THESIS APPROVAL

GRADUATE SCHOOL, KASETSART UNIVERSITY

Doctor of Philosophy (Biotechnology)

DEGREE

Biotechnology | Biotechnology

FIELD | **DEPARTMENT**

TITLE: Investigation on Suitable Pretreated Agricultural By-Products for Cellulase Production and Regulation of Cellulase Expression

NAME: Miss Nattawadee Jantasila

THIS THESIS HAS BEEN ACCEPTED BY

THESIS ADVISOR

(Associate Professor Sunee Nitisinprasert, D.Sc.)

THESIS CO-ADVISOR

(Mr. Keith Filer, Ph.D.)

DEPARTMENT HEAD

(Assistant Professor Suttipun Keawsompong, Ph.D.)

APPROVED BY THE GRADUATE SCHOOL ON _____

DEAN

(Associate Professor Gunjana Theeragool, D.Agr.)

THESIS

INVESTIGATION ON SUITABLE PRETREATED AGRICULTURAL BY-PRODUCTS FOR CELLULASE PRODUCTION AND REGULATION OF CELLULASE EXPRESSION

NATTAWADEE JANTASILA

A Thesis Submitted in Partial Fulfillment of
the Requirements for the Degree of
Doctor of Philosophy (Biotechnology)
Graduate School, Kasetsart University
2012

Nattawadee Jantasila 2012: Investigation on Suitable Pretreated Agricultural By-Products for Cellulase Production and Regulation of Cellulase Expression. Doctor of Philosophy (Biotechnology), Major Field: Biotechnology, Department of Biotechnology. Thesis Advisor: Associate Professor Sunee Nitisinprasert, D.Sc. 150 pages.

Three cheap agricultural waste sources in Thailand of rough rice bran (RRB), palm kernel meal (PKM) and cassava pulp (CP) containing lignocellulose were aimed to investigate the suitable pretreatment for production of cellulolytic enzymes from *Aspergillus niger* 386017M1 by solid state fermentation process (SSF). Comparing three different pretreatments of acid (AC), alkaline (AKL) and steam techniques (STE), the suitable pretreatment of RRB and PKM were STE providing the increasing cellobiohydrolase (CBH) activities of 6,244 and 1,626 units/g, and beta–glucosidase (BG) activities of 28,485 and 26,869 units/g, respectively. While the AC pretreatment was suitable for CP having CBH and BG activities of 7,303 and 9,349 units/g, respectively. The maximum growth of *A. niger* in solid state fermentation of both the control and suitable pretreated RRB, PKM and CP were 5 day with the pH of around 4–5. Comparing to the control, the CBH activities from SSF of STE–pretreated–PKM and AC–pretreated–CP significantly increased to 135 – 163 and 453 – 698 folds, respectively while the one of STE–pretreated–RBB was not significantly different. By chemical analysis, the lignin contents of STE–pretreated–RBB, STE–pretreated–PKM and AC–pretreated–CP were mostly reduced for 1.5, 1.4 and 1.3 folds of the control while the cellulose contents increased for 2.0, 3.2 and 1.6, respectively. This was emphasized by the Scanning Electron Microscope that showed the increasing of rough surface, open cell wall structure in all pretreated materials.

The acid pretreated CP was choosed to study in cellulase gene expression. The maximum CBH activities of 7,599 units/g substrates and growth of 113 ng/g were obtained from 5 d cultivation at pH 3.5. The expression of *cbhA* and *cbhB* were regulated by *xlnR*, *creA*, *pacC* and *ace2* which were measured at transcription level by qRT–PCR. Interestingly, the strong expression of *cbhB* was due to low catabolic repression of *creA* while the one of *cbhA* was to high expression of *xlnR* and *ace2*. The *pacC* gene repressed the alkaline–expressed gene of fungal during acid pH of SSF system. Obviously, activator elements of galactose and arabinose played a key important role in expression of *cbhB* to improve CBH production. This result allows direct studies of the novel evolution of cellulase mechanisms control and provides opportunities to obtain a broader perspective on the logic of regulatory circuits of *A. niger* on acid pretreated cassava waste.

_____ _____ ___ / ___ / ___

Student's signature Thesis Advisor's signature

ACKNOWLEDGEMENTS

I would like to express my special sincere gratitude and appreciation to my thesis advisor Associate Professor Dr. Sunee Nitisinprasert for her kindness, valuable guidance, attentive interest, understanding and support throughout the course of this research which enable me to complete this dissertation.

I am very grateful to express my special sincere gratitude appreciation to my major subject committee Assistant Profesor Dr. Suttipun Kaewsompong for his valuable suggestion, comment and assistance were essential for the completion of this thesis.

I would like to express my special deeply thankfulness to my co-advisor Dr. Keith Filer and Alltech Biotechnoloty Corp., Ltd for giving me an opportunity for studying in doctoral program and support throughout the financial support. My thanks are also express to the members of AP-Biosciences Centre, Alltech, Thailand for their great help and kindly support.

Special thanks also express to Dr. Sheena Fagan and members of European Biosciences Centre, Alltech, Ireland, who kindly acknowledge me for all about of qRT-PCR techniques and the very kindness support and kindly taking care of me during training abroad.

My thanks is extended to all graduate students, members of Biotechnology, KU, Thailand and members of BIOTEC, NSTDA, Thailand for their great helps and wonderful friendship.

Special appreciation I would like to express to my parents, brother and friends for their love and everything of my life.

Nattawadee Jantasila

April 2012

TABLE OF CONTENTS

LIST OF TABLES

LIST OF TABLES (Continued)

LIST OF TABLES (Continued)

Appendix Table **Page**

LIST OF TABLES (Continued)

Appendix Table **Page**

LIST OF FIGURES

LIST OF FIGURES (Continued)

LIST OF FIGURES (Continued)

INVESTIGATION ON SUITABLE PRETREATED AGRICULTURAL BY-PRODUCTS FOR CELLULASE PRODUCTION AND REGULATION OF CELLULASE EXPRESSION

INTRODUCTION

About 60-80% of the meat production cost was from animal feeding (Arana, 2007). The best way to lower feed cost is to find the cheap feed stuff sources which can be by products of food industry or agricultural wastes due to the huge amount left each year. In Thailand, one million tons of either palm kernel meal (PKM) or cassava pulp (CP) is generated annually. They both contained high level of non–starch polysaccharides (NSP) like cellulose and hemicelluose (Khempaka *et al.,* 2009). Another abundant agricultural by product which is available in Thailand and other Asian country is rough rice bran (RRB) which is from milling process and contains 50% or more of intact un–hulled rice seed in primary polishing called paddy kernels (USDA, 2009). It mainly contained 28–36% cellulose and 34–45% other crude fiber including hemicelluloses and lignin which cause low digestibility (Kodali and Pogaku, 2006). While PKM, being a by-product from the manufacturing of palm kernel oil by the mechanical extraction of palm kernels, contains 20–30% cellulose, 6% hemicelluloses and 2% lignin (Bono *et al*., 2009). The CP is the fibrous residual material generated from the separation process of cassava tuber into starch granule and fibrous. It consists of 20% cellulose, 19% hemicelluloses and 2–7% lignin (Fang *et al*., 2000; Saha, 2003; Howad, 2003; Kosugi *et al*., 2009; Khempaka *et al*., 2009; Wanrosli *et al*., 2011). These NSP can actually be used as feed or a carbon source for microbial cultivation. However, its limitation was the protective coat lignin rendering both cellulose and hemicellulose hydrolysis **(**Choct, 1997). Therefore, the suitable pretreatment and effective hydrolytic reaction is required to obtain valuable energy sources from those fiber sources. The challenging of pretreated materials, RRB, PKM and CP as the alternative media substrate to enhance cellulolytic enzyme production were attractive. Recently, there were a few reports relating to pretreatments of RRB, PKM or CP for cellulolytic enzyme production. While the demand of cellulases has increased tremendously in animal feed industry. To increase the yield of cellulase, both effective cellulase producing microorganisms and fermentation process are

considered. Solid state fermentation offers a low cost alternative for producing cellulases using natural polymers from agroindustrial residues (Raimbault, 1998). A number of fungal strains have been evaluated for enzymatic hydrolysis of lignocellulose. So far the cellulolytic enzymes system of *Aspergillus niger* was well known as a powerful fungus to produce several hydrolytic enzymes for lignocellulosic materials degradation, especially, cellulose. Its cellulase system consisted of three classes of endoglucanase (EC 3.2.1.4), cellobiohydrolase (EC3.2.1.91) and β–glucosidase (EC 3.2.1.21) synergistically converting complex carbohydrates which presented in lignocellulosic biomass into glucose (Aro and Penttila, 2005). However, *A. niger* can produce poisonous toxins spore (Blumenthal, 2004). In solid state fermentation, spore forming concerned to health problem. The health effects of *A. niger* include fever, chronic pulmonary, hearing problems and hearing loss (Bennett and Klich, 2003). *A. niger* 386017M1 isolated by Alltech Inc. (USA) is one of the most effective strain producing fiber digestion enzyme for animal feed industry. This strain did not produce spore during 10 day of solid state fermentation (Unpublished data). In addition, it contained 7 powerful complex enzymes of cellulase, xylanase, pectinase, β-glucanase, phytase, protease and α–amylase essential for fiber degradation by solid state fermentation (SSF) (Cheng *et al*., 2005; Min, 2009; Hooge *et al*., 2010; Mahai *et al*., 2010).

Two CBH of *A. niger* were *cbhA* and *cbhB* regulated by *creA, areA, pacC, xlnR, ace2, ace1* and HAP protein by expressing upon key role of the environment such as carbon, nitrogen, and pH (Seiboth *et al*., 1997; Murray *et al*., 2003; Shin, 2003; Tian–Hong, 2004; Ti, 2008). The understanding of cellulase gene expression is interesting to improve the cellulase production approach which may consequently reduce the investment cost. Therefore, this study aimed to investigate (i) the suitable pretreatments method of RRB, CP and PKM used for their SSF by an effective *A. niger* 386017M1 in order to achieve cellulase production and (ii) regulation of *cbhA* as well as *cbhB* to understand cellulase production mechanism.

OBJECTIVES

The objectives of this study are:

1. To investigate the suitable pretreatment of rough rice bran, plam kernel meal and cassava pulp for cellulase production from *A. niger* 386017M1 by solid state fermentation

2. To understand cellulase regulation system at transcription level by suitable pretreated–material used as carbon source for SSF system

LITERATURE REVIEW

1. Plant cell wall

Plant cell wall from various sources consist of cellulose, hemicelluloses, pectin and the phenolic polymer lignin (Table 1). Cellulose is the most abundant polysaccharide in nature especially in plant cell wall, providing its rigidity. Cellulose consists of β–1, 4 linked D–glucose units to form linear polymeric chains of about 8,000–12,000 glucose units. In crystalline cellulose, these polymeric chains are packed together by hydrogen bonds to form highly insoluble structures. (Aro and Penttila, 2005)

The fiber component of the grain consists of non-starch polysaccharides (NSP) which cover a large variety of polysaccharide molecules excluding α–glucans (starch) found as cell wall constitution of cereal grain. Based on the methodology of polysaccharide extraction, the NSP was classified into three main groups as namely cellulose, non–cellulosic polymer (pentosan, galactan, manans, xyoglucan) and pectic polysaccharides (polygalacturonic acids) (Choct, 1997).

2. Plant cell wall polysaccharides

A wide variety of polysaccharides occur in plants and can be classified according to their molecular characteristics (type, number, bonding and sequence of monosaccharides), physicochemical characteristics (water solubility, viscosity, surface activity) and nutritional function (e.g. digestible or non-digestible). Most of polysaccharides contain somewhere between 100 and several thousand monosaccharides. Some polysaccharides contain the same kind of monosaccharide (homopolysaccharides), whereas others contain a mixture of different kinds of monosaccharide (heteropolysaccharides). Some polysaccharides exist as linear chains, others exist as branched chains. Some polysaccharides can be digested to form an important source of energy like starch, while others are indigestible source such as non–starch: cellulose, hemicellulose and pectins known as dietary fiber of non–starch polysaccharide (NSP) and lignin

.

Table 1 Lignocellulose contents of common agricultural residues and wastes

Lignocellulosic material	Cellulose content (%)	Hemicellulose(%)	Lignin(%)
Hardwood stems	40-55	24-40	18-25
Softwood stems	45-50	25-30	25-35
Nut shells	25-30	25-30	30-40
Corn cobs	45	35	15
Wheat straw	30	50	15
Leaves	15-20	80-85	0
Rice straw	32.1	24	18
Cotton seeds hairs	80-95	5-20	0
Fresh bagasse	33.4	30	18.9
Waste paper	60-70	10-20	5-10
Grasses	25-40	25-50	10-30
Primary wastewater solids	8-15	NA[1]	24-29
Rice bran	39	13	6
Rough rice bran	28-36	13	16
Cassava pulp	20	18.21	6.95
Palm kernel meal	12	6	2

[1] NA = not available

Source: Howard *et al.* (2003)

NSP composed of various polysaccharide molecules excluding α-glucans (starch). The classification of NSP is based on the methodology used for extraction and isolation of polysaccharides. After alkaline extractions of cell wall materials, the residue remaining is called cellulose and the fraction of this residue solubilized by alkaline is called hemicellulose (Table 2). Fractions of NSP, such as cellulose, non-cellulosic and pectic polysaccharides can be separated by using sequential extraction and hydrolysis methods (Choct, 1997).

Table 2 Chemical properties of soluble NSP of cereal bran, tropioca root and palm

%NSP	Wheat bran	Rice bran	Cassava	Palm kernel
Arabinose	2.6	9	1.5	NA[1]
Xylose	5.2	27	4.5	NA[1]
Mannose	0.4	NA[1]	2.0	58
Galactose	0.5	31	9.7	NA[1]
Glucose	3.5	32	38.0	NA[1]

NA[1] = not available

Source: Hogberg and Lindberg (1995)

2.1 Hemicelluloses (Kennedy, 1988)

Hemicelluloses are ranked as the second most abundant (after cellulose) natural organic chemical, being present in all layers of the plant cell wall, where they occur closely associated with cellulose and lignin. The majority of the hemicelluloses are relatively small molecules consisting of between 50–2000 monosaccharide residues, whilst those from hard woods are larger molecules (150–200 residues). Some of these compounds have crystalline structures and it has been found that the backbone of the molecule of the D–xylan consists of D–xylopyranosyl residues with a rotation of 120° between successive residues.

2.1.1 L–Arabino–D–galactans: A group of highly branched molecules which can be isolated from maritime pine, sycamore and cultured tobacco cells. The main 1,3,-β-D-galactan chain is substituted at O-6 with short 1,6,-β-D-galactan side-chains to which may be attached, via O-3, L-arabinofuranosyl residues. Minor amounts of L-rhamnose, D-glucuronic acid, and D-xylose have also been detected (Kennedy, 1988).

2.1.2 L–Arabino–D–xylan: L–Arabino–D–xylans are a group of neutral polysaccharides, which occur in association with acidic polysaccharides, and come from cereal gums especially wheat bran and rice bran. These are highly branched polysaccharides in which

the β–D–xylan chains are 1, 4–linked and to which are attached, in an irregular manner, single α–L–arabinofuranosyl groups via 1,3–links hemicellulose in wheat flour. The polysaccharide isolated from cress seeds has been found to contain an L–arabino–D–xylan comprising chains of 1,5–linked α–L–arabinofuranosyl residues to which are attached L–arabinosyl and D–xylosyl residues (O'Sullivan, 1997).

2.1.3 D–mannans: D–galacto–D–mannans and D–gluco–D–mannans are a common constituent of plant polysaccharides, occurring in homopolysaccharides, or in heteropolysaccharides in conjunction with D-galactose or D-glucose. D-mannans occur in ivory nuts, green coffee beans, plam kernel meal, and a number of other plant sources and have a common carbohydrate structure consisting of linear chains of 1,4–linked β–D–mannopyranosyl residues, which differ in chain length depending on the material source such as D–galacto–D–mannans which occur in the seeds of leguminous plants such as rice bran (Dey *et al*., 1993).

2.1.4 D–xylans; D–xylans are the most common of the hemicelluloses, occurring in all parts of all land plants. The backbone of the molecule is an essentially linear chain of 1,4–linked β–D–xylopyranosyl residues. The most common hemicellulose in soft woods contain (0–acetyl–L–arabino)–(4–0–methyl–D–glucurono)–D–xylan but the most common side–chain contains 1,2–linked. A common side chain in D–xylan is 1,3–linked α–L–arabinose but these residues do not always occur as non-reducing end groups. A typical example of non-terminal L–arabinopyranosyl residues occurs in barley and rice bran and rice husk (Choct, 1977).

2.2 Pectins

Pectins are a group of substances found in primary cell walls and intercellular layers in land plants in which the principal constituent is D–galacturonic acid. Pectins can be subdivided into two groups, similar to polysacchrides in which a proportion of D–galactopyranosyluronic acid residues are present as methyl esters termed pectinic acids. Pectinic acid is very easily extracted with water and posses considerable gelling powers which are used commercially for gelatinization of fruit juices. Several types of pectic substances are classified with homopolysaccharids, D–galactans, L–arabinans, and D–galacturonans and the most common

pectic substances are heteropolysaccharides containing both acidic and neutral sugars. (Kennedy, 1988)

2.3 Lignin

Cellulose and hemicellulose are cemented together by lignin composition in plant cell wall. Lignin is responsible for integrity, structural rigidity, and prevention of swelling of lignocelluloses. It also defends enzymatic degradation by limiting the enzyme accessibility. Therefore the delignification processes can improve the rate and extent of enzymatic hydrolysis. However, in most delignification methods, part of the hemicellulose is also hydrolyzed, and hence the delignification does not compleately dissolve lignin. Lignin dissolves it is also an inhibitor of cellulase, xylanase, and glucosidase. Various cellulases differ in their inhibition by lignin, while the xylanases and glucosidase are less affected by lignin (Kennedy, 1988).

2.4 Cellulose

Cellulose is ubiquitous as the basic structural material in cell wall of plants. Besides the mechanical strength of the plant cell, cellulose is a protective component against external attack by mechanism forces. It comprises approximately 45% of dry wood weight. Crystalline cellulose is composed of long β–1, 4–glucan chain that is hydrogen bonded into water-insoluble microfibrils. The crystalline cellulose coexists with less ordered amorphous regions. Cellulose is characterized by the degree of polymerization (DP), which is the average number of glucan residues per molecules. The average DP of native cellulose is 3,500. (O'Sullsivan, 1997)

Cellulose is an unbranched polymer of β–1, 4–linked D–anhydroglucopyranose units (Figure 1) With respect to the mean plane of the pyranose ring all OH– and both the CH_2OH–group and the glycosidic bond are equatorial, while the hydrogen atoms are in the axial positions. As the surface of the glucan chains consists mainly of hydrogen atoms, the molecule becomes hydrophobic. The β–1, 4–linkage forces the alternate chain units to be rotated 180° around the main axis. Therefore the repeating unit of cellulose is an anhydrocellobiose. The

extended molecule thus forms a flat ribbon, which is further stiffened by intramolecular H–bonds. (Dey *et al*., 1993)

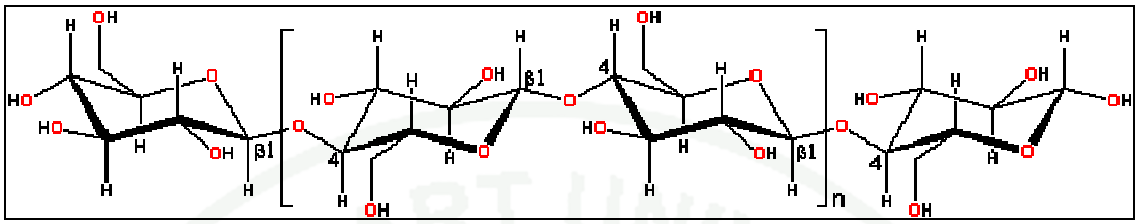


Figure 1 Polymer of β-(1⟶4)–D–glucopyranose units in $^{4}C_{1}$ conformation. The fully equatorial conformation of β–linked glucopyranose residues stabilizes the chair structure, minimizing its flexibility

Source: Nishiyama (2009)

Rough rice bran, RRB is the hard outer layer of grain and consists of combined aleurone and pericarp. It is a by–product of rice milling in the production of refined grains (Figure 2). RRB defined by U.S.D.A. as contain 50% or more of paddy kernels (28–36% cellulose and 34–45% lignin) where paddy kernels are broken or intact un–hulled rice seed in primary polishing and different from rice bran (RB).

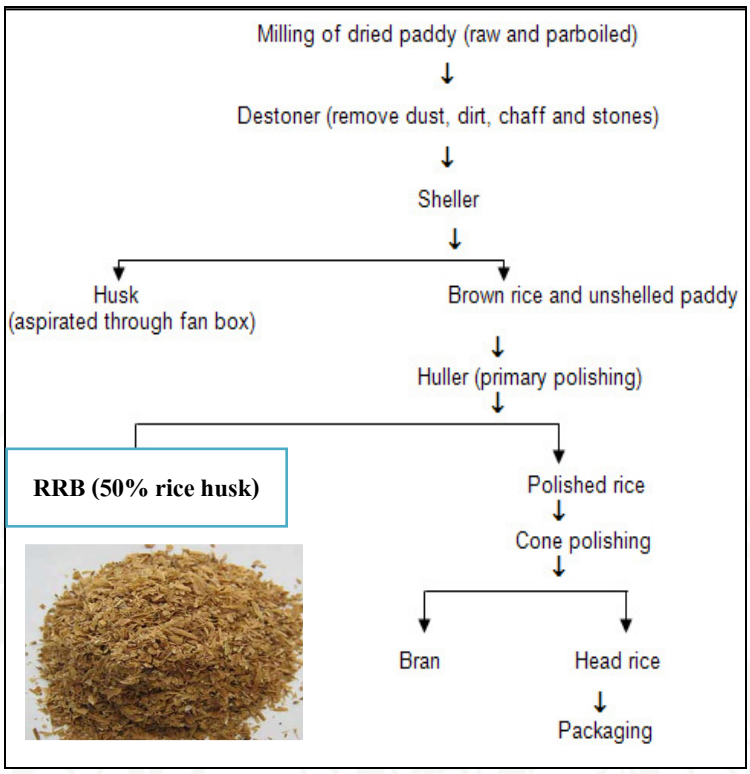


Figure 2 The rice milling process obtains rough rice bran (RRB)

Source: TanilNadu Agricultural University (2008)

Palm kernel meal (PKM) is a by-product of the kernel oil industry. It is the leftover after kernel oil is pressed out from the nut in the palm fruit (Figure 3). Palm kernel meal is commonly used as animal feed for dairy cattle because of its high protein content. However, the high fiber of 20–30% cellulose, 6% hemicelluloses and 2% lignin contents in PKM is interesting for using as carbon source for microorganism (Bono *et al*., 2009).

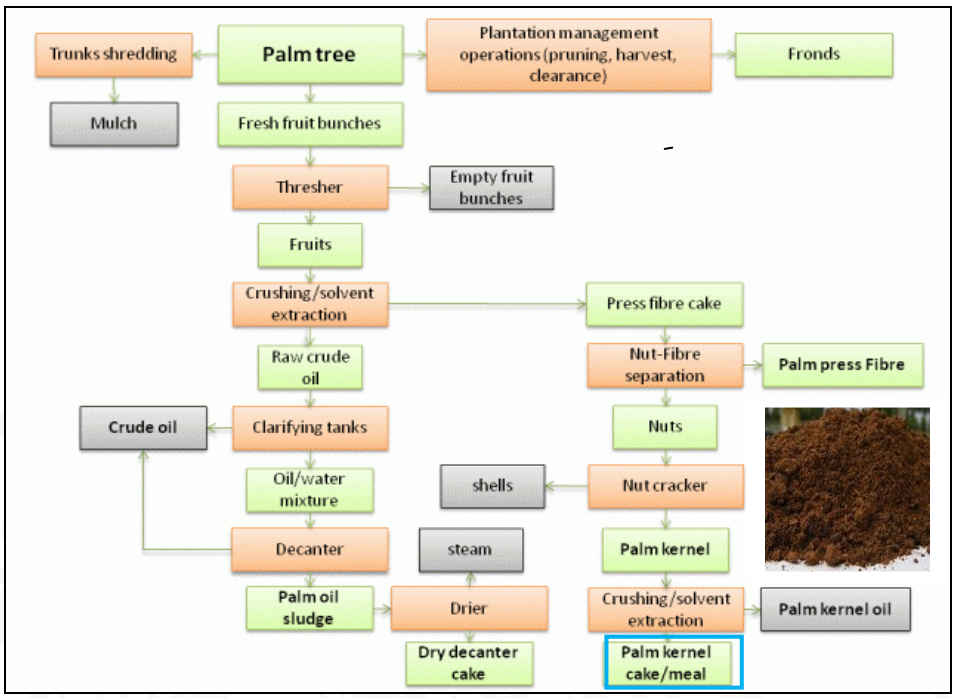


Figure 3 Palm oil milling process obtains palm kernel meal (PKM)

Source: Chin (1991)

Cassava pulp, CP is the fibrous residual material that generated from separating cassava tuber into starch granule and fiber for starch industry (Figure 4). CP contains: 20% cellulose, 19% hemicelluloses and 2–7% lignin (Kosugi *et al.*, 2009; Khempaka *et al.*, 2009; Wanrosli *et al.*, 2011).

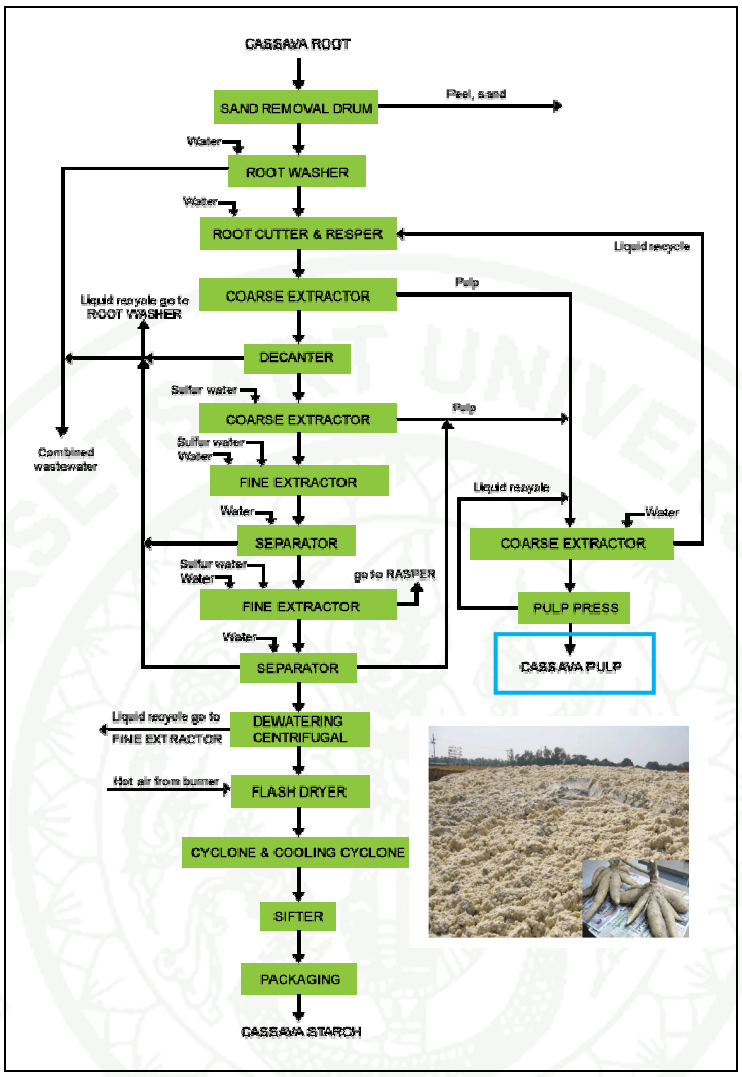


Figure 4 Cassava starch process obtains cassava pulp (CP)

Source: Sriroth *et al.* (2000)

3. Pretreatment of cellulosic materials

The structural of fiber macromolecule may simply provide an inert matrix (sugarcane bagasse, inert fibres, resins) within which the carbon and energy source (sugars, lipids, organic acids) are adsorbed. But generally, the macromolecular matrix represents the substrate and provides also the carbon and energy sources for microorganism (Raimbault, 1998). However natural cellulose is very resistant to chemical, enzymatic, and microbiological attack. The highly

crystalline structure and the presence of lignin effectively prevent those attacks. Moreover, lignin still has a significant effect on enzymatic digestibility. The reason the rate of hydrolysis improves by removal of lignin might be related to a better surface accessibility for enzymes by increasing the population of pores after removing of lignin (Taherzaden and Karimi, 2008).

Preparation and pre-treatment represent the necessary steps to convert the raw substrate into a suitable form and includes size reduction by grinding, rasping or chopping, physical, chemical or enzymatic hydrolysis of polymers to increase substrate availability by the fungus, cooking or vapour treatment for pre-degradation of macromolecular structure decreasing the relative crystalline of cellulose and decreasing the average DP (degree of polymerization) of the cellulose and elimination of major contaminants. Pretreatment of cellulosic substances can be done either by physical or chemical methods. .

3.1 Chemical treatment

3.1.1 Sodium hydroxide is a kind of alkaline treatment and appears to be the most effective method in breaking the ester bonds between lignin, hemicelluloses and cellulose. It causes swelling and separation of these structural elements. Sodium hydroxide also delignified the lignin in lignocellulosic material such as Aspen wood (Tatsumoto *et al*., 1974) with the concentration range of 1–4%NaOH. The Aspen cellulose was reached 100% digestion after 48 h while non-pretreatment Aspen was digested at 50% digestion. Therefore NaOH was used in great extent pretreated NSP in plant cell wall.

Silverstein *et al*. (2007) studied the effectiveness of sulfuric acid, sodium hydroxide, hydrogen peroxide, and ozone pretreatments for enzymatic conversion of cotton stalks. They found that sodium hydroxide pretreatment resulted in the highest level of delignification (65% with 2% NaOH in 90 min at 121°C) and cellulose conversion (60.8%).

Kodali and Pogaku (2006) studied pretreatment of rice bran to investigate the effect of cellulase production by steaming, acid and alkali upon the substrate before the fermentation process. An increase of 20% in the amount of sugar in 10 min of steaming was found to be a

standard pretreatment method of rice bran by a maximum cellulase activity of 16.7% solubilisation of cellulose.

3.1.2 Sulfuric acid, in previous research, a high concentration of acid has been used to treat waste materials that contain cellulose. Sulfuric acid was used for swelling of cellulose by using appropriate concentration at the range of 0.1–0.5%. Although acids are powerful agents used for biomass hydrolysis, concentrated acids are toxic, erosive and hazardous (Agu *et al.*,, 1997). Diluted acid hydrolysis has been successfully developed for pretreatment of cellulose material. Diluted sulfuric acid (H_2SO_4) can achieve significant results.

Srinorakutara *et al.* (2006) studied the utilization of cassava waste to produce ethanol by pretreatment with acid hydrolysis by converting starch into fermentable sugars form by *Saccharomyces cerevisiae*. The cassava waste was hydrolyzed by using 0.2–5.0 M sulfuric acid at a temperature of 60–120°C for 30 min. They found that the maximum reducing sugar of 6.1% (w/v) was obtained by the treatment of two time of 0.6 M H_2SO_4 and 120°C.

Xiao *et al.* (2004) pretreated cellulosic pyrolysate to glucose by 0.3 M H_2SO_4. The 60% conversion efficiency was achieved to yield 3.5% ethanol by yeast fermentation. The maximum glucose yield of 17.4% was obtained by hydrolysis with 0.2 M H_2SO_4, autoclave at 121°C for 20 min.

Chum *et al.* (1987) studied the pretreatment of Aspen and black cottonwood organosolv pulps by using the catalysts H_2SO_4 at pH lower than 3 resulting in a wide range concentrations of 30–70% methanol produced. This pretreatment allowed easily digestion and leads to additional solubilization of six–carbon sugars. They found the correlations between the glucan digestibility and effect of the pretreatment.

3.2 Physical treatment

The batch steam explosion process by high operating pressure (500–1,000 psig) and temperature (180–280°C), minute–range residence time is a present systematic study of

organosolv delignification. The low operating pressure was lower than 200 psig (95–180°C), and hour-range residence time still use for autohydrolysis combining with or without catalysts of dilute acid pretreatment (Chum *et al.*„ 1998).

Brownell and Saddler (1987) compared effects of initial moisture content, rate of heat transfer, pentosan solubilization and glucose yield of steam explosion and steam pretreatment to Aspen wood (*Populus tremuloides*). Treatment at 190°C with no explosion was compared with that at 240°C with explosion from full pressure. The treatment at 190°C shows at least as good solubilization of pentosan, enzymatic hydrolysis but showed greater pentosan destruction at the same degree of pentosan removal.

4. Cellulose degradation

In order to convert the cellulose polymer to other useful materials, it must first be degraded to low molecular weight entities, ideally monomeric sugar units. Polysaccharide conversion can be obtained by either chemical or enzymatic methods but the latter way is preferred owing to the highly selective mode of action, thereby eliminating the formation of unwanted by–products (Aro and Penttila, 2005).

4.1 Degradation by acid

Acid hydrolysis as an alternative to enzymatic breakdown has long been studied for analytical as well as industrial purposes. It is well known that cellulose poses special problems in the case of acid hydrolysis due to the physical and chemical properties of cellulose. Physical pre-treatment such as grinding, milling or steam explosion is essential in order to allow optimal accessibility of the acid to the fibrous material. For industrial purposes the so-called autohydrolysis procedure is of increasing importance. The autohydrolysis prior to acid hydrolysis is a stream-cracking followed by a rapid quenching, a process suitable for the elimination of hemicelluloses (Raimbault, 1998). In addition, much of the lignin becomes soluble afterwards in dilute alkali or in organic solvents. Extraction of lignin results in a relative pure cellulose for hydrolysis.

The classical cellulose hydrolysis is carried out by the Saeman method (Carvalheiro *et al.*, 2008) with 72% H_2SO_4, 41% Hydrochloric acid and 85% H_3PO_4. The action of the three concentrated acids is to dissolve the α–cellulose at low temperatures (4–5°C). Complete hydrolysis to the corresponding monomer is then done by a careful dilution of the acids to a 3–6% concentration and subsequent heating at 100–120°C for a period of 30–360 min. Increasing in temperature and pressure enhance the rate of hydrolysis and yield of glucose. However, during this process some of the sugars recombine to form oligomers, which have to be rehydrolysed by dilute sulfuric acid.

4.2 Enzymatic degradation

The term cellulase refers to the group of enzymes that contributes to the degradation of cellulose to its monomer glucose. All cellulase systems share common features. However, the latter way is preferred owning to the high selective mode of action and thereby eliminate the formation of unwanted by–products.

4.1.1 Endo–1, 4–β–glucanase (EG) cleave randomly at 1, 4–β–linkage within the cellulose chain. The endoglucanases are commonly assayed by viscosity reductions in carboxymethyl cellulose (CMC) solutions. Crystalline cellulose is not degraded by endoglucanases.

4.1.2 Exo–1, 4–β–glucanase (exo–1,4–β–D–glucan cellobiohydrolases, CBH) releases both glucose and cellobiose from the non-reducing of cellulose chains and reducing end as well. Cellobiohydrolases have virtually no activity on CMC and slowly degrade crystalline cellulose.

4.1.3 1, 4–β–glucosidases hydrolyse cellobiose to glucose, and cellobionic acid to glucose and gluconolactone.

Hydrolysis of crystalline cellulose is a complex process requiring the participation of several enzymes. The mechanism of cellulose hydrolysis was dominated and based on the initial

of Endoglucanases (EC 3.2.1.4) hydrolyzing cellulose to glucooligosaccharides on the amorphous regions of cellulose fibres. Cellobiohydrolases (EC 3.2.1.91) release cellobiose from crystalline cellulose. Glucosidases (EC 3.2.1.21) degrade the oligosaccharides to glucose. Exoglucanases release glucose from cellulose and glucooligosaccharides. The distinction between exoglucanases and cellobiohydrolases (CBH) is not always clear due to differences in the methods used to study these enzymes (Nigam and Singh, 1999). All four classes of enzymes have been identified in *Aspergillus*, although the number of isozymes produced by different species or even strains of the same species can be differ. An analysis of the production of cellulase by different species or the same species can be different in electrophoretic mobilities and physical properties for the enzyme of the different isolates (Table 3).

Fungal cellulases have frequently been reported to act synergistically in the degradation of crystalline cellulose. A commonly held model for the synergism between endoglucanases and cellobiohydrolases suggest that hydrolysis is initiated by endoglucanases that cleave random β- 1, 4-linkages within the cellulose chain. This activity is followed by cellobiohydrolases action that releases cellobiose from the non-reducing ends of the cellulose chain (Figure 5).

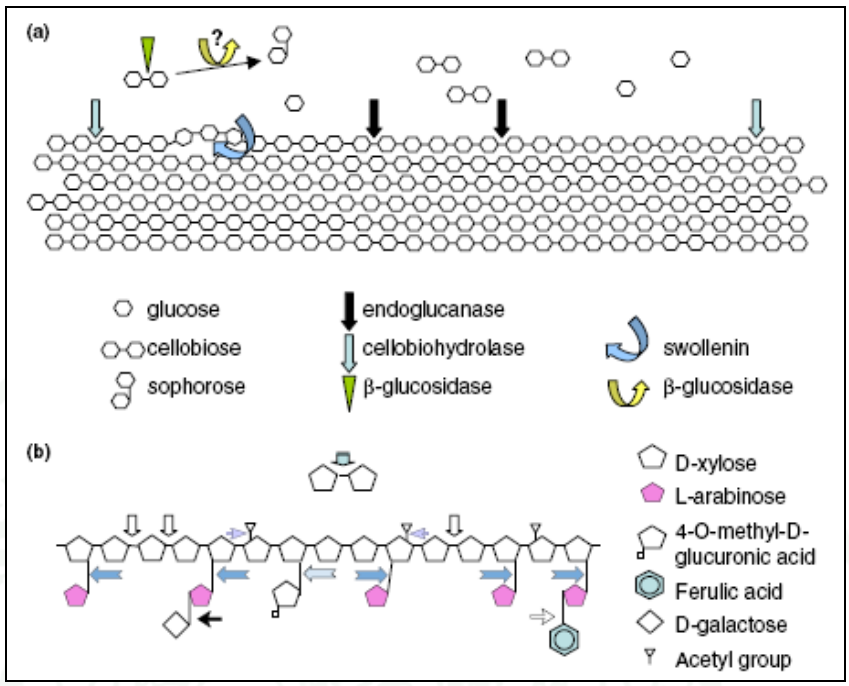


Figure 5 Degradation of cellulose and hemicellulose (a) A schematic view on cellulotic systems. (b) A schematic view on a hemicellulolytic system, degradation of arabinoxylan as an example

Source: Aro and Penttila (2005)

5. Biodegradation of plant cell wall polysaccharides by *Aspergillus*

The genus *Aspergillus* is a group of filamentous fungi with a large number of species. The first record of this fungus can be found in Micheli's *Nova Plantarum Genera*, but a more detailed description of the *Aspergilli* did not appear until the middle of the 19th century. In 1926 these fungi were firstly classified into 11 groups within the genus. A reexamination of the genus was published by Thom and Raper (1945) and proposed to be 14 distinct groups. Some of these groups consisted of pathogenic fungi (e.g., *A. fumigatus*, *A. flavus*, and *A. arasiticus*), but most important for industrial applications are some members of the group of black aspergilli (*A. niger* and *A. tubingensis*). In addition to the morphological techniques traditionally applied, new molecular and biochemical techniques have been used in the reclassification of this group of

Aspergillus. These analysis resulted in the clear distinction of eight groups of black aspergilla. There were *A. niger*, *A. tubingensis*, *A. foetidus*, *A. carbonarius*, *A. japonicus*, *A. aculeatus*, *A. heteromorphus*, and *A. ellipticus*. Products of several of these species have been proposed as a GRAS (Generally Regarded As Safe) status, which allows them to be used in food and feed applications. The black Aspergilli have a number of characteristics which make them as ideal organisms for industrial applications, such as good fermentation capabilities and high levels of protein secretion. In particular, the wide range of enzymes produced by *Aspergillus* for the degradation of plant cell wall polysaccharides are major importance to the food and feed industry (Table 4). Recently, the interesting of several *Aspergillus* spp. has increased as hosts for heterologous protein production. (Varies, 2001)

Aspergillus niger 386017M1 produced complex enzymes for cellulosic material degradation. *A. niger* 386017M1 isolated by Alltech Inc. (USA) is one of the most effective strain in fiber digestion for animal feed industry. It contained 7 power complex enzymes of cellulase, xylanase, pectinase, β–glucanase, phytase, protease and α-amylase which is essential for fiber degradation by solid state fermentation (SSF) under commercial name, Allzyme SSF. The optimum solid state fermentation is at temperature 30°C, pH 4.00 and 60% moisture content and 5 days cultivation. Optimum enzyme extraction using tween 80 for 90 mins, improved the increasing of CMCase activity for 5%. Whereas, 5% of glucose and ammonium phosphate used as carbon and nitrogen source increased CMCase activity for about 2 and 8%, respectively.

Table 3 Physical properties of endoglucanases and exoglucanases and glucosidases from *Aspergillus*

Species and enzyme type	Enzyme	Mol mass (kDa)	pH opt	*T* opt (°C)	pI
Endoglucanases					
A. aculeatus	FI-CMCase	25	4.5	50	4.8 2
A. aculeatus	FII-CMCase	66	5.0	70	4.0 3
A. aculeatus	FV-CMCase	38	4.0	65	3.4 3
A. aculeatus	Hydrocellulase	68	2.5	60	3.5 3
A. aculeatus	FI-Avicelase	109	5.5	65	4.7 3
A. aculeatus	FIII-Avicelase	112	2.5	65	4.0 3
A. nidulans	Endo-I	25	6.0	65	24
A. nidulans	Endo-II	32.5	5.0	50	24
A. nidulans	EG A	35	6.5	50	58
A. niger		26	3.8–4.0	45	158
A. niger	A	43	2–7	60	389
A. niger	B	25	2–7	60	389
A. oryzae	CelA	31	5.0	55	193
A. oryzae	CelB	53	4.0	45	193
Exoglucanases					
A. nidulans	Exo-I	29	5.5	50	24
A. niger		52.5	5.5	50	340
β-Glucosidases					
A. aculeatus	β-Gluc1	133	4.5	55	4.7 3
A. japonicus		240	5.0	65	323
A. nidulans	β-Gluco-I	26	6.0	35	24
A. nidulans	β-Gluco-II	14	5.0	65	24
A. nidulans	P-I	125	5.0	50	4.4 2
A. nidulans	P-II	50	5.5	60	4.0 2
A. oryzae	BGI	130			4.9 3
A. terreus		200	4.5	55–60	4.8 4, 313, 360
A. niger		100	4.8	65	120
A. niger	β-Glu I	49	5.0	55	3.2 4

Source: Ververis *et al.* (2004)

Table 4 Spectrum of microbial cultures for production of various enzymes in solid state fermentation systems.

Substrate	Microorganisms	Enzyme
Rice husk	*Penicillium* sp.	Cellulase
Wheat bran + rice straw	*Trichoderma* sp.	Cellulase
Agro waste	*A. niger., T. reesei*	Cellulase
Palm oil meal waste	*A. niger*	Cellulase
Cassava waste	*T. hazianum*	Cellulase
Puddy straw	*T. reesei*	Cellulase
Sweet sorghum silage	*T. reesei, A. niger*	Cellulase
Rice bran + cassava waste	*A*. sp.	Cellulase
Rice bran	*Rhizopus oligosporus.*	Acid protease
Rice bran	*R. oligosporus*	Protease
Sweet potato residue	*A. niger*	Acid protease
Rice bran, wheat bran	*Candida* sp.	Lipase
Rice bran	*C. rugosa.*	Lipase
Rice bran	*Aspergillus oryzae.*	α-amylase
Rice bran, soybean meal	*A. niger.*	Glucoamylase
Rice	*Amylomyces* sp.	Glucoamylase
Copra waste	*A. niger*	Glucoamylase
Cassava	*Rhizopus* sp.	Glucoamylase
Rice straw, soybean hull,	*A. sojae.*	Xylanase
Wheat bran, rice husk	*A. sojae.*	Xylanase
Rice straw, bagasse, wheat bran	*A. fumigatus.*	xylanase

Source : Nigam and Singh (1999)

6. Cellulase production by solid state fermentation

Solid state fermentation (SSF) holds tremendous potential for the production of enzymes. The crude fermented product may be used directly as an enzyme source. In addition to the conventional applications in the food and fermentation industries, microbial enzymes have attained significant roles in biotransformation. This system offers numerous advantages over submerged fermentation systems, including high volumetric productivity, relatively higher concentrations of the product, less effluent generation, requirement for simple fermentation equipments, etc. Presently SSF has been applied to large–scale industrial processes mainly in Japan. Traditional koji, manufactured in small wooden and bamboo trays, has changed gradually to more sophisticated processes. The usual production scale in sake or miso factories is approximately 1 or 2 tons per batch, but reactors can be made and delivered by engineering firms to a capacity as large as 20 tons (Fujiwara, Ltd.) (Muarice, 1998). Most recent research activity on SSF is being developed for the submerged fermentations, which are the major production process in the pharmaceutical and food industries in industrialized nations. SSF seems to have theoretical advantages over liquid substrate fermentation (LSF) such as lower the management cost. Nevertheless, SSF has several advantages more than LSF (Table 5).

6.1 Microorganisms used for enzyme production in SSF system

A large number of microorganism, including bacteria, yeast and fungi can produce different groups of enzymes. The selection of a suitable strain for the required purpose depends upon a number of factors, in particular upon the nature of the substrate and environmental conditions. Generally, hydrolytic enzymes, e.g. cellulase, xylanase, pectinases, etc. are produced by fungi for their growth. The genetically modified strains would hold the key to enzyme production in order to achieve high productivity with less production cost.

Table 5 Comparison between liquid and solid state fermentation

Factor	LSF	SSF
Substrates	Soluble substrates (sugar)	Polymer insoluble substrates
Aseptic condition	Heat sterilization and aseptic control	Vapor treatment, non sterile conditions
Water	High volumes of water consumed and effluents discarded	Limited consumption of water
Metabolic heating	Easy control of temperature	Low heat transfer capacity Easy aeration and high surface exchange air/substrate
Aeration	Limitation by soluble oxygen High level of air required	–
pH control	Easy pH control	Buffered solid substrates
Mechanical agitation	Good homogeneization	Static conditions prefered
Scale up	Industrial equipments available	Need for engineering & new design equipment
Incubation	Easy inoculums, continuous process	Spore inoculation, batch
Contamination	Risks of contamination for single strain bacteria	Risk of contamination for low rate growth fungi
Energetic consideration	High energy consuming	Low energy consuming
Volume of equipment	High volumes and high cost technology	Low volumes and low cost of equipment
Effluent and pollution	High volumes of polluting effluents	No effluents, less pollution

Source: Raimbault (1998)

6.2 Substrates used for the production of enzymes in SSF system

Agro–industrial residues are generally considered the best substrates for the SSF processes and use of SSF for the production of enzymes. Some of the substrates that have been used included sugar cane bagasse, wheat bran, rice bran, wheat straw, rice straw, rice husk, etc. The selection of a substrate for enzyme production in a SSF process depends upon several factors, mainly cost and availability of the agricultural substrate.

6.3 Factors affecting enzyme production in solid state fermentation system

The major factors that affect microbial synthesis of enzymes in a SSF system include: selection of a suitable substrate and microorganism; pre–treatment of the substrate; particle size (inter–particle space and surface area) of the substrate; water content and a_w of the substrate; relate humidity; type and size of the inoculum; control of temperature of fermenting matter, removal of metabolic heat, cultivation period; uniformity maintenance in the environment of the SSF system, oxygen consumption rate and carbon dioxide evolution rate. (Raimbault, 1998)

6.3.1 Moisture content and water activity (Aw), the water presenting in SSF systems exists in a complex form within the solid matrix. Free water will only occur when the saturation capacity of the solid matrix is exceeded. The moisture level at which free moisture becomes apparent varies considerably between substrates, depending upon their water binding characteristics. The moisture levels in SSF processes vary between 30 and 85%. It is now generally accepted that the water requirements of microorganism should be defined in terms of the water activity (Aw) rather than the water content of the solid substrate. Aw is a thermodynamic parameter defined in relation to the chemical potential of water.

6.3.2 Temperature and heat transfer, the stoichiometric global equation of respirations are highly exo thermic and heat generation by high levels of fungal activity within the solids leading to thermal gradients because of the limited heat transfer capacity of solid substrates. In an aerobic process, heat generation may be approximated from the rate of CO_2

evolution or O_2 consumption. Heat removal is probably the most crucial factor in large scale SSF processes. Conventional convection or conductive cooling devices are inadequate for dissipating metabolic heat due to the poor thermal conductivity of most solid substrates and results in unacceptable temperature gradients. Maintaining constant temperature and moisture content simultaneously in large scale SSF is generally difficult, but using the proper ancillary equipment can be done.

6.3.3 Control of pH and risks of contamination, the pH of a culture may change in response to metabolic activities. The secretion of organic acids such as citric, acetic or lactic acid will cause the pH to decrease. On the other hand, the assimilation of organic acids which may be presented in certain media will lead to an increase in pH. The kinetics of pH variation depends highly on microorganism. This could be useful to minimize and prevent totally contamination at low pH.

6.3.4 Oxygen uptake, aeration fulfils four main functions in SSF, to maintain aerobic conditions, to desorb carbon dioxide, to regulate the substrate temperature and to regulate the moisture level. The gas environment may significantly affect the relative levels of biomass and enzyme production. In the SSF process, oxygen supply allows free access of atmospheric oxygen to the substrate. Therefore, aeration may be easier than in submerged cultivations because the rapid rate of oxygen diffusion into the water film surrounding the insoluble substrate particles and also because of the very high surface area of contact between gas phase, substrate and aerial mycelium. By this very simple aeration process, it is also possible to induce metabolic reactions, by water stress, heat stress or temperature changes, therefore all of the process that can drastically change the biochemical or metabolic behavior of fungal.

6.3.5 Microbial growth, biomass is a fundamental parameter in the characterization of microbial growth. In fungal, the fungal hyphae penetrate into and bind tightly to the substrate. Methods that have been used for biomass estimation in SSF include measurement of metabolic product to become biomass.

a) Respiratory metabolism oxygen consumption and carbon dioxide release resulting from respiration, metabolism carbon compounds can convert into biomass and carbon dioxide. Production of carbon dioxide causes the weight of fermenting substrate to decrease during growth, and amount of growth that has occurred. Growth estimation based on carbon dioxide release or oxygen consumption assumes that the metabolism of these compounds is completely growth associated.

b) Production of extracellular enzymes or primary metabolites, another metabolic activity that may be growth associated is extracellular enzyme production.

c) Biomass components, the biomass can also be estimated from measurements of a specific component, as long as the composition of the biomass is constant and stable and the fraction of the component representative; examples include: Protein Content, Nucleic Acids, Glucosamine, Ergosterol and Physical Measurement of Biomass

d) Nutrient and their concentration, supplementary nutritional requirements for the developmental microbe–substrate system of interest may be determined by preliminary experiments in either submerged or solid-state cultures in small scale. For some brown rot fungi, cellulose and lignin degradation does not provide enough energy for enzyme induction and utilization of cellulosic constituents and may require a supplemental carbon source for growth and further lignin degradation. Solid substrates may present a different problem in that the nutrient. The growth may was limited by some other nutrient when deficiency triggers the pathway leading to secondary metabolite (Nigam and Singh, 1994).

Immanuel *et al*. (2007) investigated the cellulase production of fungal strains such as *A. niger* and *A. fumigatus* against the lignocellulosic bio waste like coir waste and saw dust at varying environmental parameters of pH (5–9) and temperature (20–50°C). The high level of CMCase production was achieved at pH 5 (0.198 IU/ml) and pH 6 (0.052 IU/ml) by *A. fumigatus* using coir waste and sawdust as substrates respectively. The high level of FPase production was obtained at pH 7 by *A. fumigatus* (0.292 IU/ml) and pH 6 by *A. niger* (0.262 IU/ml) when coir waste and sawdust used as substrate respectively.

Hegde *et al.* (2006) found that *A. niger* CFR1105 induced a greater amount of cell wall degrading enzymes for 96 h by using wheat bran and rice bran as substrate. To understand the degradation pattern of non–starch polysaccharides and phenolic acid complexes under condition of solid state fermentation; 10 g of each cereal bran and 10 ml distilled water with $1x10^8$ spores and incubated at 30°C. In both cereal bran arabinoxylans were degraded extensively in 96 h, whereas the degradation of 1,3 /1,4–β–D–glucans and cellulose was negligible. However, the cereal bran can be exploited to obtain bioactive compounds such as ferulic acid.

Hanif and Rajoka (2004) investigated the influence of carbon and nitrogen source on the cellulases production by measuring CBH yield when *A. niger* NIAB 280 utilized rice husk, corn cobs, wheat straw, wheat bran, rice bran, monosaccharides and disaccharides for improved production of CBH. Levels of CBH were minimal in the presence of even low concentrations of glucose. Wheat bran and cellulose were the most effective promoter of CBH, followed by rice bran. In a defined medium with cellobiose, the cellobiohydrolase titres were 2 to 110 fold higher with cells growing on monomeric sugars. In this organism, substantial synthesis of β–cellobiohydrolases can be induced by cellobios, cellodextrin, cellulose or cellulose and hemicellulose containing substrates which showed low volumetric substrate uptake rates and required optimum concentration of carbon, nitrogen or phosphorous. During growth of *A. niger* NIAB 280 on wheat bran, maximum volumetric productivities of β–cellobiohydrolases were 39.6 IU/h.

Kang *et al.* (2004) investigated the production of cellulases and hemicellulases from *A. niger* KK2. Solid state fermentation was performed by using 1:4 and 5:0 ratios of rice straw and wheat bran respectively. The rice straw substrate showed maximum FPase activity of 19.5 IU/g and xylanase of 5070 IU/g in 4 days. The maximum FPase and xylanase activity were obtained from the fermentation of rice straw alone. In contrast, CMCase, β–glucosidase and β–xylosidase were increased 129 IU/g, 100 IU/g and 193 IU/g, respectively, when 1:4 ratio of rice straw and wheat bran were used. The fermentation were concurrently obtained after 5–6 days.

Yamane (2002) studied production of cellulose (CEL), xylan (XYL), and pectin (PEC) degrading enzyme by *A. oryzae* under SSF and submerged system. The utilization of

available rice in the sake endosperm cell wall with the production of CEL and XYL was stimulated by decreasing the moisture content of the solid substrate. These data suggest that the production of CEL and XYL is strongly influenced by culture conditions and that water activity is one of the dominant factors in the regulation of their production.

7. Molecular genetics of fungal cellulase

7.1 Gene system

The molecular genetics of cellulolytic fungi, especially *T. reesei*, has advanced considerably in recent years (Table 6). *T. reesei* gene sequences encoding *cbh1, cbh2, egl, eg3* and β–glucosidase have been reported from both genomic and cDNA clones. *A niger* gene encoding β–glucosidase has been cloned and reporting cellulases are made of three structural regions: the catalytic 'core' which hydrolyses 1,4–β–glycosidic linkages, the extended 'hinge' which is glycosylated, and the cellulose-binding 'tail' which has also been hypothesized to help solubilize the cellulose chain prior to hydrolysis. The comparisons of amino acid sequence of *T. reesei cbh1, cbh2, egl, egl3* showed the presence of two short consecutive blocks that are conserved in all four genes. (Kinghorn and Tauner, 1992) The catalytic activity, crystalline cellulose–binding capacity, and structure of these cleavage products support the general model. *T. reesei* EGII, which requires the conserved blocks for efficient binding and hydrolysis of crystalline cellulose. The functional domains can be separated by native proteases in cellulolytic bacteria and fungi.

Relative to well–defined genetic systems, such as *S. cerevisiae, Neurospora crassa* and *A. niger*, genetic analysis of cellulolytic fungi has been extremely limited. The *A. niger* expression system was used for *T. reesei* CBHII and endoglucanase I (EGI) and cellobiohydrolase I (CBHI) gene. The *Aspergillus* system has the ability to recognize and utilize *T. reesei* transcription and intron processing. The intent here was to express and secrete CBH into a low cellulolytic back-ground (Zhang *et al.,* 1998). Base on the derived amino acid sequences, the gene products of *Aspergillus* have been assigned to different glycosidase families. Endoglucanases are assigned mainly to families 5 and 12 while the exception of CelB from *A. oryzae* was assigned to

family 7, which also contains the *Aspergillus* cellobiohydrolases (CBH). The only exoglucanase gene cloned so far was assigned to family 74. All β–glucosidases from *Aspergillus* have been assigned to family 3 of the glycosidases. All cellulose–degrading enzymes have a retaining mechanism. The exoglucanase from *A. aculeatus* (family 74) is the only enzyme for which the catalytic mechanism has not yet been determined. (Vries and Visser, 2001)

7.2 Regulation system

The regulators of plant cell wall degrading enzymes included the carbon catabolite repressor CRE, CCAAT element, and binding sites for transcriptional activators or factors modulating expression. Regulation of these genes has been shown to be subjected to many regulatory pathways (Aro and Penttila, 2001).

7.2.1 XlnR, the first transcriptional activator controlling the expression of genes encoding xylanolytic and cellulolytic enzymes in filamentous fungi was isolated from *A. niger* by cDNA library complementation of a *pyrG* genotype under the *xynA* promoter. XlnR has a zinc binuclear cluster DNA-binding domain, and it was shown to bind *in vitro* to the 5′–GGCTAATAA sequence in the xylanase *xln*A promoter. The binding site was proposed to contain a core of 5′–GGCTAR that is found in most hemicellulase and cellulase gene promoter. The expression of two endoxylanases (*xlnB* and *xlnC*), a β–xylosidase (*xlnD*), two endoglucanases (*eglA* and *eglB*), two cellobiohydrolases (*cbhA* and *cbhB*) and several genes encoding the side chain cleaving hemicellulases are co–regulated by this xylanolytic activator. The fact that both hemicellulase and cellulases encoding gene regulated by XlnR indicates the transcriptional activation mechanism in *Aspergillus* is at least partially shared between cellulases and hemicellulases. The role of *XlnR* in *A. niger* seems to be no restriction to only regulation of genes encoding secreted polysaccharide degrading enzymes but also to an intracellular enzyme involved in D–xylose catabolism. The reduction of xylose to xylitol was shown to be regulated by XlnR (Hasper *et al.*, 2002).

Table 6 Genes encoding endoglucanases, exoglucanases, cellobiohydrolases, and β-glucosidases from *Aspergillus* and their assignment to the glycosidase families *Aspergillus*

Species	Activity	Gene	Glycosidase family	Database accession no.
A. aculeatus	EGL	*cel1*	5	AF054512
A. aculeatus	EGL	*cmc2*	5	AB015510
A. aculeatus	EGL	*xeg*	12	AF043595
A. aculeatus	EGL		12	D00546
A. kawachii	EGL	*cekA*	12	D12901
A. nidulans	EGL	*eglA*	5	AB009402
A. niger	EGL	*eglA*	12	AJ224451
A. niger	EGL	*eglB*	5	AJ224452
A. oryzae	EGL	*celA*	12	D83732
A. oryzae	EGL	*celB*	7	D83731
A. aculeatus	EXG		74	AB015511
A. aculeatus	CBH	*cbhI*	7	AB002821
A. niger	CBH	*cbhA*, *cbhB*	7	AF156268, AF156269
A. aculeatus	BGL	*bgl1*	3	D64088
A. kawachii	BGL	*bglA*	3	AB003470
A. terreus	BGL		3	Z37722
A. wentii	BGL	*bglA-3*	3	P29090

Source: Ververis *et al.* (2004)

7.2.2 Ace2, the *ace2* gene was isolated from a yeast designed for isolation of transcription factors binding and activating the main cellulose promoter *cbhI* of *T. reesei*. The *ace2* encodes a zinc binuclear cluster DNA-binding protein with no clear amino acid similarity to sequences in the databases. ACEII has been shown to bind *in vitro* to 5′GGSTAA sequences in the promoters of *cbh*1 and *xyn*2 of *T. reesei*. Deletion of the *ace2* gene from *T. reesei* resulted in

the reduction of expression of all the main cellulose genes, *cbh1, cbh2, egl1, egl2* and the xylanase gene *xyn2*, when the genes were induced upon growth on cellulose as the sole carbon. Further study are required to understand to what extent the XlnR homologue XYRI of *T. reesei* to regulate the expression of cellulases and xylanases. The lack of an *ace2* homologue in *A. nidulans*, and most likely in *A. niger* as well, implies that at least partly different transcriptional regulators are used by these fungi to achieve the induced expression levels (Kinghorn and Tauner, 1992).

7.2.3 Ace1, the *ace1* gene was isolated in a similar screen as *ace2* designed for the isolation of transcription factors binding to and activating the *T. reesei cbh1* promoter in *S. cerevisiae*. *ace1* contains three Cys_2His_2–type zinc fingers and it was shown to bind *in vitro* to eight sites in the *cbh1* promoter, all which contain the core 5′–AGGCA followed by a sequence rich in A and T. The 5′–AGGCA sequence are found in nearly all of the sequence of *T. reesei* cellulase promoters but their functionality has not yet been studied. Although *ace*1 activated the *cbh*1 promoter in yeast, its deletion of *ace1* in *T. reesei* led to increase the expression of all the main cellulose and xylanase genes studied in cellulose and sophorose induced cultures, indicating that ACE1 has a negative effect on the induced expression of these genes. The *A. nidulans* gene encoding a protein showed the highest overall amino acid similarity with ACEI, *stz*A (AF202995), is deposited into the database as a gene encoding a protein that alleviates sensitivity to salt and DNA damaging agents. ACE1 may have a more general regulatory role in addition to being only a repressor for cellulase and xylanase expression (Hasper *et al.*, 2002).

7.2.4 CreA, the mechanism controlling the use of substrate such as glucose (or fructose) over other alternative carbon sources is called glucose repression or carbon catabolite repression (CCR). CreA is a zinc finger protein which binds to specific sites in the promoters of a wide range of target genes. In the presence of easily metabolizable substrates, such as glucose or fructose, CreA inhibits or decreases the expression of the target genes (Aro and Penttila, 2005). In many ascomycetous fungi, glucose repression is mediated by the Cys_2His_2 type transcription factor CreA/CRE1 that has been cloned from numerous filamentous fungi. Numerous cellulase, hemicellulase and pectinase genes have been shown to be regulated by Cre proteins in *T. reesei*

and *Aspergillus* species. In general, mutations of the *cre* gene lead to (partial) derepression of gene expression on glucose.

7.2.5 PacC, the regulatory mechanism controlling pH-dependent transcriptional regulation has been analysed in detail in *A. nidulans* and a major role has been demonstrated for the zinc finger transcription factor PacC, which acts as an activator for alkaline–expressed genes. PacC contains a DNA–binding domain with three Cys_2His_2 zinc finger and it binds to promoter sites containing core hexanucleotide sequence 5′–GCCARG. In response to a signal transduced by the *pal* gene, PacC is proteolytically processed to its functional form. This proteolytic cleavage removes the 420 C–terminal amino acid that contains the negative-acting C–terminal domain. Conformation of the full–length PacC alternates between a protease–resistant and protease-sensitive form, the latter being cleaved to the active form in alknaline conditions. Six genes (*pal*A. *pal*B, *pal*C, *pal*F, *pal*H and *pal*I) are believed to be involved in the signal transduction pathway leading to the activation of PacC (Glauce, 2011). The genes regulated by PacC or its homologous include enzymes involved in the synthesis of biologically active metabolites such as penicillin and protease. Also xylanases and an endopolygalacturonase involved in pectin degradation have been show to be under PacC control.

The schematic representation of the different fungal trans-acting factors and regulatory responses affecting cellulase and xylanase expression are presented on Figure 6. The carbon catabolite repressor CRE, activators XlnR and ACEII, the repressor ACEI, the CCAAT binding Hap2/3/5 complex, pH regulator PacC, and the nitrogen regulator AreA are shown. Enzyme load in the secretory pathway can activate a negative feed–back signal that down-regulates enzyme gene transcription (RESS). Some known and possible environmental factors affecting cellulase and xylanase expression are also listed. The gene expression activating and repressing effects of the sugars depends on their concentration.

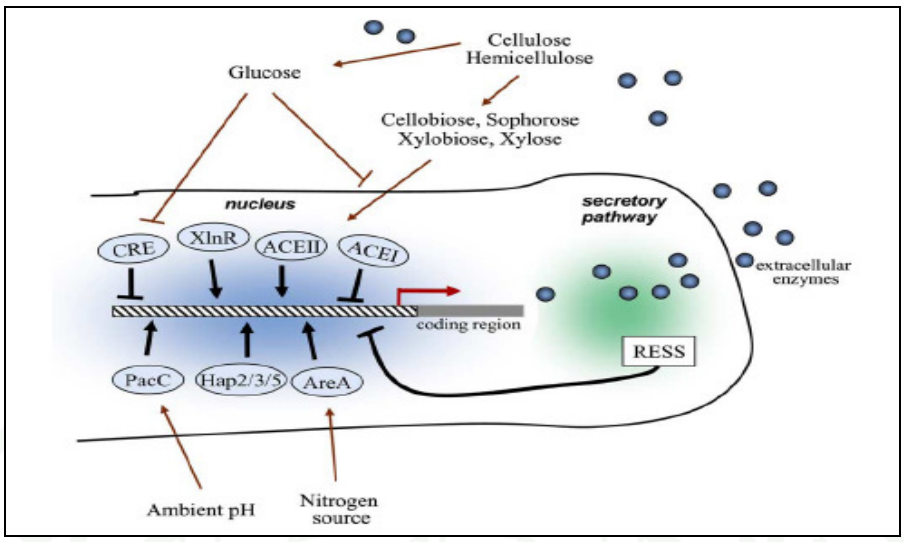


Figure 6 The gene expression activating and repressing effect of the sugars depends on their concentration

Source: Aro and Penttila (2005)

Among processes used for enzyme production, solid state fermentation (SSF) is an attractive one because it presents many advantages, especially for fungal cultivations. In SSF system, the productivity per reactor volume is much higher compared with that of submerged culture. Also, the operation costs are lower because the simpler plant, machinery and energy are required (Panagiotou *et al*., 2003). The filamentous fungi, *A. niger* and *T. reesei*, were described as a good producer of cellulose using lignocellulosic residue as a main carbon source. The fact is that the major cellulase gene cloned by differential hybridization showed that their expression is regulated at the transcriptional level of different regulators confirmed.

Kanamasa *et al.* (2003) found the improved *cbhI* gene of *A. aculeatus* by overexpressing in *A. oryzae* under the control of promoter improved by repeating the region III sequence, which is considered to be a cis–element interacting with the positive regulator for the transcription of *A. oryzae* amylase genes. The expression vector for *cbhI* was constructed; pNAN–CBH and the 1810 bp PCR product was inserted into the same sites between P–no. 8142, the promoter improved by introducing 12 copies of region III, and T-*agdA*, the terminator of the

A. oryzae amylase genes in a fungal high–level expression vector pNAN8142. The cloning strain displayed the strongest CBHI activity among all transformants produced, about 941 mg/l in liquid culture. It was comfirmed by a PCR method that the plasmid was integrated at the nia*D* locus.

Hasper *et al.* (2002) studied a novel gene, *eglC*, encoding an endoglucanase of *A. niger*.Transcription of *eglC* is regulated by *Xln*R, a transcriptional activator that controls the degradation of polysaccharides in plant cell walls. EglC is an 858–amino–acid protein and contains a conserved C-terminal cellulose–binding domain. EglC can be classified in glycoside hydrolase family 74. No homology to any of the endoglucanases from *T. reesei* was found. In the plant cell wall xyloglucan is closely linked to cellulose fibrils. They hypothesized that the EglC cellulose–binding domain anchors the enzyme to the cellulose chains while it is cleaving the xyloglucan backbone. By this action it may contribute to the degradation of the plant cell wall structure together with other enzymes, including hemicellulases and cellulases. EglC is the most active towards xyloglucan and therefore is functionally different from the other two endoglucanases from *A. niger*, EglA and EglB, which exhibit the greatest activity towards β–glucan. Although the mode of action of EglC is not known, this enzyme represents a new enzyme function involved in plant cell wall polysaccharide degradation by *A. niger*.

Gielkens *et al.* (1999) cloned and characterized two cellobiohydrolase–encoding genes (*cbhA* and *cbhB*) in *A. niger* and demonstrated that *Xln*R is also involved in the regulation of transcription of Cbh-encoding genes. The deduced amino acid sequence shows that CbhB has a modular structure consisting of a fungus-type cellulose-binding domain (CBD) and a catalytic domain separated by a Pro/Ser/Thr–rich linker peptide. CbhA consists of only a catalytic domain and lacks a CBD and linker peptide. Both proteins are homologous to fungal cellobiohydrolases in family 7 of the glycosyl hydrolases and the transcription of the *cbhA* and *cbhB* genes is induced by D-xylose but not by sophorose and in addition requires the xylanolytic transcriptional activator XlnR.

Peij *et al*. (1998) studied the expression of gene encoding enzymes involved in xylan degradation and two endoglucanases involved in cellulose degradation at the mRNA level in the filamentous fungus *A. niger*. The strain with a loss-of-function mutation in the *XlnR* gene

encoding the transcriptional activator *XlnR* and a strain with multiple copies of this gene were investigated in order to define which genes are controlled by *XlnR*. The data presented show that the transcriptional activator *XlnR* regulates the transcription of the *XlnB* and *XlnC*, and *XlnD* genes encoding the main xylanolytic enzymes (endoxylanases B and C and β–glucosidase, respectively). Also the transcription of the genes encoding the accessory enzymes involved in xylan degradation, including α-glucuronidase A, acetylxylan esterase A, arabinoxylan arabinofuranohydrolase A, and feruloyl esterase A, was found to be controlled by *XlnR*. In addition, *XlnR* also activates transcription of two endoglucanase–encoding genes, *eglA* and *eglB*, indicating that transcriptional regulation by *XlnR* goes beyond the genes encoding xylanolytic enzymes and includes regulation of two endoglucanase-encoding genes.

Hrmova´ *et al*. (1991) studied the inducing abilities of synthetic disaccharides composing of glucose and xylose from cellulose by xylan–degrading enzymes in *A. terreus* Measurement of secreted and cell–associated enzyme activities revealed the disaccharides induced synthesis of the cellulolytic and xylanolytic enzymes, 2–*0*–β–D–xylopyranosyl D–xylose, or their positional isomers, selectively induced the synthesis of cellulases and β–xylanases, respectively. An extracellular enzymes show Glcβ1–2Xyl initiated the synthesis of specific endo–1,4–β-glucanases and specific endo–1,4–xylanases identical to those produced separately in response to sopharose or Xylβ1–2Xyl. Glcβ1–2Xyl also induced specific endo–1,4–β–glucanases that hydrolysed 4–methylumbelliferyl β–lactoside at the agluconic bond. The result strengthen the concept of separate regulatory control of the synthesis of cellulases and β–xylanases. The result suggest that mixed disaccharides, composing of glucose and xylose moities, which may occur in nature, could play an important role in regulating the synthesis of wood-degrading enzymes.

8. Polymerase Chain Reaction (PCR)

8.1 PCR system

Polymerase chain reaction (PCR) is a method that allows exponential amplification of short DNA sequences (usually 100 to 600 bases) within a longer double stranded DNA

molecule. PCR entails the use of a pair of primers, each about 20 nucleotides in length, that are complementary to a defined sequence on each of the two strands of the DNA. These primers are extended by DNA polymerase so a copy is made of the designated sequence. After making this copy, the same primers can be used again, not only to make another copy of the input DNA strand but also of the short copy made in the first round of synthesis. This leads to logarithmic amplification. Since it is necessary to raise the temperature to separate the two strands of the double strand DNA in each round of the amplification process, a major step forward was the discovery of a thermo-stable DNA polymerase (Taq polymerase) that was isolated from *Thermus aquaticus,* a bacterium that grows in hot pools; as a result it is not necessary to add new polymerase in every round of amplification. After several (often about 40) rounds of amplification, the PCR product is analyzed on an agarose gel and is abundant enough to be detected with an ethidium bromide stain.

The method of Post PCR has been loaded on agar rose gel electrophoresis but still has many problems to obtain poor precision such as low sensitivity (Figure 7.), short dynamic range < 2 logs, low resolution, non–automated, size–based discrimination only, results are not expressed as numbers, ethidium bromide for staining is not very quantitative.

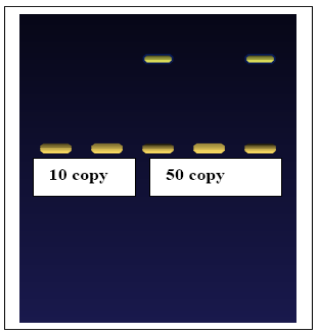


Figure 7 Post PCR process on agar rose gel with poor precision

Source: Applied Biosystem (2011)

For reasons that will be outlined below, this method of analysis is at best semi-quantitative and, in many cases, the amount of product is not related to the amount of input DNA,

making this type of PCR a qualitative tool for detecting the presence or absence of a particular DNA. In order to measure messenger RNA (mRNA), the method was extended using reverse transcriptase to convert mRNA into complementary DNA (cDNA) which was then amplified by PCR and, again analyzed by agarose gel electrophoresis. In many cases this method has been used to measure the levels of a particular mRNA under different conditions but the method is actually even less quantitative than PCR of DNA because of the extra reverse transcriptase step. Reverse transcriptase-PCR analysis of mRNA is often referred to as "RT–PCR" which is unfortunate as it can be confused with "real time–PCR".

8.2 Real Time PCR

Real-time reverse-transcriptase (RT) PCR quantitates the initial amount of the template most specifically, sensitively and reproducibly, and is a preferable alternative to other forms of quantitative RT–PCR that detect the amount of final amplified product at the end-point. Real–time PCR monitors the fluorescence emitted during the reaction as an indicator of amplicon production during each PCR cycle (in real time) as opposed to the endpoint detection. The real-time progress of the reaction can be viewed in some systems. Real-time PCR does not detect the size of the amplicon and thus does not allow the differentiation between DNA and cDNA amplification, however, it is not influenced by non–specific amplification unless SYBR Green is used. Real–time PCR quantitation (qPCR) eliminates post–PCR processing of PCR products (which is necessary in competitive RT–PCR). This helps to increase throughput and reduce the chances of carryover contamination. In comparison to conventional RT–PCR, real–time PCR also offers a much wider dynamic range, up to 10^7–fold (compared to 1000–fold in conventional RT–PCR). Dynamic range of any assay determines how much the target concentration can vary and still be quantified. A wide dynamic range means a wide range of ratios of target and normalizer can be assayed with equal sensitivity and specificity. It follows that the broader the dynamic range, the more accurate the quantitation. (Bustin *et al.,* 2005)

The real-time PCR system is based on the detection and quantitation of a fluorescent reporter. This signal increases in direct proportion to the amount of PCR product in a reaction. By recording the amount of fluorescence emission at each cycle, it is possible to monitor

the PCR reaction during the exponential phase (Figure 8.) where the first significant increase in the amount of PCR product correlates to the initial amount of target template. The higher the starting copy number of the nucleic acid target, the sooner a significant increase in fluorescence is observed. A significant increase in fluorescence above the baseline value measured during the 3–15 cycles indicates the detection of accumulated PCR product.

A fixed fluorescence threshold is set significantly above the baseline that can be altered by the operator. The parameter threshold cycle (C_T) is defined as the cycle number at which the fluorescence emission exceeds the fixed threshold. There are three main fluorescence-monitoring systems for DNA amplification: (1) hydrolysis probes; (2) hybridizing probes; and (3) DNA–binding agents. Hydrolysis probes include TaqMan probes and molecular beacons. They use the fluorogenic 5' exonuclease activity of Taq polymerase to measure the amount of target sequences in cDNA samples for light-up probes.

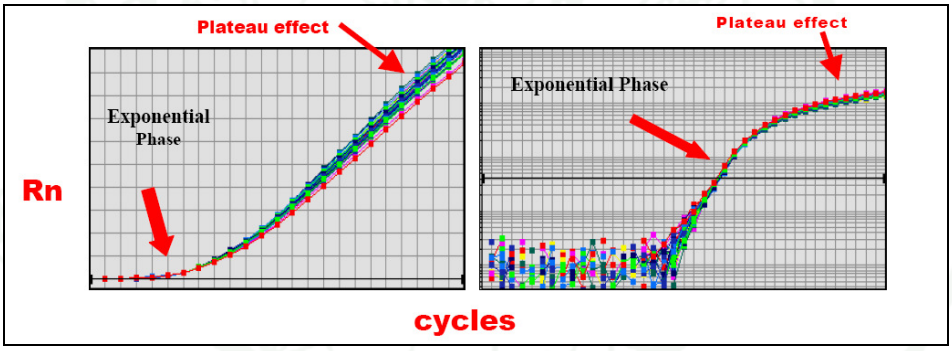


Figure 8 Signal increases in direct proportion to the amount of PCR product in a reaction. By recording the amount of fluorescence emission at each cycle

Source: Applied Biosystem (2011)

a) Optimizing a Real–Time quantitative PCR assay (qPCR)

Since real–time quantitation is based on the relationship between the initial template amount and the C_T value obtained during amplification, an optimal qPCR assay is absolutely essential for accurate and reproducible quantitation of a particular sample. The

hallmarks of an optimized qPCR assay are; a linear standard curve (R2> 0.980 or r > I-0.990I) and consistency across replicate reactions.

A powerful way to determine whether the qPCR assay is optimized is to run a set of serial dilutions of template DNA and use the results to generate a standard curve. The template used for this purpose can be a target with known concentration (for example, nanograms of genomic DNA or copies of plasmid DNA) or a sample of unknown quantity (for example, cDNA). A standard curve is constructed by plotting the log of the starting quantity of the template (or the dilution factor, for unknown quantities) against the C_T value obtained during amplification of each dilution. The equation of the linear regression line, along with Pearson's correlation coefficient (r) or the coefficient of determination (R^2), can then be used to evaluate whether the qPCR assay is optimized.

Ideally, the dilution series will produce amplification curves that are evenly spaced, as shown in Figure 9A. If perfect doubling occurs with each amplification cycle, the spacing of the fluorescence curves will be determined by the equation 2^n= dilution factor, where n is the number of cycles between curves at the fluorescence threshold (in other words, the difference between the C_T values of the curves). For example, with a 10–fold serial dilution of DNA, $2^n = 10$. Therefore, = 3.32, and the C_T values should be separated by 3.32 cycles. Evenly spaced amplification curves will produce linear standard curves, as shown in Figure 9B. The equation and r values of the linear regression lines are shown above the plot. The r or R^2 value of a standard curve represents how well the experimental data fit the regression line; that is, how linear the data are. Linearity, in turn, gives a measure of the variability across assay replicates and whether the amplification efficiency is the same for different starting template copy numbers.

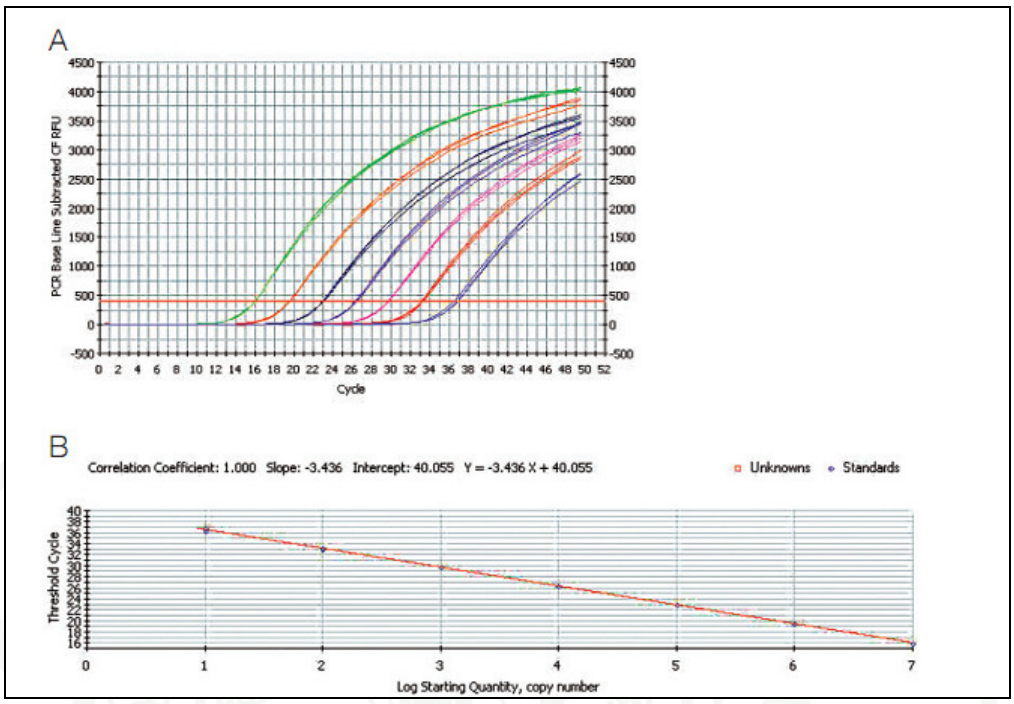


Figure 9 Generating a standard curve to assess reaction optimization. A standard curve was generated using a 10–fold dilution of a template amplified on the iCycler IQ® real–time system. Each dilution was assayed in triplicate. A. Amplification curves of the dilution series. B. Standard curve with the C_T plotted against the log of the starting quantity of template for each dilution. The equation for the regression line and the r value are shown above the graph.

Source: Bio-Rad Laboratories Inc. (2006)

b) Chemistries for monitoring Real–Time PCR

A key step in designing a qPCR assay is the chemistry to monitor the amplification of the target sequence. The variety of fluorescent chemistries available can be categorized into two major types:

• DNA–binding dyes (SYBR Green I)

• Dye–labeled, sequence specific oligonucleotide primers or probes (molecular beacons, TaqMan assays, and hybridization probes)

The most commonly used chemistries for real-time PCR are the DNA–binding dye SYBR Green I and TaqMan hydrolysis probe. SYBR Green I is a DNA dye that binds non-discriminately to double–stranded DNA (dsDNA). SYBR Green I exhibits minimal fluorescence when it is free in solution, but its fluorescence increases dramatically (up to 1000–fold) upon binding to dsDNA (Figure 10). As the PCR reaction progresses the amplified product accumulates exponentially, more SYBR Green I binds, and fluorescence increases. The advantage of using SYBR Green I is its simplicity. This is similar to the action of ethidium bromide, but unlike ethidium bromide, SYBR Green I does not interfere with DNA polymerases, so it can be added directly to a PCR reaction mixture. SYBR Green I also has less background fluorescence than ethidium bromide, is able to detect lower concentrations of double–stranded DNA, and is not hazardous.

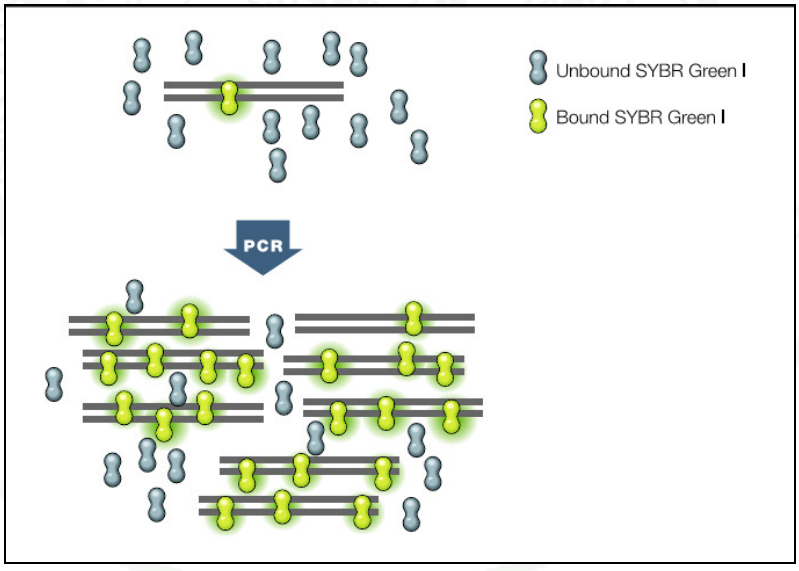


Figure 10 DNA–binding dyes in real–time PCR. Fluorescence dramatically increases when the dye molecules bind to double–stranded DNA.

Source: Bio-Rad Laboratories Inc. (2006)

The main disadvantage to the use of SYBR Green I is its nonspecificity. Since it will bind to any double–stranded DNA, if non–target sequences are being amplified, this will show up in a SYBR Green I fluorescence curve and will be indistinguishable from amplification of target sequence. For this reason, when using SYBR Green I it is prudent to verify that target DNA is being amplified; this is commonly done by running an agarose gel of the reaction products (conventional PCR). Alternatively, post amplification melt-curve analysis can be performed on the real-time PCR instrument to distinguish reaction products and analyze reaction specificity, eliminating the need for agarose gel analysis of reaction products.

c) Melt-Curve Analysis

The principle of melt-curve analysis is that the temperature increased from a low temperature (where all sequences are annealed) to a high temperature causing strand dissociation. As the dsDNA melts, SYBR Green I is released and a decrease in fluorescence is observed. Two factors are important in melting temperatures: the size of the double-stranded DNA and the GC content. The higher the GC content and the larger the strand size, the higher the melting temperature will be. By comparing the melt temperatures of known amplicons, the presence of an extra non-target amplicon or primer-dimers is easily detected. In a typical melt-curve, the fluorescence intensity is plotted against the temperature. The fluorescence decreases as the temperature increases and the dsDNA becomes denatured. There are two distinct stages to the curve: the rapid loss of fluorescence as the DNA begins to melt and the slower loss of fluorescence as the last of the dsDNA disassociates. Software can be used to plot the negative first derivative of the rate of change of fluorescence vs. temperature (-d(RFU)/dT). A characteristic peak at the amplicon's melting temperature (Tm, the temperature at which 50% of the base pairs of a DNA duplex are separated) distinguishes it from other products such as primer-dimers, which melt at different temperatures. An example of this is shown in Figure 11. The melt peak with a Tm of 89°C represents the specific product, and corresponds to the upper band in lanes 2 and 3 on the gel. The peak with a Tm of 78°C represents the nonspecific product, and corresponds to the lower bands in lanes 2 and 3 on the gel.

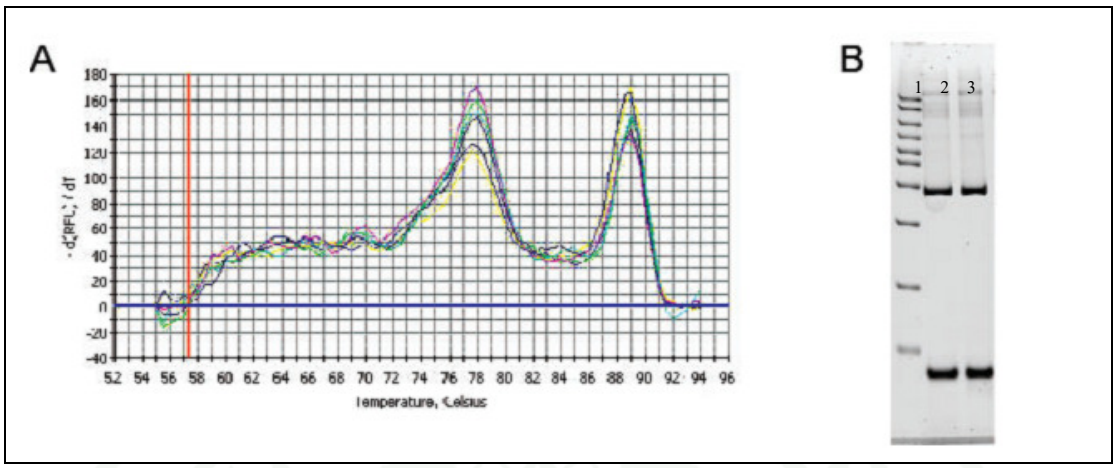


Figure 11 Melt–curve analysis of reaction product from a SYBR Green I assay. The melt-curve analysis function of real-time instruments can be used to distinguish specific products from non-specific products. A. The negative first derivative of the change in fluorescence is plotted as a function of temperature. The two peaks indicate the Tm values of two PCR products. B. Gel analysis of the qPCR products. Lane 1, AmpliSize® 50–2,000 base pairs (bp) molecular ruler; lanes 2 and 3, two replicates of qPCR product from the reaction shown in (A). The two PCR products are revealed by separate bands in the gel.

Source: Bio-Rad Laboratories Inc. (2006)

8.3 Relative quantitation

During relative quantitation, changes in sample gene expression are measured based on either an external standard or a reference sample, also known as a calibrator. When using a calibrator, the results are expressed as a target/reference ratio. There are numerous mathematical models available to calculate the mean normalized gene expression from relative quantitation assays. Depending on the method employed, these can yield different results and thus discrepant measures of standard error.

Amplification efficiency, Amplification efficiency of the reaction is an important consideration when performing relative quantitation. Past methods of calculating gene expression have assumed the amplification efficiency of the reaction is ideal, or 1, meaning the PCR product concentration doubles during every cycle within the exponential phase of the reaction. However, many PCRs do not have ideal amplification efficiencies, and calculations without an appropriate correction factor may overestimate starting concentration. Current mathematical models make assumptions of reaction kinetics and usually require its accurate measurement. Traditionally, amplification efficiency of a reaction is calculated using data collected from a standard curve with the following formula:

$$\text{Exponential amplification} = 10^{(-1/\text{slope})}$$

$$\%\text{Efficiency} = (10^{(-1/\text{slope})}-1) \text{ X } 100$$

The amplification efficiency of the reaction varies from being relatively stable in the early exponential phase and gradually declining to zero. There are several alternate methods of calculating amplification efficiency based on raw data collected during PCR. During the exponential phase, the absolute fluorescence increase at each PCR cycle for each individual sample reflects the true reaction kinetics of that sample. Consequently, data collected during the exponential phase can be log–transformed and plotted with the slope of the regression line representing the sample's amplification efficiency.

9. The relative quantification of gene expression

9.1 Standard curve method for relative quantification

The quantity of each experimental sample is first determined using a standard curve and then expressed relative to a single calibrator sample. The calibrator is designated as 1-fold, with all experimentally derived quantities reported as an n–fold difference relative to the calibrator. Because sample quantity is divided by calibrator quantity, standard curve units are eliminated, requiring only the relative dilution factors of the standards for quantification. This method is often applied when the amplification efficiencies of the reference and target genes are

unequal. It is also the simplest method of quantification because it requires no preparation of exogenous standards, no quantification of calibrator samples, and is not based on complex mathematics. However, because this method does not incorporate an endogenous control (usually a housekeeping gene), results must still be normalized.

This method requires the least amount of validation because the PCR efficiencies of the target and endogenous control do not have to be equivalent. This method requires that each reaction plate contain standard curves, and requires more reagents and more space on a reaction plate. This approach gives highly accurate quantitative results because unknown sample quantitative values are interpolated from the standard curve(s). Consider this method when testing low numbers of targets and small numbers of samples and if looking for very discrete expression changes.

9.2 Comparative Ct ($2^{-\Delta\Delta C_T}$) method (Livak method)

The comparative C_T method is a mathematical model that calculates changes in gene expression as a relative fold difference between an experimental and calibrator sample. While this method includes a correction for non-ideal amplification efficiencies (not 1), the amplification kinetics of the target gene and reference gene assays must be approximately equal because different efficiencies will generate errors when using this method. Consequently, a validation assay must be performed where serial dilutions are assayed for the target and reference gene and the results plotted with the log input concentration for each dilution on the x–axis, and the difference in C_T (target–reference) for each dilution on the y–axis. If the absolute value of the slope of the line is less than 0.1, the comparative C_T method may be used. The PCR product size should be kept small (less than 150 bp) and the reaction rigorously optimized. Because the comparative C_T method does not require a standard curve, it is useful when assaying a large number of samples since all reaction wells are filled with sample reactions rather than standards.

Standard curves are not required to run on each plate, which can result in reduced reagent usage. Gene Expression Assays have amplification efficiencies of 100%, and because of this, if using custom primers and probes, an initial validation relative standard curve is

recommended to validate the PCR efficiencies of the target and endogenous control(s), particularly when looking for low-expression-level fold changes. The comparative C_T method is useful when a high number of targets and/or number of samples are tested. Consider this method when using a high throughput strategy and when validating microarray results.

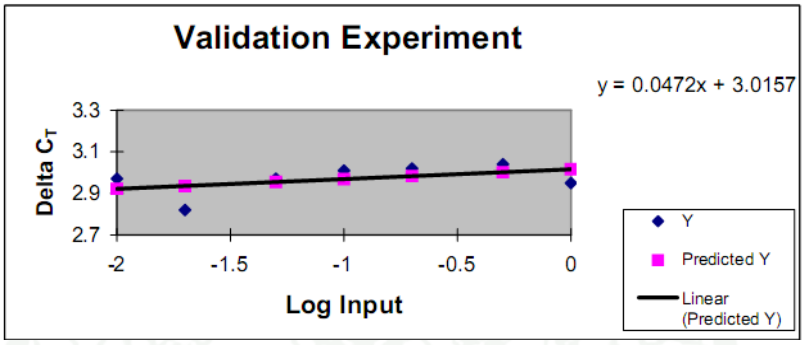
The Comparative C_T Method, also referred to as the $\Delta\Delta C_T$ Method, is similar to the Relative Standard Curve Method, except it uses arithmetic formulas to achieve the result for relative quantitation. It is possible to eliminate the use of standard curves and to use the $\Delta\Delta C_T$ Method for relative quantitation as long as the PCR efficiencies between the target(s) and endogenous control(s) are relatively equivalent. Arithmetic Formula: The amount of target, normalized to an endogenous reference and relative to a calibrator, is given by: $2^{-\Delta\Delta_{Ct}}$

For a valid $\Delta\Delta$ C_T calculation, the efficiency of the target amplification and the efficiency of the reference amplification must be approximately equal. To determine if the two amplification reactions have the same PCR efficiency, how the ΔC_T (C_T target - C_T reference) varies with template dilution. Assessing the relative efficiencies of the target amplification and the reference (endogenous control) amplification is achieved by running standard curves for each amplicon utilizing the same sample. The sample in the validation experiment must express both the target and reference genes. For example, a sample that ultimately is evaluated during experimentation (such as the calibrator) could be used. The C_T values generated from equivalent standard curve mass points (target vs. reference) are used in the ΔC_T calculation. These ΔCt values are plotted vs. log input amount to create a semi-log regression line. The slope of the resulting semi-log regression line can be used as a general criterion for passing a validation experiment. In a validation experiment that passes, the absolute value of the slope of Δ C_T vs. log input is < 0.1.

In the following example, replicates of each standard curve point were evaluated by real-time PCR. The mean and standard deviations of the replicate sample C_T values are presented in the following Figure 12.

Input Amount of Total RNA (ng)	*c-myc* Average C_T	GAPDH Average C_T	ΔC_T *c-myc* – GAPDH
1.0	25.59 ± 0.04	22.64 ± 0.03	2.95 ± 0.05
0.5	26.77 ± 0.09	23.73 ± 0.05	3.04 ± 0.10
0.2	28.14 ± 0.05	25.12 ± 0.10	3.02 ± 0.11
0.1	29.18 ± 0.13	26.16 ± 0.02	3.01 ± 0.13
0.05	30.14 ± 0.03	27.17 ± 0.06	2.97 ± 0.07
0.02	31.44 ± 0.16	28.62 ± 0.10	2.82 ± 0.19
0.01	32.42 ± 0.12	29.45 ± 0.08	2.97 ± 0.14

A



B

Figure 12 ΔC_T calculations for the validation experiment,A; Validation plot of ΔC_T vs. log input amount of RNA, B

Source: Applied Biosystem (2012)

9.3 Normalization of gene expression (Pfaffl model)

The Pfaffl model combines gene quantification and normalization into a single calculation. This model incorporates the amplification efficiencies of the target and reference (normalization) genes to correct for differences between the two assays. Normalization of gene expression, data is used to correct sample-to-sample variation. Starting material obtained from different individuals usually varies in tissue mass or cell number, RNA integrity or quantity, or experimental treatment. Under ideal conditions, mRNA levels can be standardized to cell number, but when using whole tissue samples, this type of normalization is impossible. Therefore, real–time PCR results are usually normalized against a control gene that may also serve as a positive

control for the reaction. The ideal control gene should be expressed in an unchanging fashion regardless of experimental conditions, including different tissue or cell types, developmental stage, or sample treatment. Because there is no one gene that meets this criterion for every experimental condition, it is necessary to validate the expression stability of a control gene for the specific requirements of an experiment prior to its use for normalization.

Normalize gene expression analysis could provide more advantages than Comparative gene expression and the comparison of both method was presented in Table 7.

Table 7 The comparison of relative gene expression analysis between Comparative method and Normalize method

Analysis Requirement	**Comparative method**	**Normalize method**
%efficiency closed to 100	100% efficiency needed	Use the real efficiency for better accuracy
Linearity testing	Need	No need
Reference genes	One gene	More than one gene could be added for more accuracy

10. Normalized gene expression

Normallized expression is the relative quantity of target gene normalized to the relative quantities of the reference genes. The reference gene (endogeneous control or house keeping gene) are not regulated in the experiment system. Normalized expression calculations will account for loading different or variations in cell number represented in each sample. Normalized expression can be viewed as a normalized target sequence quantity if the assay has included controls (untreated sample) for this purpose. The value is sometimes refered to as $\Delta\Delta C_T$ or dd C_T because of the equation initially introduced by Livak *et al.* (1995) to evaluate normalized expression. The software of Bio-Rad IQ5 uses a modification of this equation to use the real efficiency and also put through more than one reference gene for more accuracy, as below:

$$\text{Normalized expression}_{\text{sample (gene X)}} = \frac{\text{Relative Quantity}_{\text{sample (gene X)}}}{\text{Relative Quantity}_{\text{sample (ref gene 1)}} * \text{Relative Quantity}_{\text{sample (ref gene 1)}} * \ldots.}$$

$$\text{Relative Quantity}_{\text{(gene X)}} = (E_{\text{gene X}})^{\Delta_{\text{Ct(control-sample)}}}$$

Where E = efficiency of primer, this efficiency was calculated as follow (% efficiency X 0.01 + 1) where 100% = 2

$\mathbf{C_T}$ control = Average $\mathbf{C_T}$ for the sample which has been assigned as a control

$\mathbf{C_T}$ sample = Average $\mathbf{C_T}$ for the sample

In terms of normalization, the use of multiple housekeeping genes is the most accurate method. Nevertheless, when one has only a few genes to assay or a sample set with low diversity (such as cell culture), it may not be feasible to run multiple housekeeping genes. If a single gene is used, its stability should be validated in an assay similar to the one used to rank gene stability in the geNorm (Comparative Ct ($2^{-\Delta\Delta C_T}$) method).

Because real-time PCR is now a common method for measuring gene expression, it is increasingly important for users to be aware of the numerous choices available in all aspects of this technology. Unlike traditional PCR, there are many complexities with real-time PCR that can affect overall results. However, with a well-designed experiment performed with the proper controls, real-time PCR can be one of the most sensitive, efficient, fast, and reproducible methods of measuring gene expression.

MATERIALS AND METHODS

Materials

1. Agricultural wastes by–products

Three agricultural wastes, cassava pulp (CP), palm kernel meal (PKM) and rough rice bran (RRB) were obtained from Suppaluek Farm at Rachaburi Province, Thailand. The sizes of both RRB and CP were 0.5 mm with the moisture content of 12%, while the one of PKM was 0.2 mm with 14% moisture content.

2. Fungal strain and its cultivation

Aspergillus niger 386017M1 was obtained from the culture collection of Alltech Inc. (USA) and maintained as freeze dried form with regular subculturing at an interval of 21 d. The 1 X 10^8 spores/ml prepared by harvesting at 21 d culture on PDA medium was grown by submerged fermentation in 200 ml cultivation liquid medium (in g/100 ml, corn starch, 1.4; glucose, 1.0; bactopeptone, 1.8; KCl, 0.05; $MgSO_4 \cdot 7H_2O$, 0.15; KH_2PO_4, 0.1 and wheat bran, 2.0) at 30°C, shaking at 250 rpm for 2 d. The culture solution obtained was used as a starter for further solid state fermentation (SSF).

3. Primers for qRT-PCR

The seven primer sets used to determine the quantity of 7 target genes involving in cellulase regulation by RT-PCR were shown in Table 8.

Table 8 Primers and their sequences designed for qRT-PCR

Target Gene	Description for gene encoding protein	Acession no.	Primer sequence (5'→3') (Forward;F and Reverse; R)	Product size	Pedicted Product Tm (°C)
cbhA	1,4-β-D-glucan cellobiohydrolase A	XM_001391971	F: GCT GGA CCC ATT CTG TCA ATG R: TCC CAG GTG TTG CCA GTG T	55	56.5
cbhB	1,4-β-D-glucan cellobiohydrolase B	XM_001389539	F: GCG AGC TGC GCC TGA A R: CGA GAC CCG ATG TTC TTG CT	55	57.8
xlnR	Xylanolytic and cellulolytic activator	XM_001397073	F: GTG CGC TCA TTG CAT TGA AT R: TTG CGT TCT CGC GCA TAC	55	55
creA	Catabolite repressor	XM_001399482.1	F: ACA GCT CCA AGA CCC CTT CTA R: ACG ACG CCA TGT TGG AGT T	60	57.3

Table 8 (Continued)

Target Gene	Description for gene encoding protein	Acession no.	Primer sequence (5'⟶3') (Forward;F and Reverse; R)	Product size	Pedicted Product Tm (°C)
pacC	pH regulator in dual function	XM_001399885.1	F: CTG CGG CAA AGC CTT CA R: CAT GGG TCT TGA CGT GCT TCT	56	55.6
ace2	Transcription activator	XM_001391061.1	F: TGC AGA TCG CCG TCA TTC T R: CGC AGC CGA CTG AGC AA	52	56.8
tub	The constituent protein of microtubules	XM_001388951.2	F: GCT TCC AGT TGT TAC CAG CAC C R: GCC CCG ACA ACT TCG TCT T	51	56.7

Methods

1. Solid state fermentation system (SSF)

The SSF system was carried out by the modified method of Ravindra and Ravindra (2001). The fermentation process was performed in a 500 ml Erlenmeyer flask containing 3 ml of starter and 10 g of each agricultural substrate, RRB, PKM and CP, adjusted to gain the final moisture content of 50% by sterilized distilled water at 30 °C for 7 d. The culture samples were harvested and freeze dried using LABCONCO LYPH.LOCK6, USA at -40°C for 4–5 day and then kept at -20°C for further analysis.

2. Pretreatment of substrates

2.1 Acid pretreatment

The acid pretreatment (AC) was carried out according to the modified method of Schell *et al.* (1999). The substrate was soaked in 0.35% sulfuric acid by the ratio of 1:2 and further autoclaved at 121 °C for 1 h. The hydrolysates after acid removal by tab water were dried at 40°C overnight to obtain the moisture content of about 15%. Then, the samples were kept at -20°C until use.

2.2 Alkaline pretreatment

The alkaline pretreatment (AKL) followed the modified method of Tatsumoto *et al.* (1988). The mixture of 5% substrates and 4% NaOH (w/v) were heated to 121 °C for 1 h. The hydrolysates after alkaline removal by tab water were dried at 40°C overnight to obtain the moisture content of about 15%. Then, the samples were kept at -20°C until use.

2.3 Steam pretreatment

The steam pretreatment was examined by modified method of Brownell and Saddler (1987), the substrates were soaked in distilled water by the ratio of 1:2 and autoclaved at 140 ^{o}C for 2 h. Then, the hydrolysates were dried at 40^{o}C overnight to obtain the moisture content of about 15%. Then, the samples were kept at -20 ^{o}C until use.

3. Determination of cellulolytic Enzyme activity

3.1 Endo–1, 4–β–glucanase (CMCase)

Endo–1, 4–β–glucanase (CMCase) activity was assayed in a reaction mixture containing 1.0 ml of 1.0% carboxymethylcellulose in 50 mM acetate buffer pH 4.8 and 0.1 ml of crude enzyme solution. The reaction mixture was incubated at 50 ^{o}C for 10 min and later stopped by heating in boiling water for 5 min. The amount of reducing sugar was determined by the Somogyi–Nelson method (Somogyi, 1992). One unit of CMCase activity was defined as the amount of enzyme required to liberate 1 μmol of reducing sugar liberated per min.

3.2 Exo–1, 4–β–glucanase or cellobiohydrolase (CBH)

Exo–1, 4–β–glucanase or cellobiohydrolase (CBH) activity was determined according to the method of Laymon *et al.* (1996). The reaction mixture contained 0.1 ml of enzyme solution and 0.9 ml of 0.5 mM 4–Methylumbelliferylcellobioside in 0.1 M sodium acetate buffer, pH 5.0. After incubation at 50 ^{o}C for 2 h, the reaction mixtures were stopped by the addition of 2 ml of 0.2 M sodium carbonate solution. The liberated 4–methylumbelliferone (4–MU) was measured by fluorogenic absorbance at emission and excitation of 460 and 365 nm, respectively. One unit of CBH activity was defined as the amount of enzyme required to liberate 1 microequivalent of 4–MU per min.

3.3 1, 4–β–glucosidases (BG)

For the activity of 1, 4–β–glucosidases (BG), the assay was carried out according to the method of Macris (1984). In brief, the reaction mixture of 0.1 ml of enzyme solution and 1 ml of 2 mM ρ–nitrophenyl–β–D glucopyranoside solution in 0.1M acetate buffer, pH 5.0 were incubated at 40 °C for 5 min and then stopped by the addition of 2 ml of 1 M sodium carbonate solution. One unit of β–glucosidases activity was defined as the amount of enzyme required to liberate 1 μmol of ρ–nitrophenol per min.

4. Determination of fungal growth

The growth of *A. niger* 386017M1 was determined according to the method of Dobois *et al.* (1956). In brief, 2 ml of 60% sulfuric acid were added into 0.5 g of SSF sample and incubated at 25°C for 24 h. The reaction was then diluted by deionized water about 18 times and autoclaved at 121 °C for 1 h. After cooling, the mixture was neutralized by 3 N NaOH and made up to the final volume of 100 ml to obtain glucosamine solution. The contents of glucosamine were determined by acetylated glucosamine reaction. The mixture containing 2 ml of glucosamine sample and 2 ml of acetylacetone solution were incubated at 96 °C for 20 min. After cooling, 20 ml of 96% ethanol and 2 ml of Ehrlich reagent were added and left stand for 1 h at room temperature. The amount of N–acetyl glucosamine was measured by the absorbance at 530 nm against the standard curve of D (+) –glucosamine.

5. Analysis of cellulose and hemicellulose and lignin contents

The concentration of cellulose and hemicellulose were analysed by the modified method of Hans *et al.* (1999). One gram of sample was suspended in ethanol (4 volumes) and kept for 15 min. The reaction mixture was further incubated at 40°C for overnight. Then, 30 ml of 1% diastase was added and incubated at 25 °C for 30 min to remove all residual starch. The enzymes residues were removed by distilled water and dried at 40°C. A haft of sample was determined for its dry weight (A) while the rest was treated with 25 ml of 24% KOH at 25°C for 4 h. After removing KOH by distilled water, the sample was dried at 80 °C to obtain the dry weight (B). The

B fraction was further treated with 25 ml of 72% H_2SO_4 for 3 h and further reflux with 25 ml of 5% H_2SO_4 for 2 h to hydrolyze all the cellulose. H_2SO_4 was removed by distilled water. The sample was dried at 80 ^{o}C to obtain the dry weight called fraction C. The amount of cellulose, hemicellulose and lignin were calculated as following equation: %Cellulose = (B-C) X 100/A, % Hemicellulose = (A-B) X 100/A, %Lignin = C X100/A.

6. Analysis of monosaccharide contents

Both quantitative and qualitative of saccharides which are the composition of cellulose and hemicellulose were analysed by the modified method of Hoebler *et al.* (1989). Fifty milligram of sample was firstly hydrolyzed by 1 ml of 72% H_2SO_4 (wt/wt) for 30 min. After hydrolysis, the sample was diluted to 2 N H_2SO_4 by adding 11 ml of distilled water and further heated in a boiling water bath for 2 h. The treated sample was then filtrated (Whatman filter paper) and neutralized to pH 5–6 by calcium carbonate. Ten milliliters of aliquots were then filtered through 0.2 µm filter membrane and further analyzed by high–pressure liquid chromatography (HPLC) using Aminex HPX–87P carbohydrate column (300 X 7.8 mm) to separate each saccharide at 85°C by deionized water with the flow rate of 0.6 ml/min. Arabinose, xylose, glucose, galactose, mannose and cellobiose were used to develop standard curves at the concentration of 0.3–1.6 mg/ml. The data obtained were analyzed by SHIMADZU; HPLC LC–20A chromatographic data management system.

7. Scanning electron microscopy (SEM)

A material was completely dried by freeze drier. The dry material was mounted on a specimen stub using an adhesive by electrically–conductive double–sided adhesive tape, and sputter coated with gold before examination in the microscope. The samples were coated with gold using a vacuum sputter–coater (JEOL JSFC 1,200 series High Resolution Sputter Coater, Japan) to improve the conductivity of the sample. SEM analysis was performed with a JEOL JSM5410 series, Japan and operated at 20kV.

8. RNA extraction

8.1 Total RNA isolation

The 100 mg of either pure culture of *A. niger* 386017M1 pellet or SSF culture were frozen in liquid nitrogen and ground to powder form by cool mortar. Total RNA was isolated by RNAqueous® kit (Applied Biosystems/Ambion, Austin, Texas, USA) according to the manufacturer's instructions. The concentration of total RNA was quantified by measuring the absorbance at 260 nm (NanoDrop ND-1000, NanoDrop Technologies, North Carolina, USA). One absorbance unit at 260 nm corresponded to approximately 40 µg/ml (Sambrook and Russel, 2001). The RNA concentration of each sample was estimated in µg/ml by the following equation, [RNA] = A260 X dilution factor X 40 µg/ml Where [RNA] was RNA concentration. The RNA sample was stored at -80 °C for use in cDNA reverse transcription process. The RNA quality was measured by formamide gel electrophoresis (Appendix A). The ratio of absorbance reading at 260 and 280 nm (ratio range of 1.8–2.0) indicated the RNA good was quality.

8.2 DNase I treated total RNA

DNA contaminants in qRT-PCR reaction was eliminated by deoxyribonuclease I (Sigma, Cat no. AMP–D1) prior to sensitive reverse transcriptase–polymerase chain reaction. The 8 µl of RNA in DEPC water was added by 1 µl of 10X reaction buffer and 1µl of Amplification grade DNase I (1 unit/µl). The sample was mixed gently and incubated for 15 min at room temperature. The 1 µl Stop solution was added into the reaction to bind calcium and magnesium ions and to activate the DNase I. The reaction was heated at 70 °C for 10 min to denature both the DNase I and RNA. The sample was chilled on ice and immediately used in reverse transcription process.

9. cDNA template preparation

Total RNA used for reverse transcription was determined by Ready–To–Go T–Primed First–Stand Kit (Amersham Biosciences Corp, NJ, USA) according to manufacturer's instructions. The cDNA was stored at -20 °C for real–time PCR analysis (qRT–PCR).

10. Primer validation

The primer for the amplifcation of genes encoding cellulase of *cbhA, cbhB* and their regulatory genes of *xlnR, creA, pacC* and *ace2* were derived from the *A. niger* data bank at the National Center of Biotechnology Information (www.ncbi.nlm.nih.gov; as available in 2010) and presented in Table 8. The primer sequences were commercial supplied by Ward Medic Company, Thailand. The quantitative PCR was determined at the exponential phase after cycle optimization using iCycler iQ5 (BIO-RAD laboratories, Inc, USA). The RNA sample of *A. niger* 386017M1 grown in liquid media was first amplified by real–time PCR using gene–specific primers. The DEPC water and RNA sample of cassava material were used as non–specific template (non-template was blank) and negative control, respectively (Appendix A). The polymerase chain reaction contained the following; 10 µl 2X SsoFastEvagreen supermix; 3 µl forward primer (300 nM); 3µl reverse primer (300 nM); 2 µl cDNA (RNA sample 100 ng) and 2 µl DEPC treated water. The PCR cycling condition was as follows: 1 cycle at 95 ºC (initial denaturaion) for 20 s; 40 cycles at 95 ºC for 5 s (denaturation), 60 ºC for 45 s (primer annealing) detection of fluorescence signal process and 81 cycles at 55 ºC for 30 s for Tm analysis. PCR products were separated by agarose gel electrophoresis and visualized with UV transilluminator. The resulting PCR products were subjected to an automatic linear temperature transition from 60 to 95 ^{o}C, and the fluorescence was continuously measured. The fluorescence data were converted into melting peaks to plot the negative derivative of fluorescence over temperature (-dF/dT vs. T). The single peak of PCR products indicated to non primer–dimer interfere. Agarose gel electrophoresis was performed for size specific to the PCR product (Appendix B).

The C_T value of cDNA of pure culture *A. niger* 386017M1 was amplified by qRT–PCR in 2–fold serial dilution (100, 50, 25, 12.5 and 6.25 ng RNA sample) using gene encoding

cellulase of *cbhA, cbhB* and their regulatory system of *xlnR, creA, pacC* and *ace2*. The result could be used in capacity, tm and linearity determination.

10.1 Capacity determination

To determine cDNA loading capacity, the cDNA of pure culture *A. niger* 386017M1 was amplified by qRT–PCR in 2–fold serial dilution (100, 50, 25, 12.5 and 6.25 ng RNA sample). If perfect doubling occurs with each amplification cycle, the spacing of the fluorescence curves will be determined by the equation 2^n= dilution factor, where n is the number of cycles between curves at the fluorescence threshold (the difference between the C_T values of the curves). The standard curve by the serial dilution of DNA, $2^n = 2$ therefore, n = 1, and the C_T values should be separated by 1 cycle. The C_T value of each cDNA concentration can be log-transformed and plotted to obtain the slope of the regression line representing.

10.2 Tm determination

In a typical melt–curve, the fluorescence intensity is plotted against the temperature. The fluorescence decreases as the temperature increases and as the dsDNA becomes denatured. There are two distinct stages to the curve: the rapid loss of fluorescence as the DNA begins to melt and the slower loss of fluorescence indicating as the dsDNA disassociates. The software of iQ5 (Bio–Rad) can plot the negative first derivative of the rate of change of fluorescence vs. temperature (-d(RFU)/dT) automately after qRT–PCR process. The Tm value was presented by single peak.

10.3 Linearity determination

The linearity results were used for indicating the optimal qPCR assay which is absolutely essential for accurate and reproducible quantitation of a particular sample. The hallmark of an optimized qPCR assay is consistency across replicate reactions with the amplification efficiency of each gene approximately equal. The linearity was determined by equation $2^{-\Delta_{CT}}$ where $\Delta C_T = C_{T\ Target} - C_{T\ TUB}$. The resulting ΔC_T values of target genes were

plotted against the logarithm of cDNA and the graph slope were determined. A slope value of less than 0.1 indicated the amplification efficiencies of the target and endogenous control gene which should be approximately equal.

11. Gene expression

11.1 Efficiency determination

To obtain the %efficiency of primer amplification, the results from 10.1 was analyzed by iQ5 machine (data analysis program). The C_T data collected during the exponential phase can be log–transformed and plotted to determine the slope of the regression line representing the sample's amplification efficiency. The efficiency of 80–120 % was further used in relative qRT–PCR gene expression (Normalize expression). The iQ5 (Bio–Rad) analysis software identified lower than 80% efficiency as interrupted by seconday structure or dilution error while higher 120% efficiency was assumed as primer–dimer effect or dilution error. The amplification efficiency was calculated using data collected from a standard curve with the following formula:

$$\%\text{Efficiency} = [10^{(-1/\text{slope})} - 1] \text{ X } 100$$

11.2 Normalize gene expression

The quantification of gene expression was determined by relative quantity of target gene to the one of untreated or control group (calibrator). The RNA sample (cDNA template) of *A. niger* 386017M1 cultivation in different acid pretreated CP was amplified. The amplification of genomic DNA was prevented by DNase treatment of extracted RNA (Follow 11). Relative quantification of target genes in each treated sample and untreated sample (control or calibator) as the reference gene was determined. Relative quantification gene expression was expressed as a ratio of the target gene (*cbhA, cbhB, xlnR, creA, pacC* and *ace2*) quantity to the reference (housekeeping) gene quantity. Average $\mathbf{C_T}$ points were calculated by the Normalize expression method (Pfaffl, 2001) as the below equation. The study aim to determine the relative gene

expression of SSF cultivation during time course using different acid pretreated CP. Therefore, the templates from experimental pretreated CP cultivation of 0.1, 0.3 and 0.5% were assigned in separate 96-well plate using d 0 as control sample. The experiment was repeated by three times and all samples were analysed in 3 replicates. The results of relative gene expression values were plotted as $\log_{10}$ transformation. The relative quantity for all d 3, 5 and 7 samples and control samples d 0 will be presented.

$$\text{Normalized expression}_{(\text{gene X})} = \frac{\text{Relative Quantity}_{(\text{target gene X})}}{\text{Relative Quantity}_{(\text{reference gene})}} \quad \text{or} \quad \frac{(E_{\text{gene X}})^{\Delta \text{Ct (control-sample)}}}{(E_{\text{reference gene}})^{\Delta \text{Ct (control-sample)}}}$$

$$\text{Relative Quantity}_{(\text{gene X})} = (E_{\text{gene X}})^{\Delta \text{Ct(control-sample)}}$$

Where E = efficiency of primer, this efficiency was calculated as follow (% efficiency X 0.01 + 1) where 100% =2

C_T control = Average C_T for the sample which has been assigned as a control

C_T sample =Average C_T for the sample

12. Places

12.1 AP-Biosciences Centre, Thailand Science Park, Alltech, Thailand

12.2 Biotechnology Department, Faculty of Agro-Industry, Kasetsart University, Thailand

12.3 Europe–Biosciences Centre, Alltech, Ireland

13. Duration

The experiment were carried out from January 2008 to January 2012

RESULTS AND DISCUSSION

1. The time course of cellulase production of *A. niger* 386017M1

The three different natural lignocelluloses; RRB, PKM and CP were used for cellulase production from *A. niger* in order to compare the production of cellulase; CMCase, CBH and BG activitied during 10 days were amended.

1.1 The cellulase production of RRB

The maximum yield of CMCase, CBH and BG from SSF of RRB were day 7 (85±8 U/g, 5 (1,286±9 U/g) and 7 (4,346±1,115 U/g) respectively, at day 7 (Figure 13).

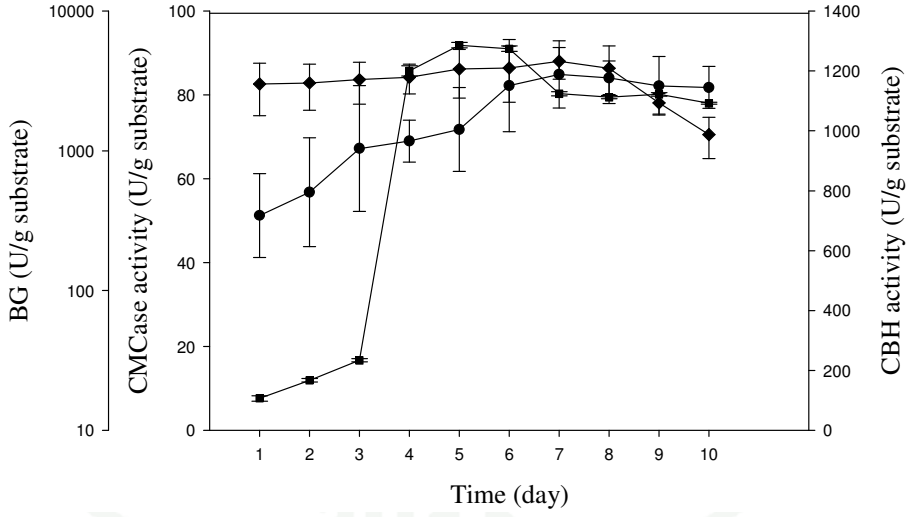


Figure 13 The cellulase production of *A. niger* 386017M1 under SSF system during 10 days by using RRB as substrate; —●— CMCase, —■— CBH, —◆— BG activity (U/g substrate)

1.2 The cellulase production of PKM

The maximum yield of CMCase, CBH and BG from SSF of PKM were day 7 (18±5 U/g), 5 (311±19 U/g) and 5 (893±52 U/g), respectively (Figure 14).

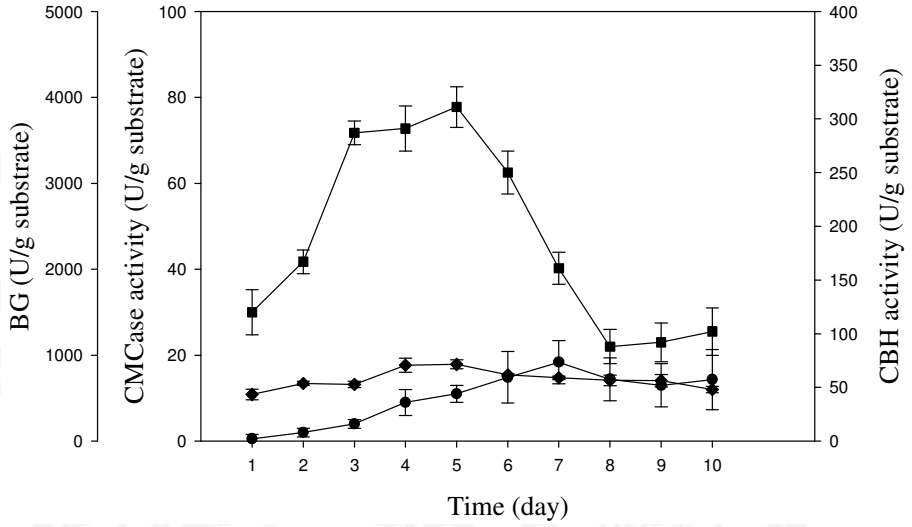


Figure 14 The cellulase production of *A. niger* 386017M1 under SSF system during 10 days by using PKM as substrate; —●— CMCase, —■— CBH, —◆— BG activity (U/g substrate)

1.3 The cellulase production of CP

The maximum CMCase, CBH and BG yield of SSF for CP were day 5 (56±6 U/g), 7 (288±18 U/g) and 4 (3,129±956 U/g) in respectively (Figure 15).

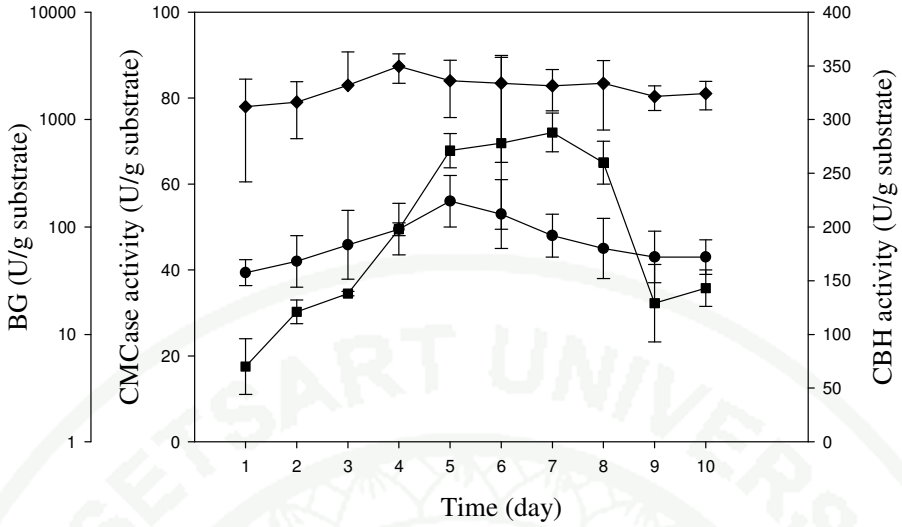


Figure 15 The cellulase production of *A. niger* 386017M1 under SSF system during 10 days by using CP as substrate; —●— CMCase, —■— CBH, —◆— BG activity (U/g substrate)

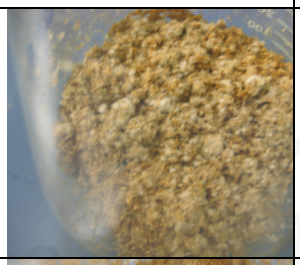
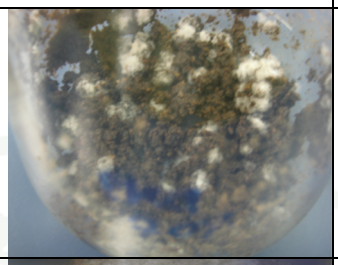
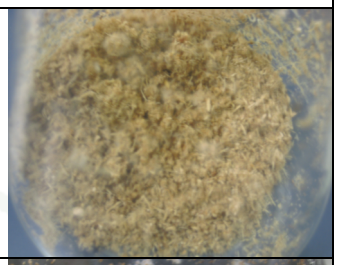
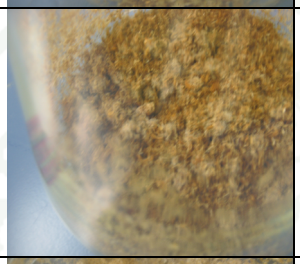
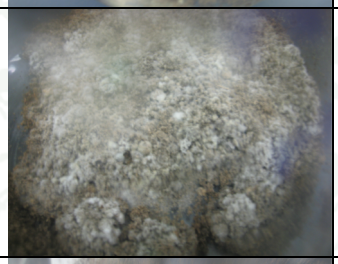
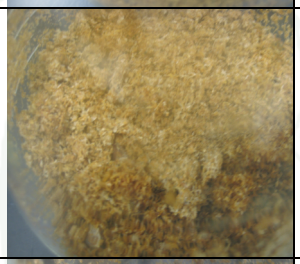
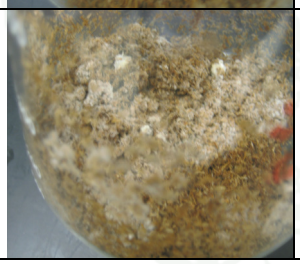
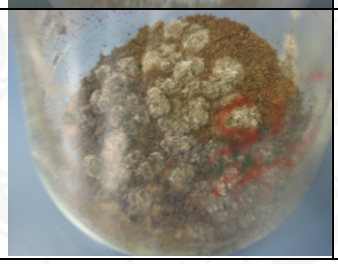
As above results, the maximum cellulase production of all material was day 5–7 and the maximum CBH acitivities of SSF for RRB and PKM was day 5 while the one for CP was day 7. Therefore 5 day fermentation was chosen for investigation of cellulase production from SSF of three materials.

2. Effect of pretreatment process on cellulase production

2.1 Suitable pretreatment of rough rice bran, palm kernel meal and cassava pulp for enzyme production

The pretreatment was generally required to improve the utilizability of lignocellulosic material, however the investment cost in process was considerable. The three pretreatment methods under mild condition were examined for cellulase production under SSF system (during 5 day) compared to untreated material as control. The morphology of *A. niger* on different pretreated material were showed in Table 9, there were no spore–forming during fermentation and these cultivation was extracted for cellulase determination.

Table 9 The morphology of *A. niger* on various pretreated materials under SSF at day 5

Materail/ Condition	RRB	PKM	CP
Control			
Acid			
Alkaline			
Steam			

Three pretreatments of acid, alkaline and stream process of 3 material sources of RRB, PKM and CP were carried out. The qualities of these pretreated substrates were assessed through measurement of three cellulase activities of cellulase; endoglucanase (CMCase), cellobiohydrolase and β–glucosidase as shown Table 10. The extracts from the pretreated RRB contained low activity of CMCase which were no significant different to the control (P>0.5) except alkaline treatment showed much lower activity than the control (P<0.5). Gossett *et al.* (1982) reported that an important aspect of alkali pretreatment is that biomass itself consumes

some alkali and causes solubilization, distribution and condensation of lignin and hemicellulose which can counter this positive effect. The results of this study were supported by Brijwani and Vadlani (2011) reporting that cellulolytic enzyme production in both mono and mixed cultures of *T. reesei* and *A. oryzae* significantly reduced in alkali–pretreated soybean hulls comparted to the native stem. However, SSF of acid and stream pretreated RRB provided the increasing activities of both CBH and BG for 2.9–3.7 and 1.4–1.6 times while the ones of PKM provided for 55–135 and 3.5–3.7 times compared to the control, respectively.

Considering to three pretreatment of CP, the activities of both CMCase and BG were higher than the control with no significant difference ($P>0.5$). Only the extract from acid pretreated CP in SSF provided the maximum CBH activity which was higher than the control for 521 times. These findings elucidated that the suitable pretreatments were needed for those agricultural wastes, especially, CP and PKM. However when the activities of these enzymes produced by different pretreatment were taken into account, it seems that both acid and stream pretreatment of RRB could provide the highest activities of CMCase, CBH and BG which might be due to excess nutrient sources for the fungal growth.

2.2 Solid state fermentation of pretreated cassava pulp, palm kernel meal and rough rice bran

According to enzyme production from different pretreated substrates by SSF, the suitable pretreatment of RRB, PKM and CP were stream, stream and acid process, respectively which were selected for further study. The composition of both pretreated and non–pretreated materials were analyzed as shown in Table 11. The amounts of cellulose from pretreated RRB, PKM and CP increased to 2.0, 3.2 and 1.6 times compared to the control, respectively while the ones of hemicellulose and lignin decreased to 1.3–1.5 times. It was presumed that the higher lignin removal of pretreated substrate would provide the accessible structure for enzyme digestion and further produce suitable carbon sources for fermentation to produce higher yield of CBH activity. By scanning electron microscope, the structure of this pretreated materials were changed to crack and rough surface of cell wall as shown in Figure 16-18. This confirmed that the pretreatment used affected the outer surface of materials.

Table 10 Effect of pretreatment methods to cellulase production by *A. niger*386017M1 at 5 day solid state fermentation.

Treatment	Rough rice bran			Palm kernel meal			Cassava pulp		
	CMCase (U/g)	CBH (U/g)	BG (U/g)	CMCase (U/g)	CBH (U/g)	BG (U/g)	CMCase (U/g)	CBH (U/g)	BG (U/g)
Control	70±20 [a]	2,126±672 [b]	20,864±838 [b]	10±6 [a]	12±2 [c]	7,171±2,220 [b]	9±1 [a]	14±3 [b]	3,817±821 [a]
Acid	44±27 [ab]	7,809±3,401 [a]	33,072±5,715 [a]	10±7 [a]	660±242 [b]	25,377±16,310 [ab]	11±2 [a]	7,303±648 [a]	9,349±5,546 [a]
Alkaline	17±13 [b]	235±124 [b]	8,042±1,981 [c]	8±5 [a]	431±21 [b]	13,119±3,153 [b]	8±4 [a]	584±85 [b]	4,931±1,915 [a]
Steam	58±8 [a]	6,244±3,121 [a]	28,485±5,352 [ab]	14±9 [a]	1,626±101 [a]	26,869±7,1544 [a]	11±3 [a]	13±5 [b]	9,517±1,260 [a]

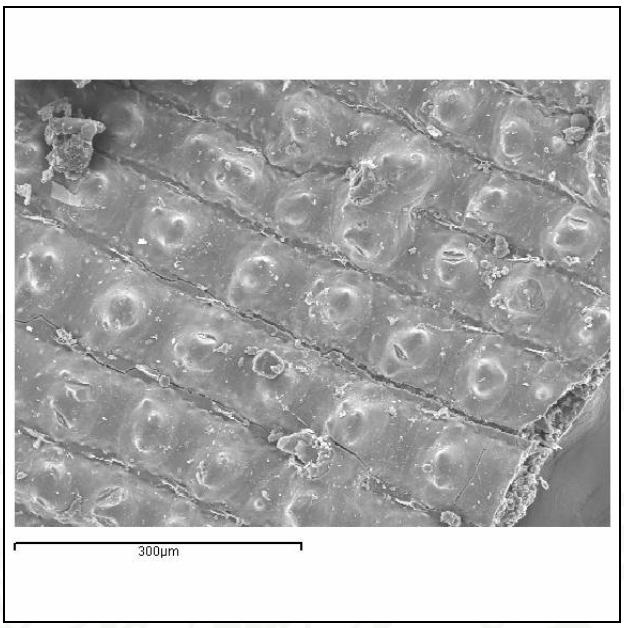
The values given above are means of three determinations ±SD. Different letters in each row indicate significant differences between the samples ($p \leq 0.05$) by RCBD, Random Complete Block Design of SPSS.

Table 11 Chemical composition of the native and pretreatment materials. RRB, rough rice bran; PKM, palm kernel meal; CP, cassava pulp.

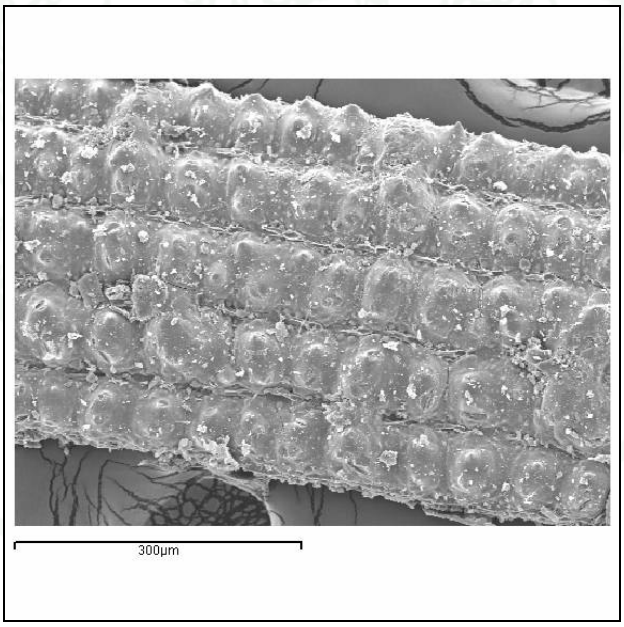
Material	% (w/w as dry basis)			
	Cellulose	Hemicellulose	Lignin	Other
Native RRB	28±8	26±5	26±3	20±4
Steam pretreated RRB	57±6	19±6	17±3	7±1
Native PKM	14±4	38±9	28±4	20±7
Steam pretreated PKM	45±7	27±6	20±9	8±4
Native CP	31±5	37±4	21±4	11±3
Acid pretreated CP	50±6	27±4	16±8	7±3

Both pretreated and native materials were further carried out for SSF by *A. niger* 386017M1 in order to investigate maximum cellulolytic enzyme production during cultivation. The results were shown in Figure 19-21. The growth of *A. niger* indicating as glucosamine concentration in SSF for the pretreated materials tended to be lower than the one in SSF for the native materials during 3–7 d. The maximum growth of *A. niger* 386017M1 from all SSF were 5 d. The pH decreased to around 4–5. CBH activities from SSF for these three pretreated materials were significantly higher than the one for the native ones ($P<0.05$). While the pretreated materials used for SSF did not significantly effect to the yields of CMCase and β–glucosidase at 3, 5 and 7 d. Production of CBH from SSF of pretreated PKM and CP exhibited at 3 d and kept rather stable during 7 d cultivation while the one of the control showed low activities of 8±0.8–13±5 units/g. The activities of CBH from SSF of pretreated PKM and CP were significantly increased to 135–146 and 434–698 folds, respectively compared to the control during 3–7 d. These finding showed that *A. niger* could be able to ferment only pretreated form and produce the high yields of CBH in SSF. Considering to SSF for RRB, CBH activity of the pretreated RRB displayed at 3 d and kept rather stable during 7 d cultivation as well. However, its high activity of 2,127±676 units/g from the control occurred at 5 d. This caused the CBH activities of the SSF for pretreated RRB increased to 568 and 3.4 folds at 3 and 5 d cultivation, respectively. It seems that CBH could be produced in SSF of both native and pretreated RRB but its production rate by native RRB fermentation was lower.

The reducing sugar left during fermentation as shown in Table 15 were lower in the pretreated RRB and PKM cultivation. The higher reducing sugar concentration left in the native RRB and PKM culture might concern to the rate liming step of CBH production. However, high glucose concentration of 376±28 mg/g substrate of pretreated CP culture left was higher than the native CP for 1.4 times (271±31 mg/g). These finding indicated that such glucose concentration left did not concern to catabolic repression of CBH production proposed by other works (Nakari-Seta´la´ and Penttila, 1995; Hanif and Rajoka, 2004; Gautam *et al*., 2011). These results were also supported by McKelvey and Murphy. (2010) reporting that addition of 1, 5 and 10% glucose in wheat bran fermentation of *A. niger* BFJS had no effect to the increasing of CMCase activity. Interestingly, high galactose concentration of 58±7 mg/g were detected from SSF for pretreated CP while it was not from the one for native CP. Karafta *et al.* (2006) reported D–galactose adding, may be an inducer at low dilution rate and not concern to carbon catabolic repression in *Hypocrea jecorina*. In addition, it was found that D–galactose induced cellobiohydrolase 2 (*cbh2*) gene transcription and lead to endoglucanase; Cel 7A and Cel6A protein accumulation under promoter region of *A. niger* in synthetic medium (Karafta *et al*., 2006). This may presume that galactose detected from the pretreated CP culture may involve in enhancing CBH production.

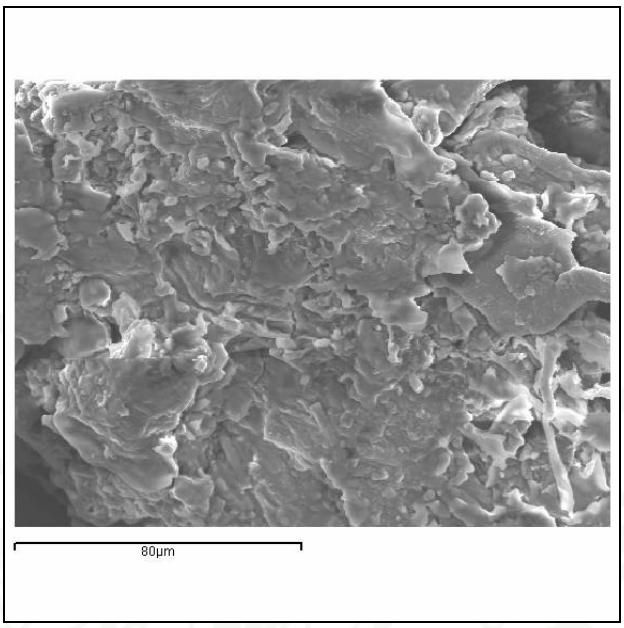


A

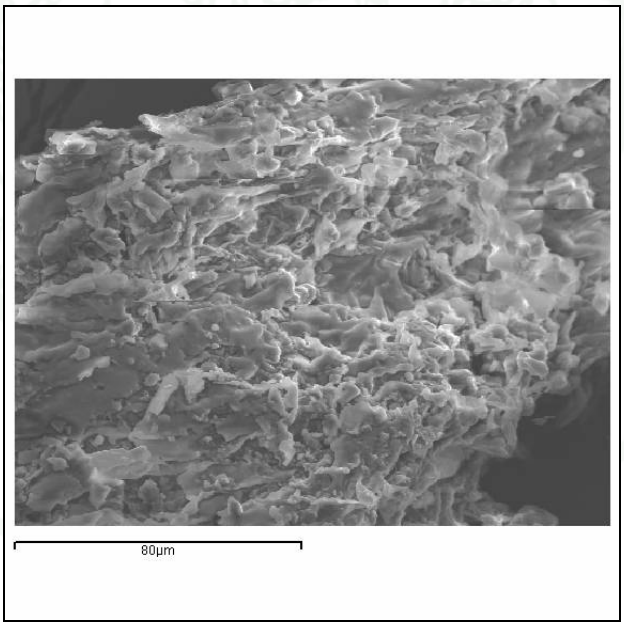


B

Figure 16 Photographs of native and pretreatment of rough rice bran (RRB), by scanning electron microscope. A, the native RRB (200X); B, the steam pretreated RRB (200X)

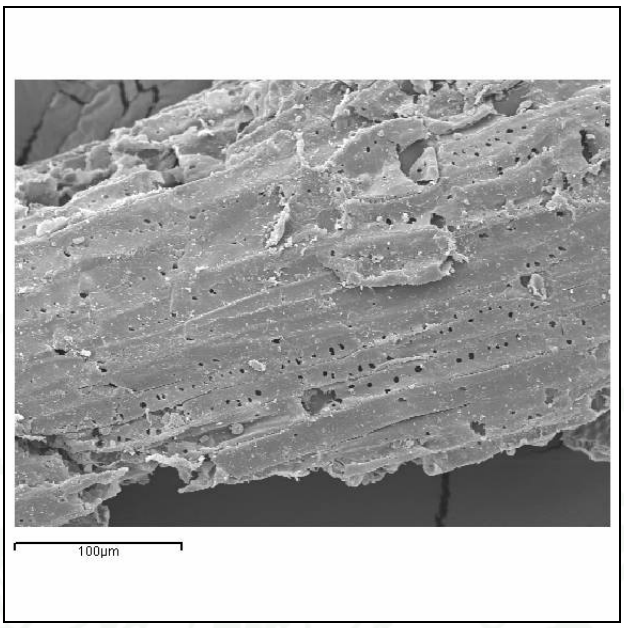


A

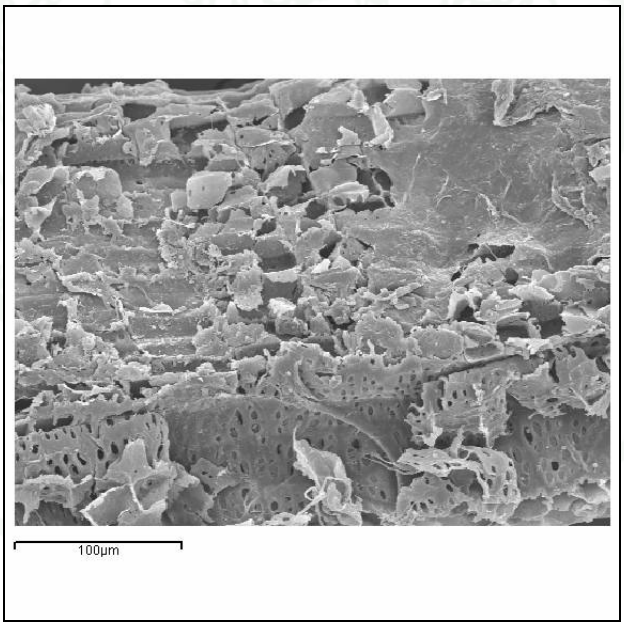


B

Figure 17 Photographs of native and pretreatment of palm kernel meal (PKM) by scanning electron microscope. A, the native PKM (750X); B, the steam pretreated PKM (750X)



A



B

Figure 18 Photographs of native and pretreatment of cassava pulp (CP) by scanning electron microscope. A, the native CP (350X) and B, the acid pretreated CP (350X).

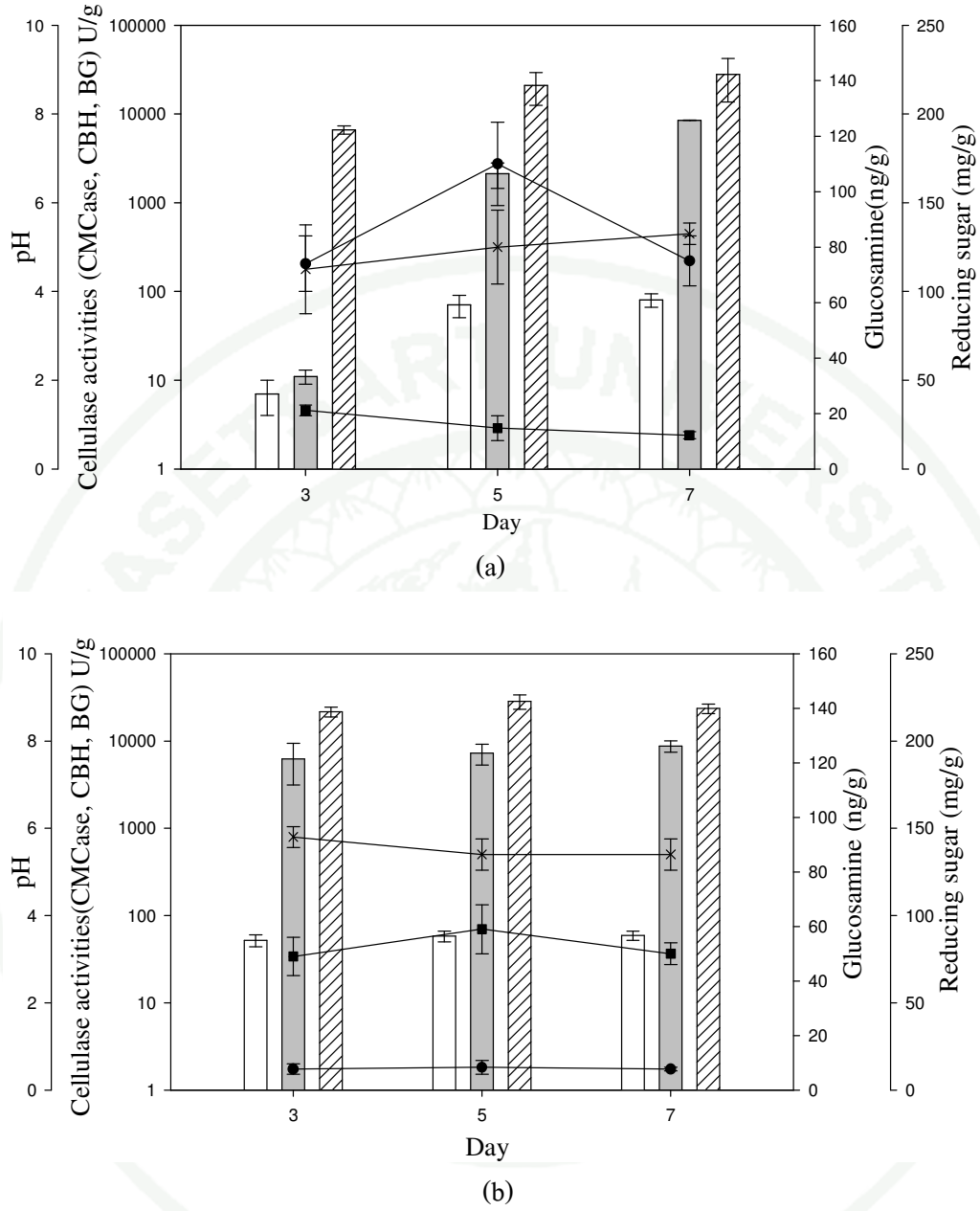


Figure 19 Solid state fermentation of the native and pretreated materials by *A.niger* 38017M1. (a), SSF for native rough rice bran; (b), SSF for steam pretreated rough rice bran; Growth of *A. niger* 38017M1 determined as glucosamine concentration ( ), CMCase activity ( ), CBH activity ( ), BG activity ( ), reducing sugar ( ) and pH ( ).

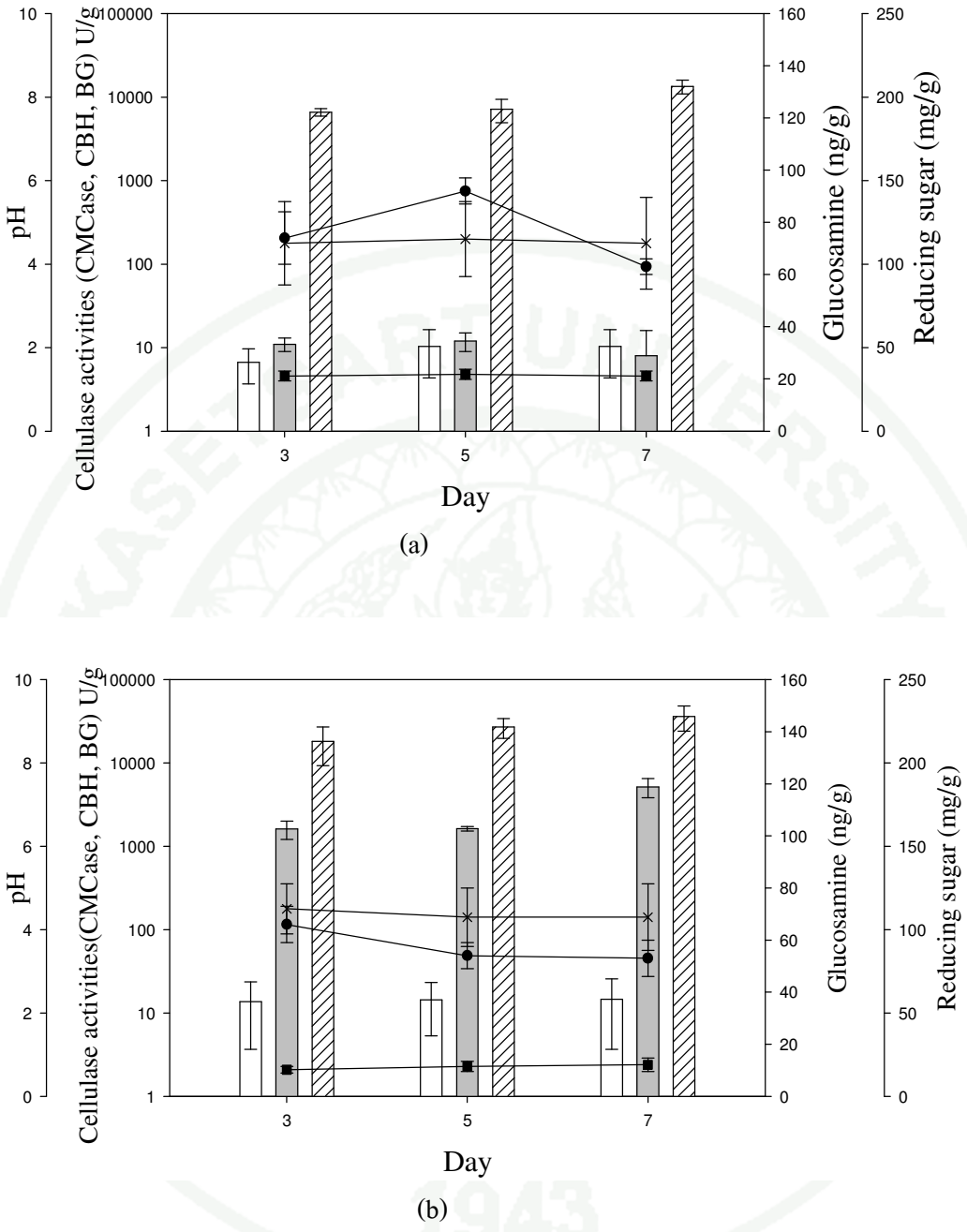


Figure 20 Solid state fermentation of the native and pretreated materials by *A.niger* 38017M1. (a), SSF for native palm kernel meal; (b), SSF for steam pretreated palm kernel meal. Growth of *A. niger* 38017M1 determined as glucosamine concentration ( ), CMCase activity ( ), CBH activity ( ), BG activity ( ), reducing sugar ( ) and pH ( ).

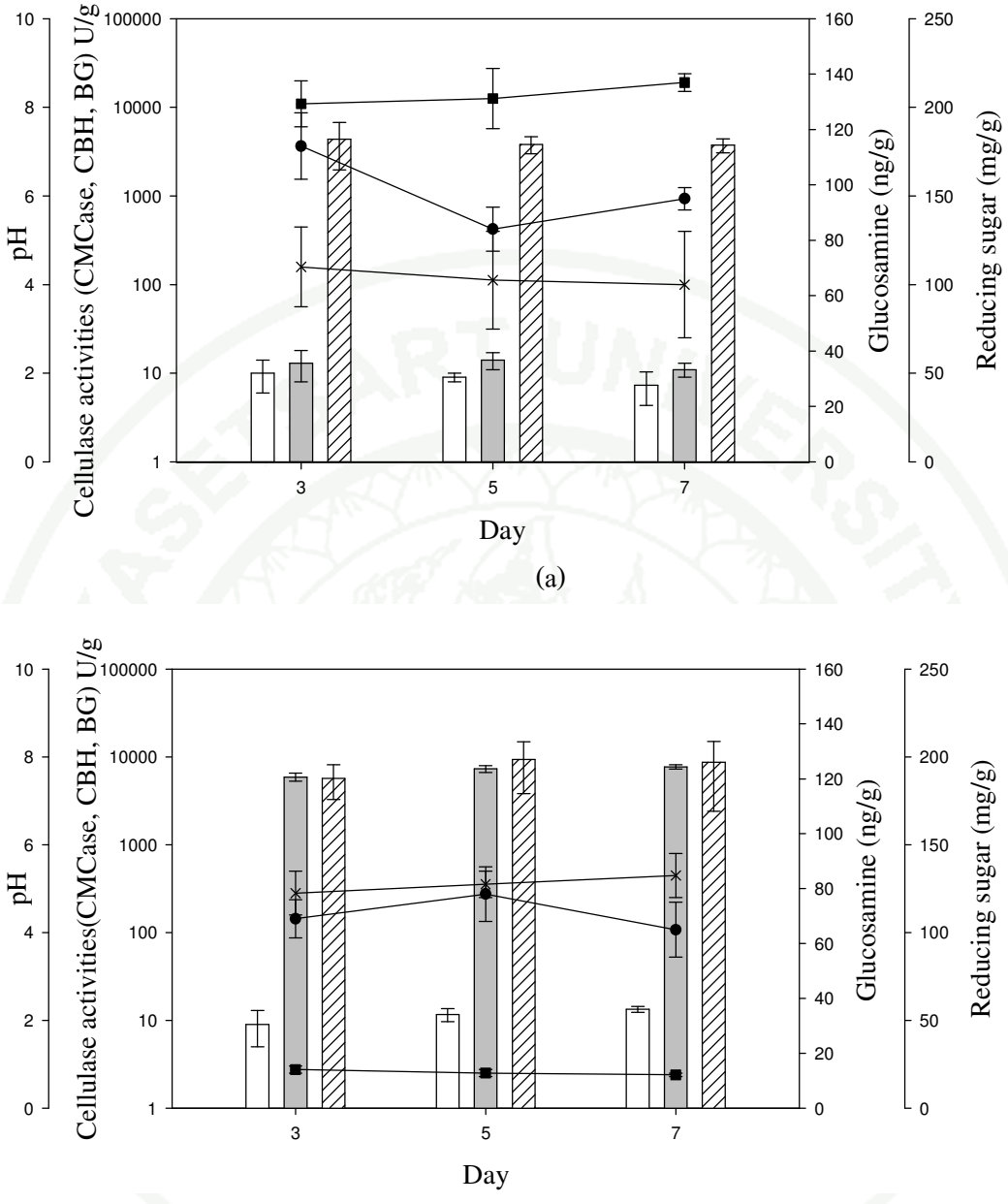


Figure 21 Solid state fermentation of the native and pretreated materials by *A.niger* 38017M1. (a), SSF for native cassava pulp; (b), acid pretreated cassava pulp; SSF. Growth of *A. niger* 38017M1 determined as glucosamine concentration ( ), CMCase activity ( ), CBH activity ( ), BG activity ( ), reducing sugar ( ) and pH ( ).

The enzyme activity of fungal in gram dry weight was showed in Table 12-14. This indicated that the fungal can produce the higher amount of cellulase activities in pretreated materials than the native one of all materials.

Table 12 The cellulase comparison, CMCase, CBH and BG (U/g cell) of *A. niger* by using suitable pretreated RRB

Day	Native RRB Cellulase (U/g cell)			Steam pretreated RRB Cellulase (U/g cell)		
	CMCase	CBH	BG	CMCase	CBH	BG
3	0.61±0.29	3.15±1.15	163.78±19.80	1.06±0.16	65.75±0.36	211.26±17.90
5	0.64±0.18	19.33±6.16	189.67±19.00	0.98±0.14	56.76±0.47	258.95±25.40
7	1.07±0.18	113.07±0.34	373.04±22.70	1.18±0.14	116.83±25.70	314.25±17.60

Table 13 The cellulase comparison, CMCase, CBH and BG (U/g cell) of *A. niger* by using suitable pretreated PKM

Day	Native PKM Cellulase (U/g cell)			Steam pretreated PKM Cellulase (U/g cell)		
	CMCase	CBH	BG	CMCase	CBH	BG
3	0.06±0.03	0.10±0.02	58.12±9.60	0.20±0.15	14.11±5.90	158.90±74.00
5	0.12±0.05	0.14±0.02	85.37±11.70	0.18±0.17	19.36±1.80	319.87±68.00
7	0.11±0.08	0.08±0.01	141.44±51.00	0.23±0.10	54.27±25.10	380.23±25.00

Table 14 The cellulase comparison, CMCase, CBH and BG (U/g cell) of *A. niger* by using suitable pretreated CP

Day	Native CP Cellulase (U/g cell)			Acid pretreated CP Cellulase (U/g cell)		
	CMCase	CBH	BG	CMCase	CBH	BG
3	0.14±0.03	0.18±0.04	58.89±5.70	0.14±0.04	79.66±8.90	76.99±30.00
5	0.10±0.01	0.15±0.03	41.49±8.40	0.22±0.01	79.38±8.30	101.62±27.00
7	0.12±0.02	0.17±0.01	59.19±8.50	0.25±0.05	121.94±7.05	137.83±17.00

Table 15 The contents of residual sugars from 5 days solid state fermentation of both native and pretreated materials. RRB, rough rice bran; PKM, palm kernel meal; CP, cassava pulp.

Material	mg/g as dry basis					
	Cellobiose	Glucose	Xylose	Galactose	Arabinose	Mannose
Native RRB	ND	245±17	11±2	11±4	17±2	5±1
Steam pretreated RRB	ND	78±8	ND	5±1	11±1	ND
Native PKM	108±12	254±39	79±6	2±1	67±7	14±2
Steam pretreated PKM	ND	120±24	8±2	8±4	2±1	16±1
Native CP	ND	271±31	ND	ND	8±2	14±2
Acid pretreated CP	ND	376±28	ND	58±7	16±4	16±3

ND = Not detectable

2.3 The comparison of pretreatment effect on CBH improvement

The CBH production of *A. niger* on different pretreated material were compare to untreated material. The results showed that SSF for acid pretreated CP provided maximum folds of CBH activity for about 521 times compared with native CP while others material obtained 3.4 and 135 times (Table 16). The SSF for acid pretreated CP was chosen as best nutrient source to produce CBH for further study.

Table 16 Comparison on CBH production of *A. niger* from SSF for pretreated materials and the native ones at day 5

Pretreated Material	CBH (U/g)	Changing rate (time)
RRB		
Native RRB	2,127	3.4
Steamed pretreated RRB	7,543	
PKM		
Native PKM	12	135
Steamed pretreated PKM	1,627	
CP		
Native CP	14	521
Acid pretreated CP	7,303	

3. Effect of various acid concentration of cassava pulp on cellulase production

The effect of three different sulfuric (H_2SO_4) concentration of 0.1, 0.3 and 0.5% to the growth, pH, sugar, chemical composition and cellulase production were carried out.

3.1 Chemical composition of various acid pretreated CP

The results were shown in Table 17. The maximum reduction of hemicelluloses and lignin was found from SSF for 0.3 and 0.5% acid pretreated CP for about 1.4 (30%), 4.3 (77%), respectively, compared to the control. Moreover, the cellulose content of both 0.3 and 0.5% acid pretreated CP was improved for about 121% and 160%, respectively.

Table 17 Physical composition of acid pretreated Cassava Pulp (CP) in various condition

%(w/w as dry basis)				
Pretreated CP (% Acid)	Cellulose (%)	Hemicellulose (%)	Lignin (%)	Other (%)
0	28±2	26±1	26±2	20±5
0.1	23±2	20±2	26±2	31±2
0.3	62±0	18±1	12±2	8±0
0.5	73±4	6±2	13±2	8±4

3.2 The effect of acid pretreatment on growth

The maximum production of fungal biomass expressed as glucosamine of SSF for pretreated CP with acid concentration of 0, 0.1, 0.3 and 0.5%were at day 7, 7, 5 and 3, respectively as shown in Figure 22. The highest growth of 120±9 ng/g was from 0% acid pretreated CP at d 7 however, the growth at d 3 of all condition showed at least 1 fold higher than d 0 and then stable after.

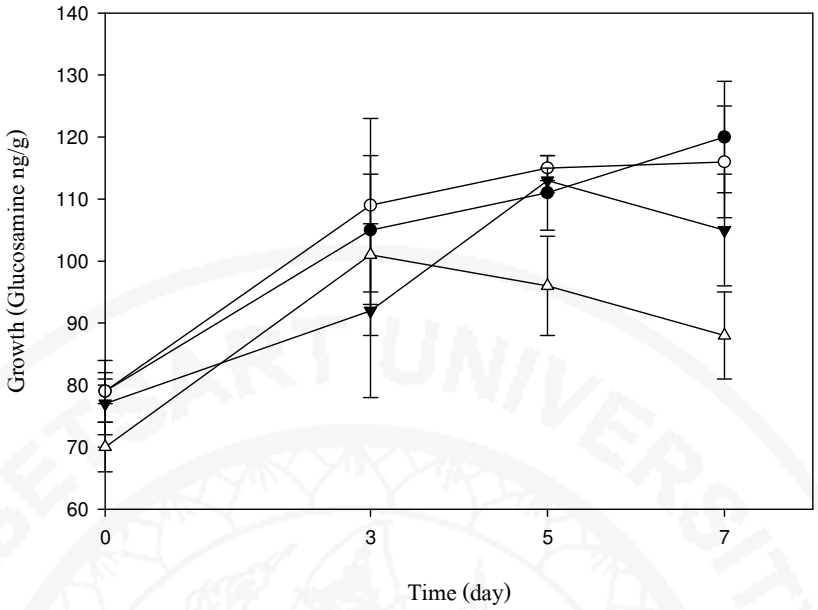


Figure 22 Effect of acid pretreatment on fungal growth of *A. niger* 386017M1 under SSF system by using; —●— 0%, —○— 0.1%, —▼— 0.3% and —△— 0.5% acid pretreated-CP during day 0, 3, 5 and 7

3.3 The effect of acid pretreatment on reducing sugar

The sugar left during cultivation was measured as shown in Figure 23. The trend of reducing sugar concentration increased during 3 d fermentation and then reduced. The reduction of reducing sugar might be because of the fungal utilization during fermentation.

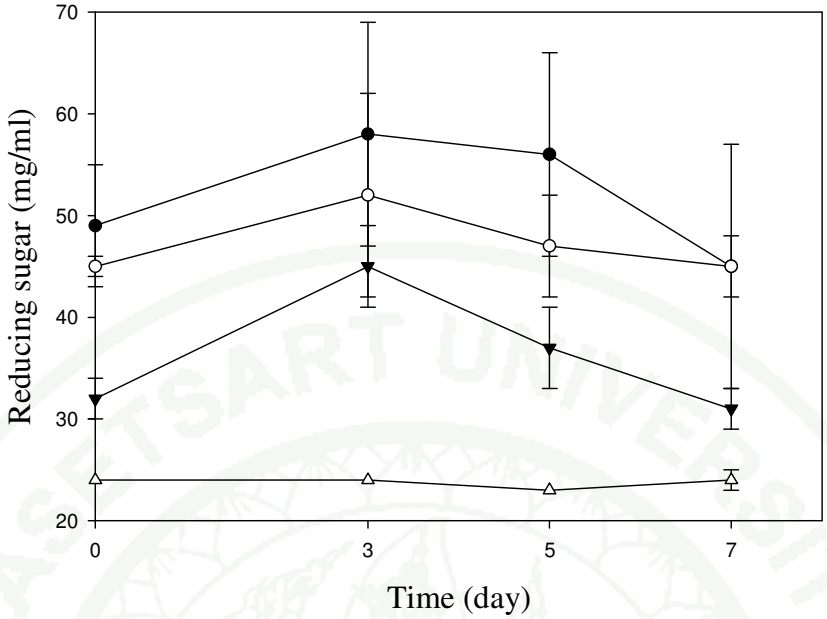


Figure 23 Effect of acid pretreatment on reducing sugar of *A. niger* 386017M1 under SSF system by using; 0%, 0.1%, 0.3% and 0.5% acid pretreated-CP during day 0, 3, 5 and 7

3.4 The effect of acid pretreatment on pH

The pH of fungal cultivation on SSF system decreased from the beginning of 5.0-5.9 to 3.4-3.9 (Figure 24), These might be because of acid formation of acetic acid and others acid compound generating to fungal fermentation system (Dalboge *et al*., 1997; Lynd *et al*., 2002; Heiss-Blanquet *et al*., 2011). However, these low pH had no significantly effected to CBH production during fermentation. These results were supported by the previous reports of Ilmen *et al.* (1997), Sriroch (2000); Gautam *et al*. (2011**).**

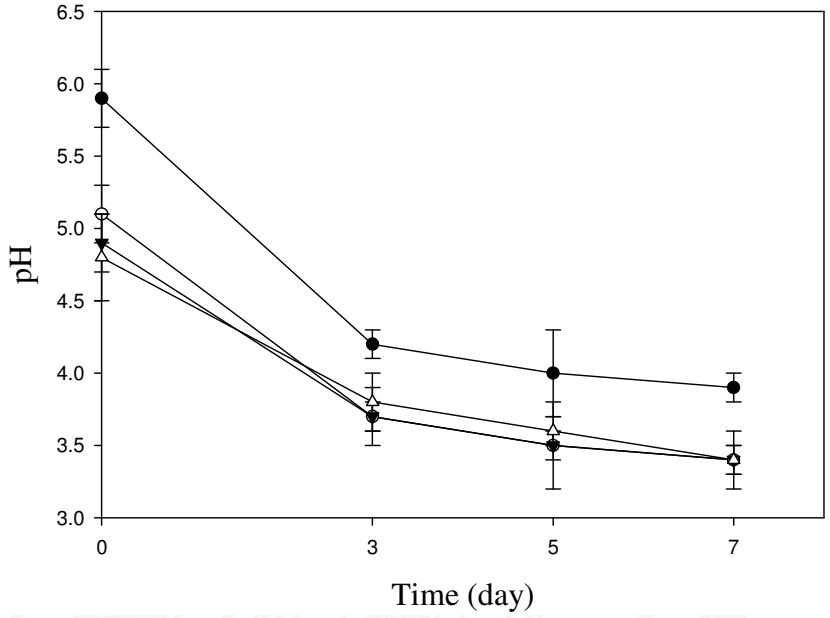


Figure 24 Effect of acid pretreatment on pH of *A. niger* 386017M1 under SSF system by using; 0%, 0.1%, 0.3% and 0.5% acid pretreated-CP during day 0, 3, 5 and 7

3.5 The effect of acid pretreatment on sugars obtained during fermentation

The residual sugar of pretreated CP for CBH production of *A. niger* during time course (0, 3, 5 and 7 d) was determined in different concentration of acid pretreated CP as showed in Table 18-21. The results showed that glucose, galactose and arabinose obtained from the control increased for 10, 1.4 and 1.2 times compared to 0 d. SSF for 0.1% acid pretreated CP showed high reduction of glucose and arabinose for about 9.8 and 1.7 times, respectively compared to d 0. Interestingly, the trends of residual sugar of the 0.3% acid pretreated CP at d 5 provided the different result, glucose was increased for 1.6 times (180 mg/g) whereas the increasing of galactose and arabinose reached to about 1.7 (67±8 mg/g) and 2.9 (44±6 mg/g) times respectively compared to d 0. The residual glucose of SSF for 0.5% acid pretreated CP at d 5 increased for about 2.4 times whereas galactose and arabinose increased for about 1.5 times compared to d 0. The results showed that cultivation of 0.3% acid pretreated CP provided lower glucose though higher galactose and arabinose content than the other treatments.

Table 18 The contents of residual sugars from 7 days solid state fermentation of native CP

mg/g as dry basis						
SSF 0% CP Day	Cellobiose (mg/g)	Glucose (mg/g)	Xylose (mg/g)	Galactose (mg/g)	Arabinose (mg/g)	Mannose (mg/g)
0	ND	45±6	ND	27±9	19±1	ND
3	ND	95±8	ND	26±6	15±3	ND
5	ND	195±19	ND	24±4	12±2	ND
7	ND	485±42	ND	39±7	23±5	27±4

ND = not detectable

Table 19 The contents of residual sugars from 7 days solid state fermentation of 0.1% acid pretreated CP

mg/g as dry basis						
SSF 0.1% CP Day	Cellobiose (mg/g)	Glucose (mg/g)	Xylose (mg/g)	Galactose (mg/g)	Arabinose (mg/g)	Mannose (mg/g)
0	ND	413±19	ND	36±8	22±1	ND
3	ND	562±31	ND	36±6	13±1	ND
5	ND	373±26	ND	39±2	20±2	ND
7	ND	42±12	ND	ND	13±4	ND

ND = not detectable

Table 20 The contents of residual sugars from 7 days solid state fermentation of 0.3% acid pretreated CP

	mg/g as dry basis					
SSF 0.3% CP Day	Cellobiose (mg/g)	Glucose (mg/g)	Xylose (mg/g)	Galactose (mg/g)	Arabinose (mg/g)	Mannose (mg/g)
0	ND	66±12	ND	40±3	15±2	ND
3	ND	286±26	ND	35±5	15±1	ND
5	ND	180±14	ND	67±8	44±6	22±2
7	ND	283±28	ND	26±4	19±7	ND

ND = not detectable

Table 21 The contents of residual sugars from 7 days solid state fermentation of 0.5% acid pretreated CP

	mg/g as dry basis					
SSF 0.5% CP Day	Cellobiose (mg/g)	Glucose (mg/g)	Xylose (mg/g)	Galactose (mg/g)	Arabinose (mg/g)	Mannose (mg/g)
0	ND	76±12	ND	ND	15±1	ND
3	ND	110±18	ND	ND	13±3	ND
5	ND	200±26	ND	24±3	13±2	ND
7	ND	186±19	ND	24±2	23±7	ND

ND = not detectable

3.6 The effect of acid pretreatment to cellulase production

The results were shown in Table 22. The greater CBH activity was obtained from SSF for 0.3% (H_2SO_4) acid pretreated CP. The maximum activities of CBH and BG from SSF for 0.3% acid pretreated CP were 7,599±22 and 6,572±2,122 U/g which were higher than the control for 30 and 3.8 folds, respectively. However, the minimal strength of acid pretreated CP (0.1% H_2SO_4) presented improved enzyme activities by 14 fold CBH (777±23 U/g) and 2.4 fold BG (4,096±288 U/g) compared to the control. The high CBH yields obtained might be because of high carbon sources of starch and cellulose left for fermentation (Kajiwara and Maeda, 1983). The SSF for 0.5% H_2SO_4 pretreated CP provided less activities of CBH and BG for 2.4 fold (386±194 U/g) and 2.6 fold (3,147±1,503 U/g) compared to control, respectively. These were probably because of high inhibitor contents of furan, lignin and phenolic compound released from irreversible conversion of pentose and hexose by high acid concentration (Martin *et al*., 2007; Kosugi *et al*., 2009). The CMCase activity of SSF for acid pretreated CP was no significantly different compared to control. This would be because of endoglucanase significantly decrease the specific viscosity of CMC with little hydrolysis due to intramolecular cleavages of the carboxymethyl substitutions structure on CMC make some glycosidic bond less susceptible to enzyme action (Percival, 2006).

Table 22 Effect of acid pretreatment on CP for cellulase production by *A. niger*386017M1 under solid state fermentation system for 7 days

	C MCase (U/g)				CBH (U/g)				Betaglucosidase (U/g)			
Day	0	0.1	0.3	0.5	0	0.1	0.3	0.5	0	0.1	0.3	0.5
0	12±4^{b}	12±4^{a}	12±5^{b}	13±5^{b}	16±2^{c}	18±1^{b}	15±1^{c}	11±5^{c}	419±96^{b}	116±304^{a}	1,777±2,054^{b}	0±115^{b}
3	19±7^{a}	17±6^{a}	15±5^{ab}	16±6^{ab}	96±8^{bc}	293±130^{b}	167±34^{c}	802±158^{a}	1,143±77^{a}	1,814±375^{a}	3,430±1,176^{ab}	3,163±733^{a}
5	22±8^{a}	23±7^{a}	15±5^{ab}	16±6^{ab}	157±70^{b}	777±23^{b}	7,599±22^{a}	386±194^{b}	1,127±429^{a}	4,096±2,887^{a}	6,572±2,322^{a}	3,147±1,503^{a}
7	20±7^{a}	14±5^{a}	18±7^{a}	17±6^{a}	246±63^{a}	3,553±811^{a}	2,834±22^{b}	272±72^{b}	1,692±288^{a}	4,062±2,547^{a}	5,104±1,584^{ab}	2,310±295^{a}

The values given above are means of three determinations ±SD. Different letters in each row indicate significant differences between the samples ($p \leq 0.05$) by RCBD; Random Complete Block Design

4. Regulation of the cellobiohydrolase (*cbh*) and five major regulatory genes on variable pretreated substrates

During the recent year, CBH is regulated by many regulator elements previously characterized (Aro *et al.*, 2001). XlnR and ACEII play a role in enhancing activity while Cre displays as catabolic repression. In addition, another element of PacC relating to pH regulator is also involved. From the previous experiment of acid pretreated CP, it was found that acid concentration of 0.3% caused the highest CBH activity at 5 d. Therefore, it was interesting to investigate its regulation by identification and quantification of those regulator elements.

4.1 Quanlification of RNA extracted by test kit

The total RNA was extracted by using test kit, RNAqueous® kit (Applied Biosystems/Ambion, Austin, Texas, USA). The RNA quality of *A. niger* from SSF for both control and acid pretreated CP were determined by formamide gel electrophoresis (Figure 25) (Appendix B). The results of both 18S RNA and 28S RNA size were around 2 and 3.8 kb, respectively according to the ribosomal RNA size of *Aspergillus* strain (Sampson, 1994). The smear band found indicated different sizes of RNA occurred. These RNA samples from different conditions were further analyzed for their regulartory system by qRT-PCR.

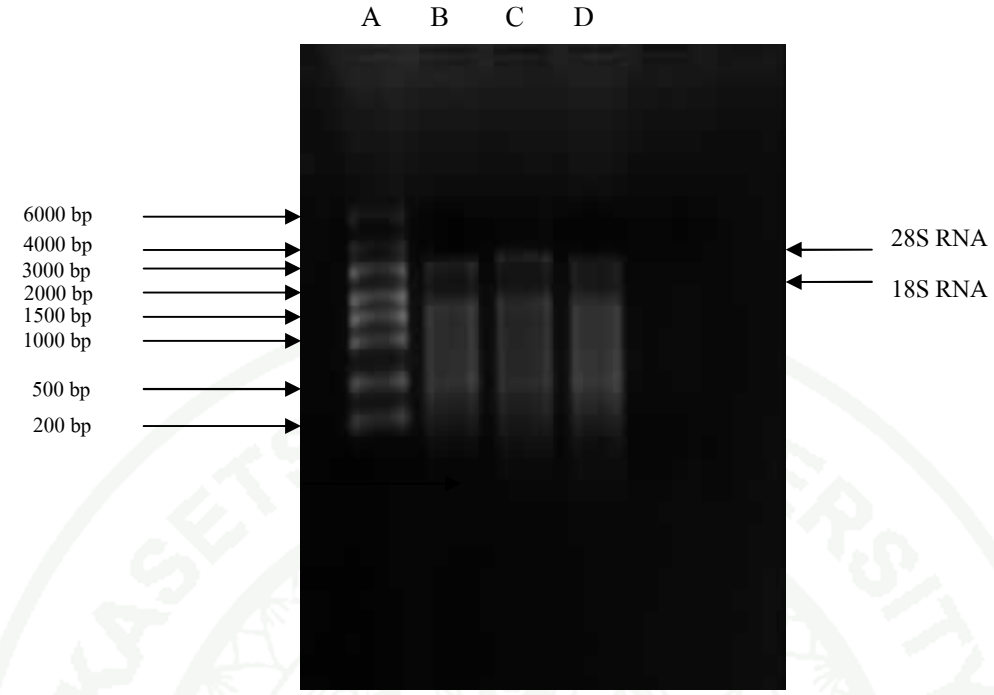


Figure 25 RNA extraction was run by 1% formamide gel electrophoresis under 100 V, 45 min in 1X MOPS buffer. The pure culture of *A. niger* 386017M1 on liquid media (Lane B), culture of fungal by SSF (lane C), acid pretreated CP material (lane D) and 4 µl RNA marker (120 ng/µl) (lane A)

4.2 qRT-PCR product

To confirm PCR product from qRT-PCR system, amplified PCR products from each sample were analysed by agarose gel electrophoresis. The size of all amplified PCR products were around 50–60 bp as expected (Figure 26).

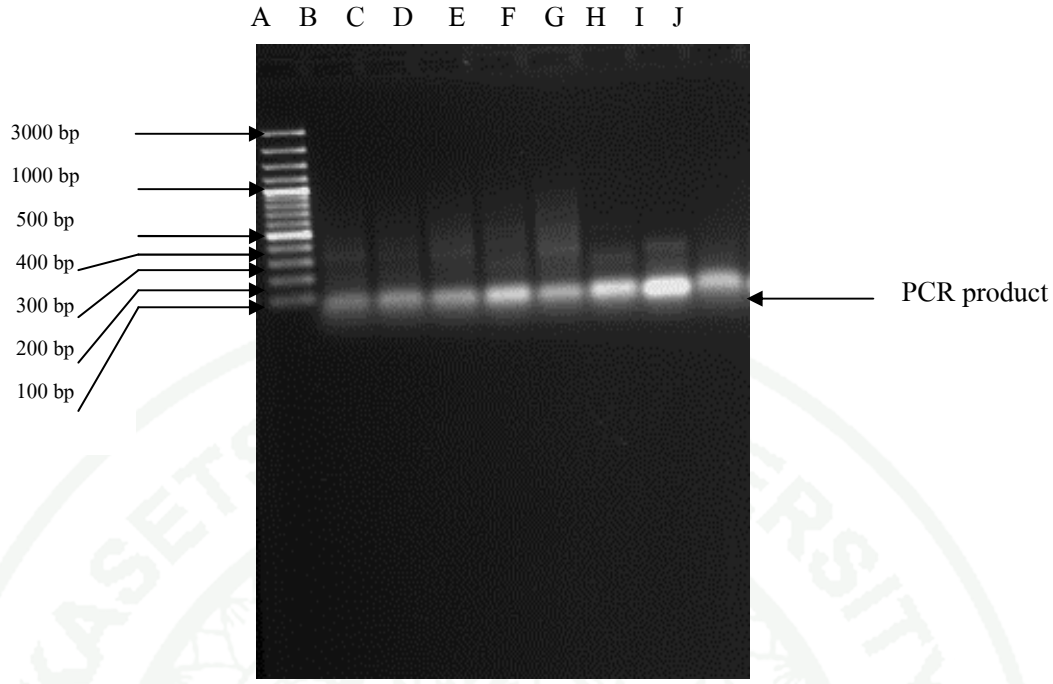


Figure 26 Gel electrophoresis of PCR product of the amlification of specific primer; *cbhA* (lane B), *cbhB* (lane C), *xlnR* (lane D), *ace2* (lane E), *pacC* (lane F), *areA* (lane G) and *creA* (lane H) and endogenous control gene (*tub*) (lane I and J) was examined using real-time PCR and SsoFast SYBR Green detection. 0.5µg of DNA marker (lane A). The 1.2% agarose gel electrophoresis was run under 100 V, 30 min in 1X TBE buffer.

4.3 Primer validation

The normalized method determined by the relative expression of *cbhA* and *cbhB* and their regulatory genes of *xlnR, ace2, pacC* and *creA* was validated by the parameters of capacity, Tm, linearity and efficiency. These factors can be determined by performing a dynamic range test of 2 folds serial dilution, 100, 50, 25, 12.5 and 6.25 ng of RNA sample (cDNA template) of *A. niger* grown in liquid media.

4.3.1 Loading capacity

To determine cDNA loading capacity during qRT–PCR, the standard curve by the 2–fold dilution of DNA, $2^n = 2$ therefore, n = 1, and the Ct values should be separated by 1 cycle.

The results were analyzed by iQ5 qRT-PCR series of Bio–Rad machine, Ct value of each RNA dilution during the exponential phase can be log–transformed and plotted to obtain the slope of the regression line. The standard curve of all genes showed the slope approximately around -3.3 to -3.9 (Figure 27–29) indicating high capacity at the wide range of 6.25–100 ng. They all showed good linearity across many different targets with increasing templates inputs. The slopes of all treatments were -3.63, -3.87, -3.17, -3.86, -3.92 and -3.39 in *cbhA, cbhB, xlnR, ace2, pacC* and *creA* which were around -3.3 according to the efficiency equation ($E = (10^{-1/slope} - 1) \times 100$). The slopes values which were less than -3.3 (more negative) indicated a loss of efficiency with increasing RNA mass (Vandesompele *et al.*, 2002).

4.3.2 Efficiency

The efficeincy of 100% indicated the high consistency of qRT-PCR process. The %efficiency of *cbhA*, *cbhB*, *xlnR*, *ace2*, *pacC*, *creA* and endogenous control gene (*tub*) obtained were about 88.4, 81.1, 106.6, 81.4, 79.8, 97.1 and 81.1%, respectively (Figure 27–29). These high % efficiency (amplification efficiency) indicated high percentage of RNA molecules that are converted along the entire transcript length in different concentration and could be predicted to allow the use of less RNA sample (cDNA template) for low transcripts.

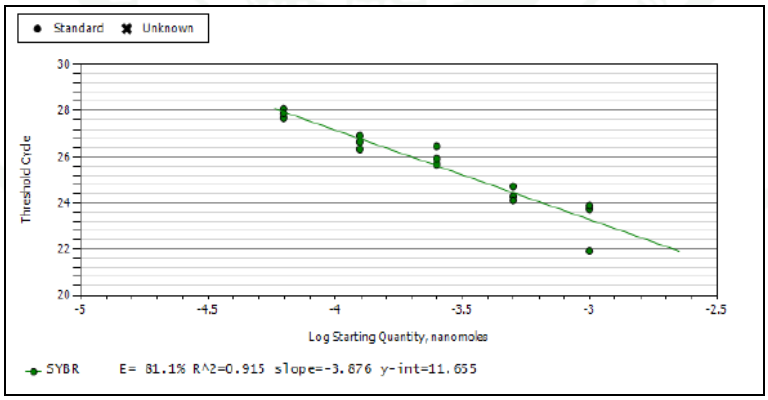
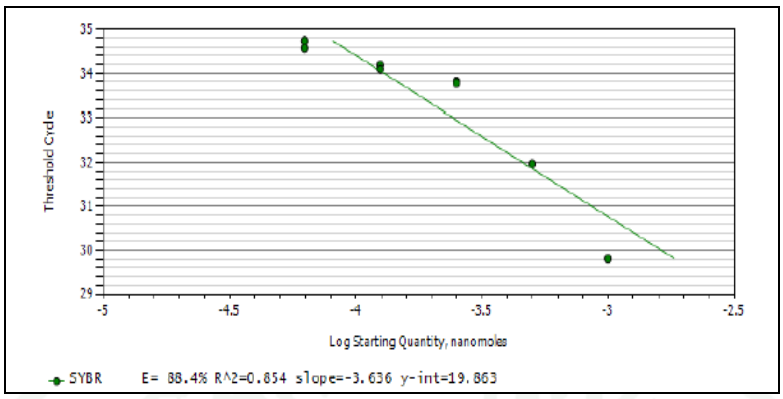
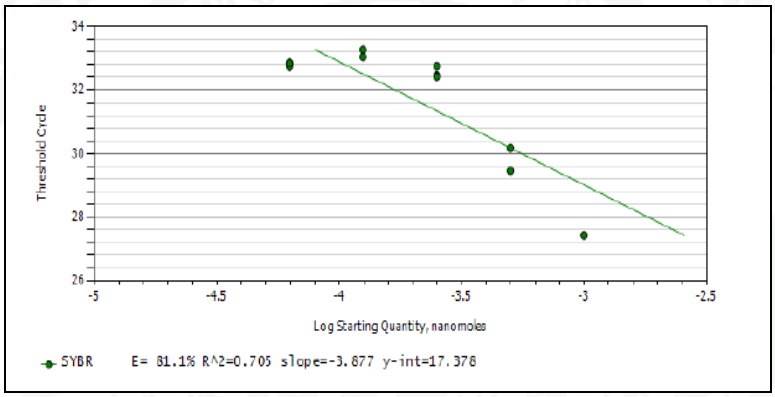


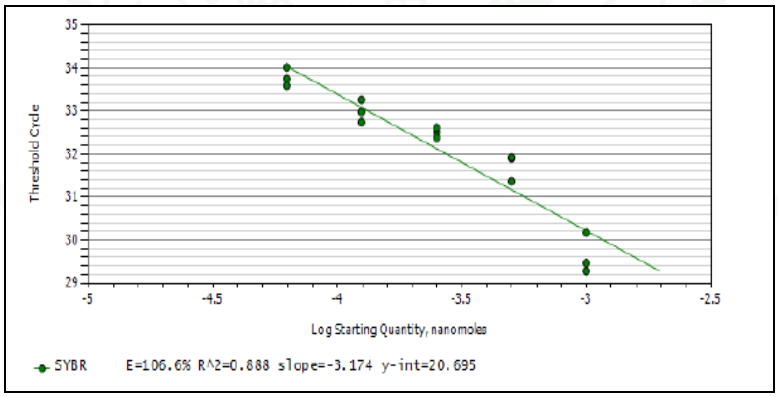
Figure 27 The standard curve of gene (*tub*) used as a control. The efficiency of each amplification was indicated as equation in figure.



(a)

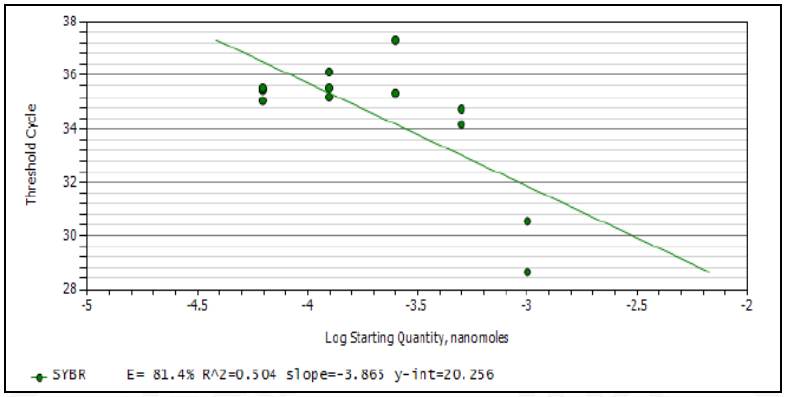


(b)

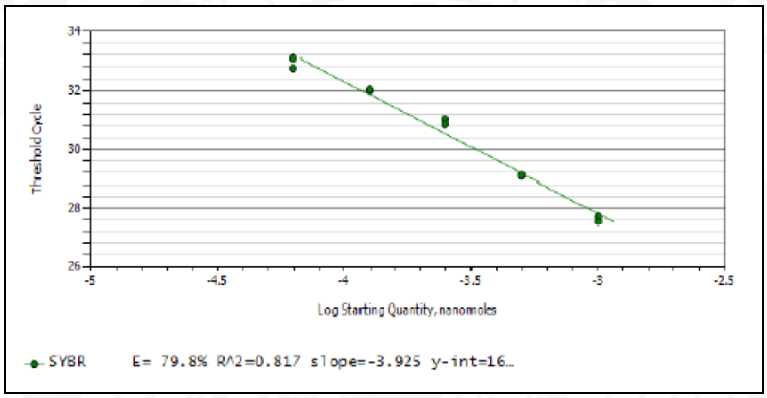


(c)

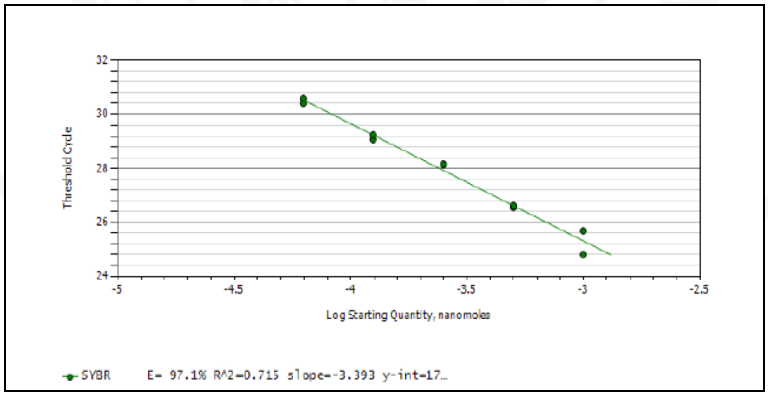
Figure 28 The standard curve of *cbhA* (a), *cbhB* (b) and *xlnR* (c). The efficiency of each amplification was indicated as equation in figure.



(a)



(b)



(c)

Figure 29 The standard curve of *ace2* (a), *pacC* (b) and *creA* (c). The efficiency of each amplification was indicated as equation in figure.

4.3.3 Tm

Tm was one mornitoring factor for primer selection to indicate primer-dimer formation from qRT-PCR reaction based on melting temperature distinguish. As the results, during the dsDNA melts, SsoFast™Evagreen® dye (SYBR Green I) was released and a decrease in fluorescence was observed. The software of iQ5 (Bio-Rad) can plot the negative first derivative of the rate of change of fluorescence vs. temperature (-d(RFU)/dT). The Tm results of all PCR products showed that the lower RNA sample concentration of 50, 25, 12.5 and 6.25 ng showed more than one peak as shown in Table 23–29, Appendix D. Low RNA concentration of 50 ng of *cbhB* showed two Tm values of 76.5 and 81.5 (Table 24) while the one of 100 ng resulted in no primer-dimer performed by RT-PCR reaction and could predict cDNA loading as template for relative RT-PCR gene expression assay. It seems that more than one peak could perform at low concentration of RNA sample due to non-specfic PCR reaction. Therefore, to avoid the interference affect, the 100 ng RNA sample (cDNA template) was used to determine the relative qRT-PCR gene expression.

Table 23 Tm value of PCR product, *cbhA* target gene

RNA sample (ng)	Identifier	Melt Temp. (°C)
100	*cbhA*	79.00
50	*cbhA*	79.00
25	*cbhA*	78.50
12.5	*cbhA*	78.50
6.25	*cbhA*	78.50

Table 24 Tm value of PCR product, *cbhB* target gene

RNA sample (ng)	Identifier	Melt Temp. (°C)
100	*cbhB*	81.50
50	*cbhB*	76.50
	cbhB	81.50
25	*cbhB*	81.50
	cbhB	84.50
12.5	*cbhB*	63.50
	cbhB	81.00
6.25	*cbhB*	76.50
	cbhB	81.00

Table 25 Tm value of PCR product, *xlnR* target gene

RNA sample (ng)	Identifier	Melt Temp. (°C)
100	*xlnR*	81.00
50	*xlnR*	81.00
25	*xlnR*	58.00
	xlnR	81.00
12.5	*xlnR*	80.50
6.25	*xlnR*	58.50
	xlnR	80.50

Table 26 Tm value of PCR product, *ace2* target gene

RNA sample (ng)	Identifier	Melt Temp. (°C)
100	*ace2*	86.50
50	*ace2*	86.50
25	*ace2*	79.00
	ace2	86.50
12.5	*ace2*	86.50
	ace2	86.50
6.25	*ace2*	86.00
	ace2	86.50

Table 27 Tm value of PCR product, *pacC* target gene

RNA sample (ng)	Identifier	Melt Temp. (°C)
100	*pacC*	81.50
50	*pacC*	81.50
25	*pacC*	81.50
12.5	*pacC*	64.00
	pacC	81.50
6.25	*pacC*	59.50
	pacC	64.00
	pacC	81.50

Table 28 Tm value of PCR product, *creA* target gene

RNA sample (ng)	Identifier	Melt Temp. (°C)
100	*creA*	81.00
50	*creA*	81.00
25	*creA*	81.00
12.5	*creA*	81.00
6.25	*creA*	81.00

Table 29 Tm value of PCR product, *tub* target gene

RNA sample (ng)	Identifier	Melt Temp. (°C)
100	*tub*	81.50
50	*tub*	81.50
25	*tub*	81.50
12.5	*tub*	81.00
6.25	*tub*	81.00

4.3.4 Linearity

The linearity results were used to indicate the optimal qPCR assay which is absolutely essential for accurate and reproducible quantitation of particular sample. The hallmark of an optimized qPCR assay is consistency across replicate reactions. The β-tubulin gene (*tub*) was used as endogenous control gene for quantitative qRT-PCR assay which should be constant across all test samples of *cbhA, cbhB, xlnR, ace2, pacC* and *creA* (Bohle *et al*., 2007; Marisa, 2005). The linearity was determined by the C_T value of standard curve, the expression of the target gene normalised to endogenous control gene was presented as $2^{-\Delta CT}$ where $\Delta C_T = C_{T\ Target} - C_{T\ TUB}$.

The linearity result was the maintenance of constant amplification efficiency. In terms of normalization, the use of multiple housekeeping genes is the most accurate method.

Single housekeeping gene is used in this study therefore its stability was validated in an assay similar to the one used to rank gene stability in comparative method (Marisa, 2005). The amplification kinetics of the target gene and reference gene assays must be approximately equal. As the result of 2 fold serial dilutions standard curve, the ΔC_T value of target gene and reference gene were showed in Table 30-32. The results plotted with the log concentration of ng of RNA sample for each dilution on the x-axis, and the difference in Ct of target and reference for each dilution on the y-axis. If the absolute value of the slope of the line is less than 0.1, the Normalize expression method may be used for further study of gene expression. The linearity results presented that all genes (*cbhA, cbhB, xlnR, ace2, pacC* and *creA*) had absoluted value of the slope of line lower than 0.1 ($\leq$0.1) (Figure 30-31) indicated that the qRT-PCR system was optimum in term of maintenance of constant amplification efficiency. These primers and optimum qRT-PCR system could be used for further study in relative gene expression (Vandesompele *et al.*, 2002).

Table 30 The C_T value of *pacC* and *xlnR* compare to endogenous control gene (*tub*) at each RNA concentration

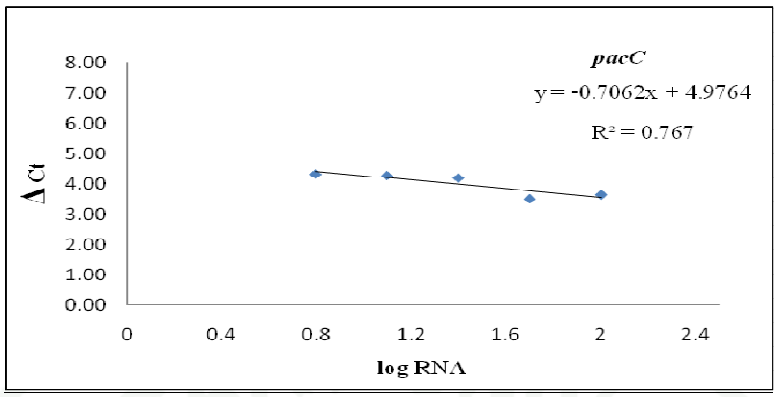
Gene	RNA sample (ng)	C_T value	Log RNA conc.	ΔC_T (target gene-*tub*)
pacC	100	28.65	2.00	3.65
	50	29.11	1.70	3.50
	25	30.94	1.40	4.20
	12.5	32.03	1.10	4.28
	6.25	32.93	0.80	4.32
xlnR	100	29.81	2.00	4.81
	50	31.97	1.70	6.37
	25	32.80	1.40	6.06
	12.5	34.14	1.10	6.38
	6.25	34.65	0.80	6.04
Tub	100	25.00		
	50	25.60		
	25	26.74		
	12.5	27.75		
	6.25	28.61		

Table 31 The C_T value of *cbhA, cbhB* and *creA* compare to endogenous control gene (*tub*) at each RNA concentration

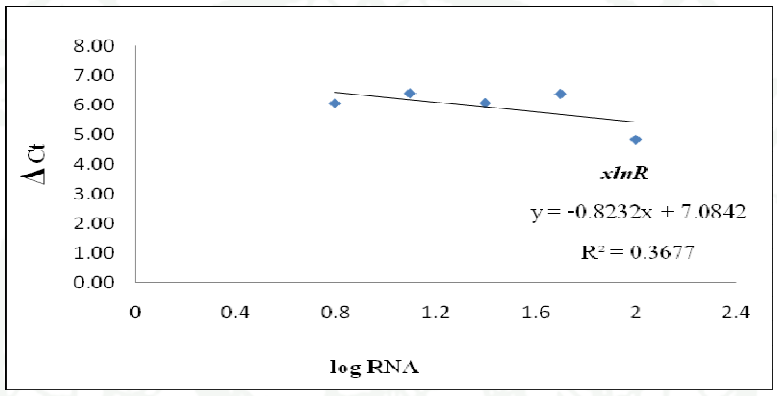
Gene	RNA sample (ng)	C_T value	Log RNA conc.	Δ C_T (target gene-*tub*)
cbhA	100	29.64	2.00	6.46
	50	30.03	1.70	5.65
	25	32.50	1.40	6.48
	12.5	32.99	1.10	6.36
	6.25	33.98	0.80	6.10
cbhB	100	27.42	2.00	4.25
	50	29.81	1.70	5.44
	25	32.55	1.40	6.53
	12.5	33.16	1.10	6.53
	6.25	33.81	0.80	5.93
creA	100	25.24	2.00	2.07
	50	26.58	1.70	2.21
	25	28.14	1.40	2.13
	12.5	29.15	1.10	2.52
	6.25	30.49	0.80	2.61
Tub	100	23.18		
	50	24.38		
	25	26.01		
	12.5	26.63		
	6.25	27.88		

Table 32 The C_T value of *ace2* compare to endogenous control gene (*tub*) at each RNA concentration

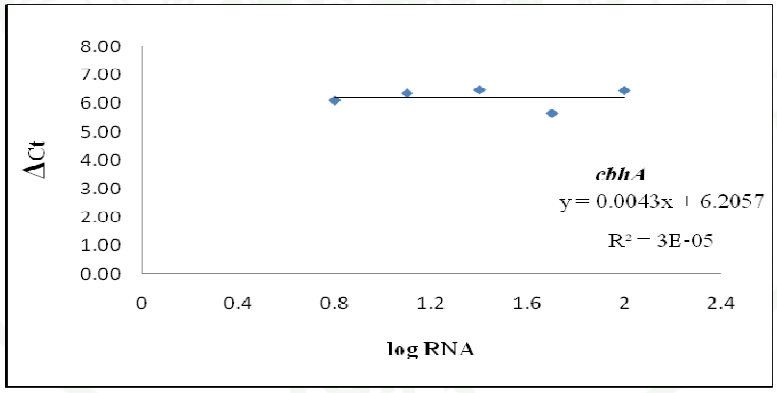
Gene	RNA sample (ng)	C_T value	Log RNA conc.	Δ C_T (target gene-*tub*)
ace2	100	25.86	2.00	1.44
	50	27.36	1.70	1.85
	25	28.03	1.40	1.34
	12.5	29.56	1.10	1.88
	6.25	30.35	0.80	1.77
Tub	100	24.42	2.00	
	50	25.51	1.70	
	25	26.69	1.40	
	12.5	27.68	1.10	
	6.25	28.57	0.80	



(a)

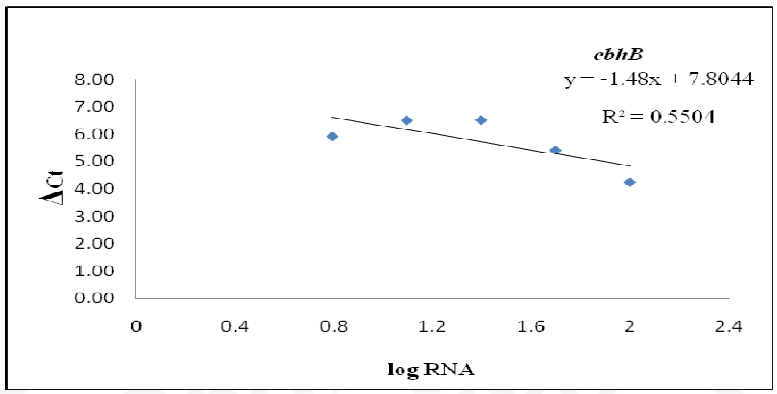


(b)

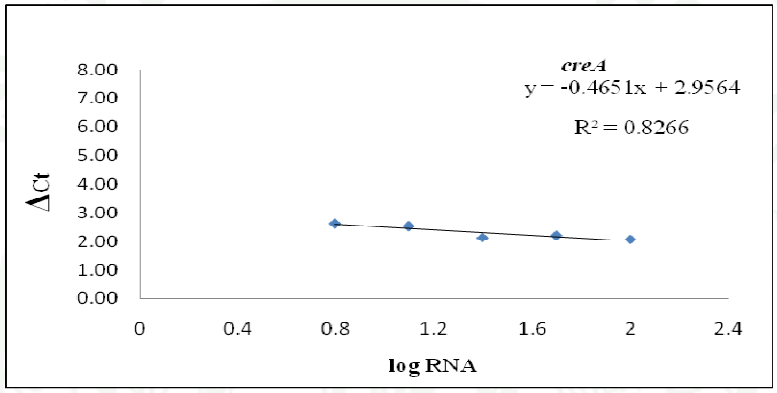


(c)

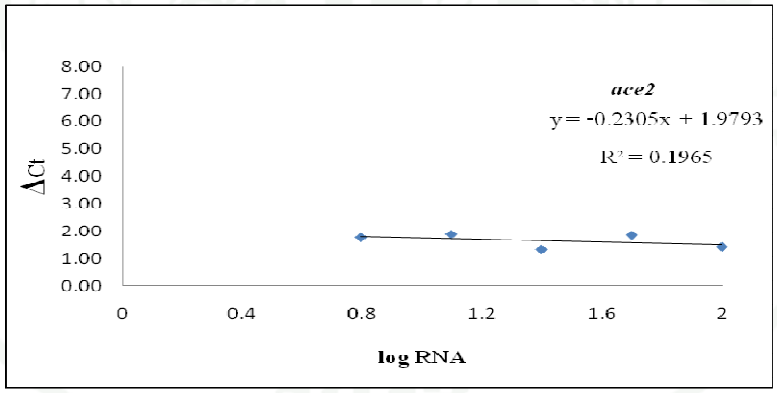
Figure 30 Primer validation, linarity of the $2^{-\Delta CT}$ method using RNA of *A. niger* 386017M1. The amplification of target genes (*cbhA* (a), *cbhB* (b), *xlnR* (c) and endogenous control gene (*tub*) was examined using real-time PCR and SsoFast Evagreen detection. The 2 fold dilutions of RNA sample were amplified by real-time PCR using gene specific primers. The ΔCT ($C_{T\ Target}$ - $C_{T\ TUB}$) value was calculated for each RNA dilution and plotted against the logarithm of RNA concentration.



(a)



(b)



(c)

Figure 31 Primer validation, linarity of the 2 $^{-\Delta C_T}$ method RNA of *A. niger* 386017M1. The amplification of target genes *ace2* (a), *pacC* (b) and *creA* (c) and endogenous control gene (*tub*) was examined using real-time PCR and SsoFast Evagreen detection. The 2 fold dilutions of RNA sample were amplified by real-time PCR using gene specific primers. The ΔCT ($C_{T\ Target}$ - $C_{T\ TUB}$) value was calculated for each RNA dilution and plotted against the logarithm of RNA concentration.

The assessment of primer was validated by the following parameters; capacity, %efficiency, Tm and linearity. In conclusion, the standard curve slope showed value over -3.3, %efficiency around 81–106%, Tm presented one peak without primer-dimer effect at 100 ng RNA sample and linearity was consistency at ΔC_T slope of less than 0.1. The values of each parameter used for primer validation were concluded in Table 33. These results indicated that all primer could be used in next experiment of relative qRT-PCR as Normalize method.

Table 33 The conclusion of primer validation for relative quantification gene expression

Target gene	Standard curve slope	Primer-dimer (Tm)	% Efficiency	ΔC_T slope	RNA sample (ng)
cbhA	-3.63	No	106.6	0.0043	100
cbhB	-3.87	No	81.1	-1.4800	100
xlnR	-3.17	No	88.4	-0.8232	100
ace2	-3.86	No	81.41	-0.2365	100
pacC	-3.92	No	79.8	-0.0706	100
creA	-3.39	No	97.1	-0.4651	100

5. Cellulase gene expression of various acid pretreated CP

Normalize method was determined as the relative quantity of target gene *cbhA, cbhB, xlnR, ace2, pacC* and *creA* to the one of the reference gene (*tub*). The relative quantities of each target gene at d 0 was assigned to 1 called as control sample. The values presented were from the mean of three separate PCR experiments using the same RNA samples. The results of relative gene expression values were plotted as $\log_{10}$ transformation. The relative quantity for all d 3, 5 and 7 samples were also shown in Appendix A.

5.1 Regulation system of CBH at 3 day solid state fermentation

The SSF for 0.5% acid pretreated CP provided the highest activity of CBH for 802±158 U/g while their expression of both *cbhA* and *cbhB* were oppositely lower than 0.3% acid pretreated CP SSF for 0.3% as shown in Figure 30. It seems that the high CBH activities were not from only expression of *cbhA* and *cbhB*.

5.2 Regulation system of CBH at 5 day solid state fermentation

The maximum CBH activity of 7,599±22 U/g appeared at 5 day cultivation of SSF for 0.3% acid pretreated CP. Its transcription of both *cbhA* and *cbhB* were significantly higher than the treatment of 0.1% and 0.5% for 3.5-4 and 1.8-2.1 times, respectively as shown in Figure 31. In addition, the expression level of *cbhB* was also higher than the one of *cbhA* for 1.6 times. This high expression was positively regulated by 4 regulators of *xlnR, ace2, pacC* and *creA*. It was seem that catabolic repression of *creA* had no effect to the expression of both *cbh.*

5.3 Regulation system of CBH at 7 day solid state fermentation

Both SSF for 0.1 and 0.3% acid pretreated CP provided the activities of CBH for 3,553±811 and 2,834±22 U/g. However their expression of *cbhA* and *cbhB* were oppositely under different regulation system. The expression of *cbhA* was higher in the treatment of 0.1% acid while the one of 0.3% and 0.5% acid were *cbhB* (Figure 32-34). The high expression of *cbhA* was positively regulated by those 4 regulators of *xlnR, ace2, pacC* and *creA* whereas the one of *cbhB* was by only *pacC* and *creA*. These findings could be concluded that the lower expression of *xlnR* and *ace2* might suppress the expression of *cbhA* from 0.3% acid treatment. In the other hand, the high expression of *cbhA* from 0.1% acid treatment was highly positive regulated by both *pacC* and *creA*. Considering to SSF for 0.5% acid pretreated CP, the CBH activity was lower to only 272±72 U/g. Its low activities were negatively regulated by *creA*.

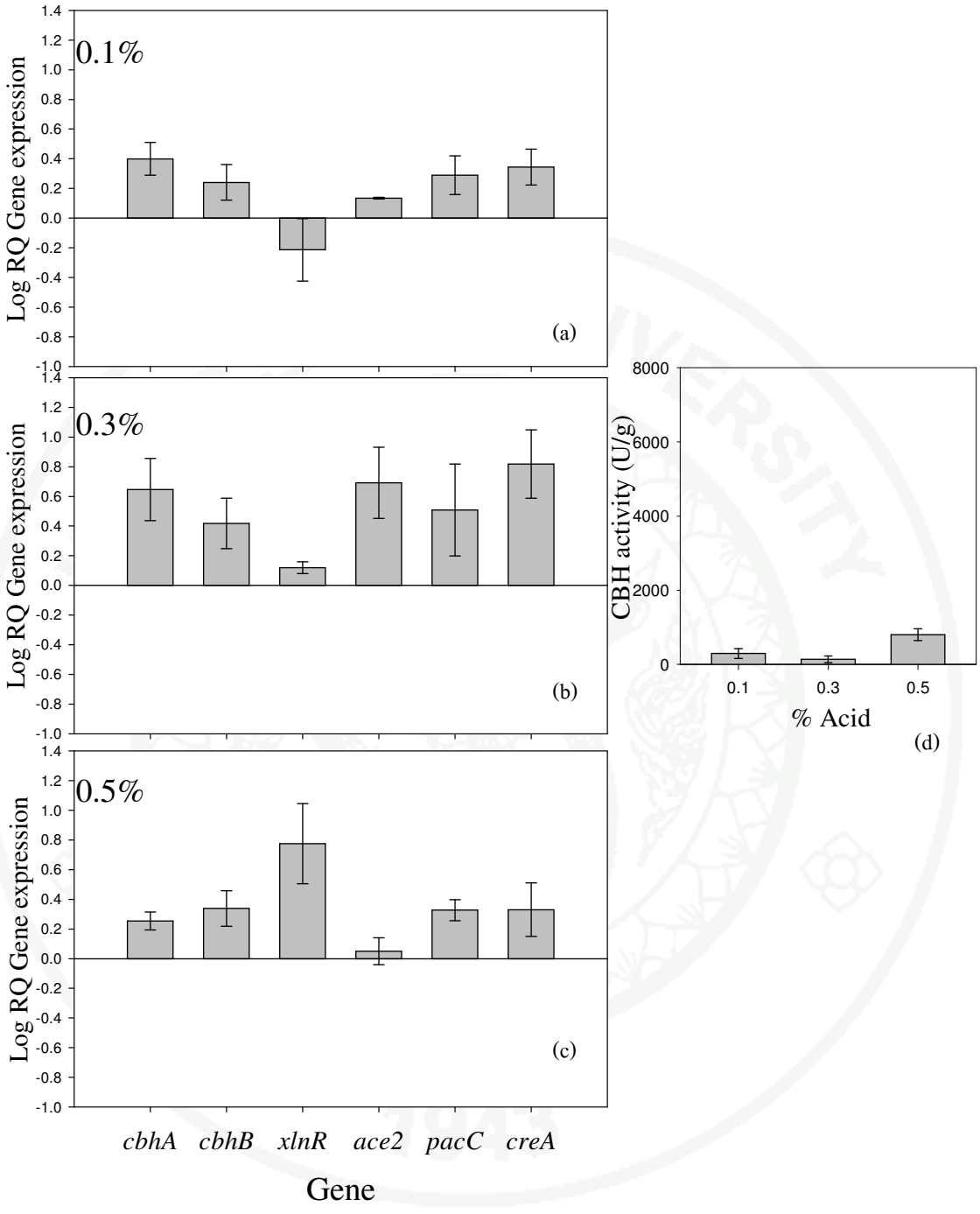


Figure 32 The relative of cellulase regulatory genes expression of *A. niger* 386017M1 under SSF system using 0.1, 0.3 and 0.5% acid pretreated CP at day 3

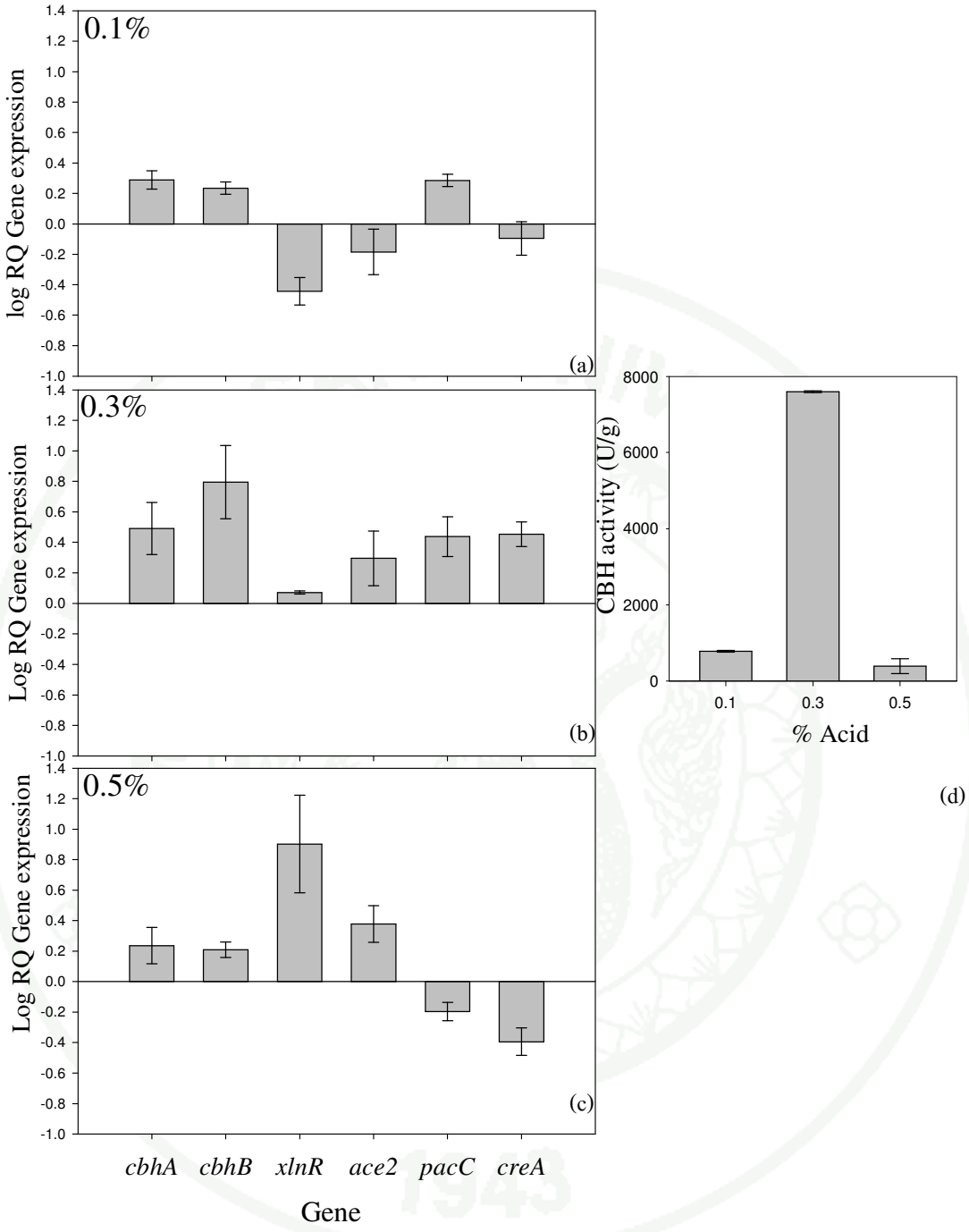


Figure 33 The relative of cellulase regulatory genes expression of *A. niger* 386017M1 under SSF system using 0.1, 0.3 and 0.5% acid pretreated CP at day 5

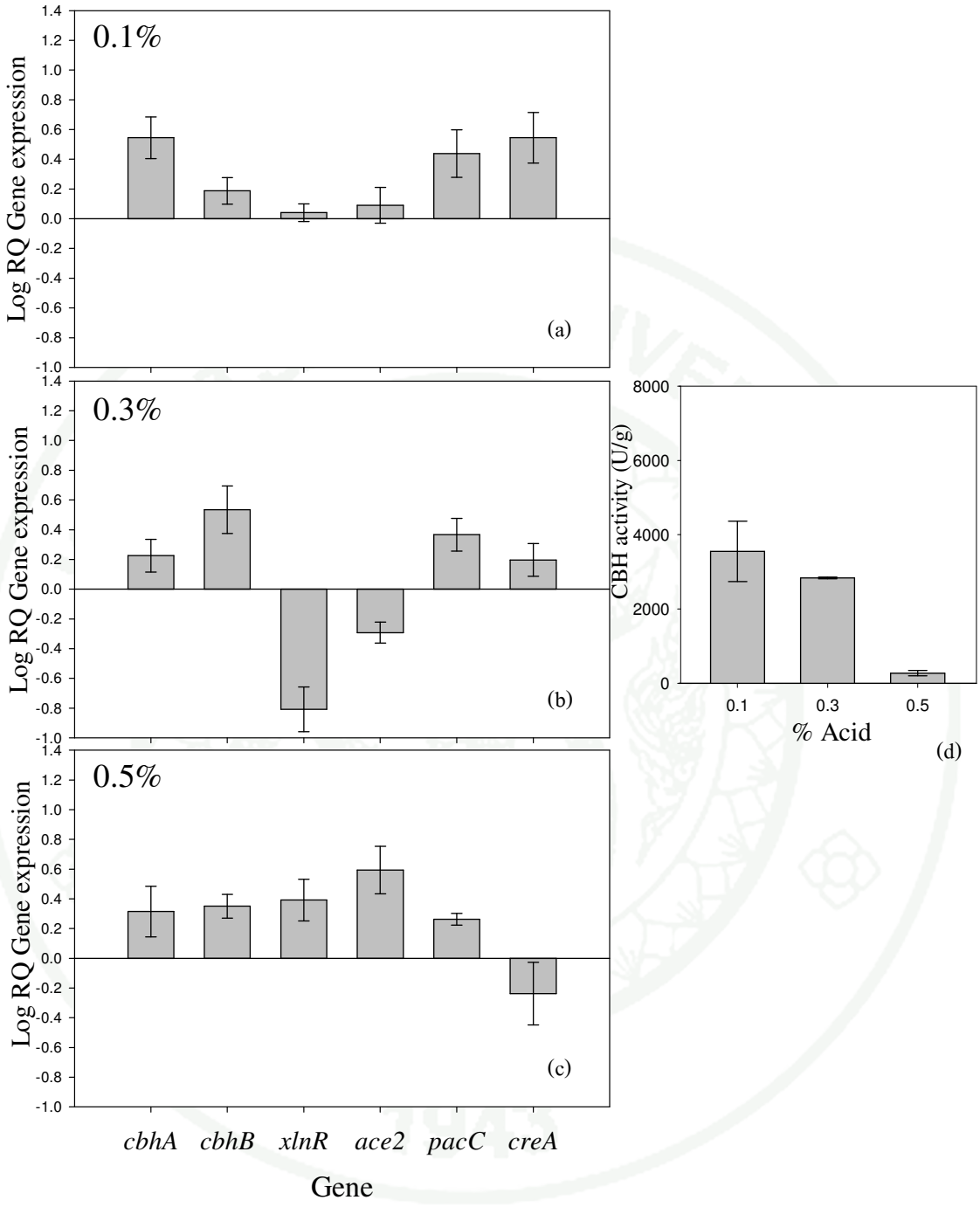


Figure 34 The relative of cellulase regulatory genes expression of *A. niger* 386017M1 under SSF system using 0.1, 0.3 and 0.5% acid pretreated CP at day 7

These findings showed that *creA* was a main regulator for a strong expression of *cbh* which supported the results of Caddick *et al.* (2006). The gene *creA* was regulatory gene frequently subjected to autogenous regulation and controlled by other factors during transcription (Marzluf *et al*., 1997). The *creA* gene encode catabolic repressor protein, CreA, that results in repression of cellulase for utilizing carbon sources in the presence of easily metabolite substrate such as glucose, xylose or fructose (Vries and Visser, 2001; Suto and Tomita, 2001). According to analysis of sugar left during fermentation, only low glucose concentration of 42±6-562±31 mg/g was detected. This would not affect the repression function of CreA.

According to the remain-sugars in SSF system (Table 18–21), CBH enzyme production in cultivation of 0.3% acid pretreated CP was regulated by high *cbhB* expression. The reduction of glucose content for about 1.6 (180±14 mg/g) times and the increasing of galactose and arabinose for about 1.9 (67±8 mg/g) and 2.9 (44±6 mg/g) times increased from d 3 to d 5 (Table 20). This expression of *cbhB* at high up-regulation of both galactose and arabinose were supported by previous work of Karafta *et al*. (2006).

These findings indicated that high CBH enzyme was improved and regulated by glucose catabolic repressor and other sugars of galactose and arabinose that remained in system.

As the results, *xlnR* and *ace2* would rather up-regulated expression to both *cbhA* and *cbhB* and parallel to the maximum CBH production. The *cbhB* was not strong regulated by *xlnR* and *ace2* comparing to *cbhA*. The up-regulated expression of *xlnR* and *ace2* supported *cbh* gene consequence to improve CBH production because *xlnR* encode transcriptional activator of XlnR, regulating both xylanolytic and cellulolytic gene expression in *A. niger* (Stricker *et al*., 2006; Hasper *et al*., 2004; Peij *et al*., 1998). Both *xlnR* and *ace2* were regulators for positive regulation of *cbh* promoter involving in transcription of cellulase expression together (Stricker *et al*., 2006; Nina, 2002; Nina, 2001). Therefore, the high up-regulate expression of *xlnR* and *ace2* would enhance *cbh* gene to provide maximum CBH production (Lockington *et al*., 2002).

In SSF system, fermentation of *A. niger* 368017M1 provided the metabolites like acetic acid to decrease the pH during cultivation resulting in improved CBH production as similar as some research (Stewart and Parry, 1981). The gene *pacC* expressed up-regulated the *cbhA* and *cbhB* gene on acid pH. Interestingly, the *pacC* gene in *Aspergillus* normally acts as activator by express in alkaline pH. However, *pacC* gene encoding PacC protein has a dual function of pH-dependent expression (Aro and Penttela, 2005; Vries and Visser, 2001) and response to ambient pH. Therefore, the high expression of *pacC* in acid pH assumed that it activated acid-expressed gene though repressed alkaline-expressed gene.

As the results, the strong expression of *cbhB* would be because the *cbhB* contained both cellulose-binding domain (CBD) and catalytic domain while *cbhA* contained only catalytic domain and lack CBD (Gielkens *et al*., 1999). In addition, the other interesting regulatory genes involving in CBH production were *ace1* and *areA* expressing for inhibitor and nitrogen metabolic repression, respectively. The *areA* was subjected to be a nitrogen catabolic repression to control second metabolites of nitrogen sourc such as nitrate, nitrite, purines, most amino acid and protein during starvation of certain nitrogen compound. These regulators might support a strong expression of either *cbhA or* cbhB which need for further study.

In addition, the synergistic of *cbhA, cbhB* and other gene encoding endo-glucanase and β-glucosidase contain putative binding site for cellulase regulatory genes in variance number and unfortunately some of gene was not contain the activator binding site therefore the transcription of cellulase may have switch itself during enzyme production. Moreover, transcription, mRNA stability, CBD binding site, translation, posttranslational modification, and direct protein-protein interactions are involved. To understand more in their expression system, these factors may need for further study.

CONCLUSIONS

Three pretreatment methods of plant based materials, RRB, PKM and CP, containing lignocellulose have been studied in order to improve cellulase production in this study. These findings revealed that the increasing of CBH production by *A. niger* 386017M1 were successful by steam pretreatment of RRB and PKM as well as dilute acid pretreatment of CP. Maximum CBH activities were obtained from the steam pretreated RRB, steam pretreated PKM and acid pretreated CP. Interestingly, the reduction of lignin content after pretreatment of steam pretreated RRB, steam pretreated PKM and acid pretreated CP reduced to 1.5, 1.4 and 1.3 folds even without alkaline pretreatment. This would be an available pretreatment technique for commercial use by economic cost of abundant agricultural waste. According to the highest CBH production, the steamed pretreated RRB might be the best substrate for cellulase production in term of enzyme production and cost investment. However, the variable cost of materials always changed by the annual yield of crop and energy for industrial process. Therefore, the decision need to concern in supply volume in industrial scale.

The lack of comprehensive understanding in the nature of intracellular inducers, signalling and cofactors that mediate the induction or repression for cellulase production, the SSF of *A. niger* 386017M1 for acid pretreated CP was studied as basis information to improve cellulase production. The component of CP containing abundance of starch and cellulosic fiber was hydrolyzed to oligosaccharides and glucose by powerful acid pretreatment (0.3% H_2SO_4) and used as inducible sources of cellulase genes regulation. The 0.3% acid pretreatment provided higher cellulose content but less hemicelluloses and lignin. These indicated that *A. niger* has more ability to utilize the pretreated CP material with the increasing of galactose and arabinose but consuming more glucose. In addition, *creA* which plays as catabolic repression probably enhanced CBH production. In the view of industry, not only pretreatment method for high enzyme production but also other concerns in term of cost investment and comfortable production. Therefore, the cheap price, high fiber content, best nutrient material source and the potential microorganism were the important factors involving in maximization of enzyme production.

LITERATURE CITED

Abdeshahian, P., N. Samat, A.A. Hamid and W.M.W. Yusoff. 2011. Solid substrate fermentation for cellulase production using palm kernel cake as a renewable lignocellulosic source in packed–bed bioreactor. **Biotechnol. Bioproc. E.** 16: 238–244

Acebal, C., M.P. Castillon, P. Estrada, I. Mata, E. Costa, J. Aguado, D. Romero and F. Jimenez. 1986. Enhanced cellulase production from *Trichoderma reesei* QM 9414 on physically treated wheat straw. **Appl. Microbiol. Biothecnol**. 24: 218–223

Agu, R.C., A.E. Amadife, C.M. Ude, A. Onya, E.O. Ogua and E. Zejiofor. 1997. Combined heat treatment and acid hydrolysis of cassava grate waste (CGW) biomass for ethanol production. **Waste. Manage.** 17(1): 91–96

Akarachranya, A., J. Kesornsit, N. Leepipatpiboon, T. Srinorahutara, V. Kitpreechavanich and V. Tolieng. 2011. Evaluation of the waste from cassava starch production as a substrate for ethanol fermentation by *Sacharomyces cerevisiae*. **Ann. Microbiol**. 61: 431–436

Alriksson, B., S.H. Rose, W.H. van Zyl, A. Sjode, N-O. Nilvebrant and L.J. Jonssan. 2009. Cellulase production from spent lignocelluloses hydrolysates by recombinant *A. niger*. **Appl. Environ. Microbiol**. 75(8): 2366–2374

Antoinette, O'Sullivan. 1997. Cellulose: thestructureslowlyunravels. **Cellulose**. 4: 173–207

Applied Biosystem. 2011. **Real-Time PCR Vs. Traditional PCR**. Available Source: http://www6.appliedbiosystems.com/support/tutorials/pdf/rtpcr_vs_tradpcr.pdf, Febuary 2, 2012.

Applied Biosystem. 2012. **Guide to Performing Relative Quantitation of Gene Expression Using Real-Time Quantitative PCR**. Available Source: http://www.biotec.uiuc.edu/centers/Keck/Functional_genomics/taqman/Guide%20to%20relative%20quantitation.pdf, Febuary 2, 2012.

Arana, M. 2007. **Solving the By-products Nutrition Mystery**. Available Source: http://www.dairybusiness.com/western/Oct00/byproducts2.htm, April 19, 2007.

Aro, N., Pakula T. and Penttila M. 2005. Transcriptional regulation of plant cell wall degradation by filamentous fungi. **Fems. Microbiol. Rev.** 29:719–739

Aro, N., A. Salohime, M. Ilmen and M. Penttila. 2001. ACEII, a novel transcriptional activator involved in regulation of cellulase and xylanase genes of *Trichoderma reesei*. The **J. Biol. Chem.** 276(26): 24309–24314

Aspinall, G.O. and T.N. Krishnamurthy. 1991. Base-catalyzed degradation of carbohydrates. VII. Alkaline degradation of 3, 6-Di-*O*-substituted Hexoses. **Can. J. Chem**. 53: 2171–2177

Atif, H., A. Yasmeen and M.I. Rajoka. 2004. Induction, repression, and de–repression of exoglucanase synthesis in *A. niger*. **Bioresource. Technol.** 94: 311–319

Bennett, J.W. and M. Klich. 2003. Mycotoxin. **Clin. Microbiol. Rev**. 16(3): 497–516

Bigelow, M. and C.E. Wyman. 2002. Cellulase production on bagasse pretreated with hot water. **Appl. Biochem. Biotech.** 98: 921–934

Bio-Rad Laboratories Inc. 2006. **Real-Time PCR Application Guide**. Source: http://www.gene-quantification.de/real-time-pcr-guide-bio-rad.pdf, Febuary 2, 2012.

Blumenthal, C.Z. 2004. Production of toxin metabolite in *Aspergillus niger, Aspergillus oryzae* and *Trichoderma reesei*: justification of mycotoxin testing in food grade enzyme preparations derived from the three fungi. **Food Grade Enzyme**. 39: 214–228

Bohle K., A. Jungeblound, Y. Gocke, A. Dalpiaz, C. Cordes, H. Horn and D.C. Hempel. 2007. Selection of reference genes for normalization of specific gene quantification data of A. niger. **J. Biotechnol.** 132: 353–358

Bono, A., P.H. Ying, F.Y. Yan, C.L. Mui, R. Sarbatly and D. Krishnainh. 2009. Synthesis and characterization of caboxymethyl cellulose from palm kernel cake. **Adv. Nat. Appl. Sci**. 3(1): 5–11

Bustin, S.A., V. Benes, T. Nolan and M.W. Pfaffl. 2005. Quantitative real-time RT-PCR-a perspective. **J. Mol. Endocrinol**. 34(3): 597–601

Brijwani, K. and P.V. Vadlani. 2011. Cellulolytic enzymes production via solid–state fermentation: effect of pretreatment methods on physicochemical characteristics of substrate. **Enzyme Research**. Article ID 860134. Doi:10.4061/2011/860134

Brownell, H.H. and J.N. Saddler. 1987. Steam pretreatment of lignocellulosic material for enhanced enzymatic hydrolysis. **Biotechnol. Bioeng.** 29: 228–235

Caddick, M.K., M.G. Jones and J.M. van Tonde. 2006. Opposing signals differentially regulate transcript stability in *A. nidulans*. **Mol. Microbiol**. 62: 509–519.

Carvalheiro, F., L.C. Duarte and F.M. Gírio. 2008. Hemicellulose biorefineries: a review on biomass pretreatments. **J. Sci. Ind. Res.** 67: 849-864

Chandel, A.K., E.S. Chan, R. Rudravaram, M. L. Narasu, L.V. Rao and P. Ravindra. 2007. Economics and environment impact of bioethanol production technologies: an appraisal. **Biotechnol. Molec. Biol. Rev.** 2(1): 014–032

Cheng, J.I., Y.B. Wu, C. Miao, R.M. King and M. Porser. 2005. The effect of a novel enzyme complex on the performance and nutrient digestibility in laying hens fed a corn–soy diet. **Aust. Poult. Sci.** Symposium 17th

Christakopoulos, P., W. Goodenough, D. Kekos, B.J. Macris, M. Claeyssens and M. Bhat. 1994. Purification and characterization of an extracellular β-glucosidase with transglycosylation and exo-glucosidase activities from *Fusarium oxysporum.* **Eur. J. Biochem.** 224: 379–385

Choct, M. 1997. Feed non-starch polysaccharides: Chemical Structrues and Nutritional Significance. **Feed Milling International.** 13–26

Chum, H. L., D.K. Johnson, S. Black, J. Baker, K. Grohmann, K.V. Sarkanen, K. Wallace and H.A. Schroeder. 1987. Organosolv pretreatment for enzymatic hydrolysis of poplars: I. Enzyme hydrolysis of cellulosic residues. **Biotechnol. Bioeng**. 31(7): 643–649

Dalboge, H. 1997. Expression cloning of fungal enzyme genes; a novel approach for efficient isolation of enzyme genes of industrial relevance. **FEMS. Microbiol. Rev.** 21: 29–42

Dashtban, M., H. Schraft and W. Qin. 2009. Fungal bioconversion of lignocellulosic residues; Opportunities & Perspectives. **Int. J. Biol. Sci.** 5(6): 578–595

Dey, P. M. 1993. **Methods in Plant Biochemistry**. Acedemic press. Verginia, U.S.A.

Dobois, M., K.A. Gilles, J.K. Hamiton, P.A. Rebers and F. Smith. 1956. Colorimetric method for determination of sugars and related substrates. **Anual Chemistry**. 28: 350–356

Dowzer, C.E. and J.M. Kelly. 1991. Analysis of the *creA*, a regulator of carbon catabolite repression in *A. nidulans*. **Mol. Cell. Biol.** 11(11): 5701–5709

Eyini, M., V. Rajapandy, K. Parahi and M. Wong. 2004. Effect of different pretreatment methods on the bioconversion of rice bran into ethanol. **Mycobiology.** 32(4): 170–172

Fang, J.M., R.C. Sun and J. Tomkinson. 2000. Isolation and characterization of hemicelluloses and cellulose from rye straw by alkaline peroxide extraction. **Cellulose.** 7: 87–107

Farm, Y.Y., D. Krishniah, M. Rajin and A. Bono. 2009. Cellulose extraction from palm kernel cake using liquid phase oxidation. **J. Eng. Sci. Technol.** 4(1): 57–68

Foreman, P.K., B. Doug, L. Dankmeyer, R. Dean, S. Diener, N.S. Dunn-Coleman, F. Goedegebuur, T.D. Houfek, G.J. England, A.S. Kelley, H.J. Meerman, T. Mitchell, C. Mitchinson and H.A. Olivare. 2003. Transcriptional regulation of biomass-degrading enzymes in the filamentous fungus *Trichoderma reesei*. **J. Biol. Chem.** 278(34): 31988–31997

Fowler, T. 1993. **Deletion of the *T. reesei* β-glucosidase gene, *bgl*II**, pp. 56-65 *In* Molecular Biology. Washington D. C., USA.

Garcia, E., D. Johnston, J.R. Whitaker and S.P., Shoemaker. 1993. Assessment of endo–1, 4–beta–D–gluconase activity by a rapid colorimetric assay using disodium 2, 2′-bicinchoninate. **J. Food. Biochem**. 17: 135–145

Gary, E.H. and C.P. Kubicek. 1998. ***Trichoderma* and *Gliocladium*** . Taylor & Francis Ltd. New York, U.S.A.

Gautam, S.P., P.S. Bundela, A.K. Pandey, J. Khan, M.K. Awasthi and S. Saraiya. 2011. Optimization for the production of cellulase enzyme from municipal solid waste residue by two novel cellulolytic fungi. **Biotechnol. Res. Int.** 2011: 1–8

Gielkens, M.M.C., E. Dekkers, J. Visser and L.H. de Graaff. 1999. Two cellobiohydrolase-encoding genes form *A. niger* require D–xylose and the xylanolytic transcriptional activator XlnR for their expression. **Appl. Environ. Microb.** 65(10): 4340–4345

Glauce, L.T., E.H.D. Oliveira, T.A.N. Peres, A.H.S. Cruz and N.M. Martinez–Rossi. 2011. Transcription of *Aspergillus nidulans pacC* is modulated by alternative RNA splicing of palB. **FEBS. Letters**. 585: 3442–3445

Gupta, R., Y.P. Khasa and R.C. Kuhad. 2011. Evaluation of pretreatment methods in improving the enzymatic saccharification of cellulosic materials. **Carbohyd. Polym.** 84: 1103–1109

Hanif, A., A. Yasmeen and M.I. Rajoka. 2004. Induction, production, repression, and de-repression of exoglucanase synthesis in *A. niger*. **Bioresouces. Technol.** 94: 311–319

Hans, F.L., J.F. ackson Peter and J.S. Bain. 1999. **Analysis of Plant Waste Material**. Sprinker Inc.

Hasper, A., E. Dekkers, M.V. Mil, P.J.I. de Vondervoort and L.H. van de Graaff. 2002. EglC, a new endoglucanase from *Aspergillus niger* with major activity towards xyloglucan. **Appl. Environ. Microb.** 68(4): 1556–1560

Hegde, S., S. Kavitha, M.C. Varadaraj and G. Muralikrishna, 2006. Degradation of cereal bran polysacchride–phenolic acid complexes by *A. niger* CFR1105**. Food. Chem.** 96: 14–16

Heiss-Blanquet, S., D. Zheng, N.L. Ferreira., C. Lapierre and S. Baumberger. 2011. Effect of pretreatment and enzymatic hydrolysis of wheat straw on cell wall composition, hydrophobicity and cellulase adsorption. **Bioresources. Technol.** 102: 5938–5946

Hoebler, C., J.L. Barry, A. David and J. Delort–Laval. 1989. Rapid acid hydrolysis of plant cell wall polysaccharides and simplified quantitative determination of their neutral monosaccharides by gas–liquid chromatography. **J. Agric. Food. Chem.** 37: 360–365

Hogberg, A. and J.E. Lindberg. 2004. Influence of cereal non-starch polysaccharides on digestion site and gut environment in growing pigs. **Livest. Prod. Sci.** 87(2): 121–130

Hongbo, Y., X. Zhang, L. Song, J. Ke, C. Xu, W. Du and J. Zhang. 2010. Evaluation of white-rot fungi-assisted alkaline/oxidative pretreatment of corn straw undergoing enzymatic hydrolysis by cellulase. **J. Biosci. Bioeng**. 110(6): 660–664

Hooge, D. M., J. L. Picrce, K. W. McBride and P. J. Rigolin. 2010. Meta- analysis of broiler chicken trials using diets with or without AllzymeSSF enzyme complex. **Int. J. Poul. Sci.** 9(9): 819–823

Hossain, S., M.I. Khalil and M.K. Alam. 2009. Upgrading of animal feed by solid state fermentation by *Pleurotus sajor–caju*. **Eur. J. Appl. Sci.** 1(4): 53–58

Howard, R.L., E. Abotsi, E.L.J. van Rensburg and S. Howard. 2003. Lignocellulose biotechnology: issues of bioconversion and enzyme production. **Afr. J. Biotechnol.** 2(12): 602–619

Hrmova´, M., E. Petrakova and P. Biely. 1991. Induction of cellulose and xylan-degrading enzyme systems in *Aspergillus terreus* by homo– and heterodisaccharides composed of glucose and xylose. **J. Gen. Microbiol.** 137: 541–547

Hsu, T.A. 1996. **Pretreatment of Biomass. Hanbook of Biotechnol: Production and Utilization**. Washington DC, U.S.A.

Ilmen, M., M.L. Saloheime, A. Onnela and M.E. Penttila. 1997. Regulation of cellulase gene expression in the filamentous fungus *T. reesei*. **Appl. Environ. Microb.** 63(4): 1298–1306

Immanuel, G., C.M.A. Bhagavath, P.L. Raj, P. Esakkiraj and A. Palavesam. 2007. **Production and partial purification of cellulalse by *Aspergillus niger* and *A. fumigatus* fermented in coir waste and sawdust.** Available Source: http://www.ispub.com/ostia/index.php:xmlFilePath=journals/ijmb/vol3n1/cellulase.xml, April 27, 2007.

Kajiwara, S. and H. Maeda. 1983. The monosaccharide composition of cell wall material in cassava tuber (*Manihot utilissima*). **Agric. Biol. Chem**. 47(10): 2335–2340

Kalogeris, E., P. Christakopoulos, D. Kekos and B.J. Macris. 1998. Studies on the solid-state production of thermostable endoxylanases from *Thermoascus aurantiacus* characterization of two isoenzymes. **J. Biotechnol.** 60: 155–163

Kanamasa, S., M. Mochizuki, G. Takada, T. Kawaguchi, J.I. Sumitani and M. Arai. 2003. Overexpression of *Aspergillus aculeatus* cellobiohydrolase I in *Aspergillus orezae*. **J. Biosci. Bioeng.** 95(6): 627–629

Kang, S.W., Y.S. Park, J.S. Lee, S.I. Hong and S.W. Kim. 2004. Production of cellulases and hemicellulases by *A. niger* KK2 from lignocellulosic biomass. **Bioresources. Technol.** 91: 153–156

Karafta, L., E. Fekete, C. Gamauf, A. Szentinai, C.P. Kubicek and B. Seiboth. 2006. D-galactose induces cellulase gene expression in *Hypocrea jevorina* at low growth rate. **Microbiology.** 152(5): 1507–1514

Kennedy, J. F. 1988. **Carbohydrate Chemistry**. Clarendon Press.

Khempaka, S., W. Molee and M. Guillaume. 2009. Dried cassava pulp as an alternative feedstuff for broilers: Effect on growth performance, carcass traits, digestive organs, and nutrient digestibility. **J. Appl. Poult. Res**. 18: 487–493

Kinghorn, J. K. and G. Tuaner. 1992. **Applied Melecular Genetics of Filamentous Fungi**. New York, Chapman & Hall.

Kodali, B. and R. Pogaku. 2006. Pretreatment studies of rice bran for the effective production of cellulase. **Electron. J. Environ. Agric. Food. Chem**. 5(2): 1253–1264

Kosugi, A., A. Kondo, M. Ueda, Y. Murata, P. Vaithanomsat, W. Thanapase, T. Arai and Y. Mori. 2009. Production of ethanol from cassava pulp via fermentation with a surface engineered yeast strain displaying glucoamylase. **Renew. Energ**. 34: 1354–1358

Kuhls, K., E.E. Lieckfeldt, G.J. Samuels, W. Kvacs, W. Meyer, O. Petrini, W. Gams, T. Borner and C.P. Kubicek. 1996. Melecular evidence that the asexual industrial fungus *Trichoderma reesei* is a clonal derivative of the ascomycete *Hypocrea jecorina***. Proc. Natl. Acad. Sci.** 93: 7755–7760

Kumar, L. R. Chardra and J. Saddler. 2011. Influence of steam pretreatment severity on post-treatment used to enhance the enzymatic hydrolysis of pretreated softwoods at low enzyme loading. **Bioethanol. Bioeng.** 108(10): 2300–2311

Laymon, R.A., W.S. Adney, A. Mohagheghi, M.E. Himmel and S.R. Thomas. 1996. Cloning and expression of full-length *T. reesei* cellobiohydrolase I cDNAs in *E. coli*. **Appl. Biochem. Biotechnol.** 57(58): 389–397

Levente, K., E. Fekete, C. Gamau, A. Szentirmai, C.P. Kubicek and B. Seiboth. 2006. D-Galactose induces cellulase gene expression in *Hypocrea jecorina* at low growth rates. **Microbiology.** 152: 1507–1514

Lineback, D.R. 1982. **Food Carbohydrates.** AVI publishing company.

Lockington, R.A., L. Rodbourn, S. Barnett, C.J. Carter and J.M. Kelly. 2002. Regulation by carbon and nitrogen sources of a family of cellulases in *A. nidulans*. **Fungal. Gnet. Biol.** 37: 190–196

Lu, T., T. Wang, X. Li and X. Liu. 2008. Improved heterologous gene expression in *Trichoderma reesei* by cellobiohydrolase I gene (*cbh1*) promoter optimization. **Acta. Biophys. Sin.** 40(2): 158–165

Lynd, L.R., R.P.J. Weimer, W.H. van Zyl and I.S. Prtorius. 2002. Microbial cellulose utilization: Fundamentals and biotechnology. **Microbiol. Mol. Biol. R.** 66(3): 506–577

McKelvey, S. and R. Murphy. 2010. The effect of carbon and nitrogen supplementation on the transcriptional regulation of hydrolytic enzymes produced by *A. niger*. **Bioproc. J.** 9(1): 31–42

Macris, B.J. 1984. Production and characterization of cellulase and β-glucosidase from a mutant of *Alternaria alternata*. **Appl. Environ. Microb.** 560–565

Mahai, B.A., A. Sara, L. Panta and L. Claper. 2010. The influence of some fedder additives on the meat quality indices of broiler turkey juveniles. **Anim. Sci. Biol.** 67(1–2): 80–84

Martin, C., H.B. Klinke and A.B. Thomsen. 2007. Wet oxidation as a pretreatment method for enhancing the enzymatic convertibility of sugarcane bagasse. **Enzyme. Microb. Tech.** 40: 426–432

Marzluf, A.M. 1997. Genetic regulation of nitrogen metabolism in the fungi. **Microbiol. Mol. Biol. R.** 61(1): 17–32

Maurice, R. 1998. General and microbiological aspects of solid state fermentation. **Electron. J. Biotechnol.** 1(3): 174–188

McGlnnls, G.D., W.W. Wilson and C.E. Mullen. 1983. **Biomass Pretreated with Water and High Pressure Oxygen**. The WO process, Ind Eng Chem Prod Res Dev.

McGinnis, G.D., S.E. Prince, C.J. Bierman and J.F. Lowrimore. 1984. Wet oxidation of model carbohydrate compounds. **Carbohydrate. Res**. 128: 51–60

Meryl, A.D. and M.J. Hynes. 1991. **Regulatory Circuits in *A. nidulans***. Academic Press, Inc. London.

Min, Y.N., E. Yan, F.Z. Liu, C. Coto and P.W. Waldroup. 2009. Effect of various dietary enzymes on corn-soy digestibility of diets high in distrillers dried grain with soluble for broiler. **J. Appl. Poult. Res.** 18: 734–740

Murray, P.G., C.M. Collins, A. Grassick and M.G. Tuohy. 2003. Molecular cloning, transcriptional, and expression analysis of the first cellulase gene (*cbh2*), encoding cellobiohydrolase II, from the moderately thermophilic fungus *Talaromyces emersonii* and structure prediction of the gene product. **Biochem. Bioph. Res. Co.** 301: 280–286

Nakari-Seta´la´, T. and M. Penttila. 1995. Production of *T. reesei* cellulases on glucose-containing media. **Appl. Environ. Microb.** 61(1): 3650–3655

Nakari-Seta´la´, T. and M. Penttila. 1995. Production of *Trichoderma reesie* cellulases on glucose-containing Media. **Appl. Environ. Microb.** 61(10): 3650–3655

Nigam, P. and D. Singh. 1994. Solid-state (substrate) fermentation systems and their applications in biotechnology. **J. Basic. Microb.** 34(6): 405–423

Nina, A., A. Saloheimo, M. Ilmen and M. Penttila. 2001. AceII, a novel transcriptional activator involved in regulation of cellulase and xylanase genes of *T. reesei*. **J. Biol. Chem.** 276(26): 24309–24314

Nina, A., T. Pakula and M. Penttila. 2005. Transcriptional regulation of plant cell wall degradation by filamentous fungi. **FEMS. Microbiol. Rev.** 29: 719–739

Noel, N. M.E. van P., J. Visser and L.H. de Graaff. 1998. Isolation and analysis of *xlnR*, encoding a transcriptional activator co-ordinating xylanolytic expression in *A. niger*. **Mol. Microbiol.** 27(1): 131–142

Noel, N.M.E. van P., M.C.M. Gielkens, R.P. de Vries, J. Visser and L.H. de Graaff. 1998. The transcriptional activator XlnR regulates both xylanolytic and endoglucanases gene expression in *A. niger*. **Appl. Environ. Microb.** 64(10): 3615–3619

Oriol, E., M. Raimbault, S. Roussos and G. Viniegra-Gonzales. 1988. Water and water activity in the solid state fermentation of cassava starch by *A. niger.* **Appl. Microbiol. Biot.** 27: 498–503

Panagiotou, C., D. Kekos, B.J. Macris, M. Claeyssens and M. Bhat. 1995. Purification and characterization of nonrandomly acting endo–1,4–β–glucanase from the culture filtrates of *Fusarium oxysporum.* **Arch. Biochem. Biophys.** 316: 428–433

Panagiotou, G., D.B. Kekos, J. Macris and P. Christakopoulos. 2003. Production of cellulolytic and xylanolytic enzymes by *Fusarium oxysporum* on corn stover in solid state fermentation. **Ind. Crop. Prod.** 18: 37–45

Peij, N.N., M.M. Gielkens, R.P. Vries. R.P. 1998. The transcriptional activator XlnR regulates both xylanolytic and endoglucanase gene expression in *Aspergillus niger*. **Appl. Environ. Microbiol**. 64(10): 3615–3619

Pfaffl M.W. 2001. A new mathematical model for relative quantification in real-time RT–PCR. **Nucleic. Acids. Res.** 29: 45

Puls, J., K. Poutanen, H–U. Korner and L. Viikari. 1985. Biotechnical utilization of wood carbohydrates after steaming pretreatment. **Appl. Microbiol. Biotechnol.** 22: 416–423

Puls, J. and K. Poutanen. 1989. Mechnisms of enzymic hydrolysis of hemicelluloses (xylans) and procedures for determination of the enzyme activities involved, P. 37–52. *In* Coughlan, M. P. (Ed.), Enzymes Systems for Lignocellulose Degradation. **Appl. Biochem. Biotec.**

Raimbault, M. 1998. General and microbiological aspects of solid substrate fermentation. **J. Biotechnol.** 1(3): 174–188

Rajoka, M.I. 2004. Influence of various fermentation variables on exo-glucanase production in Cellulomonas flavigena. **Electron. J. Biotechno**. 7(3): 256–263

Ravindra, A. and P. Ravindra. 2001. Studies on production of single cell protein by *Aspergillus niger* in solid state fermentation of rice bran. **Braz. Arch. Biol. Techn.** 44(1):79–88

Sae-Lee, N. 2007. The production of fungal mannanase, cellulase and xylanase using palm kernel meal as a substrate. **Walailak J. Sci & Tech**. 4(1): 67–82

Saha, B.C. 2003. Hemicellulose bioconversion. **J. Ind. Microbiol. Biotechnol**. 30: 279–291

Sampson, R.A. 1994. **Taxonomy and Genetics to Industrial Application.** N.Y: Plenum Press, New York, U.S.A.

Sanchez, C. 2009. Lignocellulosic residues : biodegradation and bioconversion by fungi. **Biotechnol. Adv.** 27: 185–194

Schell, J.D., M. F. Ruth and M.P. Tucker. 1999. Modeling the enzymatic hydrolysis of dilute acid–pretreated Douglas fir. **Appl. Biochem. Biotech.** 77: 67–81.

Schell, J.D. 2003. Dilute-Sulfuric acid pretreatment of corn stover in pilot-scale reactor. **Appl. Biochem. Biotechnol**. 69: 105–108

Seiboth, B., S. Hakola, R.L. Mach, P.L. Suominen and C.P. Kubicek. 1997. Role of four major cellulases in triggering of cellulase gene expression by cellulose in *Trichoderma reesei.* **J. Bacteriol.** 179(17): 5318–5320

Seip E. R., C. P. Woloshuk, G. A. Payne and S. E. Curtis. 1990. Isolation and sequence analysis of a β-Tubulin gene form Aspergillus flavus and its use as a selectable marker. **Appl. Environ. Microb.** 56(12): 3686–3692

Shin, K., M. Mochizuki, G. Takada, T. Kawaguchi, J–I. Sumitani and M. Arai. 2003. Overexpression of *A. aculeatus* cellobiohydrolaseI in *A. oryzae*. **J. Biosci. Bioeng.** 95(6): 627–629

Shuai, L., Q. Yang, J.Y. Zhu, F.C. Lu, P.J. Weimer, J. Ralph and X.J. Pan. 2010. Comparative study of SPORL and dilute–acid pretreatments of spruce for cellulosic ethanol production. **Bioresource. Technol.** 101: 3106–3114

Shyamala, H., S. Kavitha, M.C. Varadaraj and G. Muralikrishna. 2006. Degradation of cereal bran polysaccharide–phenolic acid complexes by *A. niger* CFR 1105. **Food. Chem.** 96: 14–19

Silverstein, R.A., Y. Chen and R.R. Sharma-Shivappa. 2007. A comparison of chemical pretreatment methods for improving saccharification of cotton stalks. **Bioresource. Technol.** 98(16): 3000-3011

Somogyi, M. and N. Nelson. 1952. Determination of reducing sugars by Nelson-Somogyi method. **J. Biol. Chem**. 200: 245–245

Srinorakutara, T., L. Kaewvimol and L-A. Saengow. 2006. Approach of cassava waste pretreatments for fuel ethanol production in Thailand. **Chulalongkorn Journal**. 31(1): 1–8

Sriroth, K., S., R. Chollakup, S. Chotineeranat, K. Piyachomkwan and C.G. Oates. 2000. Processing of cassava waste for improved biomass utilization. **Bioresources. Technol.** 71: 63–69

Stewart, J.C. and J.B. Parry. 1981. Factors influencing the production of cellulases by *A. fumigates*. **J. Gen. Microbiol.** 125: 33–39

Stricker, A.R., P. Trefflinger, N. Aro, M. Penttila and R.L. Mach. 2006. Role of Ace2 (activtator of cellulase 2) within the *xyn2* transcriptsome of *Hypocrea jecorina*. **Fungal. Genet. Biol.** 45: 436–445

Sundu, B. and J. Dingle. 2003. **Use of Enzymes to Improve the Nutritional Value of Palm Kernel Meal and Copra Meal**. Proceed Quensland Poultry Science Symposium, Gatton, Queensland, Australia. 24 July 2003. 11(14): 1–15

Suto, M. and F. Tomita. 2001. Induction and catabolite repression mechanisms of cellulase in fungi. **J. Biosci. Bioeng.** 92(4): 305–311

Szengyel, Z. and G. Zacchi. 2000. Effect of acetic acid and furfural on cellulose production of *T. reesei* RUT C30. **Appli. Biochem. Biotechnol**. 89: 31–42

Taherzaden, M.J. and K. Karimi. 2008. Pretreatment of lignocellulosic wastes to improve ethanol and biogas production: A review. **Int. J. Mol. Sci**. 9: 1621–1651

Tatsumoto, K., J.O. Baker, M.P. Tucker, K.K. Oh Mohagheghi, A.K. Grohmann and M.E. Himmel. 1988. Digestion of pretreated aspen substrates: hydrolysis rates and adsorptive loss of cellulase enzymes. **Appl. Biochem. Biotech.** 18: 159–174.

Teoh, Y.P. and M.D. Mashitah. 2010. Cellulase production by *Pycnoporus sanguineus* on oil plam residues through pretreatment and optimization study. **J. Appl. Sci.** 10(12): 1036–1043

Thom, C., and Raper, K.B. 1945. **A Manual of the *Aspergilli***. Williams & Wilkins. Baltimore, U.S.A

Torget, R.W., R.W. Hayward and T.K. Elanda. 1997. **Total Hydrolysis of Lignocellulosic Biomass Using Very Dilute Acid Employing a Novel Shrinking Bed Reactor Configuration.** 19th symposium on biotechnology for fuels and chemicals, Springs. Co. Ltd. Colorado, U.S.A

Updegraff, D.M. 1969. Semimicro determination of cellulose in biological materials. **Anal. Biochem.** 32: 420–424

U.S.D.A. 2009. **United States Standards for Rough Rice.** Available Source: http://www.gipsa.usda.gov/fgis/standards/ricestandards-rough_rice.pdf, November 27, 2012.

Vandesompele, J., K. De Preter, F. Pattyn, B. Poppe, N. Van Roy, A. Paepe and F. Speleman. 2002. Accurate normalization of real–time quantitative RT–PCR data by genometric averageing of multiple internal control genes. **Genome. Biol.** 3, research 0034

Ververis, C., K. Georghiou, N. Christodoulakis, P. Santasa and R. Santas. 2004. Fiber dimensions, lignin and cellulose content of various plant materials and their suitability for paper production. **Ind. Crop. Prod.** 19: 245–254

Vries, R.D. and J. Visser. 2001. *Aspergillus* enzymes involved in degradation of plant cell wall polysaccharides. **Microbiol. Mol. Biol. R.** 65(4): 497–522

Walker, H. G., Jr. B. Lai, W. C. Rockwell and G. O. Kohler. 1970. Preparation and evaluation of popped grains for feed use. **Cereal. Chem**. 47: 513- 521

Wang, T-H., T. Liu, Z–H. Wu, S–L. Liu, Y. Lu and Y–B. Qu. 2004. Novel cellulase profile of *Trichoderma reesei* strains constructed by *cbh1* gene replacement with *eg3* gene expression cassette. **Acta. Bioch. Bioph. Sin.** 36(10): 667–672

Wanrosli, W.D., M.H.M. Kassim and A. Seeni. 2011. Cellulose phosphate from oil palm biomass as potential biomaterials. **Bioresources.** 6(2): 1719–1740

Whealan, W.J. 1969. **Hydrolysis with α-amylase**. P. 252-260 *In* Methods Carbohydrate Chemistry.

Willfor S., R. Sjoholm, C. Laine, M. Roslund, J. Hemming and B Holmbom. 2003. Characterisation of water-soluble galactoglucomannans from Norway spruce wood and thermomechanical pulp**. Carbohyd. Polym.** 52(2): 175–187

Xia, L. and P. Cen. 1999. Cellulase production by solid state fermentation on lignocellulosic waste from the xylose industry. **Process. Biochem.** 34: 909–912

Xiao, Z., X. Zhang, D.J. Gregg and J.N. Saddler. 2004. Effect of sugar inhibition on cellulase and β–glucosidase during enzymatic hydrolysis of softwood substrates. **Appl. Biochem. Biotechnol**. 115(1-3): 1115–1126

Yamane, Y.I. 2002. Production of cellulose– and xylan– degrading enzymes by a koji mold, *Aspergillus oryzae*, and their contribution to the maceration of rice endosperm cell wall. **J. Biosci. Bioeng.** 93(1): 9–14

Yan, Y.F., D. Krishniah, M. Rajin and A. Bono. 2009. Cellulose extraction from palm kernel cake using liquid phase oxidation. **J. Eng. Sci. Technol.** 4(1): 57–68

Yang, B. and C.E. Wyman. 2004. Effect of xylan and lignin removal by batch and flowthrough pretreatment on the enzymatic digestibility of corn stover cellulose. **Biotechnol. Bioeng.** 86: 88–95

Yu, Z., J. Lui, P.J. Gao, L.P. Ma, D.X. Shi and S.J. Pang. 1998. Structure investigation of cellobiohydrolase I from *T. pseudokoningii* S38 with scanning tunneling microscope. **Appl. Phys**. 67: 483–485

APPENDICES

Appendix A

Enzyme production and gene expression result

APPENDIX A

Appendix Table A1 The cellulase production of *A. niger* 386017M1 under SSF system during 10 days by using RRB, PKM and CP as substrate

	RRB			PKM			CP		
Day	CMCase (U/g)	CBH (U/g)	BG (U/g)	CMCase (U/g)	CBH (U/g)	BG (U/g)	CMCase (U/g)	CBH (U/g)	BG (U/g)
1	51±10	107±9	3,005±1,218	1±1	120±21	542±62	39±3	70±26	1,317±1,054
2	57±13	167±6	3,054±1,106	2±1	167±11	670±24	42±6	121±11	1,456±789
3	67±15	234±5	3,232±2,076	4±1	287±11	659±39	46±8	138±2	20,79±2,174
4	69±5	1,200±17	3,363±812	9±3	291±21	882±82	49±6	198±6	3,129±956
5	72±10	1,286±9	3,845±1,463	11±2	311±19	893±52	56±6	271±16	2,303±1,256
6	82±11	1,274±9	3,914±1,694	15±6	250±20	771±16	53±8	278±80	2,185±1,7844
7	85±8	1,124±7	4,368±1,115	18±5	161±15	736±21	48±5	288±18	2,062±856
8	84±4	1,112±5	3,900±2,724	14±5	88±16	706±62	45±7	260±20	2,172±1,372
9	82±7	1,122±6	2,198±363	13±5	92±18	702±73	43±6	129±36	1,638±423
10	82±5	1,092±3	1,307±429	14±7	102±22	599±37	43±4	143±17	1,745±516

Appendix Table A2 Growth, cellulase production, reducing sugar and pH of *A. niger* 38017M1 under solid state fermentation using untreated RRB as substrate

	Native RRB					
Day	CMCase (U/g)	CBH (U/g)	BG (U/g)	glucosamine (ng/g)	reducing Sugar (mg/ml)	pH
3	7±3	11±2	6,626±681	74±10	33±3	4.5±1.0
5	71±20	2,127±676	2,0864±8,384	110±15	23±7	5.0±0.83
7	80±14	8,480±26	27,978±14,299	75±9	19±2	5.3±0.24

Appendix Table A3 Growth, cellulase production, reducing sugar and pH of *A. niger* 38017M1 under solid state fermentation using steamed RRB as substrate

	Steamed RRB					
Day	CMCase (U/g)	CBH (U/g)	BG (U/g)	glucosamine (ng/g)	reducing Sugar (mg/ml)	pH
3	52±8	6,246±3,121	21,625±2,773	49±7	12±3	5.8±0.24
5	58±8	7,245±1,956	28,482±5,352	59±9	13±4	5.4±0.36
7	59±7	8,762±1,281	23,570±2,944	50±4	12±1	5.4±0.36

Appendix Table A4 Growth, cellulase production, reducing sugar and pH of *A. niger* 38017M1 under solid state fermentation using untreated PKM as substrate

Day	Native PKM					
	CMCase (U/g)	CBH (U/g)	BG (U/g)	glucosamine (ng/g)	reducing Sugar (mg/ml)	pH
3	7±3	11±2	6,626±681	74±10	33±3	4.5±1.0
5	10±6	12±3	7,171±2,220	92±5	34±3	4.6±0.9
7	10±6	8±0.8	13,437±2,511	63±3	33±3	4.5±1.1

Appendix Table A5 Growth, cellulase production, reducing sugar and pH of *A. niger* 38017M1 under solid state fermentation using steamed PKM as substrate

Day	Steamed PKM					
	CMCase (U/g)	CBH (U/g)	BG (U/g)	glucosamine (ng/g)	reducing Sugar (mg/ml)	pH
3	14±10	1,610±398	18,115±8,822	66±7	16±2	4.5±0.6
5	14±9	1,627±101	26,869±7,144	54±5	18±3	4.3±0.7
7	15±11	5,157±1,338	36,123±12,159	53±7	19±4	4.3±0.8

Appendix Table A6 Growth, cellulase production, reducing sugar and pH of *A. niger* 38017M1 under solid state fermentation using untreated CP as substrate

	Native CP					
Day	CMCase (U/g)	CBH (U/g)	BG (U/g)	glucosamine (ng/g)	reducing Sugar (mg/ml)	pH
3	10±4	13±5	4,359±2,402	114±12	202±13	4.4±0.9
5	9±1	14±3	3,817±821	84±8	205±17	4.1±1.1
7	7±3	11±2	3,729±672	95±4	214±5	4.0±1.2

Appendix Table A7 Growth, cellulase production, reducing sugar and pH of *A. niger* 38017M1 under solid state fermentation using acid pretreated CP as substrate

	Acid CP					
Day	CMCase (U/g)	CBH (U/g)	BG (U/g)	glucosamine (ng/g)	reducing Sugar (mg/ml)	pH
3	9±4	5,895±623	5,697±2,426	69±7	22±2	4.9±0.5
5	12±2	7,303±648	9,349±5,546	78±10	20±2	5.1±0.3
7	13±1	7,683±460	8,683±6,290	65±10	19±1	5.3±0.5

Appendix Table A8 Effect of acid pretreatment on fungal growth, cellulase production, reducing sugar and pH of *A. niger* 386017M1 under SSF system by using 0% acid pretreated CP as substrate

0% H_2SO_4	Cellulase (U/g)			Glucosamine	Reducing	pH
Day	CMCase	CBH	BG	(ng/g)	Sugar (mg/ml)	
0	11±4	16±2	178±963	79±2	49±6	5.9±0.2
3	20±7	96±8	1,144±77	104±12	58±11	4.2±0.1
5	23±8	158±70	1,127±429	110±6	56±10	4.0±0.3
7	21±7	247±63	1,693±288	120±9	45±12	3.9±0.1

Appendix Table A9 Effect of acid pretreatment on fungal growth, cellulase production, reducing sugar and pH of *A. niger* 386017M1 under SSF system by using 0.1% acid pretreated CP as substrate

0.1%	Cellulase (U/g)			Glucosamine	Reducing	pH
H_2SO_4 Day	CMCase	CBH	BG	(ng/g)	Sugar (mg/ml)	
0	12±4	18±1	7.6±304	79±5	45±1	4.6±0.2
3	17±6	293±130	1,814±375	108±14	52±10	3.7±0.1
5	23±7	777±23	4,096±2,887	115±2	47±5	3.5±0.3
7	14±5	3,553±811	4,062±3,547	116±9	45±3	3.4±0.1

Appendix Table A10 Effect of acid pretreatment on fungal growth, cellulase production, reducing sugar and pH of *A. niger* 386017M1 under SSF system by using 0.3% acid pretreated CP as substrate

0.3% H_2SO_4 Day	Cellulase			Glucosamine (ng/g)	Reducing Sugar (mg/ml)	pH
	CMCase	CBH	BG			
0	12±5	15±1	1577±2,057	76±3	32±2	5.1±0.2
3	16±5	137±88	3,430±1,176	92±3	45±4	3.7±0.2
5	15±5	7,599±22	6,572±2,222	113±5	37±4	3.5±0.1
7	19±7	2,834±22	5,104±1,584	104±19	31±2	3.4±0.1

Appendix Table A11 Effect of acid pretreatment on fungal growth, cellulase production, reducing sugar and pH of *A. niger* 386017M1 under SSF system by using 0.5% acid pretreated CP as substrate

0.5% H_2SO_4 Day	Cellulase			Glucosamine (ng/g)	Reducing Sugar (mg/ml)	pH
	CMCase	CBH	BG			
0	13±5	11±5	0±0	70±4	24±0	3.3±0.3
3	16±6	802±158	3,163±733	101±13	24±0	3.8±0.2
5	17±6	387±194	3,148±1503	96±8	23±0	3.6±0.1
7	17±6	272±72	2,310±295	87±7	24±1	3.4±0.2

Appendix Table A12 The relative of cellulase regulatory genes expression (Log RQ) of *A. niger* 386017M1 under SSF system using 0.1% acid CP during 7 days

0.1%/Day	*cbhA*	*cbhB*	*xlnR*	*creA*	*pacC*	*ace2*
3	0.39±0.11	0.24±0.12	-0.21±0.21	0.13±0.12	0.28±0.13	0.34±0.005
5	0.28±0.06	0.23±0.04	-0.44±0.09	-0.18±0.11	0.28±0.04	0.09±0.15
7	0.54±0.14	0.18±0.09	0.04±0.06	0.09±0.17	0.43±0.16	0.54±0.12

Appendix Table 13 The relative of cellulase regulatory genes expression (Log RQ) of *A. niger* 386017M1 under SSF system using 0.3% acid CP during 7 days

0.3%/Day	*cbhA*	*cbhB*	*xlnR*	*creA*	*pacC*	*ace2*
3	0.64±0.21	0.41±0.17	0.11±0.04	0.69±0.23	0.50±0.31	0.81±0.24
5	0.49±0.17	0.79±0.24	0.07±0.01	0.29±0.08	0.43±0.13	0.45±0.18
7	0.22±0.11	0.53±0.16	-0.80±0.15	-0.29±0.11	0.36±0.11	0.19±0.07

Appendix Table A14 The relative of cellulase regulatory genes expression (Log RQ) of *A. niger* 386017M1 under SSF system using 0.5% acid CP during 7 days

0.5%/Day	*cbhA*	*cbhB*	*xlnR*	*creA*	*pacC*	*ace2*
3	0.25±0.06	0.33±0.12	0.77±0.27	0.04±0.18	0.32±0.07	0.32±0.09
5	0.23±0.12	0.20±0.05	0.90±0.32	0.37±0.09	-0.19±0.06	-0.39±0.12
7	0.31±0.17	0.35±0.08	0.39±0.14	0.59±0.21	0.26±0.04	-0.23±0.16

Appendix Table A15 C_T value of positive; *A. niger*, negative of acid pretreated CP ; AC CP material and NTC; non-template for primer validation

Gene	C_T value		
	A. niger	AC CP	NTC
cbhA	25.36	33.18	33.95
cbhB	26.96	33.01	34.64
xlnR	26.06	35.75	35.59
ace2	24.97	36.49	33.86
pacC	30.88	35.36	34.69
area	29.09	37.00	36.06
creA	26.09	31.13	32.03
tub	23.73	35.22	36.57

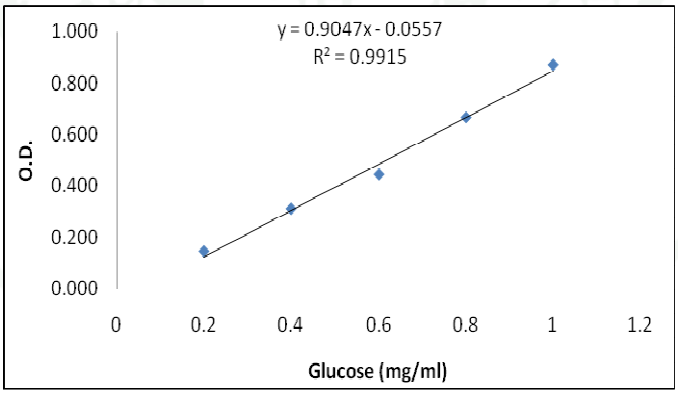
Appendix B

Calculation

APPENDIX B

1. Reducing sugar determination

A working stock solution of anhydrous glucose (10 mg/ml) was made up to .1, 0.2, 0.3, 0.4, 0.5, 0.6, 0.7, 0.8 and 0.9 mg/ml glucose. Glucose standard curve was prepared by adding 0.5 ml of each glucose dilutions to 1.0 ml of citrate buffer in a test tube. The blanks and glucose standards was stopped by addition of 3.0 ml of DNS reagent. All tubes were boiled exactly for 5.0 min. All samples, blanks, and glucose standards were boiled together and later cooled down in a cold ice-water bath. Color formation was determined by measuring absorbance against the reagent blank at 540 nm. With this dilution the glucose standards described above should give absorbance in the range of 0.1 to 1.0 A. A linear glucose standard curve using the absolute amounts of glucose (mg/ml) was plotted against absorbance at 540 nm. The data for the standard curve should closely fit a calculated straight line, with the correlation coefficient for this straight line fit being very near to one. The standard curve was verified by running a calibration verification standard. An amount of glucose independently prepared should fall about midpoint on the standard curve.



Appendix Figure A1 Standard curve of glucose (mg/ml)

3. Sugar residue calculation (g/g substrate) (HPLC)

The sugar residue as g/g substrate was obtained for standard of each sugar and moisture content follow:

10% moisture content substrate

100 g substrate real weight = 100 g-10 g (%)

= 90 g

100 g substrate real weight 90 g

0.05 g substrate (use for HPLC) = (90 X 0.05)/100

= 0.045 g

The sugar content from HPLC result presented number 0.46 g/l

1000 ml	provided	sugar 0.46 g/l
12 ml		= (0.46 X 12)/1000
(total volume of hydrolyzed sample)		= 0.0055 g/l

If use real weight of substrate	0.045 g	provided	0.0055 g/l
In	1 g		= (0.0055 X 1)/0.045
			= 0.114 g/g substrate

4. The DNaseI treatment reaction

Total RNA concentraion 100 ng/µl in 100 µl of sample solution

Maximum total RNA containing was 2,500 ng in DNaseI treated reaction

Total RNA (2,500 ng)	50	µl
10X reaction buffer	5	µl
Amplification DNaseI (1U/ µl)	5	µl
Total reaction	60	µl

Follow the instruction of test kit (assumed as 50 µl reaction)

5. cDNA Synthesis

The First stand cDNA synthesis test kit contained dNTP, reverse transcriptase, RNA guard (porcine) and universal primer was added by 60 µl of total RNA from **3** without adding more DEPC water and then follow the instruction of use. If the volume of reaction less than 33 µl, the DEPC water to final volume.

6. Standard curve formation of target gene for primer validation

Total RNA of pure culture *A. niger*	100 ng/µl (from cDNA synthesis Follow 3.)
The cDNA Template	2 µl/ reaction of qRT-PCR (Follow instruction of test kit)

The total RNA (cDNA template) was dilute with 50 µl DEPC water to obtain final concentration about 50 ng/µl

Standard curve of 5 different total RNA concentration

2 fold dilution	Log Value
100 ng	2
50 ng	1.7
25 ng	1.4
12.5 ng	1.0
6.25 ng	0.8

qRT-PCR reaction

2X sSoFastEvagreen Supermix	10	µl
Forward Primer (300 ng)	3	µl
Reverse Primer (300 ng)	3	µl
cDNA template (100 ng)	2	µl
DEPC water	2	µl
Total reaction	20	µl

7. Gel electrophoresis

Agarose gel electrophoresis was performed to check DNA of PCR product quality. Agarose was mixed with 1x Tris-acetate EDTA (TBE) buffer to 1.2% agarose gel and heated until complete solubilization. The warm agarose was poured into the casting tray. After the gel set completely, the gel was placed in an electrophoresis chamber containing 1x TAE running buffer. The 0.5 µg DNA sample and 100 bp DNA marker (GeneRuler 100 bp Plus DNA Ladder, Fermentas, Germany) were mixed with 1µl 6 X loading dye (10 mM Tris-HCl, 0.03% bromophenol blue, 0.03% xylene cyanol FF, 60% glycerol and 60 mM EDTA) and the sample mixture was loaded slowly into the well of the submerged gel. The DNA samples were running at 5 V/cm or 100 volts for 30 min. After finishing, the gel was stained in 2.5 µg/ml ethidium bromide (EtBr) solution for 15 min. The RNA bands were visualized under UV transilluminator and photographed.

8. Formamide gel electrophoresis (Denaturing agarose gel electrophoresis)

A denaturing gel system was suggested for most RNA forms because of extensive secondary structure via intramolecular base pairing in RNA, and this prevented it from migrating strictly according to its size. RNA molecular weight markers, RiboRuler^{TM}High Range RNA Ladder (Fermentas, Germany) and RNA sample could be used for this purpose. The warm of 1g garose in 72 ml water until dissolved and then cooled to 60°C. The 10 ml of 10 X MOPS running buffer and 18 ml 37% formaldehyde (12.3M) were added. The gel least 25 µl was poured into the casting tray. After the gel set completely, the gel was placed in an electrophoresis chamber containing 1x MOPS running buffer. The 1–3 µg RNA was denatured at 70°C for 10 min and then added 2 X RNA loading dye (95% formamide, 0.025% SDS, 0.025% bromnophenol blue, 0.025% xylene cyanol FF, 0.025% ethidium bromide and 0.5 mM EDTA) from RiboRuler^{TM}High Range RNA Ladder (Fermentas, Germany). The RNA sample was load on a gel and running at 5 V/cm or 100 Volts, 45 min until the bromophenol blue (the faster-migrating dye) has migrated at least 2–3cm into the gel or as far as 2/3 the length of the gel. Visualize the gel on a UV transilluminator.

Appendix C

Chemical

APPENDIX C

1. TAE buffer

The stock of 50X TAE Buffer was prepared in 500 ml; 121 g Tris base in 250 ml H2O was stired to dissolve and then added 28.6 ml acetic acid, added 50ml 0.5M EDTA pH 8.0. The solution was mixed in graduated cylinder and then added ddH2O to 500 ml. The 1X TAE Buffer was prepared for 500ml, 10ml 50X TAE buffer and 490ml ddH2O.

2. TBE buffer

The stock of 10X TAE Buffer was prepared in 1000 ml; 108g Tris base in 250 ml H2O was stired to dissolve and then added 55 g of boric acid and 40 ml of 20 mM EDTA pH 8.0. The solution was mixed in graduated cylinder and then added ddH2O to obtain 1000 ml. The 1X TAE Buffer was prepared for 1000ml by the mixture of 100ml 10X TBE buffer and 900 ml ddH2O.

3. MOPS buffer

The 10X MOPS was prepared as the following: 41.85 g 4-morpholinopropanesulfonic acid and 6.80 g sodium acetate to 800 ml DEPC treated water and stired until completely dissolved. Add 20 ml of a DEPC treated 0.5 M NaEDTA solution and adjust pH to 7.0 with 10 M NaOH or Glacial acetic acid (CH3COOH) and then adjust volume to 1 liter with DEPC treated water.

4. DECP treated water (DEPC water)

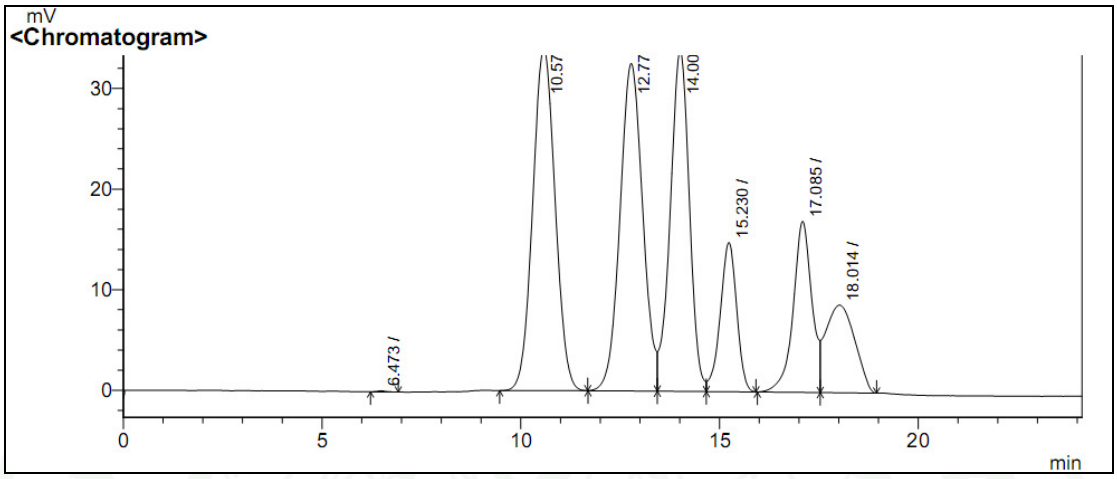
A 0.1% solution of DEPC (diethyl pyrocarbonate) was added to distilled water and then stirred at room temperature for overnight. The DEPC was eliminated by autoclaving at 121°C, 45 mins.

Appendix D

HPLC result

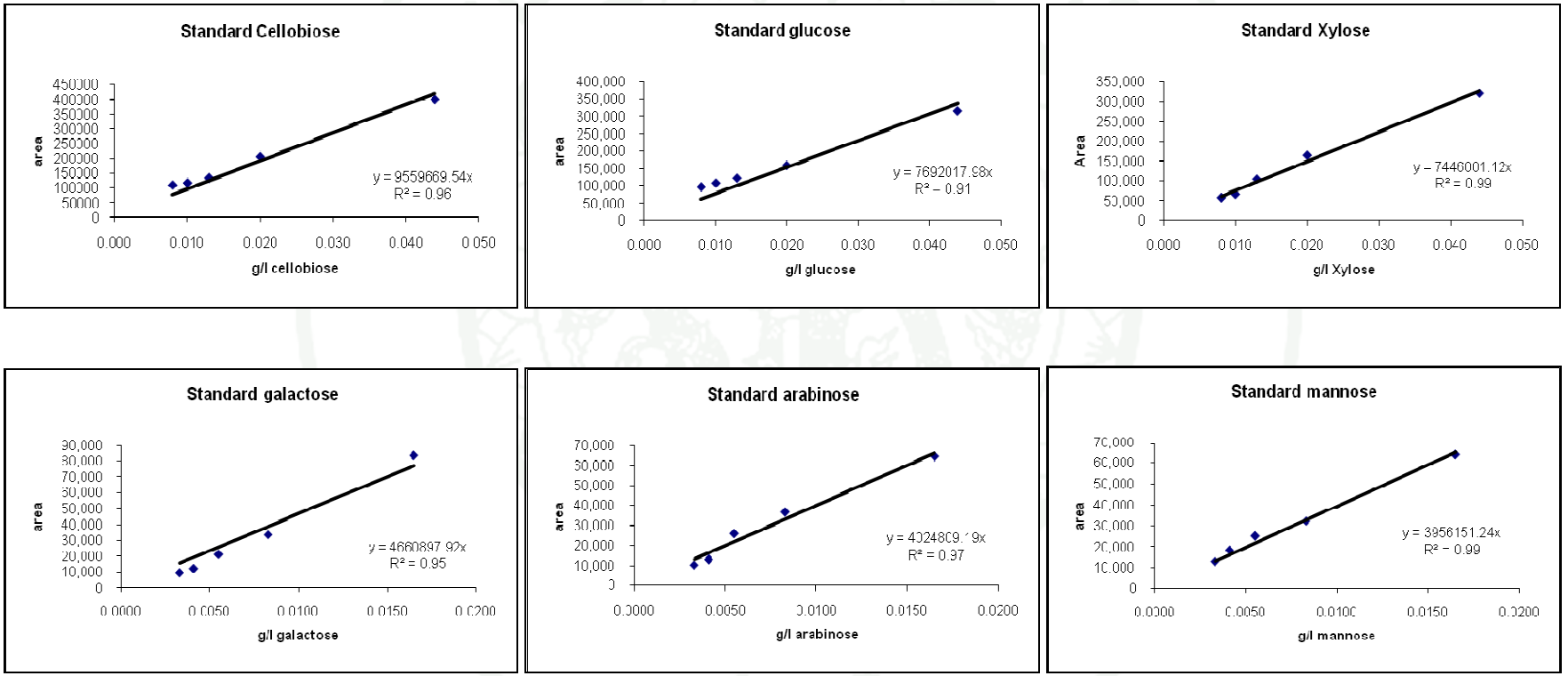
APPENDIX D

1. The chromatogram of standard sugar by HPCL analysis



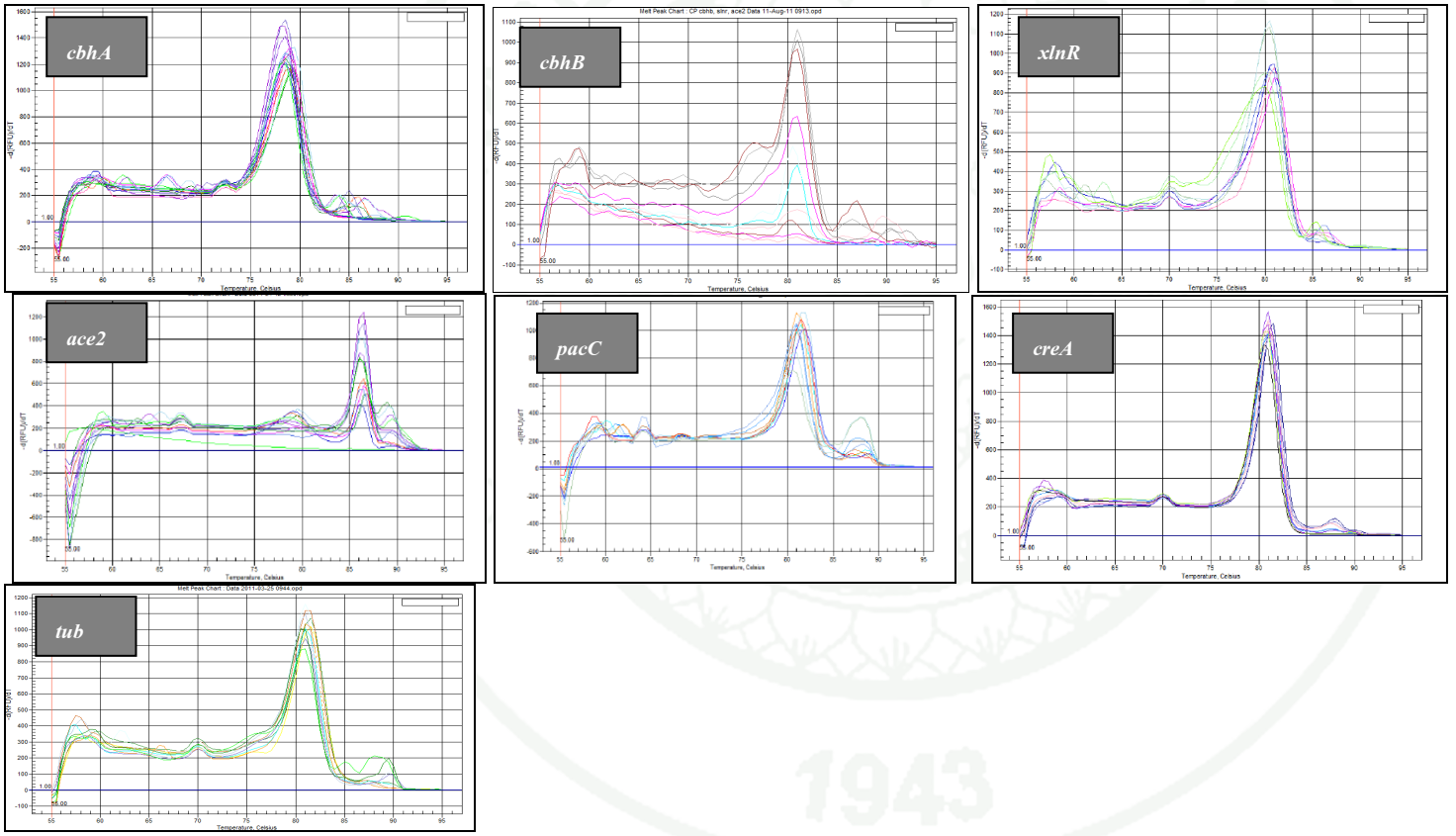
Appendix Figure A2 Chromatogram of standard mix sugar (0.8 mg/ml); cellobiose, glucose, xylose, galactose, arabionose and monnose at retention time 10.5, 12.7, 14.0, 15.2, 17.0 and 18.0 min in respectively. Analyze by high-pressure liquid chromatography (HPLC) using Aminex HPX–87P carbohydrate column (300 X 7.8 mm) to separate each saccharide at 85°C by deionized water with the flow rate of 0.6 ml/min

2. Standard curve of residual sugar (HPLC)



Appeindix Figure A3 Standard curve of sugar; cellobiose, glucose, xylose, galactose, arabinose and mannose

3. Tm of specific primer for primer validation; Single peak was identified as non primer-dimer affect



Appeindix Figure A4 Tm of PCR product from target primer; *cbhA, cbhB, xlnR, ace2, pacC, areA, creA*

CURRICULUM VITAE

NAME : Miss Nattawadee Jantasila

BIRTH DATE : March 6, 1977

BIRTH PLACE : Srisaket, Thailand

EDUCATION :

YEAR	INSTITUTION	DEGREE
1999	Ubonratchathani University	B. Sc. (Microbiology)
2003	Kasetsart University	M. S. (Biotechnology)

POSITION/TITLE : Researcher

WORK PLACE : Alltech Biotechnology Corp., Ltd., 209/1 K Tower B, 17th Floor, Sukhumvit 21 Road (Asoke), Klongtoey Nua, Wattana, Bangkok, 10110, Thailand