealed that FPase, CMCase, xylanase, pectinase and amylase were able to retain their activities 100%, except mannanase, which retained more than 85% of its activity as compared to their controls (FPase; 0.438 FPU/ml, CMCase; 2.365 U/ml, xylanase; 5.347 U/ml, mannanase; 3.207 U/ml, pectinase; 0.740 U/ml and amylase; 0.835 U/ml) after incubating at 30-40°C for 1 h. At 50°C for 1 h of incubation, CMCase and xylanase also retained about 95% (2.253 U/ml) and 90% (4.822 U/ml), respectively which is relatively stable while FPase, mannanase, pectinase and amylase still retained 78% (0.342 U/ml), 70% (2.245 U/ml), 51% (0.377 U/ml) and 69% (0.576 U/ml) of their activities

respectively. With decreasing sharply above 60°C for 1 h, no activity of xylanase and mannanase was recorded; in addition, FPase, CMCase, pectinase and amylase lost their activities about 79%, 62%, 94% and 99% respectively. BCC5776 crude enzyme showed high stability below 50°C. However, cellulolytic and hemicellulolytic enzymes that are stable at high temperatures can be used in cellulose saccharification processes at elevated temperatures to protect both substrates and products of the enzymatic reaction from microbial contamination and deterioration [82].

The pH stability (Fig. 4.10) was measured by incubating enzymes at room temperature for 1 h using different buffer systems (pH 2.0-10.0) and assayed under the same conditions of optimum activity. The results exhibited by FPase, CMCase, xylanase, mannanase, pectinase and amylase activities had a wide range between pH 4.0-8.0. The activities of FPase, CMCase, xylanase, mannanase, pectinase and amylase without treatment was used as a control (100%) in this experiment, which equaled 0.073 FPU/ml, 2.488 U/ml, 5.577 U/ml, 2.978 U/ml, 0.123 U/ml and 0.802 U/ml, respectively. As pH value diverged from the optimum level, the efficient functioning of the enzyme was affected, most probably, due to the change in active site conformation which is determined, in part, by ionic and hydrogen bonding that can be affected by pH [69]. It is also clear that BCC5776 crude enzyme extract was stable in broad pH range.

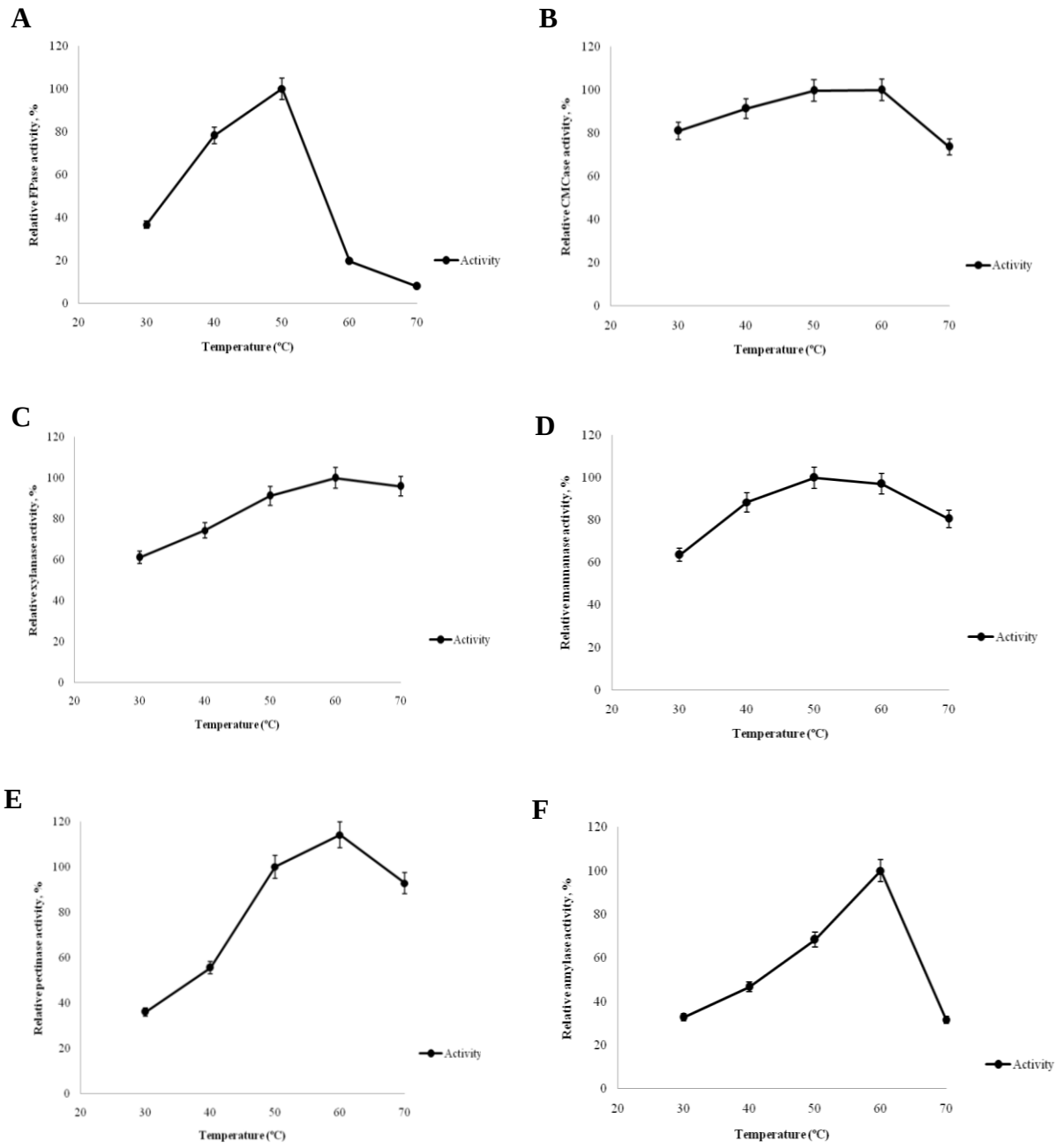

Figure 4.7 Effects of temperature on the enzyme activity of *C. globosum* BCC5776 crude enzymes from 5-L bioreactor using 0.1 M sodium acetate buffer (pH 5.5). (A) FPase; (B) CMCase; (C) xylanase; (D) mannanase; (E) pectinase and (F) amylase activities.

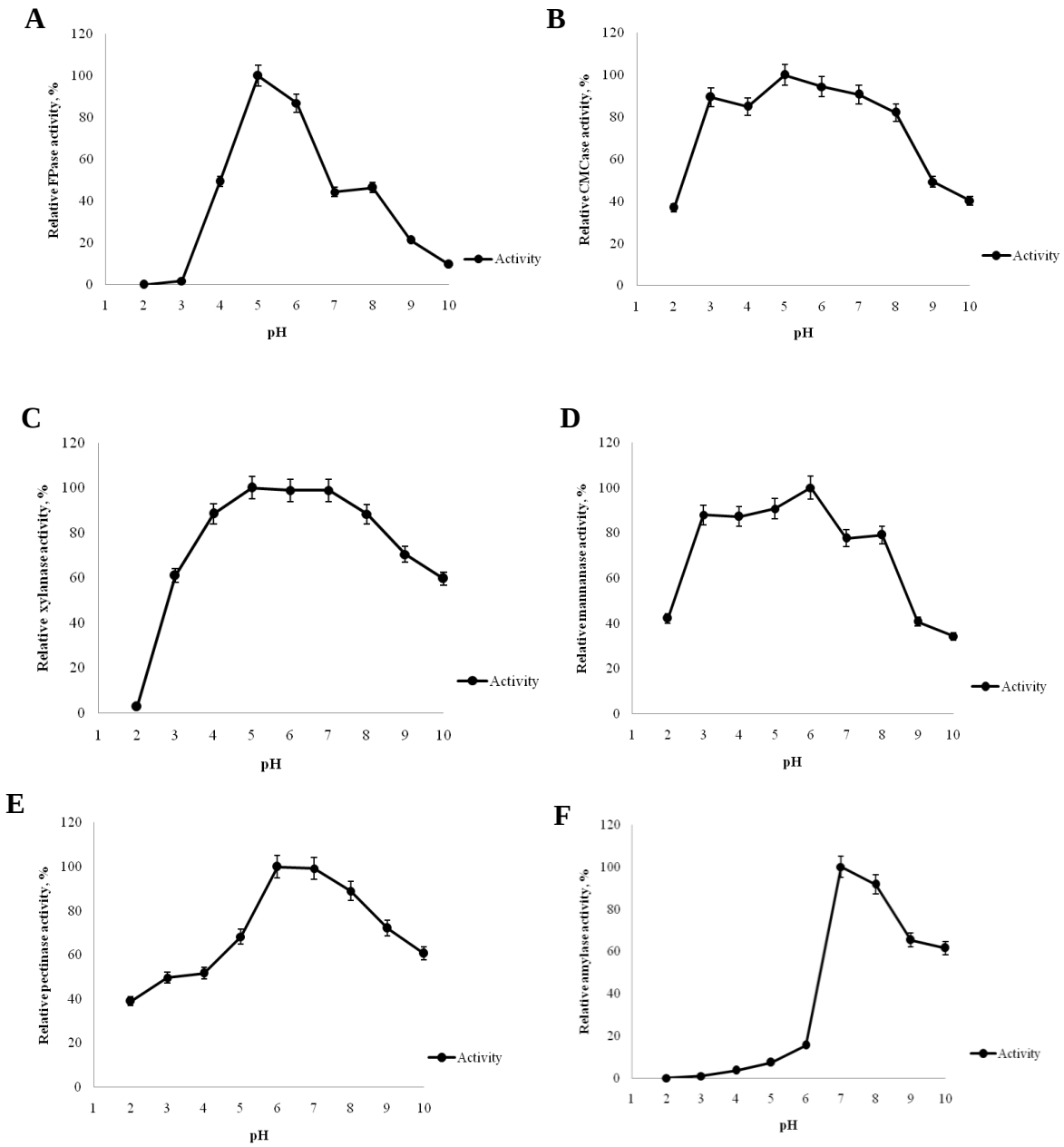

Figure 4.8 Effects of pH on the enzyme activity of *C. globosum* BCC5776 crude enzymes from 5-L bioreactor at 50 ºC with 0.1 M glycine buffer (pH 2.0-4.0), 0.1 M sodium acetate buffer (pH 4.0-6.0), 0.1 M sodium phosphate buffer (pH 6.0-8.0) and 0.1 M Tris (pH 8.0-10.0). (A) FPase; (B) CMCase; (C) xylanase; (D) mannanase; (E) pectinase and (F) amylase activities.

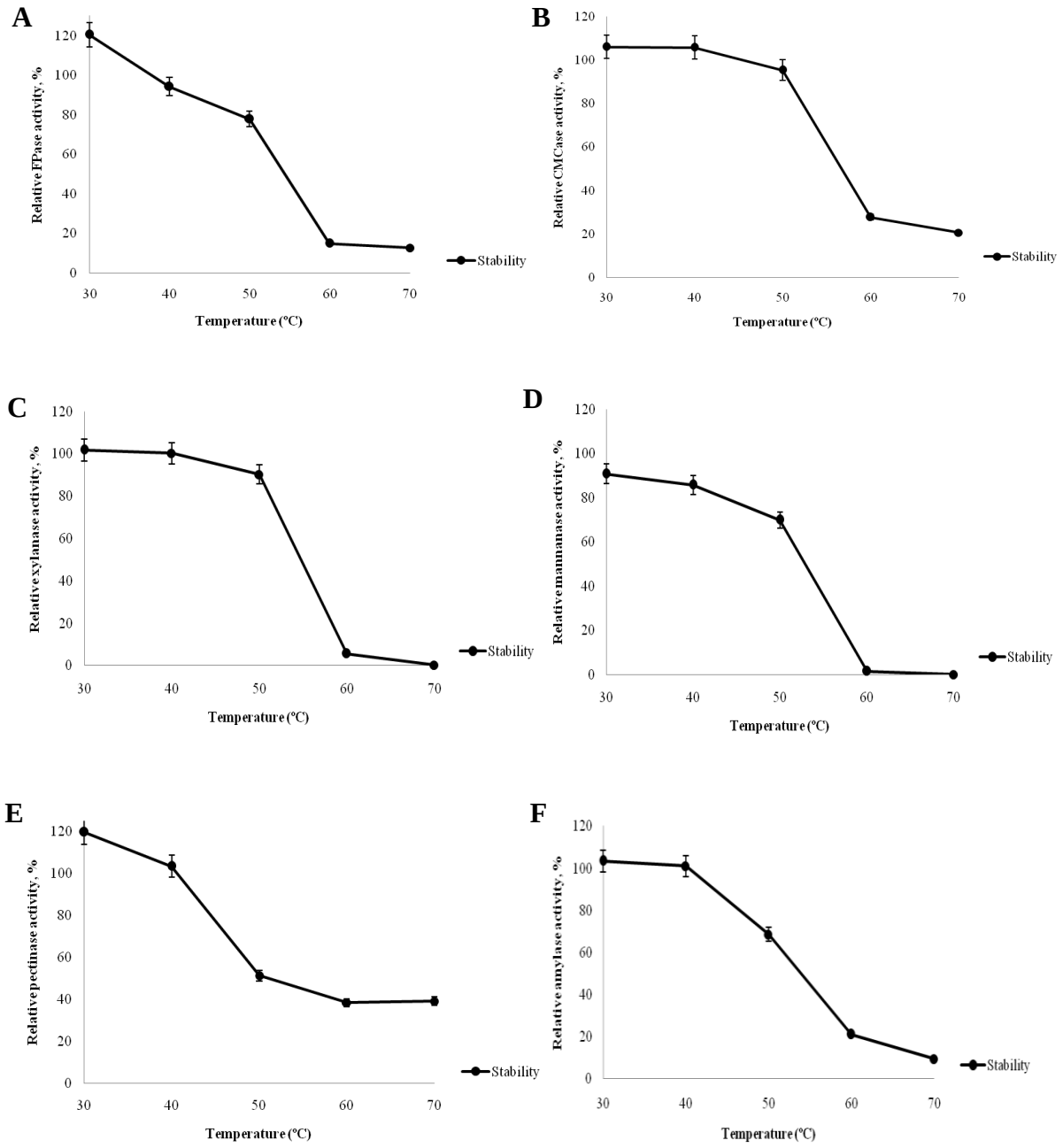

Figure 4.9 Effects of temperature on enzyme stability of *C. globosum* BCC5776 crude enzymes from 5-L bioreactor. Enzymes were incubated for 1 h at indicated temperatures under the same conditions of optimum activity assay. The activity without treatment was used as a control, 100%. Error bars represent the standard deviation of the mean calculated for three replicates. (A) FPase; (B) CMCase; (C) xylanase; (D) mannanase; (E) pectinase and (F) amylase activities.

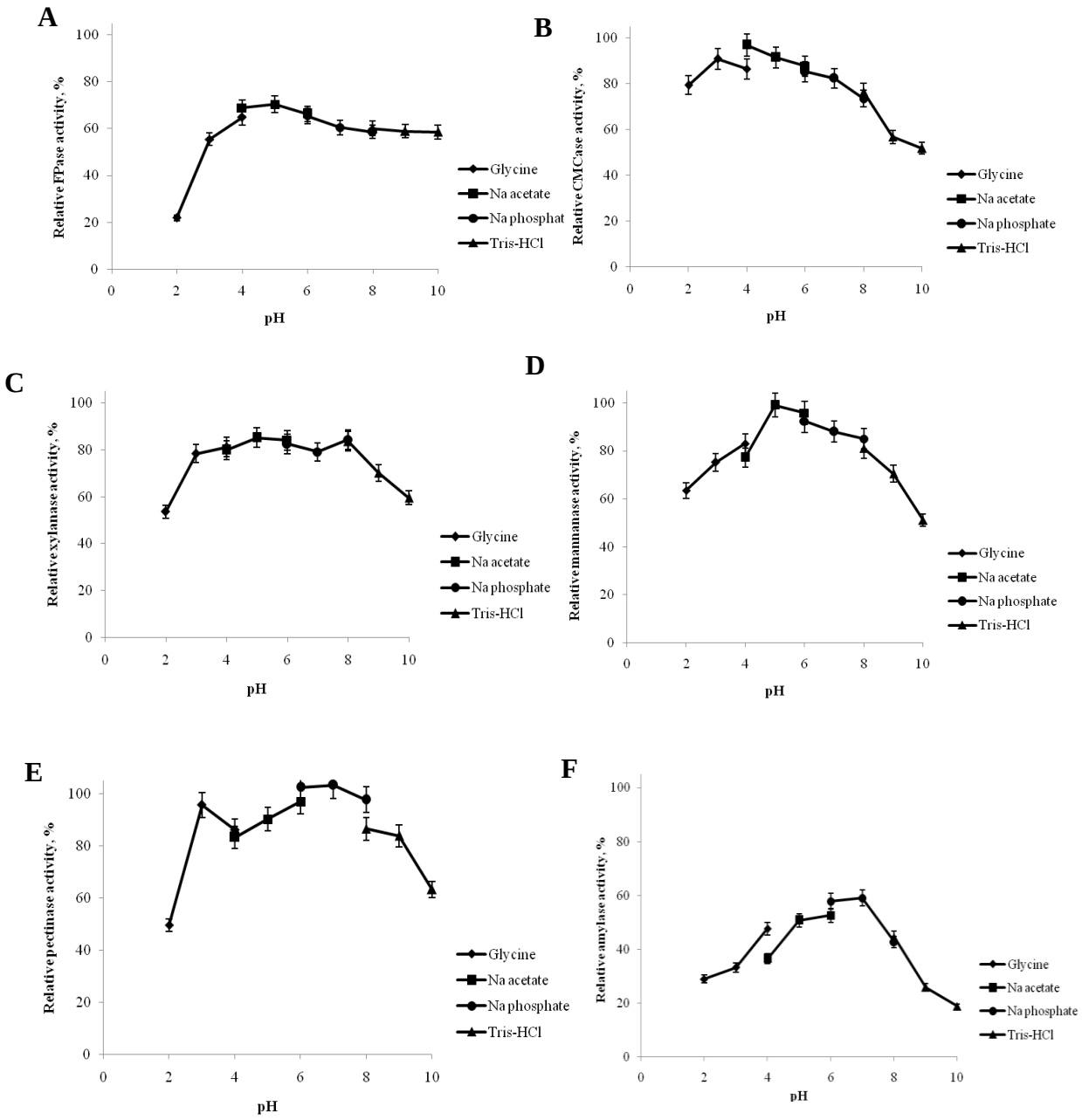

Figure 4.10 Effects of pH on enzyme stability of *C. globosum* BCC5776 crude enzymes from 5-L bioreactor. Enzymes were incubated for 1 h at room temperature with varying pHs (pH 2.0-10.0). At pH 2.0-4.0 using 0.1 M glycine buffer, pH 4.0-6.0 using 0.1 M sodium acetate buffer, pH 6.0-8.0 using 0.1 M sodium phosphate buffer and pH 8.0-10.0 using 0.1 M Tri-HCl. The activity without treatment was used as a control, 100%. Each enzyme activity was measured under the same conditions of optimum temperature and pH activity assay. (A) FPase; (B) CMCase; (C) xylan0ase; (D) mannanase; (E) pectinase and (F) amylase activities.

4.5.3 Protein characterization and identification

C. globosum BCC5776 fungal growth at 144 h of fermentation in 5-L fermenter with 30ºC incubated temperature was selected for total protein extraction. The BCC5776 crude enzyme extracts separated from cells had a protein content of 0.77 mg protein/ml. The amount of crude extracts used was 30 µl (0.023 mg protein) for analysis of 10% SDS-PAGE.

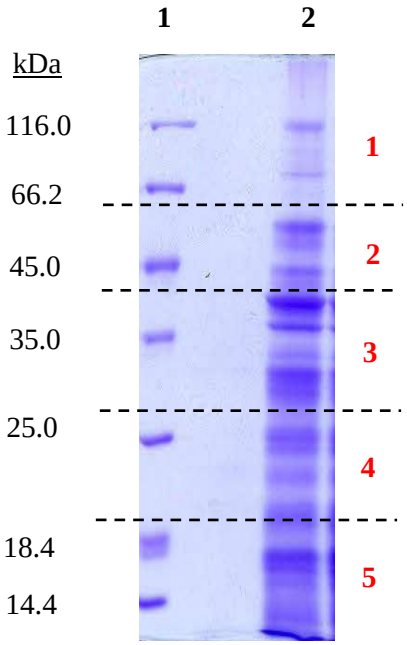

Figure 4.11 SDS-PAGE analysis of the BCC5776 crude enzyme extracts. Lane 1- Marker proteins; Lane 2 –the BCC5776 crude enzyme extracts stained with Comassie brilliant blue.

As shown in Fig. 4.11, diverse proteins bands appeared after staining with staining buffer containing Comassie brilliant blue, methanol and acetic acid. The bands corresponded to different molecular weight when compared to the standard marker proteins from 14.4 to 116.0 kDa. LC/MS/MS was introduced to identify the proteomic profile of the BCC5776 crude enzyme extract. The MS/MS spectra were applied to the Mascot® database search engine (Matrix Science), which employed the acquired data to query the NCBI genome database. Most of the proteins identified in the BCC5776 crude enzyme extract demonstrated majority cellulolytic enzymes, which are core enzymes produced from the BCC5776, as well as hemicellulolytic enzymes with a substantial fraction of non-cellulosic polysaccharide hydrolyzing enzymes.

Several cellulolytic enzymes were identified in *C. globosum* crude enzyme extract (Table 4.4), including 4endoglucanases (cleaves randomly at 1,4-β-linkages within the cellulose chain; creating more chain ends for the exo-1,4-β-glucanase), 8 cellobiohydrolases (attacks reducing, CBH I or non-reducing, CBH II end of cellulose chain; releasing cellobiose units),1 carbohydrate binding module (carbohydrate-active enzymes) and 1 cellobio dehydrogenase (oxidizes soluble cellodextrins, mannodextrins and lactose). These cellulolytic enzymes were involved in degradation of cellulose.

A variety of hemicellulolytic enzymes were also presented in the BCC5776 enzyme extract. These included a number of endo- andexo-acting hemicellulases e.g., xylanase (degrade the linear polysaccharide β-1,4-xylan into xylose), β-1,3-xylanase (random endo-hydrolysis of 1,3-β-D-glycosidic linkages in 1,3-β-D-xylan), β-galactosidase (hydrolyzes β-galactosides into monosaccharides), β-glucosaminidase (catalyze the hydrolysis of chitin to amino sugar),β-1,4-galactanase (specifically hydrolyzes 1,4-β-D-galactosidic linkages in type I arabinogalactans), β-1,3-galactosidase (catalyzes the hydrolysis of 1,3-β and 1,6-β- linked D-galactopyranosyl residues from oligosaccharides) and α-mannosidase (cleaves the alpha form of mannose).

Other hydrolases were also displayed in the BCC5776 secretome, including different enzymes attacking starch and other non-cellulosic polysaccharides, suggesting that *C. globosum* has the capability to produce various hydrolytic enzymes for biotechnological utilizations. The proteomic profiles of the BCC5776 enzyme extract thus provided evidence for synergism based on activity complementation of enzyme systems.

The total number of proteins identified in the BCC5776 enzyme extract was 33. Out of this number, as shown in Fig. 4.12A, 90% were functionally characterized proteins and only 10% were hypothetical proteins. Out of twenty-seven functionally characterized proteins, twenty-three functionally characterized proteins matched with hydrolytic enzymes involved in lignocellulose degradation. Twenty-seven functionally characterized proteins were also classified matching with different fungal species(Fig. 4.12B). Most of functionally characterized proteins matched with *Neofusicoccum parvum*. Out of five functional proteins showed closest simplicity to protein from *Macrophomina phaseolina* (strain MS6). The rest of fourteen functional proteins resembled those in *Gibberella fujikuroi* (strain IMI 58289), Phialophora *spp*., Colletotrichum *spp*., *Irpex lacteus,Aspergillus oryzae* (strain ATCC 42149), *Thielavia terrestris* (strain NRRL 8126),

Volvariella volvacea, *Togninia minima* (strain UCR-PA7), *Heterobasidion irregular* (strain TC 32-1), *Sclerotinia sclerotiorum* (strain ATCC 18683) and uncultured fungus. Functional classification of the extracellular proteins of the BCC5776 enzyme extract is illustrated in Fig.4.13.

Table 4.4 The identification of the lignocellulolytic enzymes from the secreted proteins of *Chaetomium globosum* BCC5776 using proteomic analysis with LC/MS/MS.

No.	GI number	Gene Name	Protein name	Organism	Mascot score	Prot mass
Cellulases						
1	343435330	*cbh1*	Cellobiohydrolase I	Uncultured fungus	111	18482
2	343441380	*cbh1*	Cellobiohydrolase I	Uncultured fungus	60	17704
3	517325505	*FFUJ_11390*	Endo-β-1,4-glucanase / cellulase (EC 3.2.1.4)	*Gibberella fujikuroi* (strain IMI 58289)	59	42508
4	343435350	*cbh1*	Cellobiohydrolase I	Uncultured fungus	55	18629
5	485920895	*UCRNP2_6495*	Endoglucanase (EC 3.2.1.4); cellobiohydrolase (EC 3.2.1.91)	*Neofusicoccum parvum*	167	47768
6	310801037	*GLRG_11038*	Endo-β-1,4-glucanase (EC 3.2.1.4); reducing end-acting cellobiohydrolase (EC 3.2.1.176); chitosanase (EC 3.2.1.132); endo-β-1,3-1,4-glucanase (EC 3.2.1.73)	*Colletotrichum graminicola* (strain M1.001)	150	49663
7	46395332	*cex1*	Cellobiohydrolase	*Irpex lacteus*	111	56112
8	407917740	*MPH_12047*	Endo-β-1,4-glucanase / cellulase (EC 3.2.1.4)	*Macrophomina phaseolina* (strain MS6)	95	32163
9	477528227	*Cob_01073*	Exoglucanase type c	*Colletotrichum orbiculare* (strain MAFF 240422)	109	52197
10	367046256	*THITE_118360*	1,4-β-D-glucan cellobiohydrolase C (EC 3.2.1.91)	*Thielavia terrestris* (strain NRRL 8126)	84	56119
11	49333363	*cbhI-I*	Cellobiohydrolase I-I	*Volvariella volvacea*	82	55320
12	343435720	*cbh1*	Cellobiohydrolase I	*Uncultured fungus*	75	18463
13	575066085	*cbp1*	Carbohydrate-binding module family 1 protein	*Heterobasidion irregulare* (strain TC 32-1)	68	35482
14	485929653	*UCRNP2_206*	Putative cellobiose dehydrogenase protein	*Neofusicoccum parvum*	99	89743
Hemicellulases						
15	407926573	*MPH_03404*	β-galactosidase (EC 3.2.1.23); exo-β-glucosaminidase (EC 3.2.1.165); exo-β-1,4-galactanase (EC 3.2.1.-); β-1,3-galactosidase (EC 3.2.1.-)	*Macrophomina phaseolina* (strain MS6)	204	107902
16	407929733	*MPH_00117*	Mannosyl-oligosaccharide 1,2-α-mannosidase (EC 3.2.1.113)	*Macrophomina phaseolina* (strain MS6)	122	57629
17	74664704	*xynF3, xlnF3*	Endo-1,4-β-xylanase (EC 3.2.1.8); endo-1,3-β-xylanase (EC 3.2.1.32)	*Aspergillus oryzae* (strain ATCC 42149)	101	34903
18	407926897	*MPH_02878*	Endo-1,4-β-xylanase (EC 3.2.1.8); endo-1,3-β-xylanase (EC 3.2.1.32)	Macrophomina phaseolina (strain MS6)	189	34499
19	485916263	*UCRNP2_9647*	Endo-1,4-β-xylanase (EC 3.2.1.8); endo-1,3-β-xylanase (EC 3.2.1.32)	*Neofusicoccum parvum*	185	35125
20	485916633	*UCRNP2_9407*	Endo-1,4-β-xylanase (EC 3.2.1.8); endo-1,3-β-xylanase (EC 3.2.1.32)	*Neofusicoccum parvum*	130	34638
21	485919833	*UCRNP2_7241*	Endo-β-1,4-xylanase (EC 3.2.1.8); endo-β-1,3-xylanase (EC 3.2.1.32)	*Neofusicoccum parvum*	87	23690
22	500259512	*UCRPA7_2184*	Endo-β-1,4-xylanase (EC 3.2.1.8); endo-β-1,3-xylanase (EC 3.2.1.32)	*Togninia minima* (strain UCR-PA7)	84	23642
Miscellaneous						
23	485916757	*UCRNP2_9363*	Putative carboxypeptidase s1 protein	*Neofusicoccum parvum*	78	61265
24	485919267	*UCRNP2_7651*	Putative leucyl aminopeptidase protein	*Neofusicoccum parvum*	147	40795
25	485924582	*UCRNP2_3892*	Putative tripeptidyl-peptidase 1 protein	*Neofusicoccum parvum*	68	65131
26	407926489	*MPH_03319*	Polysaccharide deacetylase	*Macrophomina phaseolina* (strain MS6)	66	26890
Unkhown function						
27	325683994	*cel*	Glycoside hydrolase	*Phialophora sp.* G5	161	50242

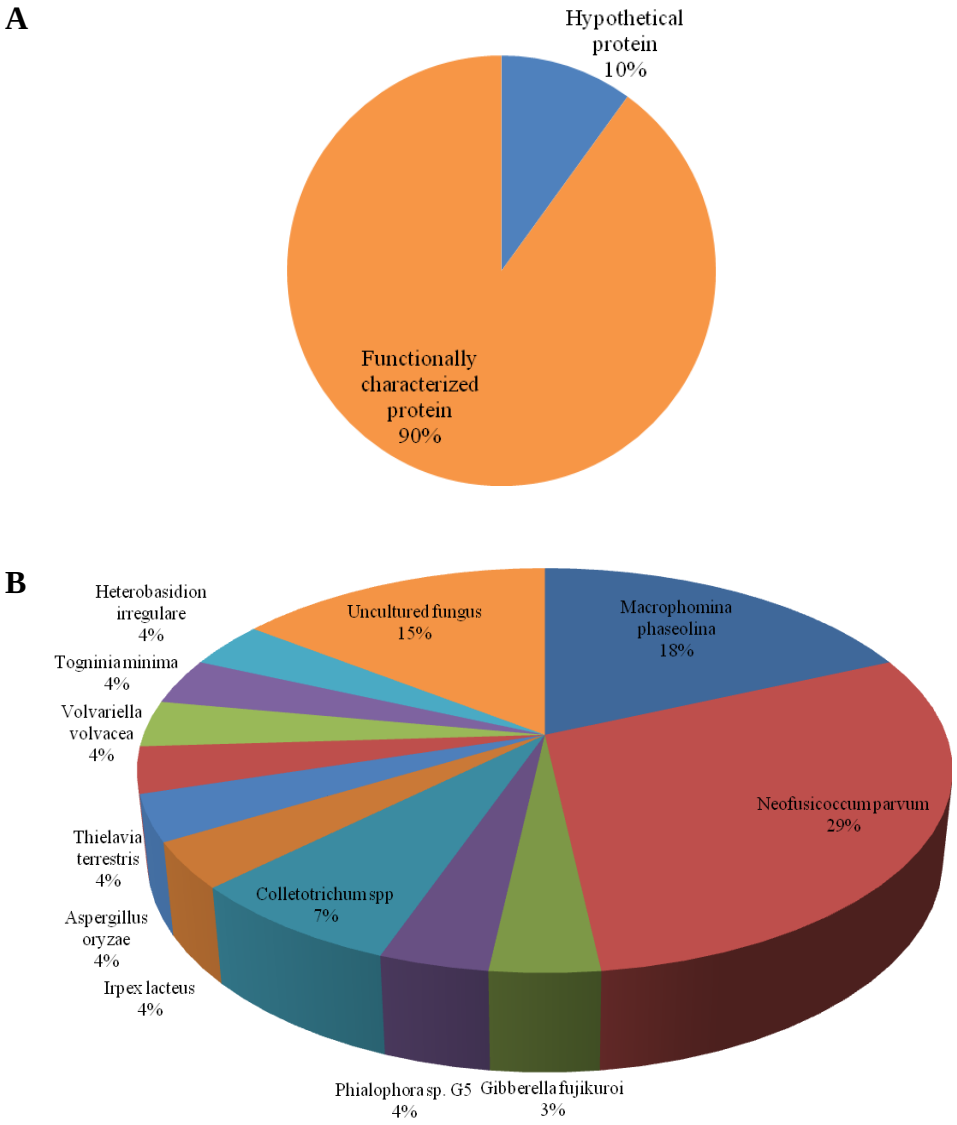

Figure 4.12 Venn diagrams of the *Chaetomium globosum* BCC5776 proteins identified through LC/MS-MS, (A) classification of functionally characterized proteins and hypothetical proteins, and (B) classification of functionally characterized proteins in different species.

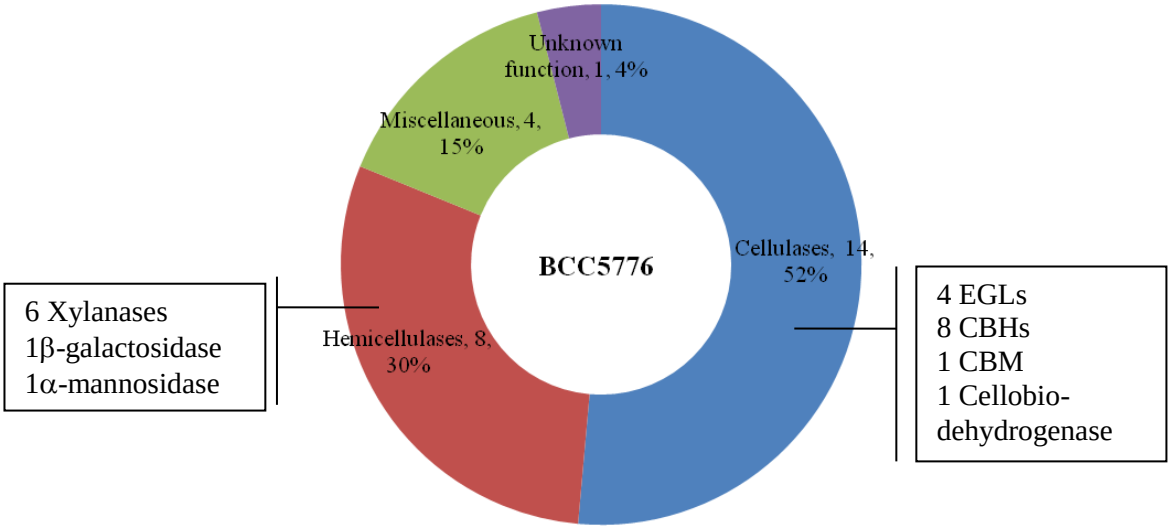

Figure 4.13 The functional distribution of the proteins identified from the LC/MS-MS analyses of BCC5776 enzyme extracts based on their putative lignocellulose-degrading action.

4.6 Enzymatic hydrolysis

4.6.1 Compositions of rice straw

Rice straw has cellulose, hemicellulose and lignin as the major components with substantial higher ash content than other agricultural by-products. The composition of rice before pretreatment was determined according to the standard methods of the National Renewable Energy Laboratory [56]. The chemical compositions of rice straw used as starting material in this study and other potent local biomass are shown in Table 4.5

Table 4.5 Chemical composition of rice straw.

Component	Biomass composition (%)		
	Rice straw[(a)]	Sugarcane bagasse [(b)]	Corn stover [(b)]
Cellulose	35.51	43.4	40
Hemicellulose	13.78	26.3	27.7
Lignin	25.03	24.4	23.1
Ash	22.01	1.9	6.5

(a) Current study (b) From the review paper (NREL methods)

Table 4.5presents the main components of rice straw, which are 35.51% cellulose, 13.78% hemicellulose, 25.03% lignin and 22.01% ash. According to the component analysis, rice straw contained a relatively lower content of cellulose and hemicellulose with

higher ash content compared with other biomass e.g. sugarcane bagasse and corn stover. This results in a unique characteristic of rice straw in bioprocessing to produce fermentable sugars and other fermentation products.

4.6.2 Alkaline pretreatment of rice straw

Rice straw which is approximately 8 million tons/year in the country represents a by-product obtained from agriculture. It is an important under-utilized biomass making it a promising biomass for biorefineries. The rice straw in this study was physically processed and pretreated with 10% (w/v) NaOH at 80°C for 90 min at ratio 1 g per 3 ml liquid as described in the method section. The pretreated rice straw was washed with water until its pH decreased to neutral (7.0). The solid biomass fraction was then air-dried at 60ºC overnight and kept in air-tight plastic bags for further study.

Table 4.6 Composition of native rice straw and rice straw after pretreatment.

Component	% (w/w)	
	native rice straw	pretreated rice straw
Cellulose	35.51	74.31
Hemicellulose	13.78	13.40
Lignin	25.03	1.36
Ash	22.01	2.90

The pretreatment step led to a noticeable reduction in the lignin content and a substantial decrease in hemicelluloses, while the cellulose content markedly increase in the pretreated rice straw. The pretreated rice straw (Table 4.6) consisted of 74.31% cellulose, 13.61% hemicellulose, 1.36% lignin and 2.90% ash. In comparison with the chemical components, alkaline pretreatment increased the proportion of cellulose by 109.26% and decreased the proportion of hemicellulose and lignin by 2.76% and 94.55%, respectively. Substantial decrease in hemicellulose content was due to alkaline hydrolysis of the hemicelluloses fraction. Compared to other pretreatment methods previously reported for rice straw e.g. ammonia treatment, acid pretreatment, organic solvent pretreatment and steam pretreatment which showed various effects to chemical and physical characteristics of lignocellulosic biomass. Alkaline pretreatment has been considered as one of the most widely used pretreatment methods which could markedly enhance digestibility of lignocellulose. These combined effects thus provide the basis for increased susceptibility of the alkaline pretreated biomass to subsequent enzymatic hydrolysis.

4.6.3 Screening for commercial enzymes with activity complementing crude enzyme *C. globosum*BCC5776

In the first step, four commercial enzymes, including Accellerase® XY, Accellerase® 1500, Optimash® BG and Novozyme® 188,were selected to screen for their synergistic actions with the BCC5776 enzyme. These enzymes were selected based on their pre-screened cellulolytic and hemicellulolytic enzyme profiles, as shown in Table 4.7. All commercial enzymes were also determined in Table 4.8 for total protein content.

Table 4.7 The composition activity enzyme profiles of different commercial enzymes.

Name	IUPAC (U/ml)				
	FPase	CMCase	Xylanase	β-glucosidase	β-xylosidase
Accellerase® 1500	22.86	1157.41	277.78	62.09	28.64
Optimash® BG	0.27	1851.85	3174.60	112.71	36.93
Novozyme® 188	0.03	5.94	14.34	436.45	5.70
Accellerase® XY	0.10	78.80	1010.10	41.73	33.09

Table 4.8 Total protein content of differentcommercial enzymes.

Name	Dilution factor (D.F)	mg protein/ml
Accellerase® 1500	20	16.26
Optimash® BG	80	51.3
Novozyme® 188	80	45.22
Accellerase® XY	20	9.87

The first screen was performed by combining 0.29mg of proteins of each commercial enzymes with equal mg proteins of the BCC5776 crude enzyme extract produced in 5-L fermenter. The enzyme candidates were examined for the hydrolysis of the alkali-pretreated rice straw after 24 h. From the preliminary screening, different combinations of enzymes increased the reducing sugar yield of that obtained with an equal amount of the commercial enzymes and the BCC5776 used except Accellerase® 1500. A substantial increase in the reducing sugar yields was observed for the BCC5776 crude enzyme extract mixed with Accellerase® XY, Optimash® BG or Novozyme® 188, all of which showed more than a fold increase relative to the BCC5776 native non-concentrated enzyme or commercial enzymes alone at 0.58 mg protein (Fig. 4.14).

Increases in the enzyme hydrolysis efficiency were observed, leading to the final reducing sugar yields of 452.40–521.09 mg/g pretreated rice straw (Fig. 4.15). The enzyme

mixtures among the BCC5776,Accellerase® XY and Novozyme® 188 exhibited synergistic activity at enzyme loading equal to 3 FPU/g. *Chaetomium* has previously been reported to be a potent cellulolytic enzyme producer for the degradation of plant polysaccharides for biomass [83, 84]. The BCC5776 crude enzyme extracts were concentrated by ultra-filtration. The concentrated enzymes were equally diluted to 1 FPU/ml as an enzyme stock.

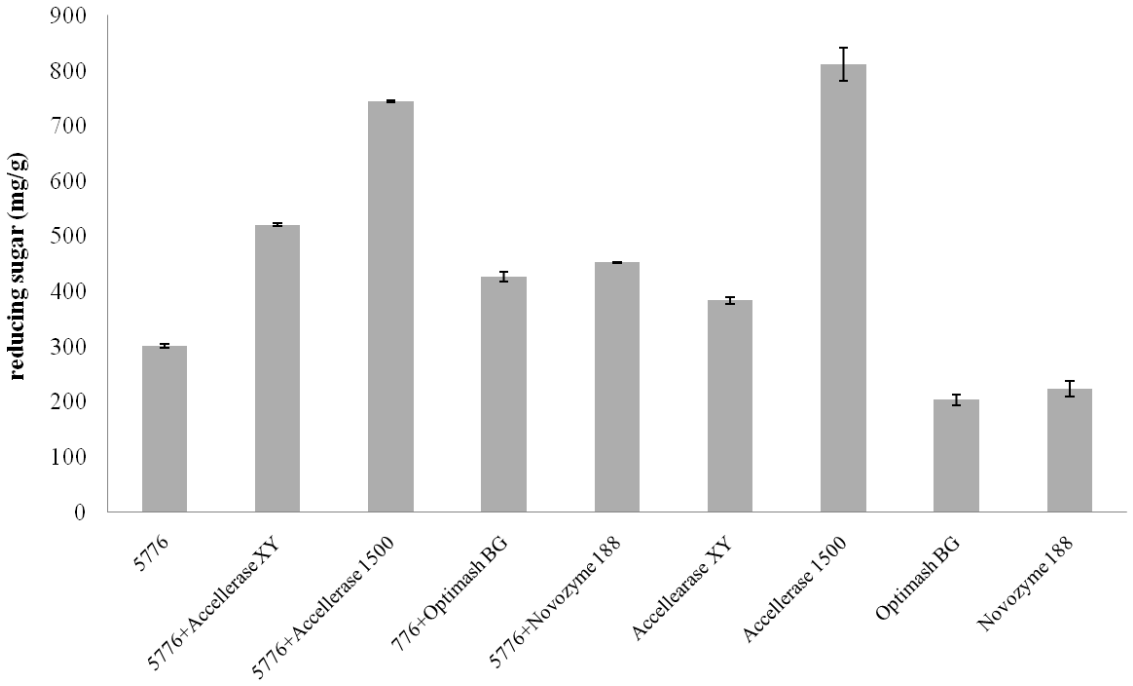

Figure 4.14 The primary screen of different combinations of commercial enzymes and crude fungal BCC5776 enzyme extracts. A total of 0.29 mg protein of the BCC5776 enzyme extracts and 0.29 mg proteins of different commercial enzymes were mixed for a total enzyme dosage of different FPU/g loading of pretreated rice straw. The commercial enzymes control reaction contained 0.58 mg protein.

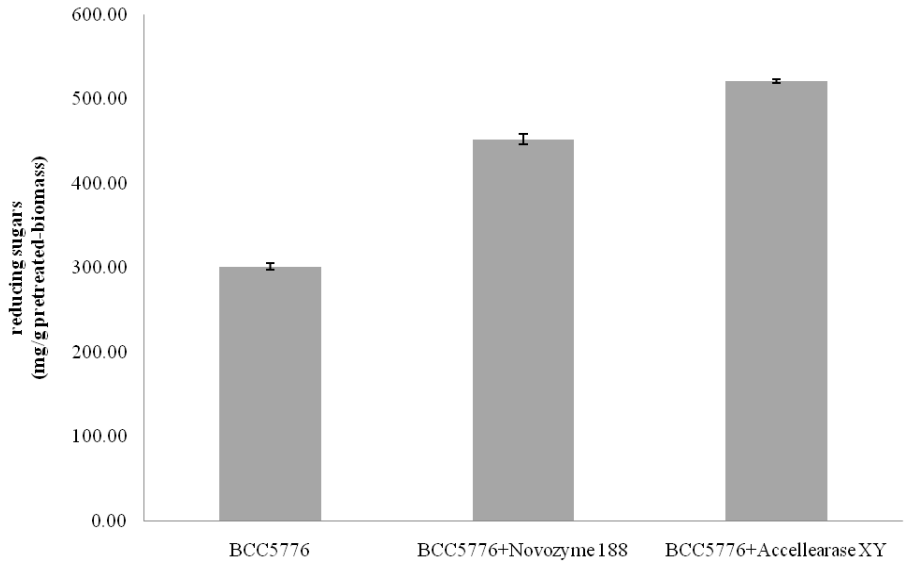

Figure 4.15 The synergistic effects of Novozyme® 188and Accellerase® XY on BCC5776 enzyme extract mixture on pretreated rice straw hydrolysis. The reactions contained 3 FPU/g of BCC5776 blend with the indicated amount of Novozyme®188 (6.41 µl) and Accellerase® XY (29.38µl). The reactions were incubated at 50 ºC for 24 h.

After the selection of synergistic commercial enzymes with BCC5776 native non-concentrated enzyme, each component of Accellerase® XY and Novozyme® 188 was further varied in the amount of enzyme dosage (U/g) in order to indicate the suitable amount for each enzyme. Accellerase® XY and Novozyme® 188 were varied the amount of enzyme loading in range of 500-2500 XU/g and 10-100 BU/g, respectively (Fig. 4.16). The results exhibited that Accellerase® XY enzyme loading at 1500 XU/g (74.25 µl) mixed with BCC5776 concentrated enzyme at 10 FPU/g (773.75 µl) gave the maximum reducing sugar yield of 555.29 mg/g pretreated rice straw. After increasing enzyme loading, the reducing sugar slightly decreased while Novozyme® 188 synergistic with BCC5776 concentrated enzyme at 25 BU/g (2.86 µl) provided the maximum reducing sugar yield of 516.73mg/g pretreated rice straw. The surplus of enzyme dosage was not necessary. Furthermore, the design of experiment (DOE) was used for synergistic activity studies among these enzymes including BCC5776 enzyme extract, Accellerase® XY and Novozyme® 188.

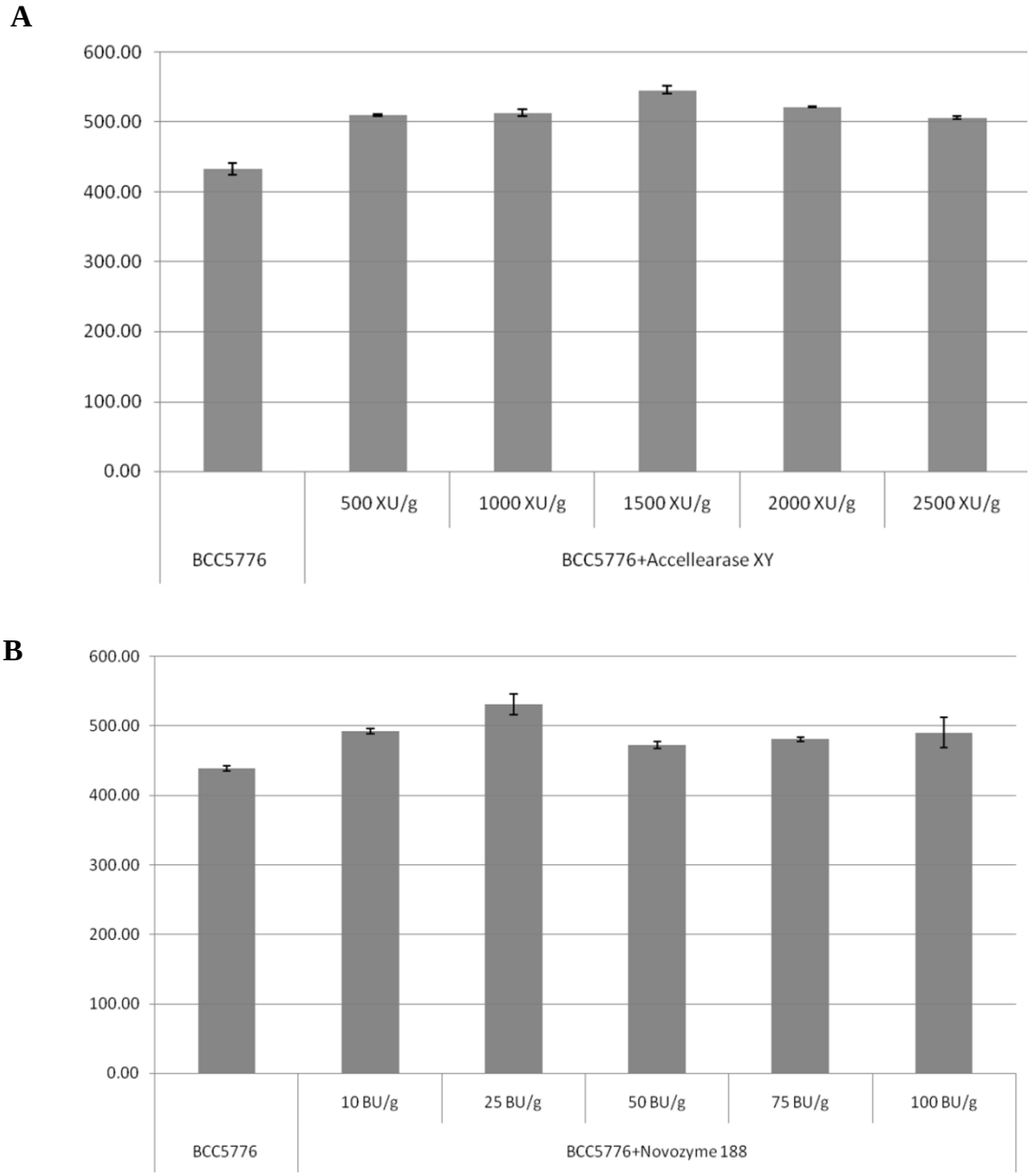

Figure 4.16 The synergistic effects of (A) Accellerase® XY and (B) Novozyme® 188 at different enzyme dosages on BCC5776 enzyme extract mixture in pretreated rice straw hydrolysis. The reactions contained 10 FPU/g of BCC5776 blend with varying amount of Accellerase® XY (500-2500 XU/g) and Novozyme®188 (10-100 BU/g). The reactions were incubated at 50 ºC for 24 h.

4.6.4 Optimization of the ternary enzyme mixture using a systematic mixture design

In order to optimize the BCC5776-Accellerase® XY-Novozyme® 188 composition for efficient saccharification of rice straw and to understand the interaction of each component in the hydrolysis process, a systematic approach of process optimization known

as mixture design was applied [55]. According to this method, the response is a function of the proportion of each component existing in the mixture. The total reducing sugar from the saccharification of rice straw was proposed to depend significantly on the proportion of the three components, i.e. the BCC5776 crude enzyme extract, Accellerase® XY, and Novozyme® 188. The {3,3}-simplex lattice experiment was created using the Minitab 16.0software. All 13 experimental points are described in Table 4.9. Each optimization experiment has a total component loading equal to 100%. However, it should be noted that although the amount of total component loading was 100%, the FPU loading/g substrate of the experimental points was different, depending on the amount of the cellulolytic components. Briefly, all experiments were performed in 1.5 ml tube with a reaction volume of 1 ml containing pretreated rice straw in 100 mM sodium acetate buffer, pH 5.5 hydrolyzed with total amount of enzyme mixtures equal to 400 µl at 50°C for 48 h. Each point was performed in quadruplicate to minimize experimental variation.

Table 4.9 Design of experiment for enzymatic hydrolysis of pretreated rice straw and the associated response data.

Run no.	%			Reducing sugar (mg/g pretreated rice straw)	
	BCC5776	Novozyme® 188	Accellerase® XY	Average	SD
1	100.00	0.00	0.00	500.89	2.61
2	66.67	33.33	0.00	493.08	2.19
3	66.67	0.00	33.33	525.40	0.50
4	33.33	66.67	0.00	421.22	4.25
5	33.33	33.33	33.33	535.56	2.60
6	33.33	0.00	66.67	519.30	3.22
7	0.00	100.00	0.00	69.19	3.37
8	0.00	66.67	33.33	205.27	4.11
9	0.00	33.33	66.67	233.27	4.58
10	0.00	0.00	100.00	204.95	4.89
11	66.67	16.67	16.67	567.76	1.72
12	16.67	66.67	16.67	426.06	1.43
13	16.67	16.67	66.67	483.53	2.13

After this optimization experiment, each desired product (reducing sugar) was then entered into the Minitab 16.0 software to analyze the experiments with mixtures and to determine the optimal conditions for maximizing reducing sugar using a statistical approach. According to Table 4.9,the reducing sugar of each experimental point after the

hydrolysis of pretreated rice straw for 48 h was illustrated. The standard deviation of all the data points was in a satisfy yield range(0–5%). When each individual component was considered, BCC5776 (No. 1) demonstrated a higher reducing sugar than Novozyme® 188 (No. 7) and Accellerase® XY (No. 10) since the composite enzyme activity profiles of both Novozyme® 188andAccellerase® XY showed deficient amount of the cellulolytic components.

The binary combinations No. 2 and 4exhibited that the synergistic action between BCC5776 and Novozyme® 188slightly decreased the reducing sugar compare to No. 1. In contrast to No.7, the reducing sugar obtained from No. 2 and No.4 significantly increased because the composite enzyme activity profile of Novozyme® 188 lacked cellulolytic activity particularly FPase which plays an important role in degradation of plant polysaccharides for biomass.

However, complete degradation of cellulose to glucose requires β-glucosidase (EC 3.2.1.21). These results indicate a positive interaction between BCC5776 and Novozyme® 188. The synergism among BCC5776, Novozyme® 188, and Accellerase® XY was investigated in the ternary combinations(No. 5 and No. 10–13). No.5 and No.11 showed an improvement in the reducing sugar compared to the binary mixture No.2-4 and No.6. Nevertheless, No.10 and No.12-13 presented a decrease in the reducing sugar relative to the binary mixture No.2-4 and No.6. The highest reducing sugar of 567.76 mg/g pretreated rice straw was obtained with the ternary mixture comprising a 4:1:1 ratio of BCC5776: Novozyme® 188:Accellerase® XY.

This result showed the enhancing effect amongBCC5776, Novozyme® 188, and Accellerase® XY when combined with the synergistic, ternary enzyme mixture on rice straw hydrolysis after 48 h, which resulted in an increased reducing sugar and consequently, a reduction of the core cellulolytic enzyme loading by 33% (No. 11). The response data for the reducing sugar were then analyzed sequentially using a multiple regression analysis from the linear to the full cubic model to find the fitted model. The full cubic model was observed to be the best fitted model for the reducing sugar (R^2 = 97.30%, $P_{Model} < 0.01$).

Table 4.10 The regression model analysis of the {3,3} full cubic model developed for the reducing sugar (mg/g pretreated biomass).

Factor	Coefficient	SE	T	*p*-value
BCC5776	482.7	14.3	*	*
Novozyme188	72	14.3	*	*
Accellerase XY	205	14.3	*	*
BCC5776*Novozyme188	786.6	63.94	12.3	0.000
BCC5776*Accellerase XY	845.1	63.94	13.22	0.000
Novozyme188*Accellerase XY	377.3	63.94	5.9	0.000
BCC5776*Novozyme188*Accellerase XY	2534.8	416.85	6.08	0.000
BCC5776*Novozyme188*(-)	-586.8	122.48	-4.79	0.000
BCC5776*Accellerase XY*(-)	-528.2	122.48	-4.31	0.000
Novozyme188*Accellerase XY*(-)	78.8	122.48	0.64	0.523
S = 28.6660	PRESS = 47389.9			
R^2 = 97.30%	R^2 (pred) = 96.29%		R^2 (adj) = 96.72%	

The statistically significant values of the full cubic model regression analysis are illustrated in Table 4.10. These coefficients gave understanding into ranking the importance of each component, and the interaction between pairs of components. For a single factor, the BCC5776,Novozyme® 188, Accellerase® XY coefficients presented a positive relation to the reducing sugar. For pairwise interactions, a robust synergism between BCC5776 and Novozyme® 188 or Accellerase® XY can be observed (786.6 and 845.1), while the small interaction between Novozyme® 188 and Accellerase® XY can also be investigated. The highest coefficient was observed for the BCC5776*Novozyme® 188*Accellerase® XY term. This indicated that an extreme interaction occurred among these three components. Based on the full cubic model, all of the factors were statistically significant ($p < 0.05$) except for the Novozyme188*Accellerase XY*(-) term. This unimportant term was then deleted from the equation. Finally, the reduced equation for the reducing sugar yield based on the component amount was:

Reducing sugar (mg/g) = 4.82685*BCC5776 + 0.72036*Novozyme® 188 + 2.04999*Accellerase® XY + 0.07866*BCC5776* Novozyme® 188 + 0.08451*BCC5776*Accellerase® XY + 0.03772*Novozyme® 188*Accellerase® XY + 0.00253*BCC5776*Novozyme® 188*Accellerase®XY(-) 0.00059*BCC5776*Novozyme®188(-) 0.00053*BCC5776*Accellerase®XY(-)

Upon obtaining the reduced equation for the full cubic model, the responses of the reducing sugar with respect to the various component combinations were clearly represented as ternary mixture contour plots (Fig. 4.17).In the graph, the area that emphasized the greatest reducing sugar was in the middle of the BCC5776 and Accellerase® XY axes, and near the bottom of the Novozyme®188vertex. This implies that a high level of reducing sugars could be achieved when an intensive amount of the BCC5776was consumed while a less amount ofNovozyme®188 and Accellerase® XY were nearly equal. To indicate the optimal formulation, the Minitab 16.0 software was used to predict the optimal combination points for maximizing the reducing sugar yield. The combination with the highest efficiency based on these criteria was determined to be 44.4% BCC5776, 20.6% Novozyme®188, and 35.0% Accellerase® XY with the predicted reducing sugar yield of 591.22 mg/g pretreated rice straw.

To validate this prediction, an experiment was conducted using these conditions. The experimental reducing sugar yield was 572.68 mg/g pretreated rice straw, which was in agreement with the predicted value. This result confirmed the effectiveness of this model. This work showed the use of experimental design for the optimization of efficient synergistic biomass-degrading enzyme systems for target lignocellulosic biomass substrates. The optimal ternary enzyme mixtures were then used in the subsequent experiments to enhance the reducing sugar yield.

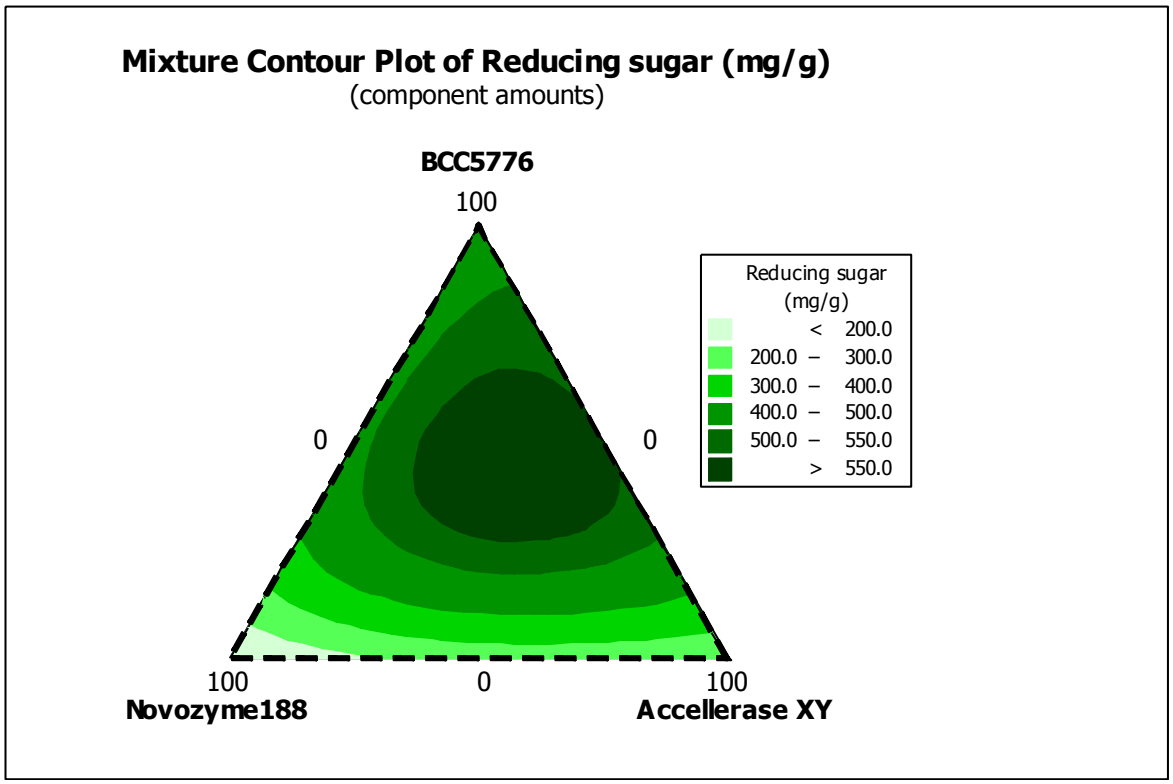

Figure 4.17 The ternary plots of the experimental design optimization of the ternary enzyme complex. The contour plot represented the different levels of reducing sugar. 100% component amount always equals to 400 µl.

The increase in the reducing sugar yield from the hydrolysis of alkali-pretreated rice straw was further investigated using optimal ternary mixtures compared with single components, as shown in Table 4.11. The total reducing sugar yield of the optimal ratio was approximately 572.68 mg/g pretreated rice straw (No.4) which higher than the single enzyme (No.1-3 and No.8). When increasing amounts of enzyme usage as 2 times (2x), the total reducing sugar yield of 642.75 mg/g pretreated rice straw (No.5) was obtained. To extend enzyme dosage of 3x and 4x, the BCC5776 concentrated enzyme extract which was diluted equivalent to 2 FPU/ml as enzyme stock before use was required. The highest total reducing sugar yield of approximately 764.69 mg/g pretreated rice straw (No.7) was observed with the ternary combination (4x).

These results were also examined using HPLC to categorize sugar components, including glucose, xylose and arabinose. Glucose derived from cellulose was the major product of the saccharification of rice straw in all of the enzyme combinations except Novozyme®188 and Accellerase® XY because their composite activity enzyme profiles play dominant β-glucosidase and xylanase additional with β-xylanase, respectively, while

all of them lack core cellulolytic group degrading cellulose. Pentoses (xylose and arabinose) were the major composite sugars from hemicellulose hydrolysis. Therefore, the use of single Accellerase® XY exhibited a high amount of xylose and arabinose. In conclusion, the ternary enzyme mixtures were able to improve glucose, xylose and arabinose yields from the hydrolysis reaction while the amount of each enzyme dosage was optimized to maintain synergistic activity of the enzyme components. Fig. 4.18 presents the total reducing sugar (No.5-7) derived from the saccharification of alkali-pretreated rice straw using optimal ratio in ternary combinations after 48 h. Increasing amount of enzyme dosage was relative to high FPU level in biomass hydrolysis reaction resulting in higher total reducing sugar.

Table 4.11 The composite sugar analysis of the hydrolysis products of the pretreated rice straw digested by various enzyme combinations at 48 h.

No.	Enzyme components	Total reducing sugar (mg/g)	Glucose (mg/g)	Xylose (mg/g)	Arabinose (mg/g)
1	BCC[a]	396.89	143.67	73.92	18.14
2	Novo.188[a]	49.07	19.76	16.62	12.68
3	Acc.XY[a]	173.61	20.50	68.41	74.37
4	BCC+Novo.188+Acc.XY (1x)[b]	572.68	332.50	114.14	68.27
5	BCC+Novo.188+Acc.XY (2x)[c]	642.57	383.27	127.11	87.32
6	BCC+Novo.188+Acc.XY (3x)[d]	703.64	423.30	131.89	100.77
7	BCC+Novo.188+Acc.XY (4x)[e]	764.69	474.84	153.84	116.27
8	Acc.1500[a]	520.18	423.26	81.36	3.65

[a]Abbreviations: BCC, BCC5776 (100% = 3.55 FPU/g); Novo.188, Novozyme® 188 (100% = 0.01 FPU/g); Acc.XY, Accellerase® XY (100% = 0.80 FPU/g); Acc.1500, Accellerase® 1500 (100% = 3.55 FPU/g).

[b-e] Ternary mixture at % optimal ratio (44.4:20.6:35.0)

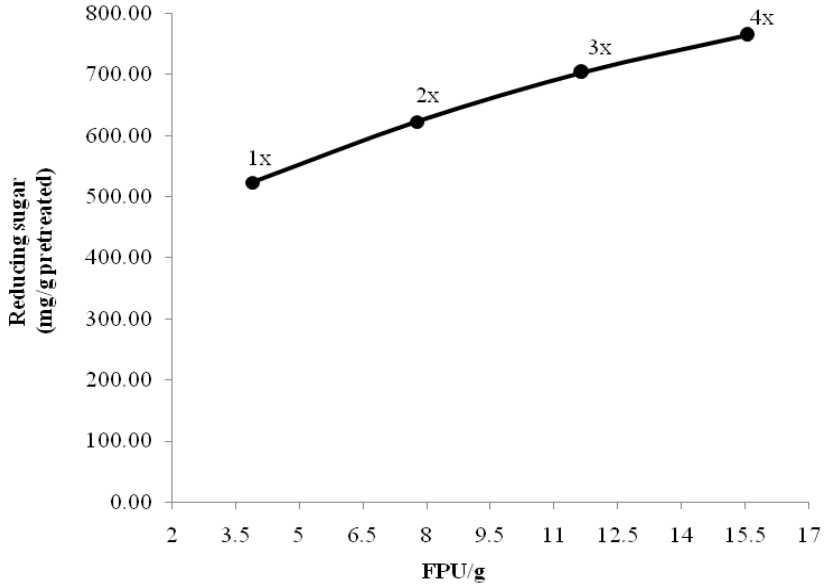

Figure 4.18 The total reducing sugars versus FPU loading/g in pretreated rice straw hydrolysis. The reactions contained different FPU loading/g pretreated rice straw of a 44.4:20.6:35.0 (optimum ratio) BCC5776:Novozyme® 188:Accellerase® XY enzyme extract. The reactions were incubated at 50 ºC for 48 h.

CHAPTER 5

CONCLUSION

In this study, an efficient fungal biomass-degrading enzyme mixture from *C. globosum* BCC5776 was developed in order to ameliorate cellulase production with the focus on filter paper cellulase activity (FPase). Many fermentation parameters including carbon sources, medium types, and nitrogen sources were studied sequentially using one factor at a time (OFAT) analysis. It was found that 4% (w/v) microcrystalline cellulose AVICEL® supplemented with 1% (w/v) soybean meal in distilled water can enhance cellulase activity to 0.253 FPU/ml. In addition, optimization of fermentation conditions was further studied using multivariate analysis. A total of 8 experimental run with different combinations of three factors i.e. concentration of AVICEL®, inducer, and initial pH and 1 experimental run as a control were studied.

The data showed that addition of 0.1% (v/v) lactose as an inducer, decrease of AVICEL® concentration to 2% (w/v) and control of initial pH to 5.8 can improve the cellulase yield. Under the optimized condition, the maximum cellulase yield was 0.322 FPU/ml which was higher than 55% of the control. Besides, addition of OPEFB for enhancing yield of cellulases was observed. The result showed that 1% (w/v) OPEFB as a co-substrate could increase the yield of cellulases to 0.400 FPU/ml. This optimal condition was used as the most efficient enzyme production. SmF process with high volumetric cellulolytic enzymes also yielded the maximum of 0.398 FPU/ml and 0.359 FPU/ml in 1-L Erlenmeyer flask and 5-L bioreactor, respectively.

This study could provide the basis for up-scaling the production of the volumetric enzyme in submerged fermentation process using inexpensive substrate. Furthermore, the development of hyper-producing mutant strains using ultraviolet (UV) radiation was also studied along with medium optimization. The mutants showed the improvement of cellulase production; however, the stability of the mutant strain need further improvement.

The alkaline pretreatment process of rice straw was used to improve the enzymatic digestibility of substrate by lignin removal to less than 2% wt in the pretreated biomass with partial hydrolysis of hemicelluloses. Synergistic enzyme system for the hydrolysis of alkaline pretreated rice straw was optimized based on the synergy of the BCC5776 crude enzyme extract with various commercial enzymes (Accellerase® 1500, Accellerase® XY,

Optimash® BG and Novozyme® 188). Among 4 commercial enzymes, Novozyme® 188 and Accellerase® XY exhibited the highest level of synergy with BCC5776. This synergy was based on their cellulolytic and hemicellulolytic enzymes activity profiles. Using the full cubic model, the optimal formulation of the enzyme mixture was predicted to the percentage of BCC5776:Novozyme® 188:Accellerase® XY = 44.4:20.6:35.0,which produced 572.68 mg reducing sugar/g pretreated rice straw. Higher total reducing sugar was obtained when the amount of enzyme dosage was increased to4x. The highest total reducing sugar was approximately 764.69 mg reducing sugar/g pretreated rice straw using enzyme loading equivalent to 15.53 FPU/g. This reducing sugar yield was corresponding to Suwannarangsee *et al.* (2012) [19] who reported that synergistic enzyme system for the hydrolysis of alkaline pretreated rice straw provided maximum yield of 769 mg reducing sugar/g biomass.

This work demonstrated the use of an efficient systematic approach for the design and optimization of a synergistic enzyme mixture comprising the local enzymes and complemented commercial enzymes for lignocellulosic degradation. The reducing sugar would be further employed as sugar feedstock for production of ethanol or commodity chemicals.

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APPENDIX A

Composition of biomass

Procedure Title: Determination of Structural Carbohydrates and Lignin in Biomass Laboratory Analytical Procedure

1. Introduction

1.1 Carbohydrates and lignin make up a major portion of biomass samples. These constituents must be measured as part of a comprehensive biomass analysis; Carbohydrates can be structural or non-structural. Structural carbohydrates are bound in the matrix of the biomass, while non-structural carbohydrates can be removed using extraction or washing. Lignin is a complex phenolic polymer.

1.2 Portions of this procedure are substantially similar to ASTM E1758-01 "Standard method for the Determination of Carbohydrates by HPLC.

1.3 This procedure is suitable for samples that do not contain extractives. This procedure uses a two-step acid hydrolysis to fractionate the biomass into forms that are more easily quantified. The lignin fractionates into both acid soluble and insoluble materials. The acid insoluble material may also include ash and protein, which must be accounted for during gravimetric analysis. The acid soluble lignin is measured by UV-Vis spectroscopy. During hydrolysis the polymeric carbohydrates are hydrolyzed into the monomeric forms, which are soluble in the hydrolysis liquid. They are then measured by HPLC. Protein may also partition into the liquid fraction. A measure of acetyl content is necessary for biomass containing hemicellulose with a xylan backbone, but not biomass containing a mannan backbone. Acetate is measured by HPLC.

2. Scope

2.1 This procedure is appropriate for extractives free biomass, which includes biomass that has been extracted using LAP "Determination of Extractives in Biomass", as well as process solids containing no extractives. Results are reported on an oven dry weight basis. Results may be reported on a received biomass basis or an extractives-free basis, depending on the type of biomass used. LAP "Preparation of Samples for Biomass Compositional Analysis" should be used prior to this procedure.

2.2 This procedure is appropriate for biomass containing the components listed throughout the procedure. Any biomass containing other interfering components not listed must be further investigated.

2.3 A measure of acetyl content is necessary for biomass containing hemicellulose with a xylan backbone, but not biomass containing a mannan backbone.

2.4 All analyses should be performed in accordance with an appropriate laboratory specific Quality Assurance Plan (QAP).

3. Terminology

3.*1 Oven dry weight (ODW)*: the weight of biomass mathematically corrected for the amount of moisture present in the sample at the time of weighing

3.2 *Prepared biomass*: biomass prepared according to LAP "Preparation of Samples for Biomass Compositional Analysis".

3.3 *Extractives free biomass* biomass after exhaustive water and ethanol extraction (refer to LAP "Determination of Extractives in Biomass").

3.4 *Acid insoluble lignin*: the residue remaining on a medium porosity filtering crucible after a two-step hydrolysis, with correction for acid insoluble ash and acid insoluble protein, if necessary.

3.5 *Structural carbohydrates*: Polymeric carbohydrates, namely cellulose and hemicellulose.

3.6 *Non-structural components*: Non-chemically bound components of biomass that include, but are not limited to, sucrose, nitrate/nitrites, protein, ash, chlorophyll, and waxes.

4. Significance and Use

4.1 This procedure is used, possibly in conjunction with other procedures, to determine the amount of structural carbohydrates and lignin in a solid biomass sample.

5. Interferences

5.1 This procedure has been optimized for the particle size range specified in LAP "Preparation of Samples for Biomass Compositional Analysis". Deviation to a smaller particle size may result in a low bias in carbohydrate content (and consequent high lignin bias) due to excessive carbohydrate degradation. Deviation to a larger particle size may

also result in a low bias in carbohydrate content (and consequently high lignin bias) due to incomplete hydrolysis of polymeric sugars to monomeric sugars.

5.2 Samples containing extractives are not suitable for this procedure. Extractives will partition irreproducibly, resulting in a high lignin bias.

5.3 Samples with ash content above 10 wt% may not be suitable for this procedure, as the sample may contain soil or other minerals that will interfere with appropriate acid concentrations and may catalyze side reactions.

5.4 Samples with moisture content above 10 wt% may not be suitable for this procedure, as the excess moisture will interfere with appropriate acid concentrations. Samples should be dried (air-dried or oven dried at less than 40 ºC) prior to this procedure.

5.5 Samples containing protein will bias the acid insoluble lignin high unless the protein is accounted for in the gravimetric determination of acid insoluble material. An independent nitrogen analysis is required to estimate the protein content of the residue. The protein estimate is then subtracted from the acid insoluble residue measurement. Physical separation of the acid insoluble protein from the acid insoluble lignin is beyond the scope of this procedure.

5.6 This procedure is not suitable for samples containing added acid, base, or catalyst.

5.7 Certain guard columns for carbohydrate quantification may cause artifact peaks. Individual carbohydrates should be run on new columns and guard columns to verify the absence of artifact peaks.

6. Apparatus

6.1 Analytical balance, accurate to 0.1 mg

6.2 Convection drying oven, with temperature control of 105 ± 3 ºC

6.3 Muffle furnace, equipped with a thermostat, set to 575 + 25 °C or equipped with optional ramping program

6.4 Water bath, set at 30 + 3 °C

6.5 Autoclave, suitable for autoclaving liquids, set to 121 + 3 °C

6.6 Filtration setup, equipped with a vacuum source and vacuum adaptors for crucibles

6.7 Desiccator containing desiccant

6.8 HPLC system equipped with refractive index detector and the following columns:

6.8.1 Shodex sugar SP0810 or Biorad Aminex HPX-87P column (or equivalent) with ionic form H+/CO3- deashing guard column

6.8.2 Biorad Aminex HPX-87H column (or equivalent) equipped with an appropriate guard column

6.9 UV-Visible spectrophotometer, diode array or single wavelength, with high purity quartz cuvettes of pathlength 1 cm

6.10Automatic burette, capable of dispensing 84.00 ml water, optional

7. Reagents and materials

7.1 Reagents

7.1.1 Sulfuric acid, 72% w/w (specific gravity 1.6338 at 20 ºC)- (also commercially available as a reagent for the determination of fluorine, from Fluka #00647)

7.1.2 Calcium carbonate, ACS reagent grade

7.1.3 Water, purified, 0.2 μm filtered

7.1.4 High purity standards : D-cellobiose, D(+)glucose, D(+)xylose, D(+)galactose, L(+)arabinose, and D(+)mannose

7.1.5 Second set of high purity standards, as listed above, from a different source (manufacturer or lot), to be used to prepare calibration verification standards (CVS)

7.2 Materials

7.2.1 QA standard, well characterized, such as a National Institute of Standards and Technology (NIST) biomass standard or another well characterized sample of similar composition to the samples being analyzed

7.2.2 Pressure tubes, minimum 90 ml capacity, glass, with screw on Teflon caps and O-ring seals (Ace glass # 8648-30 tube with #5845-47 plug, or equivalent)

7.2.3 Teflon stir rods sized to fit in pressure tubes and approximately 5 cm longer than pressure tubes

7.2.4 Filtering crucibles, 25 ml, porcelain, medium porosity, Coors #60531 or equivalent

7.2.5 Bottles, wide mouth, 50 ml

7.2.6 Filtration flasks, 250 ml

7.2.7 Erlenmeyer flasks, 50 ml

7.2.8 Adjustable pipettors, covering ranges of 0.02 to 5.00 ml and 84.00 ml

7.2.9 pH paper, range 4-9

7.2.10 Disposable syringes, 3 ml, fitted with 0.2 μm syringe filters

7.2.11 Autosampler vials with crimp top seals to fit

8. ES&H Considerations and Hazards

8.1 Sulfuric acid is corrosive and should be handled with care.

8.2 Use caution when handling hot pressure tubes after removal from the autoclave, as the pressurized tubes can cause an explosion hazard.

8.3 When placing crucibles in a furnace or removing them, use appropriate personal protective equipment, including heat resistant gloves.

8.4 Operate all equipment in accordance with the manual and NREL Safe Operating Procedures

8.5 Follow all applicable NREL chemical handling procedures

9. Sampling, Test Specimens and Test Units

9.1 Care must be taken to ensure that a representative sample is taken for analysis.

9.2 LAP "Preparation of Samples of Biomass Compositional Analysis" should be performed prior to this analysis. Samples must have a minimum total solid content of 85%.

9.3 LAP "Determination of Extractives in Biomass" should be performed prior to this analysis if extractives are present in the sample.

9.4 LAP "Determination of Solids in Biomass" should be performed at the same time that samples for this analysis are weighed out.

9.5 This procedure is suitable for samples that have been air dried or lyophilized. Samples dried at a temperature of 45°C or higher are not suitable for this procedure.

9.6 Steps 9.2 to 9.4 should be applied to the QA standard.

10. Procedure

10.1 Prepare the sample for analysis and hydrolysis.

10.1.1 Place an appropriate number of filtering crucibles in the muffle furnace at 575 +25 °C for a minimum of four hours. Remove the crucibles from the furnace directly into a desiccator and cool for a specific period of time, one hour is recommended. Weigh the crucibles to the nearest 0.1 mg and record this weight. It is important to keep the crucibles in a specified order, if they are not marked with identifiers. Permanent marking decals are available from the Wale Apparatus. Do not mark the bottom of the filtering crucible with a porcelain marker, as this will impede filtration.

10.1.2 Place the crucible back into the muffle furnace at 575 ± 25^{o}C and ash to constant weight. Constant weight is defined as less than ± 0.3 mg change in the weight upon one hour of re-heating the crucible.

10.1.3 Weigh 300.0 + 10.0 mg of the sample or QA standard into a tared pressure tube. Record the weight to the nearest 0.1 mg. Label the pressure tube with a permanent marker. LAP "Determination of Total Solids in Biomass" should be performed at the same time, to accurately measure the percent solids for correction. Each sample should be

analyzed in duplicate, at minimum. The recommended batch size is three to six samples and a QA standard, all run in duplicate.

10.1.4 Add 3.00 ml (or 4.92 + 0.01 g) of 72% sulfuric acid to each pressure tube. Use a Teflon stir rod to mix for one minute, or until the sample is thoroughly mixed.

10.1.5 Place the pressure tube in a water bath set at 30 + 3 °C and incubate the sample for 60 + 5 minutes. Using the stir rod, stir the sample every five to ten minutes without removing the sample from the bath. Stirring is essential to ensure even acid to particle contact and uniform hydrolysis. 0.01 ml (or 4.92 + 0.01 g) of 72% sulfuric acid to each pressure tube. Use a Teflon stir rod to mix for one minute, or until the sample is thoroughly mixed.

10.1.6 Upon completion of the 60-minute hydrolysis, remove the tubes from the water bath. Dilute the acid to a 4% concentration by adding 84.00 + 0.04 ml deionized water using an automatic burette.

Note: The volume of the 4% solution will be 86.73 ml, as demonstrated in the following calculations. 0.04 g of purified water using a balance accurate to 0.01 g. Screw the Teflon caps on securely. Mix the sample by inverting the tube several times to eliminate phase separation between high and low concentration acid layers.

Density 72% H_2SO_4= d72% H_2S0_4= 1.6338 g/ml

Density H_20= dH_20= 1.00 g/ml

Density 4% H_2SO_4 = d4% H_2SO_4 = 1.025 g/ml

1. The weight of 3.00 ml 72% H_2SO_4 is:3.00 ml 72% H_2SO_4 x d72% H_2S0_4= 4.90 g 72% H_2SO_4

2. The composition of 3 ml of 72% H2SO4 is:

4.90 g 72% H_2SO_4 x 72% (acid wt) = 3.53 g acid

4.90 g 72% H_2SO_4 x 28% (water wt) = 1.37 g water

3. The concentration of H_2SO_4 after dilution is:

3.53 g acid / (84.00 g H_2O+ 4.90 g 72% H_2SO_4) = 3.97 % H_2SO_4 (w/w)

4. The total volume of solution present after dilution is:

(4.90 g H_2SO_4 + 84.00 g H_20) x (d4% H_2S0_4)-1 = 86.73 ml

10.1.7 Prepare a set of sugar recovery standards (SRS) that will be taken through the remaining hydrolysis and used to correct for losses due to the destruction of sugars during dilute acid hydrolysis. SRS should include D-(+)glucose, D-(+)xylose, D-(+)galactose,L(+)arabinose, and D-(+)mannose. SRS sugar concentrations should be chosen to most closely resemble the concentrations of sugars in the test sample. Weigh out

the required amounts of each sugar, to the nearest 0.1 mg, and add 10.0 mL deionized water. Add 348 μL of 72% sulfuric acid. Transfer the SRS to a pressure tube and cap tightly.

10.1.7.1 A fresh SRS is not required for every analysis. A large batch of sugar recovery standards may be produced, filtered through 0.2 μm filters, dispensed in 10.0 mL aliquots into sealed containers, and labeled. They may be stored in a freezer and removed when needed. Thaw and vortex the frozen SRS prior to use. If frozen SRS are used, the appropriate amount of acid must be added to the thawed sample and vortexed prior to transferring to a pressure tube.

10.1.8 Place the tubes in an autoclave safe rack, and place the rack in the autoclave. Autoclave the sealed samples and sugar recovery standards for one hour at 121°C, usually the liquids setting. After completion of the autoclave cycle, allow the hydrolyzates to slowly cool to near room temperature before removing the caps. (If Step 10.2 is not performed, draw a 10 ml aliquot of the liquor for use in Step 10.5.)

10.2 Analyze the sample for acid insoluble lignin as follows

10.2.1 Vacuum filters the autoclaved hydrolysis solution through one of the previously weighed filtering crucibles. Capture the filtrate in a filtering flask.

10.2.2 Transfer an aliquot, approximately 50 ml, into a sample storage bottle. This sample will be used to determine acid soluble lignin as well as carbohydrates, and acetyl if necessary. Acid soluble lignin determination must be done within six hours of hydrolysis. If the hydrolysis liquor must be stored, it should be stored in a refrigerator for a maximum of two weeks. It is important to collect the liquor aliquot before proceeding to Step 10.2.3.

10.2.3 Use deionized waters to quantatively transfer all remaining solids out of the pressure tube into the filtering crucible. Rinse the solids with a minimum of 50 ml fresh deionized water. Hot deionized water may be used in place of room temperature water to decrease the filtration time.

10.2.4 Dry the crucible and acid insoluble residue at 105 + 3°C until a constant weight is achieved, usually a minimum of four hours.

10.2.5 Remove the samples from the oven and cool in a desiccator. Record the weight of the crucible and dry residue to the nearest 0.1 mg.

10.2.6 Place the crucibles and residue in the muffle furnace at 575 + 25°C for 24 hours.

10.2.6.1 A furnace with temperature ramping may also be used

Furnace Temperature Ramp Program:

Ramp from room temperature to 105°C
Hold at 105°C for 12 minutes
Ramp to 250 °C at 10°C / minute
Hold at 250 °C for 30 minutes
Ramp to 575 °C at 20°C / minute
Hold at 575 °C for 180 minutes
Allow temperature to drop to 105°C
Hold at 105 °C until samples are removed

10.2.7 Carefully remove the crucible from the furnace directly into a desiccator and cool for a specific amount of time, equal to the initial cool time of the crucibles. Weigh the crucibles and ash to the nearest 0.1 mg and record the weight. Place the crucibles back in the furnace and ash to a constant weight. (The amount of acid insoluble ash is not equal to the total amount of ash in the biomass sample. Refer to LAP "Determination of Ash in Biomass" if total ash is to be determined.)

10.3 Analyze the sample for acid soluble lignin as follows

10.3.1 On a UV-Visible spectrophotometer, run a background of deionized water or 4% sulfuric acid.

10.3.2 Using the hydrolysis liquor aliquot obtained in Step 10.2.2, measure the absorbance of the sample at an appropriate wavelength on a UV-Visible spectrophotometer. Refer to Section11.3 for suggested wavelength values. Dilute the sample as necessary to bring the absorbance into the range of 0.7 – 1.0, recording the dilution. Deionized water or 4% sulfuric acid may be used to dilute the sample, but the same solvent should be used as a blank. Record the absorbance to three decimal places. Reproducibility should be + 0.05 absorbance units. Analyze each sample in duplicate, at minimum. (This step must be done within six hours of hydrolysis.)

10.3.3 Calculate the amount of acid soluble lignin present using calculation 11.3.

10.4 Analyze the sample for structural carbohydrates

10.4.1 Prepare a series of calibration standards containing the compounds that are to be quantified, referring to Table 1 for suggested concentration range. Use a four point calibration. If standards are prepared outside of the suggested ranges, the new range for these calibration curves must be validated.

10.4.1.1 Table 1- Suggested concentration ranges for 10.4.1 calibration standards Component

Component	Suggested concentration range (mg/ml)
D-cellobiose	0.1-4.0
D(+)glucose	0.1-4.0
D(+)xylose	0.1-4.0
D(+)galcatose	0.1-4.0
L(+)arabinose	0.1-4.0
D(+)mannose	0.1-4.0
CVS	Middle of linear range, concentration not equal to a calibration point (2.5 suggested)

10.4.1.2 A fresh set of standards is not required for every analysis. A large batch of standards may be produced, filtered through 0.2 μm filters into autosampler vials, sealed and labeled. The standards and CVS samples may be stored in a freezer and removed when needed. Thaw and vortex frozen standards prior to use. During every use, standards and CVS samples should be observed for unusual concentration behavior. Unusual concentrations may mean that the samples are compromised or volatile components have been lost. Assuming sufficient volume, standards and CVS samples should not have more than 12 injections drawn from a single vial. In a chilled autosampler chamber, the lifetime of standards and CVS samples is approximately three to four days.

10.4.2 Prepare an independent calibration verification standard (CVS) for each set of calibration standards. Use reagents from a source or lot other than that used in preparing the calibration standards. Prepare the CVS at a concentration that falls in the middle of the validated range of the calibration curve. The CVS should be analyzed on the HPLC after each calibration set and at regular intervals throughout the sequence, bracketing groups of samples. The CVS is used to verify the quality and stability of the calibration curve(s) throughout the run.

10.4.3 Using the hydrolysis liquor obtained in Step 10.2.2, transfer an approximately 20 ml aliquot of each liquor to a 50 ml Erlenmeyer flask.

10.4.4 Use calcium carbonate to neutralize each sample to pH 5 – 6. Avoid neutralizing to a pH greater than 6 by monitoring with pH paper. Add the calcium carbonate slowly after reaching a pH of 4. Swirl the sample frequently. After reaching pH 5 – 6, stop calcium carbonate addition, allow the sample to settle, and decant off the

supernatant. The pH of the liquid after settling will be approximately 7. (Samples should never be allowed to exceed a pH of 9, as this will result in a loss of sugars.)

10.4.5 Prepare the sample for HPLC analysis by passing the decanted liquid through a 0.2 μm filter into an autosampler vial. Seal and label the vial. Prepare each sample in duplicate, reserving one of the duplicates for analysis later if necessary. If necessary, neutralized samples may be stored in the refrigerator for three or four days. After this time, the samples should be considered compromised due to potential microbial growth. After cold storage, check the samples for the presence of a precipitate. Samples containing a precipitate should be refiltered, while still cold, through a 0.2 μm filter.

10.4.6 Analyze the calibration standards, CVS, and samples by HPLC using a Shodex sugar SP0810 or Biorad Aminex HPX-87P column equipped with the appropriate guard column.

HPLC conditions:

Injection volume: 10 – 50 μl, dependent on concentration and detector limits

Mobile phase: HPLC grade water, 0.2 μm filtered and degassed

Flow rate: 0.6 ml / minute

Column temperature: 80 - 85°C

Detector temperature: as close to column temperature as possible

Detector: refractive index

Run time: 35 minutes

Note: The de-ashing guard column should be placed outside of the heating unit and kept at ambient temperature. This will prevent artifact peaks in the chromatogram.

10.4.7 Check test sample chromatograms for presence of cellobiose and oligomeric sugars. Levels of cellobiose greater than 3 mg/ml indicate incomplete hydrolysis. Fresh samples should be hydrolyzed and analyzed.

10.4.8 Check test sample chromatograms for the presence of peaks eluting before cellobiose (retention time of 4-5 minutes using recommended conditions). These peaks may indicate high levels of sugar degradation products in the previous sample, which is indicative of over-hydrolysis. All samples from batches showing evidence of over-hydrolysis should have fresh samples hydrolyzed and analyzed.

10.5 Analyze the sample for acetyl content if necessary

10.5.1 Prepare 0.005 M (0.01 N) sulfuric acid for use as a HPLC mobile phase. In a 2L volumetric flask, add 2.00 mlof standardized 10 N sulfuric acid and bring to volume with HPLC grade water. Filter through a 0.2 μm filter and degas before use. If 10N sulfuric

acid is not available, concentrated sulfuric acid may also be used. 278 µl concentrated sulfuric acid brought to volume in a 1-L volumetric flask with HPLC grade water will also produce 0.005 M sulfuric acid.

10.5.2 Prepare a series of calibration standards containing the compounds that are to be quantified. Acetic acid is recommended, formic acid and levulinic acid are optional. A range of 0.02 to 0.5 mg/ml is suggested. An evenly spaced four point calibration is suggested. If standards are prepared outside of the suggested ranges, the new range for these calibration curves must be validated.

10.5.3 Prepare an independent calibration verification standard (CVS) for each set of calibration standards, using components obtained from a source other than that used in preparing the calibration standards. The CVS must contain precisely known amounts of each compound contained in the calibration standards, at a concentration that falls in the middle of the validated range of the calibration curve. The CVS should be analyzed on the HPLC after each calibration set and at regular intervals throughout the sequence, bracketing groups of samples. The CVS is used to verify the quality and stability of the calibration curve(s) throughout the run.

10.5.4 Prepare the sample for HPLC analysis by passing a small aliquot of the liquor collected in step 10.2.2 through a 0.2 µm filter into an autosampler vial. Seal and label the vial. If it is suspected that the sample concentrations may exceed the calibration range, dilute the samples as needed, recording the dilution. The concentrations should be corrected for dilution after running.

10.5.5 Analyze the calibration standards, CVS, and samples by HPLC using a Biorad Aminex HPX-87H column equipped with the appropriate guard column.

HPLC conditions:

Sample volume: 50 µl

Mobile phase: 0.005 M sulfuric acid, 0.2 µm filtered and degassed

Flow rate: 0.6 ml / minute

Column temperature: 55 -65°C

Detector temperature: as close to column temperature as possible

Detector: refractive index

Run time: 50 minutes

APPENDIX B

Alkaline pretreatment

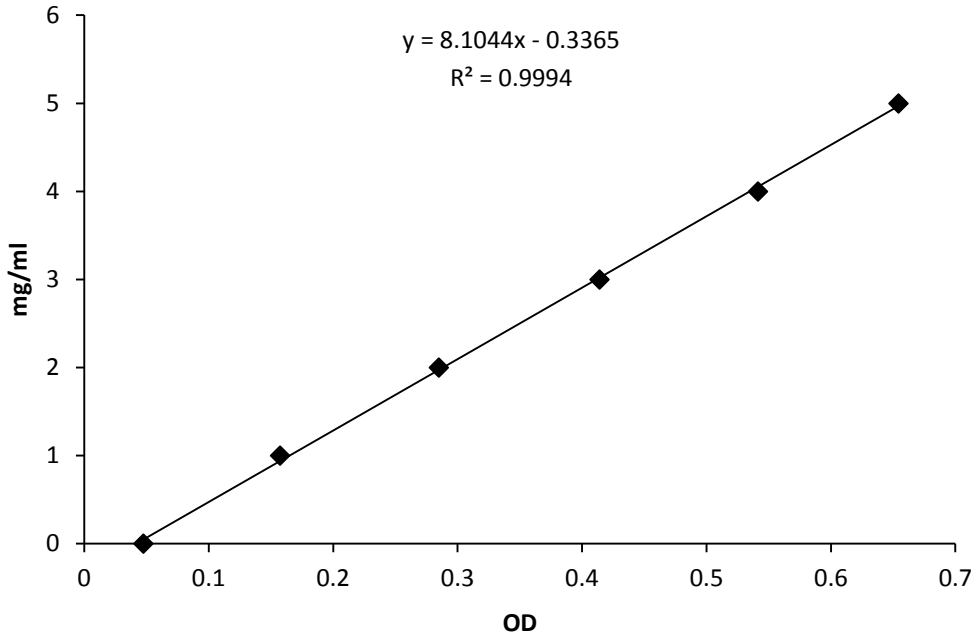

Figure A.1. Standard curve for analysis of the concentration of reducing sugar with DNS method (OD 560 nm)

Sample	Dry weight (g)	DF	blank	mg	OD540 mg/ml	mg/g sub
1	0.0516	10	0.046	0.016	33.5546	33.5706
2	0.0545	10	0.046	0.016	35.423	35.439
3	0.0541	10	0.046	0.016	35.1171	35.1331