SACCHARIFICATION OF CASSAVA WASTE BY *Trichoderma reesei* FOR ETHANOL PRODUCTION

Jutarat Kesornsit

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แซ็กคาริฟิเคชันของกากมันสำปะหลังโดย *Trichoderma reesei* เพื่อการผลิตเอทานอล

จุฑารัตน์ เกษรสิทธิ์

วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาวิทยาศาสตรมหาบัณฑิต
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Thesis Title	SACCHARIFICATION OF CASSAVA WASTE BY *Trichoderma reesei* FOR ETHANOL PRODUCTION
By	Miss Jutarat Kesornsit
Field of Study	Industrial Microbiology
Thesis Advisor	Associate Professor Ancharida Akaracharanya, Ph.D.
Thesis Co-advisor	Teerapatr Srinorakutara, Ph.D.

Accepted by the Faculty of Science, Chulalongkorn University in Partial Fulfillment of the Requirements for the Master's Degree

…….. Dean of the Faculty of Science
(Professor Supot Hannongbua, Ph.D.)

THESIS COMMITTEE

…….. Chairman
(Associate Professor Sirirat Rengpipat, Ph.D.)

…….……. Thesis Advisor
(Associate Professor Ancharida Akaracharanya, Ph.D.)

…….. Thesis Co-advisor
(Teerapatr Srinorakutara, Ph.D.)

…….. External Member
(Associate Professor Vichien Kitpreechavanich, Ph.D.)

…….. Member
(Associate Professor Pairoh Pinphanitchakarn, Ph.D.)

จุฑารัตน์ เกษรสิทธ์ : แซ็กคาริฟิเคชันของกากมันสำปะหลังโดย *Trichoderma reesei* เพื่อการผลิตเอทานอล (SACCHARIFICATION OF CASSAVA WASTE BY *Trichoderma reesei* FOR ETHANOL PRODUCTION) อ. ที่ปรึกษา : รศ.ดร. อัญชริดา อัครจรัลญา, อ. ที่ปรึกษาร่วม : ดร. ธีรภัทร ศรีนรคุตร, 66 หน้า.

การเจริญของ *T. reesei* TISTR 3080 ซึ่งผลิตทั้งเซลลูเลสและอะไมเลสในกากมันสำปะหลัง (15% น้ำหนักเปียก/ปริมาตร) ที่เติม $(NH_4)_2SO_4$ เป็นเวลา 3 วัน ก่อให้เกิดน้ำตาลรีดิวซ์ 13.21 มก./มล. การปรับภาวะให้เหมาะสมคือ ใช้กากมันสำปะหลัง (15% น้ำหนักเปียก/ปริมาตร) $(NH_4)_2SO_4$ (0.2%) ค่าความเป็นกรด-ด่าง 6.0 บ่มที่ 35°ซ (150 รอบ/นาที) เป็นเวลา 3 วัน ทำให้น้ำตาลรีดิวซ์ที่ได้เพิ่มเป็น 14.07 มก./มล. หลังจากนั้นหยุดการเจริญของ *T. reesei* โดยเพิ่มอุณหภูมิขึ้นเป็น 60°ซ และเติมกากมันสำปะหลังลงไปเพิ่มทำให้ปริมาณน้ำตาลรีดิวซ์ในน้ำเลี้ยงเชื้อ (cassava waste hydrolysate) เพิ่มขึ้นเป็น 17.59 มก./มล. ผลการหมักน้ำเลี้ยงเชื้อนี้ซึ่งมีกลูโคส 13.62 มก./มล. ด้วย *S. cerevisiae* เป็นเวลา 72 ชม. ได้เอทานอล 1.91% (น้ำหนัก/น้ำหนัก) ของกากมันสำปะหลัง

นำกากใยซึ่งมี *T. reesei* เจริญอยู่ หลังการแยกน้ำเลี้ยงเชื้อ (cassava waste hydrolysate) ออกไปแล้ว มาย่อยเป็นน้ำตาลโดยแบ่งเป็น 2 ขั้นตอนคือ ผลิตเซลลูเลสจากกากใยแล้วนำเซลลูเลสที่ได้มาย่อยกากใย ภาวะที่ให้ปริมาณเอนไซม์สูงสุด เอนโดกลูคาเนส (0.34 ยูนิต/มล.) คือเติมสารอาหารที่ปราศจากเชื้อลงไปผสมกับกากใยที่มี *T. reesei* เจริญอยู่แล้วบ่มต่อให้เชื้อเจริญชนิดและความเข้มข้นของสารอาหารที่เติมและภาวะที่เหมาะสมต่อการเจริญคือ กากใย 15% (น้ำหนักเปียก/ปริมาตร) $(NH_4)_2SO_4$ 0.3%, KH_2PO_4 0.4 %, $MgSO_4.7H_2O$ 0.025%, yeast extract 0.05%, ความเป็นกรด-ด่าง 6.0 บ่มที่ 35°ซ (150 รอบ/นาที) เป็นเวลา 3 วัน เมื่อใช้เอนโดกลูคาเนสที่ผลิตได้ (15.98 หน่วยเอนไซม์) ย่อยกากใย (90% น้ำหนักเปียก/ปริมาตร) 60°ซ ค่าความเป็นกรด-ด่าง 6.0 เป็นเวลา 6 ชม. จะได้น้ำตาลรีดิวซ์ (54.60 มก./มล.) ซึ่งเป็นน้ำตาลกลูโคส (26.86 มก./มล.) ในน้ำกากใย (cassava fiber hydrolysate) ผลการนำน้ำกากใยนี้มาหมักด้วย *S. cerevisiae* เป็นเวลา 72 ชม. จะได้เอทานอล 1.84% (น้ำหนัก/น้ำหนัก) ของกากใย

โดยวิธีการนี้ กากมันสำปะหลังย่อยเป็นน้ำตาลกลูโคส 2.83% (น้ำหนัก/น้ำหนัก) ของกากมันสำปะหลังและได้เอทานอล 3.11% (น้ำหนัก/น้ำหนัก) ของกลูโคสที่ได้

ภาควิชา	จุลชีววิทยา	ลายมือชื่อนิสิต……..
สาขาวิชา	จุลชีววิทยาทางอุตสาหกรรม	ลายมือชื่ออาจารย์ที่ปรึกษา.....
ปีการศึกษา	2550	ลายมือชื่ออาจารย์ที่ปรึกษาร่วม……..

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KEY WORD: CASSAVA WASTE / *Trichoderma reesei* / CELLULASE

JUTARAT KESORNSIT: SACCHARIFICATION OF CASSAVA WASTE BY *Trichoderma reesei* FOR ETHANOL PRODUCTION. THESIS ADVISOR: ASSOC. PROF. ANCHARIDA AKARACHARANYA, PH. D., THESIS CO-ADVISOR: TEERAPATR SRINORAKUTARA, Ph.D. 66 pp.

Cultivation of *T. reesei* TISTR 3080 which produced both cellulase and amylase in cassava waste (15% wet w/v) supplemented with $(NH_4)_2SO_4$ for 3 days released 13.21 mg/ml of reducing sugar. After optimization of the conditions; 15% (wet w/v) cassava waste, 0.2% $(NH_4)_2SO_4$, pH 6.0, incubated at 35°C (150 rpm), 3 days; the reducing sugar increased to 14.07 mg/ml. To discontinue reducing sugar consumed by *T. reesei*, the incubating temperature was rapidly increased to 60°C. Addition of cassava waste into the culture incubated at 60°C, the reducing sugar increased to 17.59 mg/ml. Fermentation of this cassava waste hydrolysate which contained 13.62 mg/ml glucose to ethanol by *S. cerevisiae* for 72 h resulted in ethanol 1.91% (w/w) of cassava waste.

T. reesei grown-cassava fiber separated from the cassava waste hydrolysate was also saccharified. Two steps method was performed: *T. reesei* cellulase production and hydrolysis of the cassava fiber by the cellulase produced. Non decontaminated cassava fiber suspended in sterile optimized nutrients for *T. reesei* cellulase production, then directly incubated without inoculation gave maximum endoglucanase (0.34 U/ml). The optimal nutrient concentrations and incubation conditions used for *T. reesei* cellulase production were 15% (wet w/v) cassava fiber, 0.3% $(NH_4)_2SO_4$, 0.4% KH_2PO_4, 0.025% $MgSO_4.7H_2O$, 0.05% yeast extract), pH 6.0, at 35°C (150 rpm) for 3 days, respectively. The endoglucanase produced (15.98 units) was used to saccharify the *T. reesei* grown-cassava fiber: 90% (wet w/v) at 60°C, pH 6.0 for 6 h. Then, cassava fiber hydrolysate which contained 54.60 mg/ml reducing sugar or 26.86 mg/ml glucose was fermented to ethanol by *S. cerevisiae*. Ethanol (1.84% w/w of fiber) was obtained after 72 h.

By this procedure cassava waste was saccharified to glucose at 4.23% (w/w) of cassava waste, and ethanol at 3.11% w/w of glucose was produced.

Department	Microbiology	Student's signature……
Field of study	Industrial Microbiology	Advisor's signature……
Academic year	2007	Co-advisor's signature……..

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ABBREVIATIONS

w/v	=	weight/volume
w/w	=	weight/weight
mg/ml	=	milligram/milliliter
mM	=	millimolar
N	=	normality
nm	=	nanometer
min	=	minute
h	=	hour
U	=	unit
ft	=	feet
ha	=	hectare
lb/in^2	=	pounds/square inch
°C	=	degree celsius
rpm	=	round per minutes
$A_{520\ nm}$	=	$Absorbance_{520\ nm}$

CHAPTER I

INTRODUCTION

Energy consumption has been increasing steadily with the population growth and industrial development. Fossil fuel has difficulty in meeting the increase of energy demand. Therefore, exploring for suitable alternative energy is an urgent indispensable mission. Ethanol which is produced through fermentation of sugar by microorganisms has a highest potential for Thailand, which is one of the top net agro-industrial product exporter of the world. Currently, Thailand produces ethanol from cassava and sugar cane molasses. Price of these raw materials is a reason of an ethanol production cost to be too expensive to compete with gasoline. In order to reduce ethanol production cost, one promising way is to utilize plentiful, low-cost agricultural or agro-industrial waste as a substrate. Cassava (*Manihot esculenta* Crantz) is one of a major commercial crop of Thailand. Annually, Thailand produces approximately 16-18 millions tons of cassava. One-half of the cassava produced is exported as cassava chip and pellet. The remainder is supplied as a raw material for cassava starch production, which generates approximately 1.5 million tons of cassava waste (Office of the National Economic and Social Development Board, 2006). The cassava waste contains 60-70% (w/w) starch and 10-15% (w/w) cellulosic fiber. Therefore, cassava waste is a potentially promising low cost substrate for ethanol production (Srinorakutara, *et. al*, 2006). A major step in the conversion of starch and cellulose to ethanol is the breakdown of starch and cellulose to glucose. Two methods are currently suggested as economically feasible: acid and enzymatic hydrolysis (Saxena *et. al*., 1991). Enzyme utilizing method which is more environmental friendly, should be better. However, enzyme is expensive and cellulosic fiber in the waste efficiently absorps enzyme which leads to high amount of enzyme requirement. Saccharification of the cassava waste by starch-and cellulase producing microorganisms directly is more practical. Efficiently saccharification of

cassava waste, which cassava starch is entrapped in cellulosic fiber, occurs after treatment with fungal cellulases (DeMenezes *et. al.*, 1978).

The objective of this study is to optimize the process of cassava waste saccharification by *Trichoderma reesei*, a fungus produces cellulase and amylase, and glucose liberated was further fermented to ethanol by *Saccharomyces cerevisiae*.

CHAPTER II

LITERATURE REVIEWS

2.1 Cassava

Cassava (*Manihot esculenta* Crantz) is a root crop belonging to section fructicosae of Family Euphorbiaceae, Dicotyledonae (Jos, 1969). It is a shrubby, tropical, perennial plant, commonly known as tapioca. The plant is tall, reaching 15 ft., with leaves varying in shape and size. The edible parts are tuberous roots and leaves. The tuber (root) is dark brown in color with length up to 2 ft. depend upon cultivar and soil conditions. The plant thrives on poor soils very well and is less susceptible to locust. Fertilization is rarely necessary. However, production yield increases when planted on well drained with adequate organic matter soil. Minimum growth temperature is 25°C. Many cultivars are drought resistant. Cassava is divided into two types: sweet and bitter types. The bitter type contains higher concentration of cyanogenic glucoside, poisonous compounds that often cause headache in human, than the sweet type. Therefore, the bitter type is used as raw material in industry and as animal feed.

Cassava is one of a major commercial crop of Thailand. It is planted on approximately 1.5 million ha (Santisopasri *et. al*., 2001) and yielded 16-18 million of roots annually (Office of the National Economic and Social Development Board, 2006). One-half of these root is exported as cassava chip and pellet, and the remainder is used as raw material in starch industry.

Cassava starch is used in many industries both food and non-food. The main applications are adhesive, textile and paper industries. Utilization of cassava starch in pharmaceutical and cosmetic industries is restricted, due to high variation of starch quality (Santisopasri *et. al*., 2001).

Production of cassava starch generates approximately 1.5 million tons of cassava waste annually (Office of the National Economic and Social Development Board, 2006). The main application of the waste, after drying is as a low value animal feed or fertilizer. Poorly dried or fresh one spoils rapidly which causes serious pollution problem of the industry. Since the waste still contains 60-70% w/w of starch and 10-15% w/w of cellulosic fiber, dried weight basis (Srinorakutara *et. al*., 2006). It is a potentially promising low cost substrate for ethanol production. Improved utilization of the cassava waste, not only add value to the waste but also reduce environmental problem impact.

2.2 Major composition of cassava

Cassava is composed of 2 major components, starch and cellulose.

2.2.1 Starch

Starch is a principal carbon-reserve polysaccharide in plants and a major source of carbohydrate in the diets of both human and animal. It is a glucose polymer of α-glucan linked by α-1,4 bond and branched at α-1,6 position (Ball and Morell, 2003). Starch is a huge (0.1 $>$ 50 μm in diameter) complex quaternary structure made up of two major glucan polymers: amylose (15-30%) and amylopectin (70-85%) (Yona, 2004).

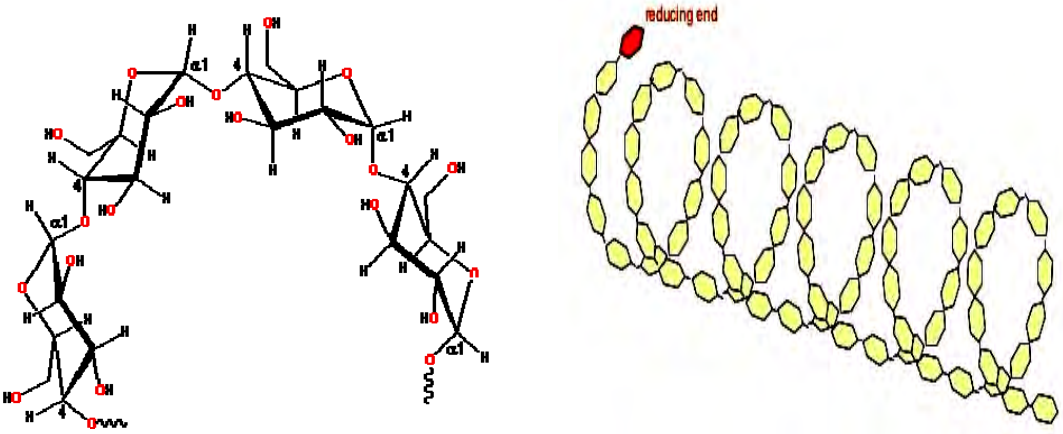
Amylose is considered to be essentially linear polymer, an unbranched, single chain polymer of 500 to 2000 glucose subunits with only the α-1,4 glucosidic bonds (Figure 1a). Although typically illustrated as a straight chain structure for the sake of simplicity, amylose is actually often helical. The interior of the helix contains hydrogen atoms and is therefore hydrophobic, allowing amylose to form complex with free fatty acid, glyceride, some alcohols, and iodine (Fennema, 1985).

Amylopectin is a major constituent of starch in plant tissues. In general, amylopectin is composed of α-1,4-linked glucose segment connected by α-1,6-linked branch points (Figure 1B). It has been estimated that about 4-6% of the linkages within

an average amylopectin molecule are α-1,6 linkages (Hood, 1982). The degree of branching in amylopectin is approximately one per twenty-five glucose units in the branched segments (Pomeran, 1991; Fennema, 1996). The behavior of branch chains of amylopectin is commonly be helical. Property of amylopectin differs from those of amylose because it is highly branched. The amylose and amylopectin contents from various plant sources are different. Some plants contain only amylose or amylopectin, and different plants contain different ratio of amylose and amylopectin (Nakamura, 1996).

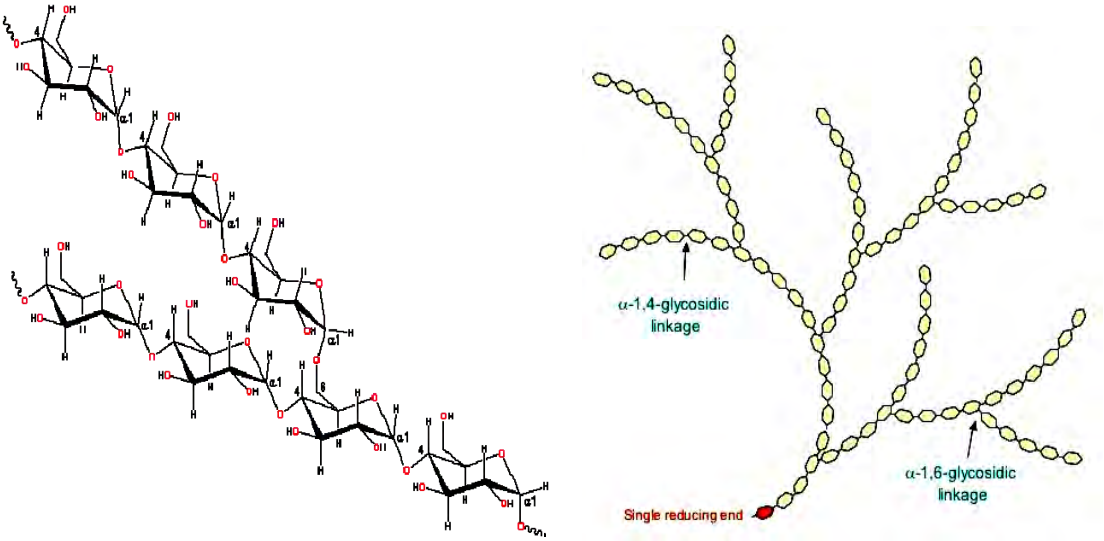
About 70% of starch granules is amorphous and 30% is crystalline. The amorphous regions consist of amylose as a major component, but together with an considerable amount of amylopectin. The crystalline regions contain primarily amylopectin (Berlitz and Grosch, 1999). Starch are generally degraded easily by amylase (Vihinen and Mäntsälä, 1989; Hoseney, 1994).

A.



Amylose, an unbranched starch

B.



Amylopectin, a branched starch

Figure 1 Structure of amylose (A) amylopectin (B)

(De Sa, 2005; Chaplin, 2007)

2.2.2 Cellulose

Cellulose is a major cell wall-component of higher plants. It functions as a structural element. Cellulose is usually forming very complex structure with hemicellulose and lignin, through covalent bonding (Scalbert *et. al*., 1985; Higuchi, 1990). Hemicellulose and lignin are amorphous substances, whereas cellulose has both crystalline and amorphous regions (Fengel and Wegener, 1984). The increasing of crystalline region increases rigidity but decreases elasticity of celluloses. In addition, the ratio of the crystalline region to the amorphous region in a cellulose structure affects an accessibility of cellulose molecule (Tripp, 1971). Cellulose is composed of linear homopolymers of D-glucose units. The smallest structural repeating unit in native cellulose is cellobiose, a disaccharide of glucose. The number of glucose units varies from 100 to 14,000 depending on the source. The structure of the cellulose chain is highly stabilized by intramolecular hydrogen bond (Atalla and Vanderhart, 1984; Michell, 1990; Atalla, 1993; Sugiyama *et. al.,* 1991). Cellulose structure is shown in Figure 2.

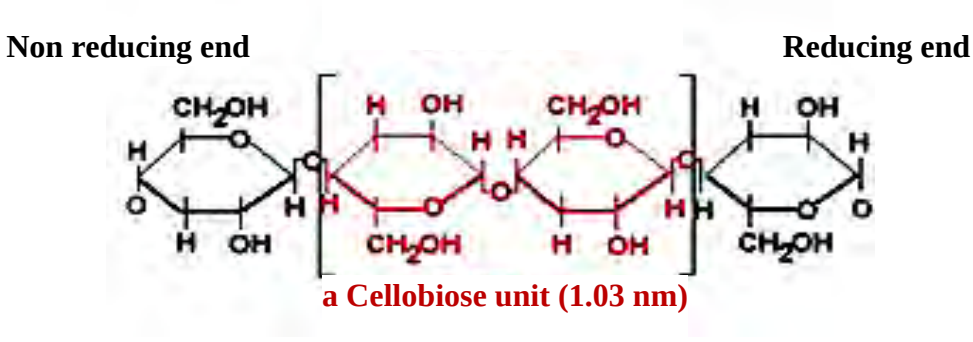


Figure 2 Cellulose structure showing the smallest repeating unit of cellobiose. (Myles, 2007)

2.3 Enzymatic degradation of starch and cellulose

2.3.1 Enzymatic degradation of starch

Amylase is a group of enzymes which hydrolyses α-1,4 and α-1,6 glycosidic linkages in starch molecule. Three different amylolytic enzymes are involved in degrading of starch to glucose **1)** α-amylase (1,4-D-glucan glucanohydrolase, EC 3.2.1.1); is an endoamylase that hydrolyzes the α-1,4-glycosidic linkage at internal position randomly, releasing dextrins and oligosaccharides. (Swift *et. al.,* 1991; Brady *et. al*., 1991; Machius *et. al*., 1995; Fugimoto *et. al*., 1998) **2)** exoamylase, an exoenzyme efficiently cleaves the α-1,4 linkage, but terminating at branch point (α-1,6 linkage) **3)** glucoamylase (β-amylase and α-glucosidase), an enzyme splits glucose unit from nonreducing ends, releasing glucose, maltose and dextrin (Gerhartz, 1990, Polakovic and Bryjak, 2003). Amylase which has primary catalytic activity for α-1,6-glycosidic linkage in amylopectin is called debranching enzyme. Pullulanase (α-dextrin 6-glucanohydrolase, EC 3.2.1.41) and isoamylase (glycogen 6-glucanohydrolases, EC 3.2.1.68) are the most widely studied debranching enzymes (Vihinen and Mäntsälä, 1989, Burton, *et. al*., 1995). Amylases and their sites of action are shown in Figure 3.

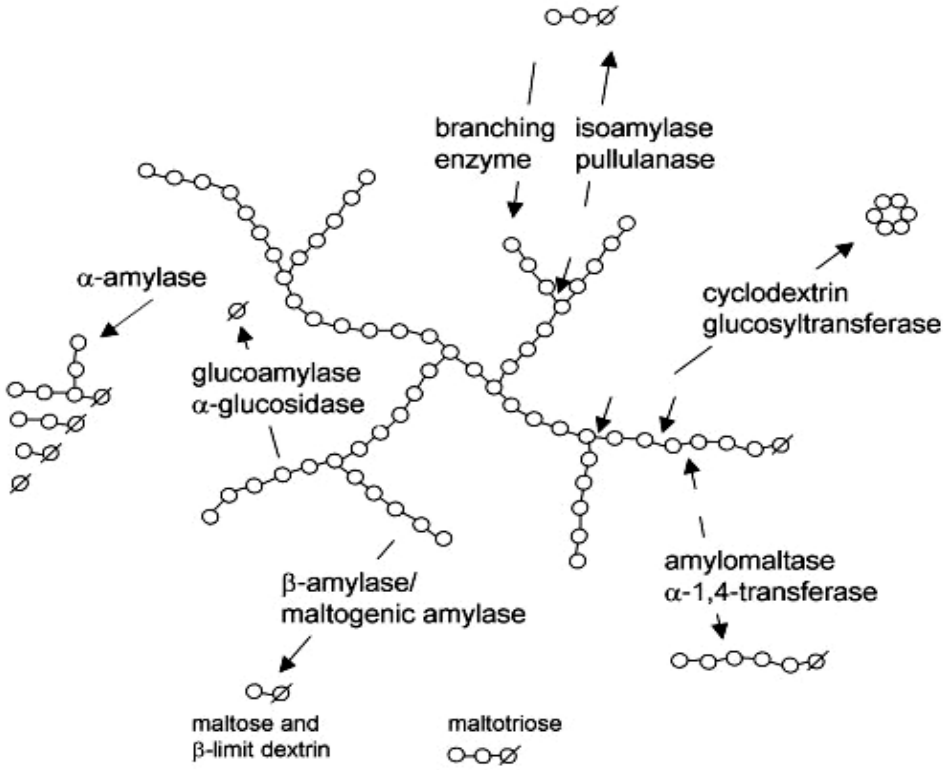


Figure 3 Starch molecule and amylase sites of action. Glucose molecules are shown as circle (○) and reducing ends are marked by a line through the circles (∅) (Turner *et. al*., 2007).

2.3.2 Enzymatic degradation of cellulose

Complete hydrolysis of cellulose to glucose requires synergistic action of exoglucanase (cellobiohydrolase), endoglucanase and β-glucosidase. Exoglucanase (1,4-β-D-glucan cellobiohydrolase, EC 3.2.1.91) is active on crystalline cellulose. Endoglucanase (1,4-β-D-glucan-4-glucanohydrolase, EC 3.2.1.4) is more active against amorphous region of cellulose and it can also hydrolyze substituted cellulose, such as carboxymethylcellulose (CMC). Exoglucanase cleaves disaccharide (cellobiose) unit either from nonreducing or reducing end, whereas endoglucanase hydrolyzes the cellulose chain internally. β-glucosidase (EC 3.2.1.21) cleaves cellobiose to glucose (Figure 4). This last step is important since high concentration of

glucose inhibits β-glucosidases activity resulted in an accumulation of cellobiose, and high concentration of cellobiose developed inhibits endoglucanase and exoglucanase activities (Béguin, 1990).

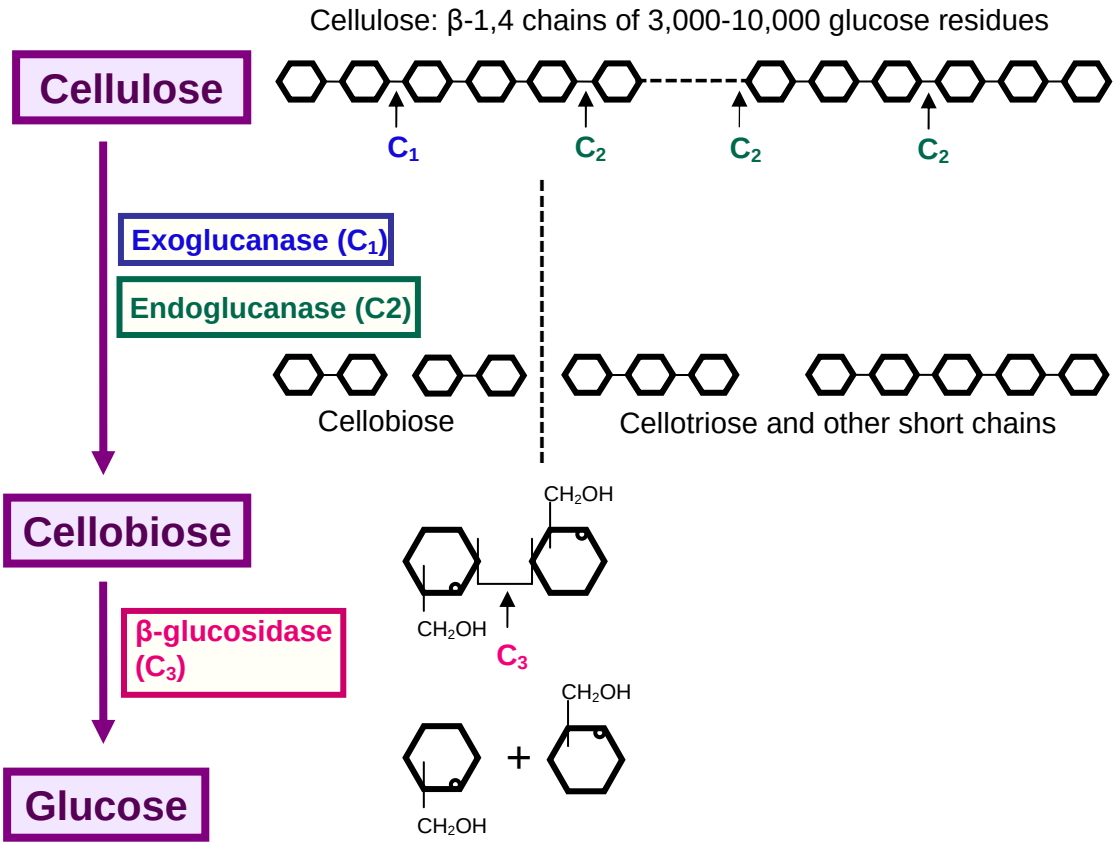


Figure 4 Enzymatic hydrolysis of cellulose (Deacon, 1997).

2.4 Amylase and cellulase

2.4.1 Source of amylase and cellulase from microorganisms

A wide variety of bacteria, fungi, yeast, and actinomycete are known to produce starch and cellulose degrading enzymes. Saprophytic fungi which are well known for their decomposition activity, possess an efficient hydrolytic system. Because they produce a pool of enzymes, including amylase, cellulase (cellobiohydrolase, endoglucanase), hemicellulase (xylanase), β-glucosidase, and

lignin-peroxidase. Some species of genus *Aspergillus, Trichoderma* are of special interest due to their enzymes produced are importance in biotechnological processes (Benoliel *et al*., 2005). Table1 shows list of enzymes produced by *Aspergillus* and *Trichoderma*.

Table 1 List of enzymes produced by *Aspergillus* and *Trichoderma*

Fungi	**Enzyme detected**										**References**
	Amylase	**Cellulase**	**DNAase**	**Xylanase**	**Lipase**	**Protease**	**Pectate lyase**	**Urease**	**Aminopeptidase**	**Phosphatase**	
Aspergillus (luchuensis)	+	+	+		+			+			Hankin and Anagnostakis, 1975
A. (fumigatus)	+		+		+		+			+	
Aspergillus sp.	+				+		+	+		+	
A. oryzae							+				Bindslev-Jensen, *et.al.*, 2006
A. niger	+	+		+	+	+					
Trichoderma viride	+			+		+					Hankin and Anagnostakis, 1975
T. reesei		+		+					+		Bindslev-Jensen, *et. al.*, 2006
Trichoderma longibrachiatum		+		+					+		
T. harzianum	+	+		+		+					De Azevedo, *et. al., 2000*

2.4.2 Industrial applications of amylase

Amylase was first industrial produced from wheat bran koji culture of *Aspergillus oryzae* by Dr. Jhokichi Takamine in 1894. Industrial production of dextrose powder and dextrose crystal from starch using α-amylase and glucoamylase began in 1959. Since then, amylases are being used for various purposes. Conversion of starch into sugar, syrup and dextrin becomes major part of starch processing industry (Marshall, 1975). The hydrolysates are used as carbon sources in fermentation as well as sources of sweetness in a range of manufactured food product and beverages. Hydrolysis of starch to products containing glucose, maltose, etc. is brought about by controlled degradation (Norman, 1978; Hurst, 1975; Slott and Madser, 1975). Some of the applications of amylase are :

- Drinking : The amylase is used to remove starch in beer, and clarify fruit juices (van der Maarel *et. al*., 2001).

- Desizing : In textile weaving, starch paste is applied. This gives strength to the textile and also prevents the loss of string by friction and cutting. It also generates softness to the surface of string. After weaving the starch on cloth has to be removed and usually α-amylase is used (Aiyer, 2005).

- Fermentation of starch to ethanol : Amylases are used in alcohol production and brewing industries.

2.4.3 Industrial applications of cellulase

Cellulase is an environmental friendly mean for utilization of cellulose which is one of the most abundant organic molecule on earth. The following is a list of processes that cellulases is used :

- Fuel ethanol production : Cellulase is used to saccharify cellulose in lignocellulosic biomass, such as agricultural and forestry residues to glucose. Then the glucose is converted to ethanol or other products (Dartmouth college, 2007).

- Textile : Cellulase has had the most impact on textile processing in recent years. Current commercial applications include "biostoning", "biopolishing" and as laundering "brightener" of cotton fabrics. (Hamlyn, 2000).

- Denim washing : Cellulase is added to desize denim in a washing machine to induce desired fading and softening by a combination of enzyme action and fabric/fabric friction, as well as machine-abrasion (Zeyer *et al*., 1994, Buchert and Heikinheimo, 1998).

An example of the cellulase applications used in textile industry are listed in Table 2.

Table 2 Applications of cellulase in textile industry

Type of fiber	Substrate	Applications	Commercial status	Ref.
Cotton	Cellulose	Biostoning	Commercial	Tyndall, 1990; Kochavi *et. al*., 1990
Lyocell, Viscose, Modal	Cellulose	Biofinishing, peach skin effect	Commercial	Kumar *et. al*., 1996; Kumar and Harnden, 1999
Wool	Cellulose impurities	Carbonisation	Research	Fornelli, 1992; Byrne and Rigby, 1995

2.5 *Trichoderma*

Trichoderma sp. belongs to the Deuteromycetes (Fungi imperfecti) which sexual cycle of reproduction has not yet been reported. It is a prototrophic eukaryote participating in the decay of organic waste, and is able to grow on an organic medium supplemented with only an organic carbon source.

It grows as long hyphae with true septa. Growth takes place at the apex with branch formation on the side. Reproduction is carried out via heavily branched conidiospores. Bottle-shaped phialides are produced individually or in clusters. Single-cell green conidia is associated in small terminal mucous lump. The surface of mycelium is cover by conidia after being exposed to light for a few seconds or when the mycelium is stressed by growth conditions. The biosynthesis of cellulase is induced by cellulose and some other inducers via catabolic repression (Montenecourt and Eveleigh, 1997).

Trichoderma cellulase also contains enzyme activity for hemicellulose hydrolysis such as xylanase, β-xylanase, glucomannanase, galactomannanase, acetylesterase, glucoronidase. Additionally several other activities such as 1,3-glucanase, protease, amylases, lactonase can be presented (Esterbauer *et. al*., 1991). Cellulase from *T. reesei* is the most commonly used cellulase (Gray *et. al*., 2006).

The *Trichoderma* genus, particularly *T. reesei* contains many cellulolytic species. Many carbon sources, either water soluble or water insoluble have been studied to determine the "best" inducer. Cellulose has usually been considered to be the best inducer for producing a well-balanced cellulase system. Cellulase production rate is slow when insoluble cellulosic substrate is used as carbon sources. The use of soluble carbon sources such as lactose, cellobiose and cellulose hydrolysate for cellulase production increases cellulase productivity. Lactose, soluble carbon sources, has been considered as the most attractive carbon source for its potential in cellulase production although it induces cellulase production in *T. reesei* to a less extent than cellulose.

2.6 Cassava waste hydrolysis

Mixture of pectinase and *Aspergillus niger* glucoamylase were successfully hydrolyze cassava waste at 37°C for 8 h (Poonparraj, 1992). Cassava waste contained 11% (w/v) of non-water soluble carbohydrate released 122.4 g/l of reducing sugar by hydrolysis with mixture-enzyme of cellulase and pectinase at 28°C for 1 h, then by α-amylase at 100°C for 2 h and finally glucoamylase at 60°C for 4 h (Srinorakutara, *et. al*., 2004). Saccharification of cassava starch contaminated with cellulolytic fiber was more efficiently after treatment with fungal cellulases (DeMenezes *et. al*., 1978). Soluble starch hydrolysate was reported as powerful inducer for *T. reesei* cellulase production (Chen and Wayman, 1992). Therefore, the aim of this study is to optimize saccharification of cassava waste contained 70% (w/w)

starch and 15% (w/w) cellulosic fiber by *T. reesei* TISTR 3080 which produced both cellulase and amylase, then glucose produced was further fermented to ethanol by *S. cerevisiae*.

CHAPTER III

MATERIALS AND METHODS

MATERIALS

3.1 Cassava waste

Cassava waste was obtained from Sanguan Wongse Industries Co., Ltd., Nakhon Ratchasima province in a frozen form (Figure 5).

3.2 Equipments

3.2.1 Analytical balance : Mettler Toledo, model AG 285, Switzerland

3.2.2 Autoclaves : Tomy, model SS- 325 and Hirayama, model HV-28, Japan

3.2.3 High Performance Liquid Chromatography (HPLC): Agilent Technology Ltd., model 1100 series, USA

3.2.4 Hot plate : E.G.O., model RK18715, Poland

3.2.5 Laminar flow : Lab service Ltd., clean model V6, Thailand

3.2.6 Platform shaker : New Brunswick Scientific, model innova 2300, USA

3.2.7 pH meter : Mettler Toledo, model SevenEasy, China

3.2.8 Precision balance : Mettler Toledo, model PB 3002, Switzerland

3.2.9 Refrigerated centrifugation : Sorvall, model Biofuge stratos, Germany (Rotor #3334, Heraeus)

3.2.10 Spectrophotometer : Spectronic Instruments, model Spectronic 20, USA

3.2.11 Water bath : Tolabo, model TW20, Germany.

3.2.12 Water bath shaker : Amerex Instrument Inc., model Gyromax 939XL, USA, and GFL, model 1086, Germany.

3.3 Chemicals

Chemicals	Company
Agar	Becton
Ammonium heptamolybdate tetrahydrate ($(NH_4)_6Mo_7O_{24}.4H_2O$)	Merck
Ammonium Sulphate ($(NH_4)_2SO_4$)	Merck
Bacto-peptone	Becton
Bovine serum albumin	Sigma
CarboxyMethylCellulose (CMC)	Sigma
Copper (II) sulfate ($CuSO_4.5H_2O$)	Merck
Disodium hydrogen arsenate ($Na_2HAsO_4.7H_2O$)	Merck
Dianisidine solution	Sigma
di-Sodium hydrogen phosphate dodecahydrate ($Na_2HPO_4.12H_2O$)	Merck
Folin phenol reagent	Sigma
Glucose ($C_6H_{12}O_6$)	Sigma
Hydrochloric acid (HCl)	Sigma
Magnesium sulfate heptahydrate ($MgSO_4.7H_2O$)	Merck
PGO enzymes	Sigma
Potassium dihydrogen phosphate (KH_2PO_4)	Merck
Potassium Sodium Tartate ($C_4H_4KNaO_6.4H_2O$)	Merck
Salicin	Sigma
Sodium carbonate (Na_2CO_3)	Merck
Sodium hydroxide (NaOH)	Merck
Sodium sulfate (Na_2SO_4)	Merck
Yeast extract	Difco

3.4 Microorganisms

3.4.1 *Trichoderma reesei* TISTR 3080

3.4.2 *Aspergillus niger* TISTR 3352

3.4.3 *Saccharomyces cerevisiae* TISTR 5596

3.5 Culture medium

3.5.1 Potato dextrose agar (PDA), Difco Laboratories, USA.

METHODS

3.6 Experiments

Flow diagram of all experiments was shown in Figure 6.

Figure 5 Cassava waste in a frozen form

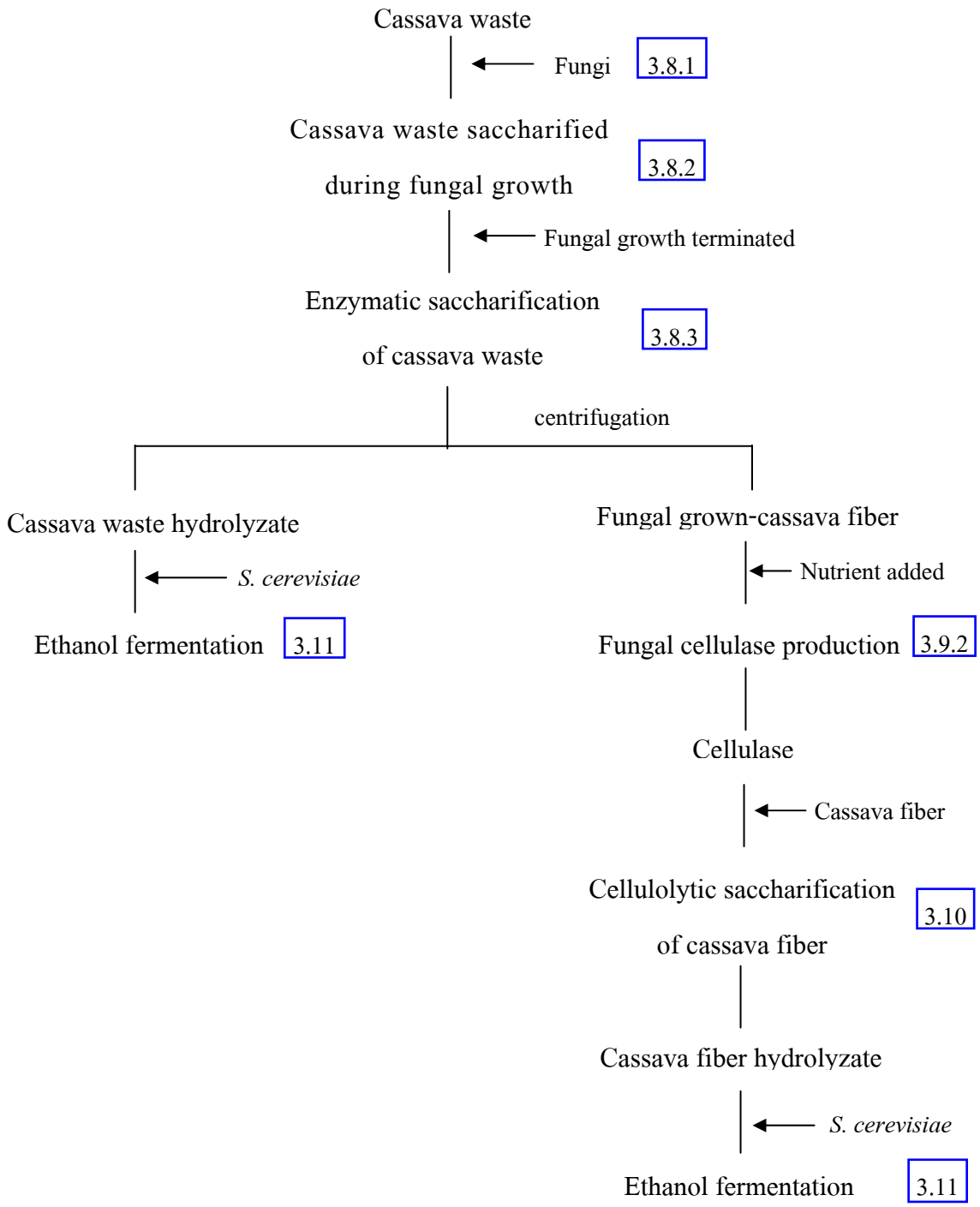


Figure 6 Flow diagram of an experiments

3.7 Microorganisms

3.7.1 Maintainance of microorganisms

Trichoderma reesei TISTR 3080, *Aspergillus niger* TISTR 3352 and *Saccharomyces cerevisiae* TISTR 5596 were obtained from Thailand Institute of Scientific and Technological Research. The cultures were long-term maintained in liquid nitrogen and also in freeze-dried form.

T. reesei and *A. niger* grown on potato dextrose agar (PDA) slant at 30°C for 7 days, and *S. cerevisiae* grown on yeast peptone dextrose (YPD) agar slants at 30°C for 2 days were kept at 4°C and subcultured every week.

3.7.2 Cultivation of microorganisms

3.7.2.1 *Trichoderma reesei* TISTR 3080 and *Aspergillus niger* TISTR 3352

T. reesei TISTR 3080 and *A. niger* TISTR 3352 were grown on potato dextrose agar (PDA) slant and incubated at 30°C for 7-10 days. Spores of the cultures were suspended in 0.1% (w/v) Tween80 and used as an inoculum at final concentration of 10^8 spores/ml.

3.7.2.2 *Saccharomyces cerevisiae* TISTR 5596

Single colony of *S. cerevisiae* TISTR 5596 grown on yeast peptone dextrose (YPD) agar at 30°C for 24 hours was inoculated into 50 ml YPD broth in 250 ml armed flask and incubated at 30°C (150 rpm) for 2 days. The culture transferred to the same medium at 1% (v/v) and incubated at the same above conditions was used as inoculum.

3.8 Cassava waste saccharification

3.8.1 Comparison of cassava waste saccharification by fungi

Trichoderma reesei TISTR 3080 at final concentration of (10^8 spores/ml) or *Aspergillus niger* TISTR 3352 (10^8 spores/ml), or *T. reesei* mixed with *A. niger* (10^8 spores/ml) was inoculated into 50 ml of 15% (wet w/v) cassava

waste containing 0.4% $(NH_4)_2SO_4$, pH 6.0 in 500 ml flask, and incubated at 35°C (150 rpm) for 3 days. Reducing sugar in supernatant obtained after centrifugation at 4°C, 11,500x g (20 min) was analysed by Somogyi-Nelson method (see 3.12.1). The fungi gave maximum reducing sugar was selected for cassava waste saccharification in latter experiments.

3.8.2 Effect of cassava waste saccharification conditions

The selected was used to saccharify cassava waste by inoculating into 15% (wet w/v) cassava waste containing 0.4% $(NH_4)_2SO_4$, pH 6.0, and incubated at 35°C (150 rpm) for 3 days. Optimization of the process was performed by varying cassava waste and $(NH_4)_2SO_4$ concentration, pH, incubation temperature, and incubation period. An optimal condition of prior experiment was used as the basis in the later experiments.

3.8.2.1 Effect of cassava waste concentration

The selected fungi was inoculated into cassava waste suspension containing 0.4% $(NH_4)_2SO_4$, pH 5.0 and incubated at 35°C (150 rpm) for 3 days. The concentration of cassava waste tested were 10, 15, 20, 30, 40 or 50% (wet w/v). Reducing sugar liberated in supernatants obtained after centrifugation at 4°C, 11,500x g for 20 min were analysed.

3.8.2.2 Effect of $(NH_4)_2SO_4$ concentration

The selected fungi was inoculated into cassava waste suspension at its optimal concentration (a result of 3.8.2.1) containing various $(NH_4)_2SO_4$ concentration (0.1, 0.2, 0.3, 0.4, 0.5 or 0.6%) and incubated at 35°C (150 rpm) for 3 days. Reducing sugar liberated in supernatants after centrifugation at 4°C, 11,500x g for 20 min were analysed.

3.8.2.3 Effect of pH

The selected fungi was inoculated into cassava waste suspension containing optimal concentration of cassava waste and $(NH_4)_2SO_4$ (a result of 3.8.2.2) at various pH (3.0, 4.0, 5.0, 6.0, 7.0 or 8.0) and incubated at 35°C (150 rpm) for

3 days. Reducing sugar liberated in supernatants obtained after centrifugation at 4°C, 11,500x g for 20 min were analysed.

3.8.2.4 Effect of temperature

The selected fungi was inoculated into cassava waste suspension containing optimal concentration of cassava waste, $(NH_4)_2SO_4$, optimal pH (a result of 3.8.2.3) and incubated at various temperature (30, 35, 40, 45, 50, 60°C) with shaking (150 rpm) for 3 days. Reducing sugar liberated in supernatants obtained after centrifugation at 4°C, 11,500x g for 20 min were analysed.

3.8.2.5 Effect of incubation period

The selected fungi was inoculated into cassava waste suspension containing optimal concentration of cassava waste, $(NH_4)_2SO_4$, optimal pH and incubated at optimal temperature (a result of 3.8.2.4) with shaking at 150 rpm for 5 days. Everyday, reducing sugar in culture supernatant collected by centrifugation at 4°C, 11,500x g (20 min) was analysed. Glucose content in the supernatant was analysed by PGO enzymes method (see 3.12.5).

3.8.2.6 Enzyme production profile

Supernatant obtained after centrifugation of the fungal-culture grown at the optimal cassava waste saccharification conditions (as mentioned in 3.8.2.5) was analysed for amylase, endoglucanase content and their temperature stability (see 3.12).

3.8.3 Enzymatic saccharification of cassava waste

After cassava waste was saccharified by the selected fungi at the optimal conditions (as mentioned in 3.8.2.5), the culture was rapidly heated to 50°C or above to terminate fungal growth, while cassava waste in the culture was continued saccharified by an enzyme produced. Effect of heating period and substrate (cassava waste) concentration were determined.

3.8.3.1 Effect of heating period

The culture was rapidly heat to temperature which fungi could not grow but the enzyme was still active. During heating period, water evaporation was protected to assure a concentration of reducing sugar liberated. Optimal heating period was the time when there was no increment of the reducing sugar liberated.

3.8.3.2 Effect of substrate concentration

Various amount of cassava waste was added to the culture (as mentioned in 3.8.2.5), then rapidly heated for a period of time (a result of 3.8.3.1). Reducing sugar liberated was analysed. Optimal substrate concentration was the concentration of cassava waste added which released maximum reducing sugar.

Glucose content of the culture supernatant at the optimal substrate concentration was analysed by PGO enzymes method (see 3.8.2.6.)

3.9 *Trichoderma reesei* TISTR 3080 cellulase production from cassava fiber

3.9.1 Cassava fiber

Cassava waste (15% wet w/v) was autoclaved at 121°C (15 lb/in^2) for 15 min, and cassava fiber separated by cloth-filtration was used as substrate.

T. reesei at final concentration of 10^8 spores/ml was inoculated into 15% (wet w/v) fiber suspension containing 0.4% $(NH_4)_2SO_4$, 0.4% KH_2PO_4, 0.05% $MgSO_4.7H_2O$, 0.05% yeast extract, pH 5.0 and incubated at 30°C (150 rpm) for 3 days. The culture was centrifuged at 4°C, 11,500x g (20 min), and supernatant was used as crude enzyme to quantify endoglucanase produced (see 3.12.2)

Optimization of the cellulase production was performed by varying concentration of fiber, nutrients added, pH, incubation temperature, and incubation period. An optimal condition of prior experiment was used as the basis in the later experiments.

3.9.1.1 Effect of fiber concentration

T. reesei was inoculated into various concentration of fiber suspension (5, 10, 15 or 20% (wet w/v)) containing nutrients as shown in 3.9.1, pH 5.0 and incubated at 30°C (150 rpm) for 3 days. Supernatant obtained after centrifugation at 4°C, 11,500x g (20 min) was used as crude enzyme to quantify endoglucanase produced.

3.9.1.2 Effect of nutrients concentration added

T. reesei was inoculated into the fiber suspension at its optimal concentration (a result of 3.9.1.1), pH 5.0 and incubated at the same above conditions. Each kind of nutrients added into the fiber suspension was step-wise varied.

Nutrients	**Concentration (% w/v)**
$(NH_4)_2SO_4$	0, 0.1, 0.2, 0.4 or 0.8
KH_2PO_4	0, 0.1, 0.2, 0.4 or 0.8
$MgSO_4.7H_2O$	0, 0.025, 0.05, or 1
yeast extract	0, 0.025, 0.05, or 1

Supernatant obtained after centrifugation at 4°C, 11,500x g (20 min) was used as crude enzyme to quantify endoglucanase produced.

3.9.1.3 Effect of pH

T. reesei was inoculated into the optimal concentration of fiber suspension containing optimal concentration of nutrients (a result of 3.9.1.2) at various pH (4.5, 5.0, 5.5, 6.0, 6.5 or 7.0), and incubated at the same above conditions. Supernatant obtained after centrifugation at 4°C, 11,500x g (20 min) was used as crude enzyme to quantify endoglucanase produced.

3.9.1.4 Effect of incubation temperature

T. reesei was inoculated into the optimized fiber suspension (a result of 3.9.1.3) and incubated at 30, 35, 40 or 45°C with shaking (150 rpm). Supernatants obtained after centrifugation at 4°C, 11,500x g (20 min) were used as crude enzyme to quantify endoglucanase produced.

3.9.1.5 *Trichoderma reesei* cellulase production profile

T. reesei was cultivated in the optimized fiber suspension and incubated at the optimized conditions (a result of 3.9.1.4) for 5 days. Everyday, endoglucanase and β-glucosidase produced in the culture supernatant was analysed.

3.9.2 Fungal grown-cassava fiber

After cassava waste was saccharified by the selected fungi, cassava waste hydrolyzate containing reducing sugar was separated from fiber by centrifugation at 4°C, 11,500x g (20 min). The fungal grown-fiber was used as substrate for *T. reesei* cellulase production. Two method of fungal grown-fiber medium were prepared.

3.9.2.1 Fungal grown-fiber was suspended in the optimized basal medium for cellulase production and steriled by autoclaving at 121°C, 15 lb/in^2, (15 min). *T. reesei* (10^8spores/ml) was inoculated and incubated at the optimal condition (a result of 3.9.1).

3.9.2.2 Fungal grown-fiber was added to steriled optimized basal medium for cellulase production, and further incubated at the optimal condition (a result of 3.9.1) without inoculation.

Endoglucanase produced in culture supernatant obtained after centrifugation at 4°C, 11,500x g (20 min) was analysed (see 3.12.2).

Endoglucanase produced by *T. reesei* grown in fiber medium containing optimal concentration of nutrients and incubated at the optimal conditions (a result of 3.9.1) was used as control.

3.9.3 Characterization of *Trichoderma reesei* TISTR 3080 cellulase

T. reesei cellulase produced by the method mentioned above was characterized.

3.9.3.1 Effect of temperature on endoglucanase and β-glucosidase activities

Endoglucanase and β-glucosidase activities were assay by the method described in 3.12.2 and 3.12.3, except reaction mixtures were incubated at various temperature (30, 35, 40, 45, 50, 55, 60, 65 or 70°C).

3.9.3.2 Effect of pH on endoglucanase and β-glucosidase activities

Endoglucanase and β-glucosidase activities were assay by the method described in 3.12.2 and 3.12.3, except pH of reaction mixtures were varied. Various kind of buffer were used to dissolve substrate and used as enzyme diluent. Citrate and phosphate buffer were used to accommodate pH 3.0, 4.0, 4.5, 5.0 and 5.0, 5.5, 6.0, 7.0 respectively.

3.9.3.3 Temperature stability

Temperature stability of the crude enzyme at 40, 45, 50, 55, 60, 65 or 70°C were analysed (see 3.12.4).

3.10 Fungal grown-fiber saccharification by *T. reesei* TISTR 3080 cellulase

T. reesei cellulase produced from fungal grown-fiber at the optimal condition (as described in 3.9.2) was used to saccharify fungal grown-fiber at the optimal pH and temperature for endoglucanase activity (a result of 3.9.3). Optimization of the process was performed by varying saccharification period and substrate concentration.

3.10.1 Effect of saccharification period

The fungal grown-fiber (15% wet w/v) was saccharified by *T. reesei* cellulase at its optimal pH and temperature (as described in 3.9.3). The reaction was centrifuged at 4°C, 11,500x g (20 min) and reducing sugar content in the supernatant was analysed every 3 hours.

3.10.2 Effect of substrate concentration

T. reesei cellulase was used to saccharify various concentration of fungal grown-fiber (15, 30, 45 or 60% w/v) at the same above conditions. The reaction was incubated to the hour when there was no reducing sugar liberated from 15% w/v fiber (a result of 3.10.1). Reducing sugar content in the supernatants obtained after centrifugation at 4°C, 11,500x g (20 min) was analysed.

3.11 Ethanol production

The cassava waste hydrolyzate obtained from cassava waste saccharified by selected fungi and the fiber hydrolyzate obtained from fiber saccharified by *T. reesei* cellulase were used as substrate for ethanol fermentation by *S. cerevisiae*.

Single colony of *S. cerevisiae* grown on YPD agar at 30°C for 24 h was inoculated into YPD broth (50 ml) in 250 ml armed flask and incubated at 30°C (200 rpm) for 2 days. The culture transferred to the same medium at 1% (v/v) and incubated at the same above conditions was used as inoculum. The inoculum was inoculated at 10% (v/v) into the above cassava waste hydrolyzate or fiber hydrolyzate supplemented with 0.2% $(NH_4)_2SO_4$ and incubated at 30°C without shaking for 72 h. Ethanol produced was analysed by HPLC (see 3.12.6).

3.12 Analytical procedure

3.12.1 Amylase activity assay (Bernfeld, 1955)

Soluble starch (1% w/v in 50 mM phosphate buffer pH 6.0) heated to boiling for 5 min was used as substrate. Reaction mixture consisted of substrate (0.5 ml) and the crude enzyme (0.5 ml) was incubated at 60°C for 30 min. The reaction was stopped by boiling in boiling water bath for 5 min. Reducing sugar liberated was quantified by Somogyi-Nelson method (Somogyi, 1952).

Sample (1 ml) mixed with Copper solution (1 ml) was boiled for 15 min and immediately cooled in ice-water. After addition of Nelson solution (1 ml), the reaction mixture was kept at room temperature for 30 min, diluted by 5 ml of distilled water, and measured an absorbance at 520 nm (A_{520nm}). Glucose was used as an authentic reducing sugar. Glucose concentration of the sample was determined from standard curve of glucose concentration versus $A_{520\ nm}$.

One unit of amylase activity was defined as an amount of enzyme liberated 1 μmole of glucose within 1 min under the assay conditions.

3.12.2 Endoglucanase activity assay (Ghose, 1987)

Carboxymethylcellulose (CMC) at 2% (w/v) in 50 mM phosphate buffer pH 6.0 was used as substrate. Reaction mixture consisted of substrate (0.5 ml) and the crude enzyme (0.5 ml) was incubated at 60°C for 30 min. The reaction was stopped by boiling in boiling water bath for 5 min. Reducing sugar liberated was quantified by Somogyi-Nelson method as above mentioned.

One unit of endoglucanase activity was defined as an amount of enzyme liberated 1 μmole of glucose within 1 min under the assay conditions.

3.12.3 β-glucosidase activity assay (Sternberg, 1977)

Salicin at 0.4% (w/v) in 50 mM phosphate buffer pH 6.0 was used as substrate. Reaction mixture consisted of substrate (0.5 ml) and the crude enzyme (0.5 ml) was incubated at 60°C for 30 min. The reaction was stopped by boiling in

boiling water bath for 5 min. Reducing sugar liberated was quantified by Somogyi-Nelson method as above mentioned.

One unit of β-glucosidase activity was defined as an amount of enzyme liberated 1 μmole of glucose within 1 min under the assay conditions.

3.12.4 Temperature stability

The crude enzyme was preheated at various temperature for 30 min. Residual amylase, endoglucanase and β-glucosidase activities of the preheated enzymes were analysed above mentioned. An activity of non-treated enzyme was set as 100%.

3.12.5 Analysis of glucose by PGO Enzymes method

The procedure to quantify glucose concentration using PGO enzymes (glucose oxidase/peroxidase) is based upon the following coupled enzymatic reactions.

$$C_6H_{12}O_6 + H_2O + O_2 \xrightarrow{\text{Glucose oxidase}} \text{Gluconic acid} + H_2O_2$$

$$H_2O_2 + \text{o-dianisidine (Colorless)} \xrightarrow{\text{Peroxidase}} \text{Oxidized O-Dianisidine (Brown)}$$

The intensity of the brown color measured at 450 nm is proportional to the original glucose concentration.

PGO enzymes reaction solution (5.0 ml) was mixed with sample (0.5 ml) and incubated at 37°C in dark for 30 min. Absorbance at 450 nm of the reaction was measured within 30 min. Distilled water and 0.05 mg/ml glucose were used as blank and standard, respectively.

3.12.6 Analysis of ethanol

Ethanol was analysed by High Performance Liquid Chromatography (HPLC); Agilent Technology Ltd., model 1100 series containing quatenary pump, online degassor, autoinjector, column thermostat, refractive index detector, and Chemstation software. HPLC conditions for ethanol analysis was as followed.

Column	:	Aminex column HPX- 87H (300x7.8 mm) (Bio-rad, Richmond, CA) and Cation H micro-guard cartridge.
Flow rate	:	0.6 ml/min
Mobile phase	:	0.1N H_2SO_4
Column temperature	:	55°C
Sample	:	50 µl

CHAPTER IV

RESULTS

4.1 Cassava waste

Chemical composition of cassava waste analysed by Kasetsart Agricultural and Agro-Industrial Product Improvement Institute using AOAC method was shown in Table 3. Major component was starch (69.90% w/w) followed by cellulosic fiber (10.61% w/w), dry weight basis.

Table 3 Chemical composition of cassava waste

Components	% (w/w)
Starch	69.90
Fiber	10.61
Protein	1.82
Fat	0.09
Ash	1.61
Moisture	78.16

4.2 Cassava waste saccharification

4.2.1 Comparison of cassava waste saccharification by fungi

Trichoderma reesei TISTR 3080 (10^8spores/ml) or *Aspergillus niger* TISTR 3352 (10^8spores/ml), or *T. reesei* mixed with *A. niger* (10^8 spores/ml) was grown in 15% (wet w/v) cassava waste suspension containing 0.4% $(NH_4)_2SO_4$, pH 6.0, and incubated at 35°C (150 rpm) for 3 days. *T. reesei* liberated maximum reducing sugar at 13.21 mg/ml (Figure 7). *T. reesei* was selected for cassava waste

saccharification in latter experiments. Figure 8 showed saccharification of cassava waste by *T. reesei* TISTR 3080.

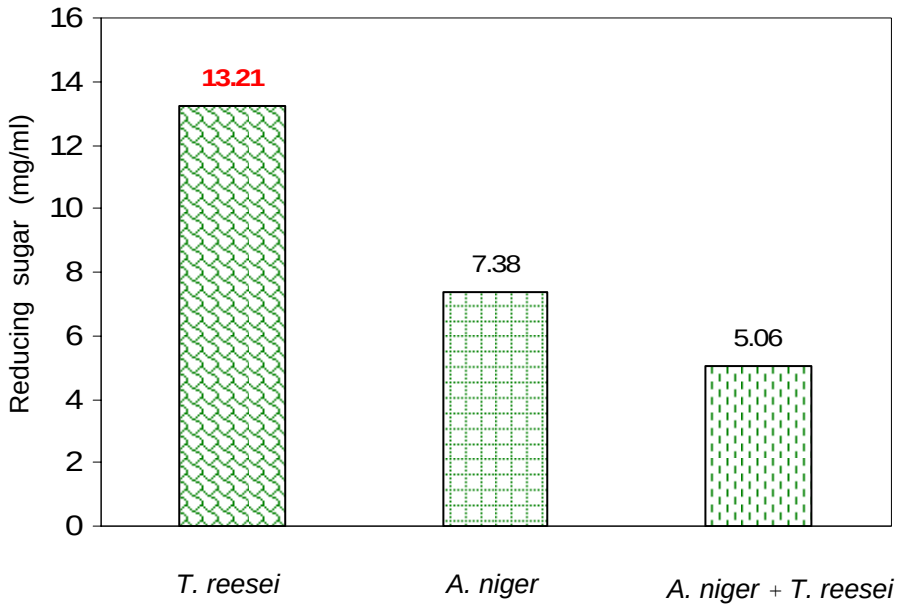


Figure 7 Comparison of cassava waste saccharification by *T. reesei* TISTR 3080, *A. niger* TISTR 3352 or their mixture.

Figure 8 Cassava waste saccharified by *T. reesei* TISTR 3080

4.2.2 Effect of cassava waste saccharification conditions

T. reesei was grown in 15% (wet w/v) cassava waste containing 0.4% $(NH_4)_2SO_4$, pH 5.0, and incubated at 35°C (150 rpm) for 3 days. Optimization of cassava waste saccharified by *T. reesei* was performed by varying cassava waste concentration, $(NH_4)_2SO_4$ concentration, pH, and growing temperature. Reducing sugar liberated in culture supernatant was analysed. The trial releasing maximum reducing sugar was selected as a condition of next experiments.

4.2.2.1 Effect of cassava waste concentration

As shown in Figure 9A, optimal concentration of cassava waste was 15% (wet w/v). Reducing sugar liberated was 13.22 mg/ml.

4.2.2.2 Effect of $(NH_4)_2SO_4$ concentration

Saccharification of 15% (wet w/v) cassava waste, an optimal concentration of $(NH_4)_2SO_4$ was 0.2%. Reducing sugar liberated was 13.63 mg/ml (Figure 9B).

4.2.2.3 Effect of pH

Optimal pH for *T. reesei* to saccharify 15% (wet w/v) cassava waste in the presence of 0.2% $(NH_4)_2SO_4$ was 6. At this pH, reducing sugar liberated was 13.70 mg/ml (Figure 9C).

4.2.2.4 Effect of temperature

Optimal temperature to saccharify 15% (wet w/v) cassava waste containing 0.2% $(NH_4)_2SO_4$, pH 6.0 by *T. reesei* was 35°C. Reducing sugar liberated at this temperature was 13.78 mg/ml (Figure 9D).

4.2.2.5 Effect of incubation time

As shown in Figure 9E, maximum reducing sugar (13.83 mg/ml) was liberated after 3 days of incubation.

Culture supernatant was also analysed for amylase activity, endoglucanase activity and their temperature stability in next experiments.

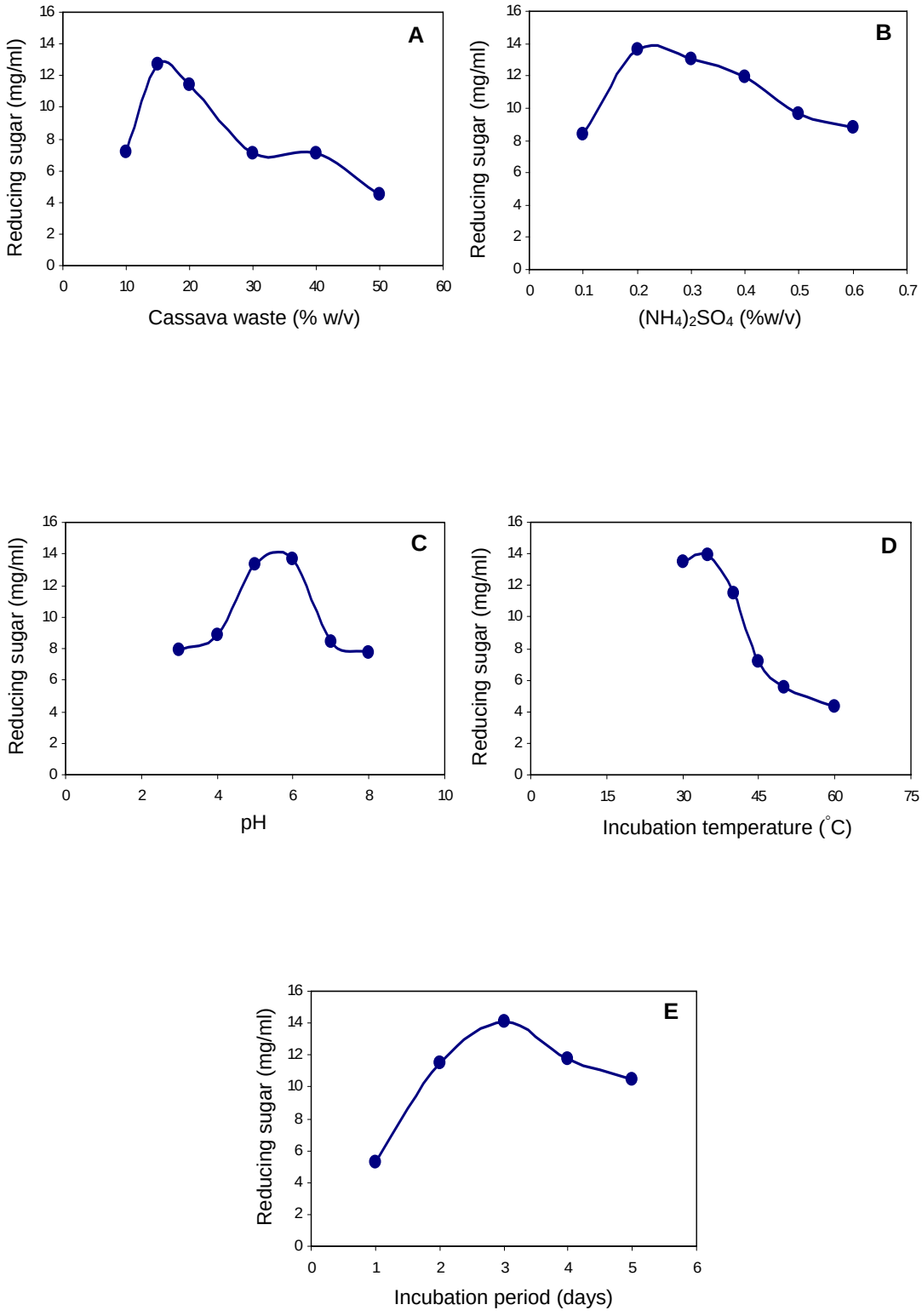


Figure 9 Optimal conditions for cassava waste saccharification by *T. reesei* TISTR 3080

4.2.2.6 Enzyme production profile

Culture supernatant of *T. reesei* grown at the optimal conditions for cassava waste saccharification (as described in 4.2.2.5) was analysed.

Amylase production

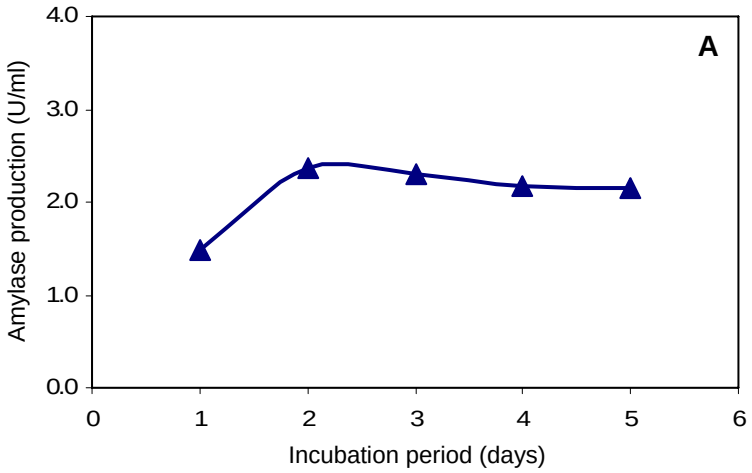
T. reesei grown in 15% (wet w/v) cassava waste containing 0.2% $(NH_4)_2SO_4$, pH 6.0 at 35°C (150 rpm) produced maximum amylase (2.37 U/ml) after 2 days of incubation. At day 3 when reducing sugar was maximum, amylase at 2.33 U/ml was produced (Figure 10A).

Endoglucanase production

Maximum endoglucanase (0.29 U/ml) was produced by *T. reesei* when grown at the optimal conditions for cassava waste saccharification (Figure 10B).

Temperature stability

After preheated the culture supernatant at the temperature which *T. reesei* could not grow (50, 60 or 65°C) for 30 min, the residual amylase and endoglucanase activities were analysed. Amylase and endoglucanase were stable up to 60°C, at this temperature 98.22% and 96.64% of activity remained, respectively (Figure 11).



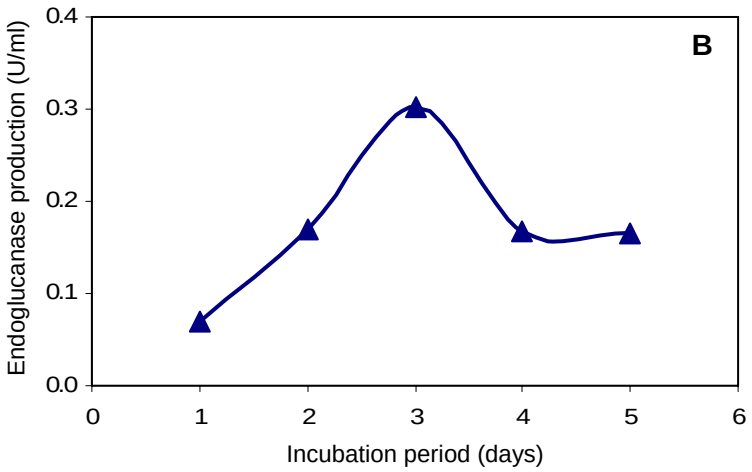
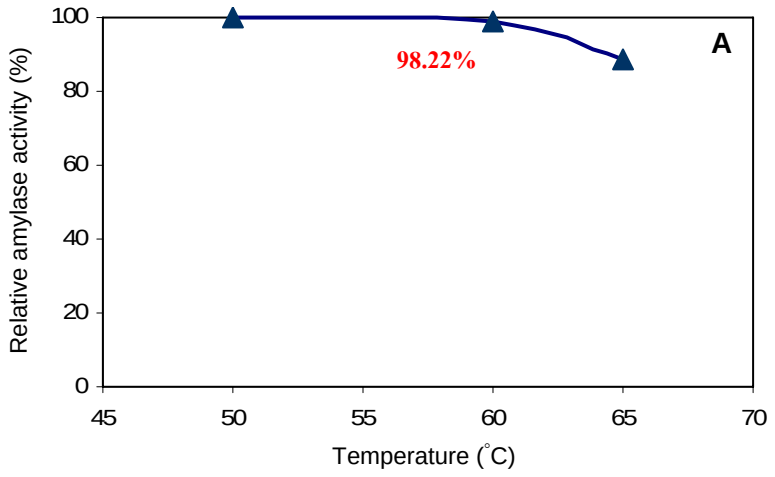


Figure 10 Enzyme production profile of amylase (A) and endoglucanase (B) produced by *T. reesei* grown at optimal conditions for cassava waste saccharification.



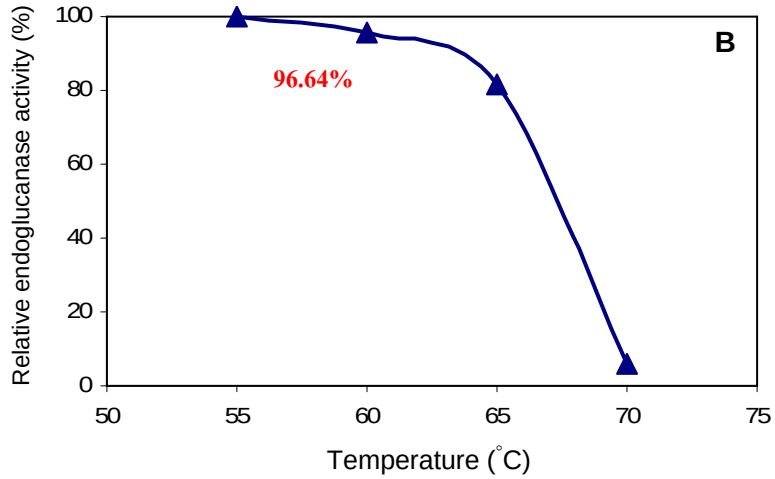


Figure 11 Temperature stability of amylase (A) and endoglucanase (B) produced by *T. reesei* grown in cassava waste.

4.2.3 Enzymatic saccharification of cassava waste

After reducing sugar liberated at the optimal conditions for cassava waste saccharification by *T. reesei* reach maximum, *T. reesei* growth was terminated by rapidly heated to 60°C then reducing sugar liberated by enzymatic hydrolysis was monitored.

4.2.3.1 Effect of heating period

When the culture rapidly heated up to 60°C, reducing sugar content increased. The reducing sugar content was stable at 14.79 mg/ml after heating for 6 h (Figure 12).

4.2.3.2 Effect of substrate concentration

Various concentration of cassava waste was added into the culture (containing amylase (2.33 U/ml) and endoglucanase (0.29 U/ml)), then rapidly heated up to 60°C for 6 h. Reducing sugar content was maximum (17.59 mg/ml) when 30% (wet w/v) of cassava waste was added (Figure 13). Glucose content in the cassava waste hydrolysate at this condition was 13.62 mg/ml. Both D-glucose and L-glucose were detected.

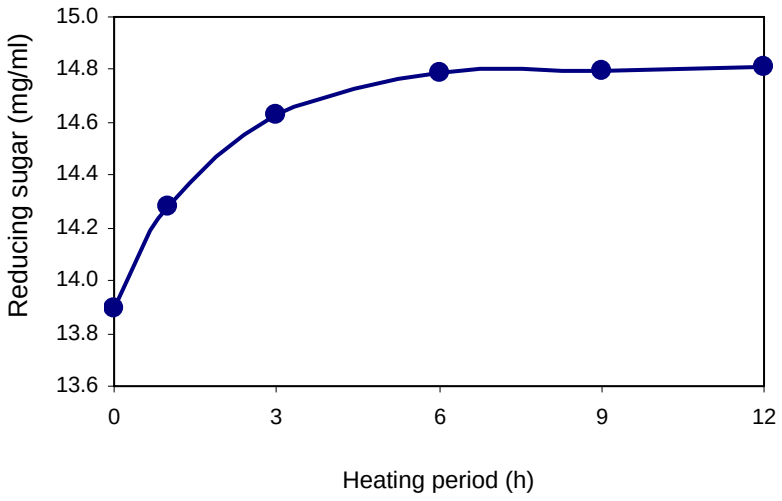


Figure 12 Effect of heating time at 60°C on enzymatic saccharification of cassava waste

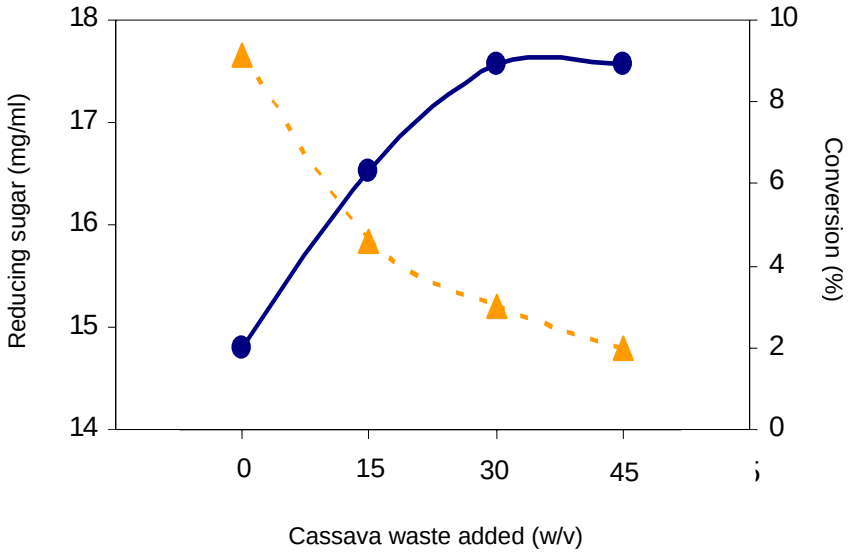


Figure 13 Effect of cassava waste concentration on enzymatic saccharification at 60°C for 6 h. Reducing sugar liberated (●), conversion of cassava waste to reducing sugar (▲).

4.3 *Trichoderma reesei* TISTR 3080 cellulase production from cassava fiber

4.3.1 *T. reesei* cellulase production from cassava fiber

Cassava fiber separated from cassava waste by autoclaving for 15 min before removing of starch solution by cloth-filtration was used as substrate for cellulase production. *T. reesei* was inoculated into 15% (wet w/v) fiber containing 0.4% $(NH_4)_2SO_4$, 0.4% KH_2PO_4, 0.05% $MgSO_4.7H_2O$, 0.05% yeast extract, pH 5.0 and incubated at 30°C (150 rpm) for 5 days. Endoglucanase produced in culture supernatant collected by centrifugation at 4°C, 11,500x g (20 min) was analysed.

4.3.1.1 Effect of fiber concentration

Concentration of fiber was varied. Maximum endoglucanase (0.31 U/ml) was produced in 15% (wet w/v) of fiber (Figure 14A).

4.3.1.2 Effect of nutrients concentration added

Concentration of $(NH_4)_2SO_4$, KH_2PO_4, $MgSO_4.7H_2O$ or yeast extract added to 15% (wet w/v) fiber was step-wise varied. An optimal concentration of prior experiment was used in latter experiments. After 3 days of incubation, maximum endoglucanase was produced in 0.2% $(NH_4)_2SO_4$ (Figure 14B), 0.4% KH_2PO_4 (Figure 14C), 0.025% $MgSO_4.7H_2O$ (Figure 14D), and 0.05% yeast extract (Figure 14E).

4.3.1.3 Effect of pH

pH of the optimized culture medium was varied. Maximum endoglucanase (0.34 U/ml) was produced in culture medium at pH 6 .0 (Figure 14F).

4.3.1.4 Effect of incubation temperature

Incubation temperature was varied. Maximum endoglucanase (0.35 U/ml) was produced at 35 $^{\circ}$C (Figure 14G).

4.3.1.5 *Trichoderna reesei* cellulase production profile

T. reesei produced maximum endoglucanase (0.36 U/ml) after 3 days of incubation as shown in Figure 14H.

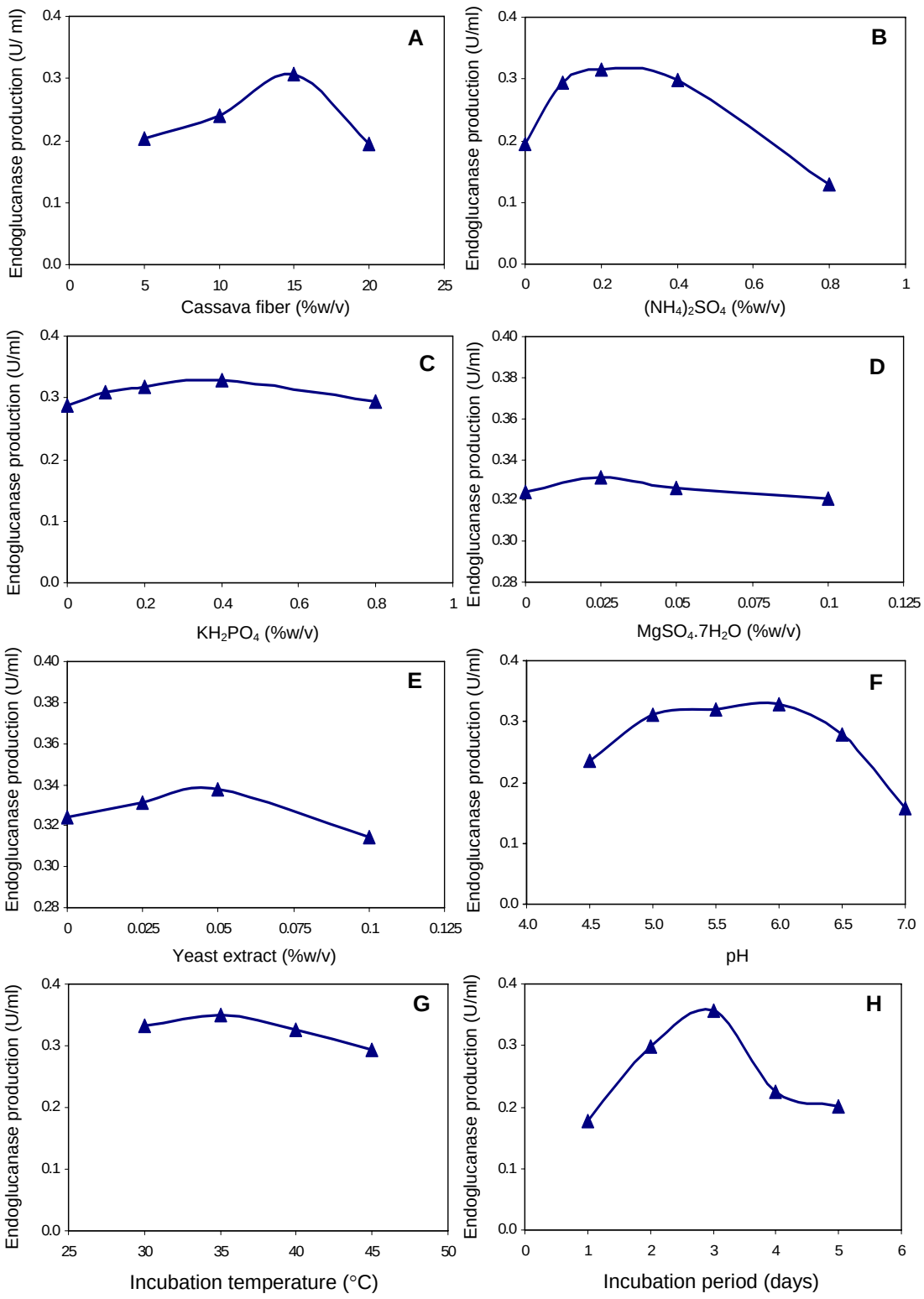


Figure 14 Optimal conditions for *T. reesei* cellulase production from cassava fiber, optimal concentration of cassava fiber (A), $(NH_4)_2SO_4$ (B), KH_2PO_4 (C), $MgSO_4.7H_2O$ (D), yeast extract (E), pH (F), incubation temperature (G), and cellulase production profile (H).

4.3.2 *T. reesei* cellulase production from *T. reesei* grown-cassava fiber

T. reesei grown in cassava waste (as described in 4.2.2) was cloth-filtered and centrifuged. Cassava waste hydrolysate (supernatant) was separated from *T. reesei* grown-cassava fiber. The *T. reesei* grown-cassava fiber was used as carbon source for cellulase production by 2 methods as described in 3.9.2.

T. reesei grown-fiber supplemented with nutrients at their optimal concentrations (a result of 4.3.1) was used as culture medium for *T. reesei* cellulase production. The culture was steriled by autoclaving then *T. reesei* was inoculated. Or, *T. reesei* grown-fiber was added to sterile nutrient solution without *T. reesei* inoculation.

As shown in Figure 15, higher endoglucanase (0.29 U/ml) was produced in *T. reesei* grown-fiber added to sterile nutrient solution without inoculation. However, endoglucanase produced was much lower than control, which cassava fiber was used (0.35 U/ml).

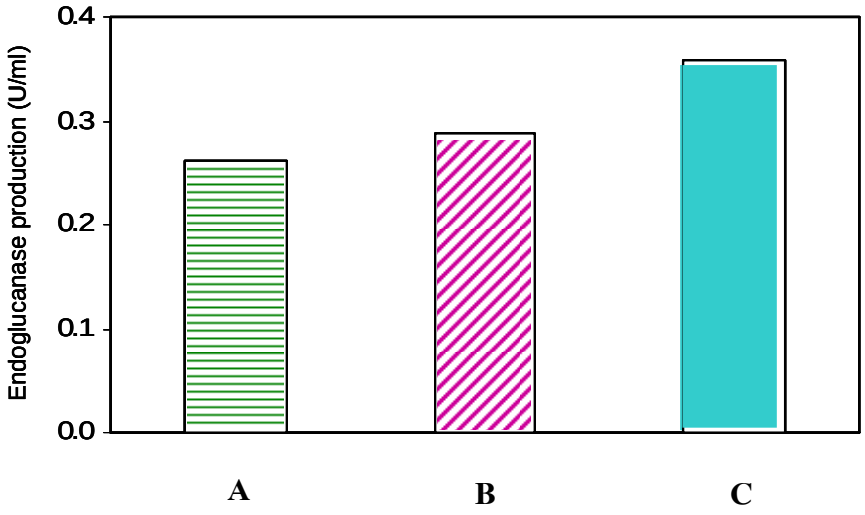


Figure 15 Effect of medium preparation method on *T. reesei* cellulase production. *T. reesei* grown-fiber or cassava fiber was used as carbon source. *T. reesei* grown-fiber was autoclaved before use and *T. reesei* was inoculated (A), *T. reesei* grown-fiber was added to sterile nutrient solution and further incubation without inoculation (B), fiber was autoclaved and *T. reesei* was inoculated (C).

To increase endoglucanase production in *T. reesei* grown-fiber, $(NH_4)_2SO_4$ concentration in the sterile nutrient solution added was varied. As shown in Figure 16, endoglucanase produced was almost the same as control when 0.3% $(NH_4)_2SO_4$ was added.

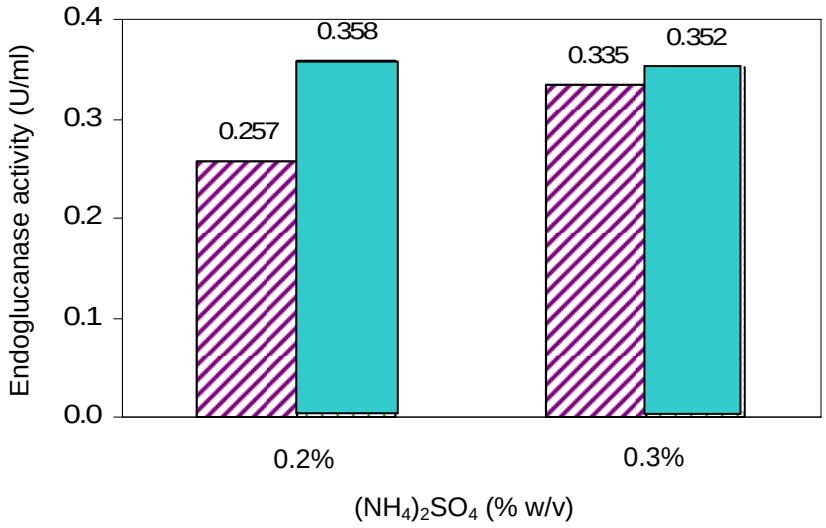


Figure 16 Effect of $(NH_4)_2SO_4$ concentration added to *T. reesei* grown-fiber on endoglucanase production. *T. reesei* grown-fiber (▨), control: cassava fiber (■).

4.4 Characterization of *Trichoderma reesei* TISTR 3080 cellulase

4.4.1 Effect of temperature on endoglucanase and β-glucosidase activities

As shown in Figure 17, optimal temperature of endoglucanase and β-glucosidase activities were 60°C and 55°C, respectively.

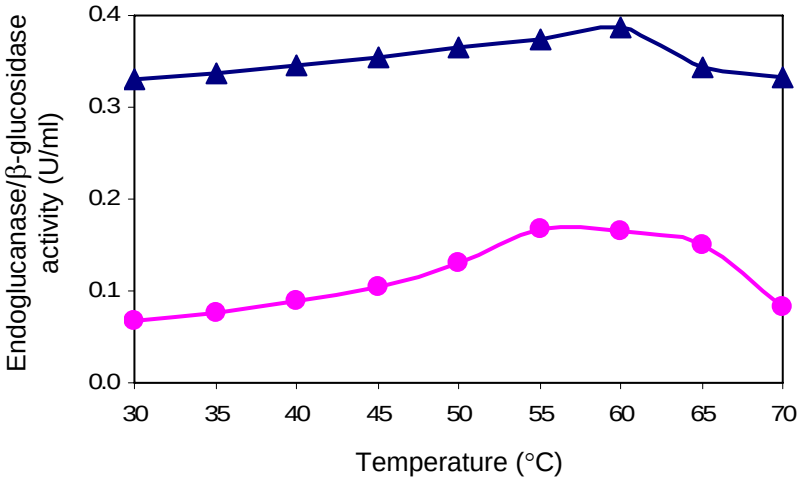


Figure 17 Optimal temperature of *T. reesei* TISTR 3080 endoglucanase activity (▲), β-glucosidase activity (●).

4.4.2 Effect of pH on endoglucanase and β-glucosidase activities

As shown in Figure 18, optimal pH for endoglucanase and β-glucosidase activities were 6.0.

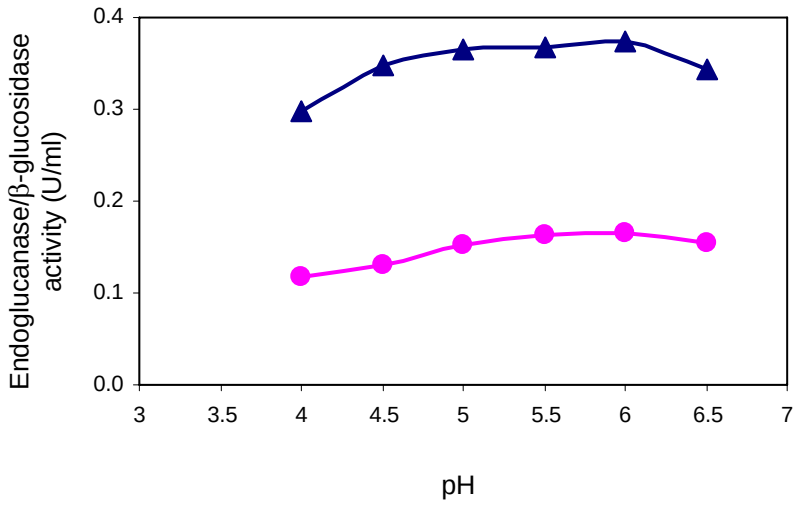


Figure 18 Optimal pH of *T. reesei* TISTR 3080 endoglucanase activity (▲), β-glucosidase activity (●).

4.4.3 Temperature stability

As shown in Figure 19, *T. reesei* TISTR 3080 endoglucanase and β-glucosidase were stable up to 60°C. After preheated at 60°C for 30 min, 96.62% and 98% of endoglucanase and β-glucosidase activities remained, respectively.

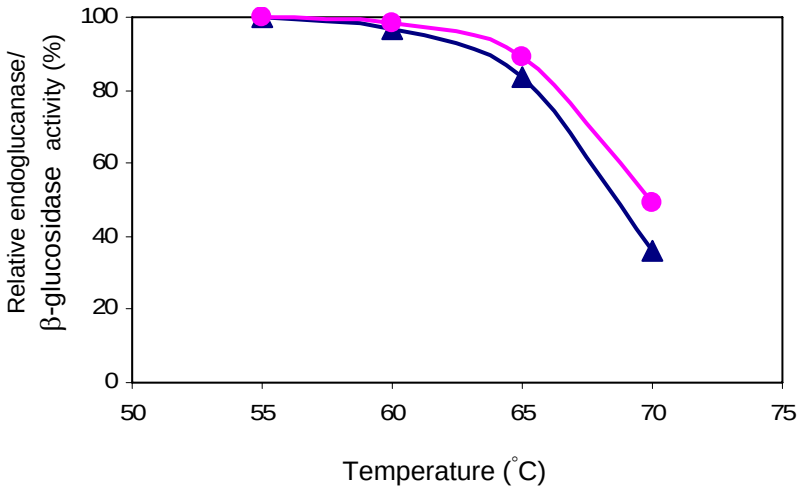


Figure 19 Temperature stability of *T. reesei* TISTR 3080 endoglucanase activity (▲), β-glucosidase activity (●).

4.5 *T. reesei* grown-fiber saccharification by *T. reesei* TISTR 3080 cellulase

4.5.1 Effect of saccharification period

T. reesei cellulase was used to saccharify *T. reesei* grown-fiber at 60°C, pH 6.0 which were optimal conditions of *T. reesei* endoglucanase activity. Reducing sugar liberated was stable after 6 h of incubation at 60°C (Figure 20).

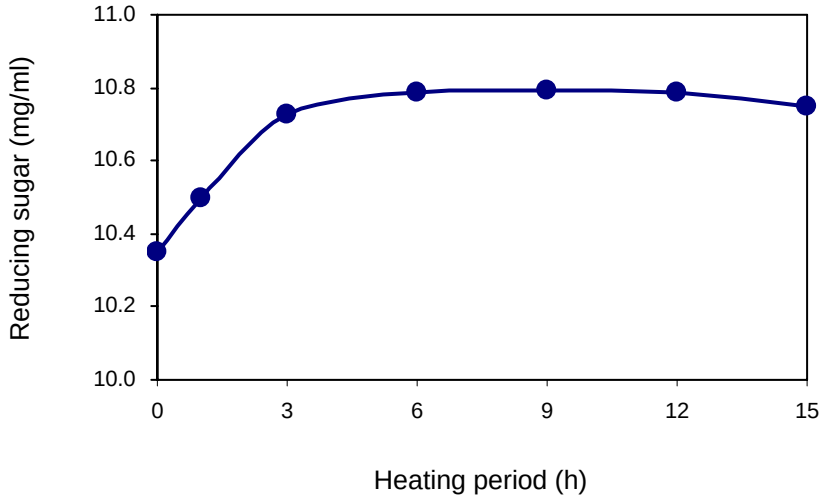


Figure 20 Effect of heating time on enzymatic saccharification of cassava fiber.

4.5.2 Effect of substrate concentration

Addition of *T. reesei* grown-fiber into *T. reesei* cellulase (0.39 U/ml) to various final concentration and incubation at 60°C for 6 h. Maximum reducing sugar (54.60 mg/ml) liberated at final concentration of 90% (wet w/v) (Figure 21). Glucose content in the fiber hydrolysate at this condition was 26.86 mg/ml.

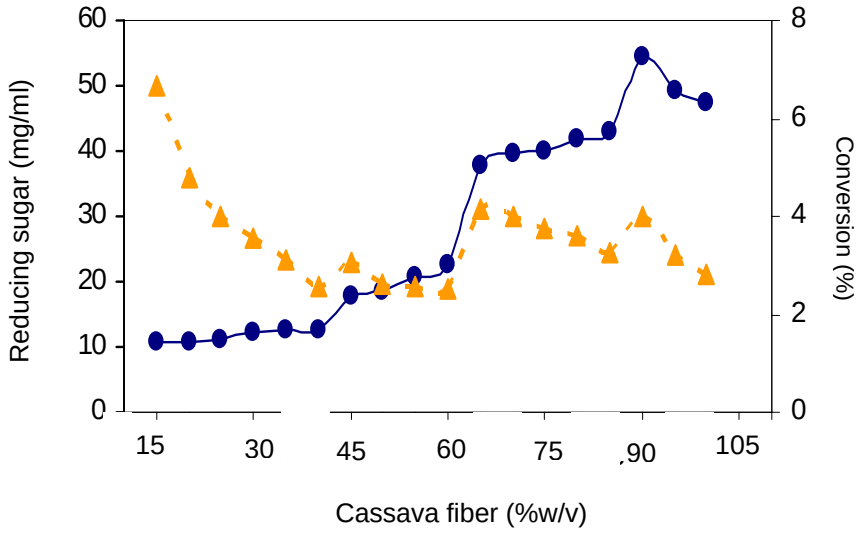


Figure 21 Effect of *T. reesei* grown-fiber concentration on enzymatic saccharification

4.6 Ethanol production

The cassava waste hydrolysate containing glucose (13.62 mg/ml) (as mentioned in 4.2.3) and the cassava fiber hydrolysate (as mentioned in 4.5.2) containing glucose (26.86 mg/ml) were fermented to ethanol by using *S. cerevisiae*.

S. cerevisiae TISTR 5596 (10% v/v) was inoculated into the cassava waste hydrolyzate and *T. reesei* grown-fiber hydrolyzate, and incubated at 30°C without shaking for 72 h.

Ethanol (1.91% w/w of cassava waste and 1.84% w/w of cassava fiber) were obtained from the cassava waste hydrolysate and the *T. reesei* grown-fiber hydrolysate, respectively.

CHAPTER V

CONCLUSIONS AND DISCUSSIONS

Comparison of cassava waste (15% wet w/v) saccharified by *T. reesei* TISTR 3080, *A. niger* TISTR 3352, or *T. reesei* TISTR 3080 and *A. niger* TISTR 3352 in the presence of 0.2% $(NH_4)_2SO_4$, pH 6.0, at 35°C (150 rpm) for 3 days, it was found that *T. reesei* liberated maximum reducing sugar at 13.21 mg/ml. Since *T. reesei* TISTR 3080 produced both amylase (2.34 U/ml) and endoglucanase (0.29 U/ml) in the saccharification conditions as shown in Figure 8. This result agreed well with Demenezes *et.al.* (1978) who reported that cassava starch contaminated with cellulotic fiber was more susceptible to amylolytic saccharified after fungal cellulase pretreatment. Saccharification by *T. reesei* was optimized by varying cassava waste and $(NH_4)_2SO_4$ concentration, pH, incubation temperature and incubation period. As shown in Figure 7, optimal conditions were 15% (wet w/v) cassava waste, 0.2% $(NH_4)_2SO_4$, pH 6.0 at 35°C (150 rpm) for 3 days.

Both amylase and endoglucanase produced by *T. reesei* were stable up to 60°C. Preheat the enzymes at this temperature for 30 min, 98.22% and 96.64% of the activity remained (Figure 9). To increase reducing sugar liberated by the above enzymes activities, reducing sugar assimilation for *T. reesei* growth was inhibited by rapidly heating up to 60°C. Reducing sugar liberated was increased from 13.83 mg/ml to 14.79 mg/ml after 6 h of incubation (Figure 10). After 6 h, reducing sugar liberated was constant. It was a responsive phenomena of substrate limitation. Since addition of cassava waste into the reaction resulted in an increase of reducing sugar to 17.59 mg/ml (Figure 11), and this amount of reducing sugar was 13.62 mg/ml of glucose. Cassava waste was saccharified to glucose at 2.42% w/w.

Cassava waste reaction mixture was centrifuged at 4°C, 11,500x g (20 min) to separate cassava waste hydrolyzate (13.62 mg/ml glucose) from cassava fiber.

Fermentation of the cassava waste hydrolyzate by *S. cerevisiae* for 72 h revealed ethanol at 3.13% w/w of glucose.

Attempt to use cassava fiber contaminated by *T. reesei* as carbon source for *T. reesei* cellulase production was performed. Firstly, cellulase of *T. reesei* was produced from cassava fiber. The cassava fiber was separated from cassava waste (15% wet w/v) which was autoclaved at 121°C, 15 lb/in^2 for 15 min. At the optimal conditions for *T. reesei* cellulase production : 15% (wet w/v) cassava fiber, 0.2% $(NH_4)_2SO_4$, 0.4% KH_2PO_4, 0.025% $MgSO_4.7H_2O$, 0.05% yeast extract, pH 6.0, incubated at 35°C (150 rpm) for 3 days : endoglucanase (0.35 U/ml) was produced (Figure 13). Second, *T. reesei* grown-cassava fiber was used as substrate for the cellulase production. It was found that addition of sterile nutrient solution at its optimal concentration as shown above to the *T. reesei* grown-cassava fiber, then further incubated at the same above conditions without inoculation gave higher reducing sugar than decontamination of the cassava fiber by autoclaving, adding nutrient solution, autoclaving again, then inoculating and incubating. This might be an effect of inhibitors released from heat treatment process of *T. reesei* metabolites. However, *T. reesei* grown-cassava fiber gave lower endoglucanase than cassava fiber. This effect was overcome by increasing of $(NH_4)_2SO_4$ added (Figure 15). *T. reesei* consumed both carbon and nitrogen source for growth, and NH_4 was depleted in *T. reesei* grown-cassava fiber.

T. reesei endoglucanase and β-glucosidase produced was characterized. Optimal temperature was 60 and 50°C, optimal pH 6.0, respectively, and they were stable up to 60°C for 30 min (Figure 16, 17, 18).

Then cellulase produced was used to saccharified *T. reesei* grown-cassava fiber at 60°C, pH 6.0. The fiber (15% wet w/v) saccharified by endoglucanase (0.34 U/ml) was saturated after 6 h. of incubation. Reducing sugar produced was 10.79 mg/ml. Increase of fiber in the reaction mixture and incubated at 60°C for 6 h, the reducing sugar increased from 10.79 mg/ml to 54.60 mg/ml when final concentration of fiber

was 90% (wet w/v). This cassava fiber hydrolyzate contained 26.86 mg/ml of glucose. The fiber was saccharified to glucose at 2.09% w/w of fiber. The cassava fiber hydrolysate was separated and fermented to ethanol by *S. cerevisiae* for 72 h. Ethanol 1.48% (w/w) of fiber or 3.09% (w/w) of glucose was produced.

As shown in Figure 20, reducing sugar obtained after addition of 30% (w/v) cassava waste and after increase of fiber concentration in saccharification reaction to 90% (w/v) increased, but conversion of glucose of cassava waste and cassava fiber to glucose was significantly reduced.

Saccharification efficiency of cassava waste or fiber to glucose will be improved, if only 15% w/w was implemented.

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APPENDICES

APPENDIX A

Culture media

1. Potato Dextrose Agar (PDA)

PDA powder (Difco)	39	g
Distilled water	1,000	ml

2. Yeast Peptone Dextrose (YPD) medium

Yeast extract	10	g
Bacto peptone	20	g
Glucose	20	g
Agar	18	g

Adjust pH to 5.0

APPENDIX B

Reagents and Buffers

1. Determination of reducing sugar

The reducing sugar was measured by the method of Somogyi (1952) using glucose as authentic reducing sugar.

1.1 Somogyi-Nelson Reagent

A. Alkaline Copper Reagents :

- Potassium sodium tartate (Rochelle salts) 40 g in 300 ml distilled water
- Disodium hydrogen phosphate dodecahydrate 71 g in 300 ml distilled water
- 10% Copper (II) sulfate 80 ml

  (8 g Copper (II) sulfate in 80 ml distilled water)
- 1N Sodium hydroxide 100 ml

  (4 g Sodium hydroxide in 100 ml distilled water)
- Sodium sulfate 180 g

Dissolve the solution above and make up volume to 1000 ml.

B. Nelson Reagent

- Ammonium molybdate 53.2 g in 500 ml distilled water
- Sulfuric acid (conc.) 21 ml

  Sulfuric acid (conc.) is added into the ammonium molybdate.
- Sodium arsenate 6 g in 50 ml distilled water

Dissolve the solution above and make up volume to 1000 ml.

1.2 Procedure

- Put proper dilution of sample 1 ml in a test tube
- Add Alkaline Copper solution 1 ml and boil in boiling water for 15 minutes. Immediately cool in ice water.
- After addition of 1 ml Nelson solution, incubate at room temperature for 30 minutes and diluted by 5 ml of distilled water.

- Absorbance (OD) of samples were measured at 520 nm. Concentration of the samples were compared to the standard curve for determination of values. Distilled water was used instead of sample as a blank.

1.3 Preparation of standard curve of glucose

Glucose standard solutions (1 mg/ml) were prepared in distilled water. Standards of 0, 20, 40, 60, 80, 100, 120, 150, 180 and 200 µg/ml were prepared from the glucose solution. The reactions were carried out by the same procedure as described previously. Absorbances were plotted against concentrations of standards.

1.4 Calculation of reducing sugar

Formula Reducing sugar (g/l) = $\frac{\text{O.D.}_{520} \text{ x dilution}}{\text{Slope}}$

2. 0.05 M Citrate buffer pH 5.0

Citric acid monohydrate	10.51	g/l
Trisodium citrate dihydrate	14.71	g/l

0.05 M citric acid (35 ml) and 0.05 M trisodium citrate (65 ml) mixed. Adjust pH to 5.0.

3. 0.05 M Citrate buffer pH 6.0

Citric acid monohydrate	10.51	g/l
Trisodium citrate dihydrate	14.71	g/l

0.05 M citric acid (11.5 ml) and 0.05 M trisodium citrate (88.5 ml) mixed. Adjust pH to 6.0.

4. 0.05 M Phosphate buffer pH 6.0

di-Sodium hydrogen phosphate	17.91	g/l
Sodium dihydrogen phosphate	7.803	g/l

0.05 M di-Sodium hydrogen phosphate (6.15 ml) and 0.05 M Sodium dihydrogen phosphate (43.85 ml) mixed. Adjust pH to 6.0.

5. 0.1% Tween 80

Tween 80	1	g
Distilled water	1000	ml

The solution was steriled by autoclaving for 15 minutes at 121°C, 15 lb/inc^2.

6. 2% Carboxymethylcellulose (CMC)

CMC	2	g
0.05 M Citrate buffer	100	ml

7. 0.4% Salicin [$C_{13}H_{18}O_7$]

Salicin	0.4	g
0.05 M Citrate buffer	100	ml

8. 1% Starch

Starch soluble	1	g
Distilled water	100	ml

Dissolve the solution above by boiling on hot plate until clearing.

9. Endoglucanase activity assay (Ghose, 1987)

- Proper dilution of enzyme sample was added to each tube containing 2% CMC (0.5 ml) and stirred to mix.

- Tubes were incubated at 60°C for 30 minutes, then stopped the reactions by boiling in boiling water for 5 minutes

- Reducing sugar liberated was quantified by Somogyi-Nelson method as described previously.

10. β-glucosidase activity assay (Sternberg, 1977)

- Proper dilution of enzyme sample was added to each tube containing 0.4% salicin (0.5 ml) and stirred to mix.

- Tubes were incubated at 60°C for 30 minutes, then stopped the reactions by boiling in boiling water for 5 minutes

- Reducing sugar liberated was quantified by Somogyi-Nelson method as described previously.

11. Calculation of enzyme unit

One unit of amylase, endoglucanase or β-glucosidase activity was defined as an amount of enzyme liberated 1 μmole of glucose within 1 min under the assay conditions :

1 unit of enzyme = 1 μmole of substrate was degraded within 1 min

= 1 μmole of glucose was released within 1 min

= 0.180 mg of glucose was released within 1 min

Amylase, endoglucanase and β-glucosidase activities **:**

0.180 mg Glucose was released within 1 min = 1 unit

1.000 mg Glucose was released within 30 min = $\frac{1}{0.180 \times 30}$ unit

= 0.185 unit

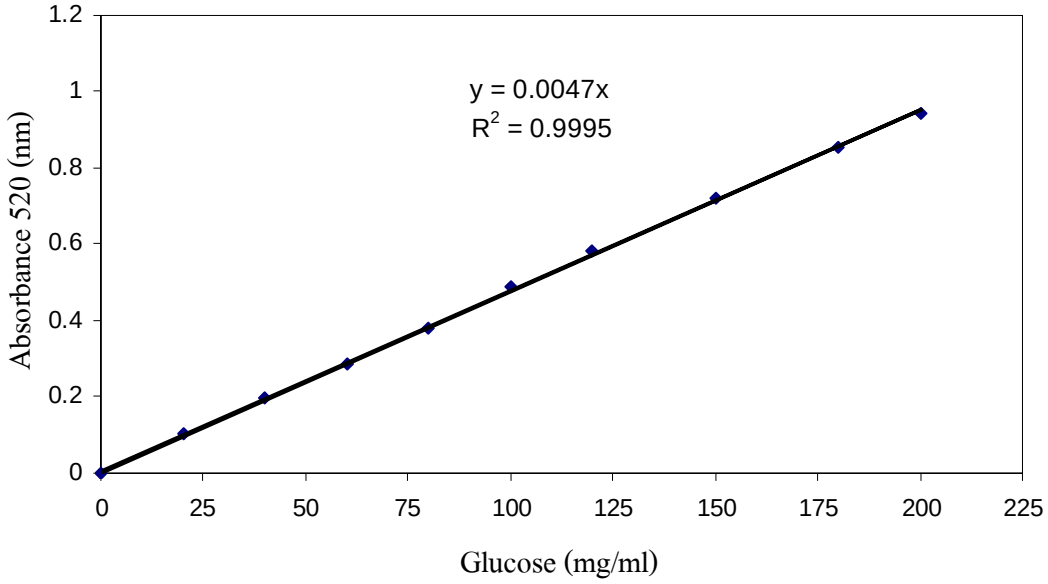
Glucose released X mg within 30 min = $\frac{(X) \times 0.185}{0.5}$ unit

or = $\frac{\text{mg Glucose} \times 0.185}{\text{ml Enzyme}}$ unit/ml

APPENDIX C

Standard curve of glucose

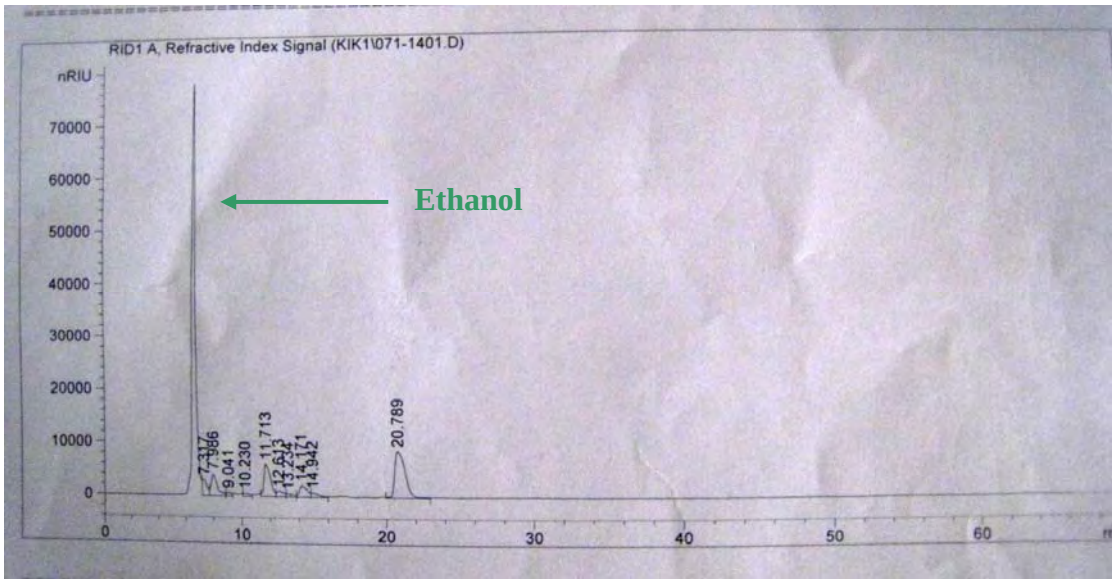
1. Standard curve of glucose



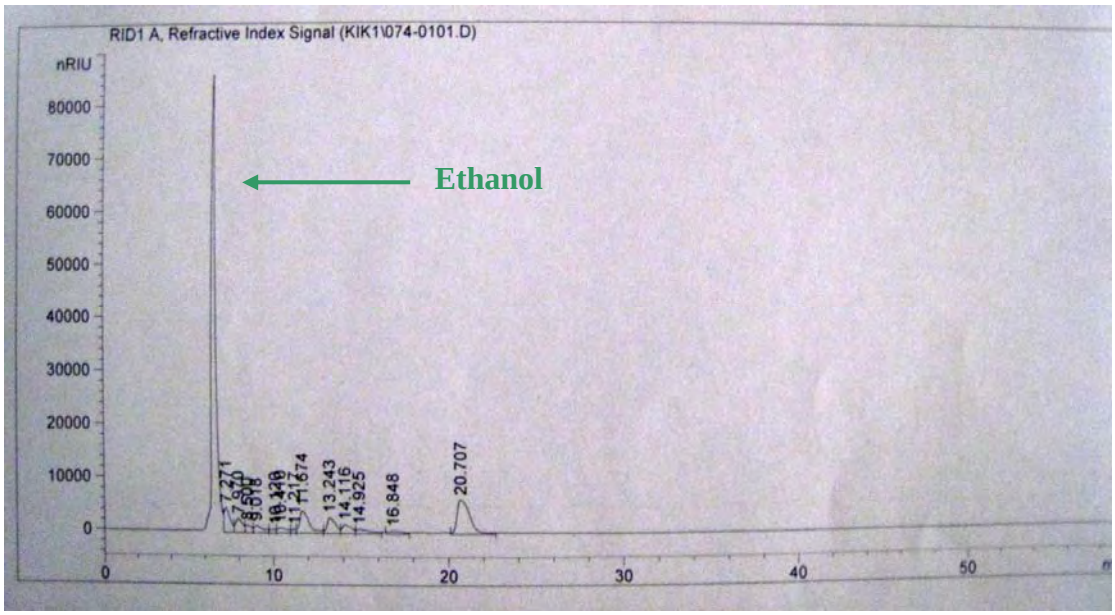
APPENDIX D

Chromatogram

1. HPLC chromatogram of ethanol from cassava waste (45% wet w/v)



2. HPLC chromatogram of ethanol from cassava fiber (90% wet w/v)



BIOGRAPHY

Miss Jutarat Kesornsit was born in September 13, 1981 in Surat Thani Province, Thailand. She has graduated from Department of Microbiology, Faculty of Science, Prince of Songkla University, Thailand with B.Sc. degree in Microbiology since 2003.