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นางสาววรพรรณ พิมพ์เอี่ยม

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UTILIZATION OF CASSAVA FIBER FOR CELLULASE AND ETHANOL PRODUCTION

Miss Woraphan Pim-iam

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Thesis Title	UTILIZATION OF CASSAVA FIBER FOR CELLULASE AND ETHANOL PRODUCTION
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วรพรรณ พิมพ์เอี่ยม : การใช้ประโยชน์จากเส้นใยกากมันสำปะหลังในการผลิตเซลลูเลสและเอทานอล. (UTILIZATION OF CASSAVA FIBER FOR CELLULASE AND ETHANOL PRODUCTION) อ. ที่ปรึกษา: รศ. ดร. อัญชริดา อัครจรัลญา, อ. ที่ปรึกษาวิทยานิพนธ์ร่วม: ผศ. ดร. ณัฐชนัย ลีพิพัฒน์ไพบูลย์, 57 หน้า.

เส้นใยกากมันสำปะหลังที่ได้จากการย่อยกากมันสำปะหลัง (15% กรัมน้ำหนักเปียก/ปริมาตร) ด้วยแอลฟา-อะไมเลส และกลูโคอะไมเลส (Spezyme, USA) ตามวิธีที่ผู้ผลิตกำหนด แล้วแยกส่วนน้ำแป้งออกโดยการปั่นเหวี่ยง เมื่อนำมาใช้เป็นแหล่งคาร์บอนสำหรับการผลิตเอนโดกลูคาเนสและบีตากลูโคซิเดสโดย *Aspergillus terreus* สายพันธุ์ 24 และ *A. niger* สายพันธุ์ 127 ตามลำดับ และใช้เป็นสารตั้งต้นสำหรับการหมักเอทานอลด้วย *S. cerevisiae* TISTR 5596 โดยวิธี แซ็กคาริฟิเคชันแบบควบคู่ (SSF) พบว่าที่สภาวะเหมาะสม *A. terreus* สายพันธุ์ 24 ผลิตเอนโดกลูคาเนส 51.6 หน่วยเอนไซม์/กรัมแห้งของเส้นใย และบีตากลูโคซิเดส 0.64 หน่วยเอนไซม์/กรัมแห้งของเส้นใย และที่สภาวะเหมาะสม *A. niger* ผลิตบีตากลูโคซิเดส 37.2 หน่วยเอนไซม์/กรัมแห้งของเส้นใย และเอนโดกลูคาเนส 1.68 หน่วยเอนไซม์/กรัมแห้งของเส้นใย เมื่อนำเอนไซม์ทั้งสองข้างต้นมาผสมกันเพื่อปรับสัดส่วนของเอนโดกลูคาเนส: บีตากลูโคซิเดส เป็น 2: 1 แล้วใช้ในกระบวนการหมักเอทานอลแบบ SSF พบว่าสภาวะที่เหมาะสมของการผลิตเอทานอลจากเส้นใยกากมันสำปะหลังโดยวิธี SSF คือหมักที่ 40°ซ 72 ชั่วโมง ใช้เอนโดกลูคาเนส 128.57 หน่วยเอนไซม์ และบีตากลูโคซิเดส 59.29 หน่วยเอนไซม์/กรัมแห้งของเส้นใย ที่สภาวะเหมาะสมนี้ได้เอทานอล 40.19% กรัม/กรัมแห้งของเส้นใย โดยวิธีข้างต้นพบว่าจะทำให้ผลผลิตเอทานอลจากการหมักกากมันสำปะหลังเพิ่มสูงขึ้นกว่าการหมักเอทานอลจากส่วนแป้งในกากมันสำปะหลังอย่างเดียว 25%

ภาควิชา	จุลชีววิทยา	ลายมือชื่อนิสิต…….....
สาขาวิชา	จุลชีววิทยาทางอุตสาหกรรม	ลายมือชื่ออาจารย์ที่ปรึกษาวิทยานิพนธ์หลัก.....
ปีการศึกษา	2550	ลายมือชื่ออาจารย์ที่ปรึกษาวิทยานิพนธ์ร่วม……...……

 # # 4972465023 : MAJOR INDUSTRIAL MICROBIOLOGY

KEY WORD : CASSAVA WASTE / ETHANOL / SSF

WORAPHAN PIM-IAM: UTILIZATION OF CASSAVA FIBER FOR CELLULASE AND ETHANOL PRODUCTION. THESIS PRINCIPAL ADVISOR: ASSOC. PROF. ANCHARIDA AKARACHARANYA, Ph.D., THESIS CO-ADVISOR: ASST. PROF. NATCHANAN LEEPIPATPIBOON, Dr. RER. NAT., 57 pp.

Cassava fiber which obtained from digestion of cassava waste (15%wet w/v) by α-amylase and glucoamylase (Spezyme, USA) using method described by manufacturer and separated from starch solution by centrifugation was used as both carbon source for endoglucanase and β-glucosidase production by *Aspergillus terreus* strain 24 and *A. niger* strain 127, respectively, and substrate for ethanol fermentation by *S. cerevisiae* TISTR 5596 via simultaneous saccharification and fermentation (SSF) method. At optimized condition, *A. terreus* strain 24 produced endoglucanase (51.6 units/g cassava fiber, dry weight basis (DS)) and β-glucosidase (0.64 units/ g DS cassava fiber), while *A. niger* strain 127 produced β-glucosidase (37.2 units/ g DS cassava fiber) and endoglucanase (1.68 units/ g DS cassava fiber). The above enzymes were mixed in order to adjust endoglucanase and β-glucosidase ratio to 2:1 and it was used in the SSF experiment. Optimal conditions for ethanol production from the cassava fiber by SSF method was 40°C, 72 h of incubation and using 128.57 units of endoglucanase and 59.29 units of β-glucosidase/g DS cassava fiber. At the optimal condition, ethanol (40.19 % g/g DS of cassava fiber) was produced. Using the method described above, ethanol production from cassava waste was 25% higher than ethanol produced from only starch component of the cassava waste.

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

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LIST OF ABBREVIATIONS

w/v	=	weight/volume
w/w	=	weight/weight
v/v	=	volumn/volumn
mg/ml	=	milligram/milliliter
mM	=	millimolar
N	=	Normality
nm	=	nanometer
min	=	minute
h	=	hour
U	=	unit
DS	=	dry substrate
lb/inc^2	=	pounds/square inch
°C	=	degree celsius
rpm	=	revolution per minutes
$A_{520\ nm}$	=	$Absorbance_{520\ nm}$

CHAPTER I

INTRODUCTION

Energy consumption has 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 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 makes ethanol production cost too expensive to compete with gasoline. In order to lower the ethanol production cost, one promising way is to utilize plentiful, low-cost agricultural or agro-industrial waste as substrate. Cassava (*Manihot esculenta* Crantz) is one of a major commercial crop of Thailand. Annually, Thailand produces approximately 20 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 2 million tons of cassava waste (Office of the National Economic and Social Development Board, 2006). The cassava waste contains 60-70% (w/w), dry weight basis (DS), of starch and 10-15% (w/w), DS, of cellulosic fiber. Therefore, cassava waste is a potentially promising low cost substrate for ethanol production. A major step in the conversion of starch and cellulose to ethanol is the breakdown of starch and cellulose to glucose.

Since cassava fiber actively adsorps enzymes used for saccharification. Therefore, enzymatic saccharification of the cassava waste requires high enzyme dose. In this study, we solve this problem by saccharifying of starch and cassava fiber separately. Saccharification of cassava fiber was maximized by raised up of β-glucosidase ratio in cellulolytic enzyme system. Feed back inhibition of glucose in cellulase system was diminished by performing an simultaneous saccharification and ethanol fermentation, where glucose was removed through ethanol fermentation by thermotolerant *Saccharomyces cerevisiae* from the system as soon as it was formed.

The objective of this study is to optimize the cassava fiber saccharification for ethanol production using cellulase prepared from fungal strain selected.

CHAPTER II

LITERATURE REVIEWS

2.1 Cassava

Cassava (*Manihot esculenta* Crantz) is a tropical root crop belonging to family Euphorbiaceae, Dicotyledonae. It is a shrubby, perennial plant, commonly known as tapioca (Fig. 2.1A). The edible part is tuberous root which is dark brown in color (Fig. 2.1B). Length of the tuberous root is depend upon cultivar and soil conditions which maximum length is up to 2 ft. 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 (Jos, 1969).

Fig. 2.1 Cassava plant (A) and its tuberous root (B).
(www.belizemagazine.com, www.pattayadailynews.com)

Cassava is one of a major commercial crops of Thailand. It is planted on over 1.5 million ha and yielded 20 million of roots annually (Office of the National Economic and Social Development Board, 2006). One-half of these roots are 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 non-food 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*., 2000).

Production of cassava starch generates approximately 2.0 million tons of cassava waste (pulp) annually (Office of the National Economic and Social Development Board, 2006). Due to the waste contains a large amount of starch (up to 50-60% (w/w), dry weight basis (DS)), and a high moisture content (60-70% w/w) (Jutart Kesornsit, 2550)). This makes the waste spoiled rapidly and caused serious pollution problem. At present, the waste has to be dried and applied as a low value animal feed and fertilizer, though the drying process is difficult and expensive because starch reacts with water at high temperature. Improved utilization of the cassava waste will lead to more efficient resource usage, more value added to the waste, and less negative environmental impact.

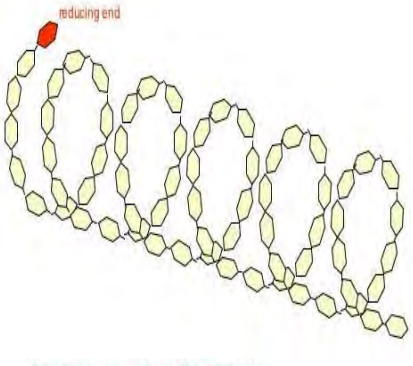
2.2 Cassava waste

Cassava waste (pulp) is a solid waste produced as a consequence of starch production. It is composed of 2 major components, starch (50-60% (w/w), DS) and (lignocellulosic) fiber (10-15% w/w, DS) (Jutart Kesornsit, 2550).

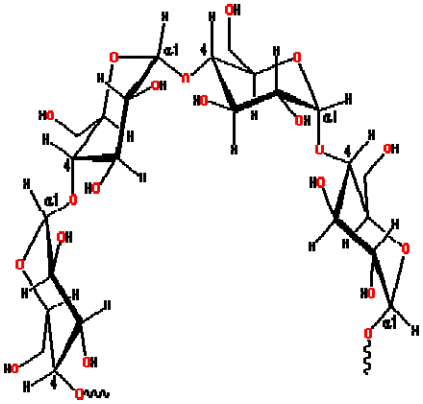
2.2.1 Starch

Starch is a glucose polymer of α-glucan linked by α-1,4 bond and branched at α-1,6 position (Ball and Morell, 2003). It is a huge (0.1>50 μm in diameter) complex quaternary structure made up of two major glucan polymers: amylose and amylopectin (Yona, 2004). Casaava starch is composed of unbranched amylose (20±5%) and branched amylopectin (80±5%) (Ejiofor *et al.*, 1996). Amylose is an unbranched single chain polymer of 500 to 2000 glucose subunits with only α-1,4 glucosidic bonds (Fig. 2.2A). Amylose is actually often helical but it is typically illustrated as a straight chain structure for the sake of simplicity. 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 composed of α-1,4-linked glucose segment connected by α-1,6-linked branch points (Fig. 2.2B). The degree of branching in amylopectin is approximately one per twenty-five glucose units in the branched segments (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.

A.



Amylose, an unbranched starch



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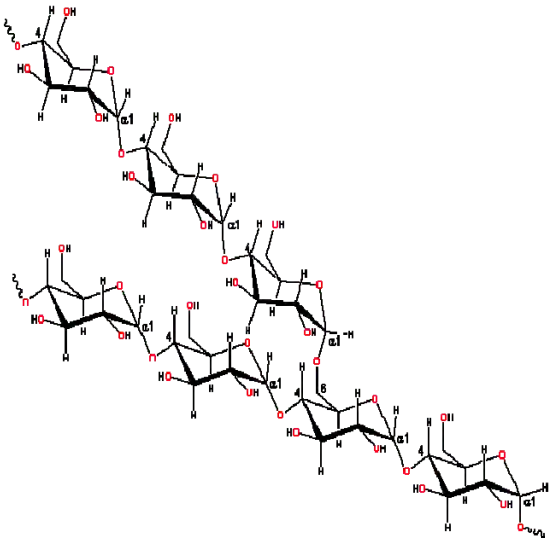
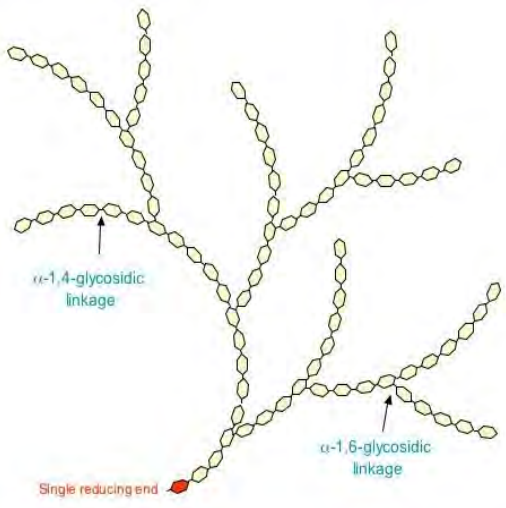




Amylopectin,a branched starch

Fig. 2.2 Structure of amylose (A) amylopectin (B) (De Sa, 2005; Chaplin, 2007)

2.2.2 (Lignocellulosic) Fiber

Lignocellulose is a major cell wall-component of higher plants. It consists of 45% cellulose, 30% hemicellulose and 25% lignin which form very complex structure 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 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 100 to 14,000 D-glucose units. The smallest structural repeating unit in native cellulose is cellobiose, a disaccharide of glucose. The cellobiose unit in cellulose structure is shown in Fig. 2.3. The cellulose structure is highly stabilized by intramolecular hydrogen bond (Atalla and Vanderhart, 1984; Michell, 1990).

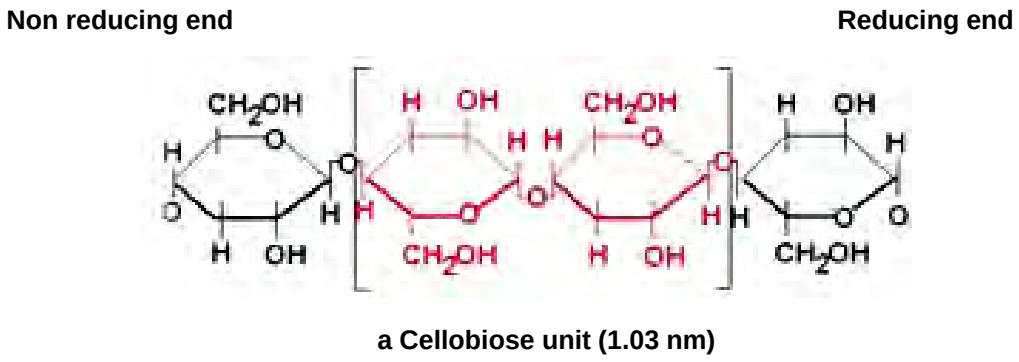


Fig. 2.3 Cellulose structure showing the smallest repeating unit of cellobiose. (Myles, 2007)

The high starch content makes the cassava waste be a potentially promising plentiful and low cost agro industrial waste substrate for ethanol production. However, a major and limiting steps are the conversion of starch and cellulose to fermentable sugar, principally glucose. Both starch and cellulose are made up of 1,4 linked D-glucopyranose unit, but starch has α-D-(1 ⟶ 4) linkages and cellulose β-D-(1 ⟶ 4) linkages. Consequently, they have very different conformations and are hydrolysed by different enzymes. Starch is hydrolysed by amylase while cellulose is hydrolysed by cellulase.

2.3 Amylase and cellulase

2.3.1 Amylase

Amylase is a group of enzymes which hydrolyses starch into glucose and/or oligosaccharides. 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. (Brady *et. al*., 1991; Machius *et. al*., 1995), **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). Pure glucoamylase are capable of hydrolyzing amylose, amylopectin, maltose and maltooligosaccharides completely. 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 Fig. 2.4.

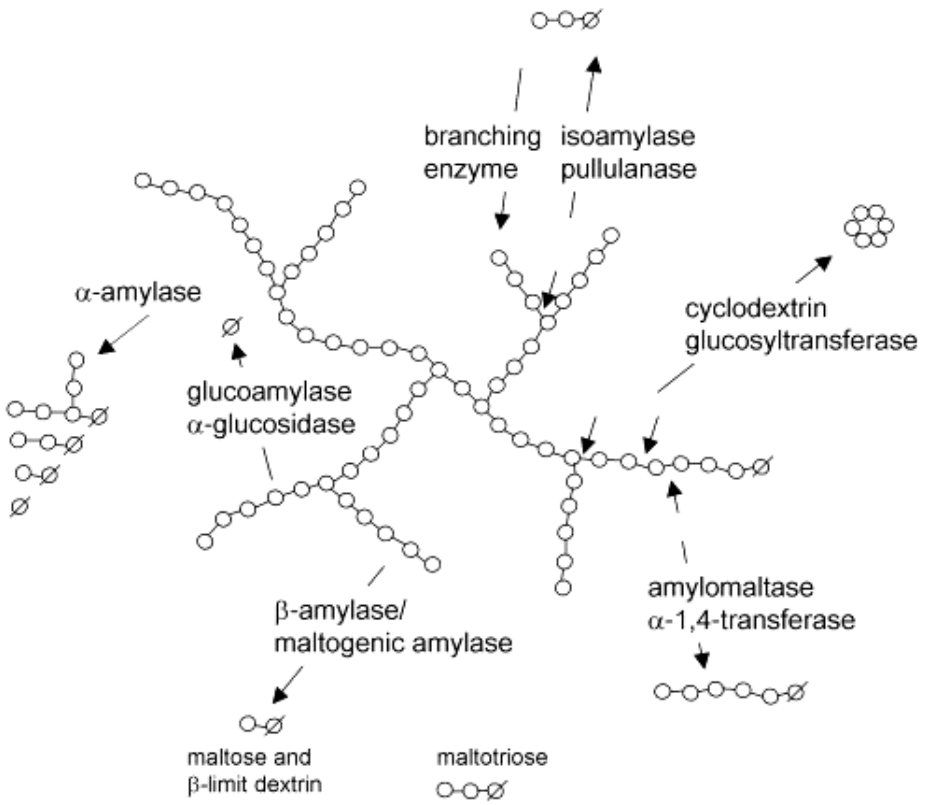


Fig. 2.4 Starch molecule and amylase sites of action. Glucose molecules are shown as circle (○) and reducing ends are marked by a line through the circles (∅) (Turner *et. al*., 2007).

2.3.2 Cellulase

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 hydrolyses the cellulose chain internally. β-Glucosidase (EC 3.2.1.21) cleaves cellobiose to glucose (Fig. 2.5). 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).

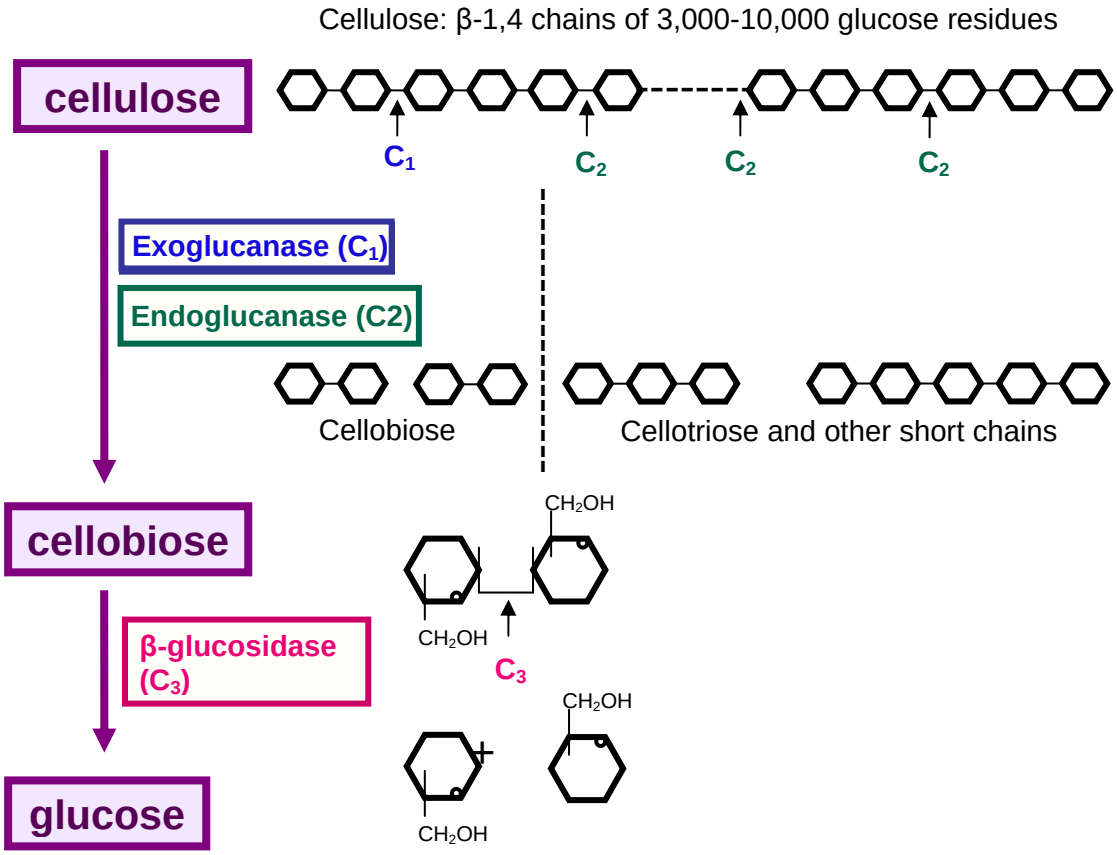


Fig. 2.5 Enzymatic hydrolysis of cellulose (Deacon, 1997).

2.3.3 Source of amylase and cellulase

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* are of special interest due to their enzymes produced are important in biotechnological processes (Benoliel *et al*., 2005; Hrmova *et al., 1989*). Table2.1 shows list of enzymes produced by *Aspergillus*. (Hankin and Anagnostakis, 1975; Bindslev-Jensen *et al*., 2006; Stefanova *et al*., 1997)

Table 2.1 List of enzymes produced by *Aspergillus*

Fungi	Enzyme detected								
	Amylase	Cellulase	DNAase	Xylanase	Lipase	Protease	Pectate lyase	Urease	Phosphatase
Aspergillus (luchuensis)	+	+	+		+			+	
A. (fumigatus)	+		+		+		+		+
Aspergillus sp.	+				+		+	+	+
A. oryzae							+		
A. niger	+	+		+	+	+			
A. terreus		+		+		+			

Agricultural materials contained both starch and cellulose can be conveniently hydrolysed to fermentable sugar by the *Aspergillus* produce a mixture of amylase and cellulase.

Shambe and Ejembi (1987) reported that stationary and shaken-submerged cultivation of *Aspergillus terreus* and *A. carneus* in medium containing 1% ground millet, starch or carboxymethylcellulose gave no difference in the yield of amylase and carboxymethylcellulase (CMCase).

Beletskaya *et al* (1985) reported that *Aspergillus terreus* produced five extracellular endoglucanases with molecular masses varying from 47 kDa to 5-7 kDa. The lower the molecular size, the lower the affinity towards insoluble and/or high polymeric cellulose substrate and the higher the hydrolysis rate for soluble celluloses and oligosaccharides.

Stefanova *et al* (1997) reported that *Aspergillus terreus* secreted two proteases, serine thiol-dependent proteinase and metalloproteinase. The extracellular metalloproteinase presented mainly during growth conditions favouring fast accumulation of low molecular mass cellulases. They suggested that the metalloproteinase promoted postsecretional proteolysis of high molecular mass cellulases into smaller enzymes of the cellulolytic complex with altered substrate specificity.

Jean (2000) proposed that *Aspergilus niger* grown in solid state fermentation conditions which closer to their natural habitats may be more capable of producing certain enzymes. Therefore, he investigated optimal conditions for cellulase production of *A. niger* 38 using mixture of wheat straw and wheat bran as substrate under solid state fermentation. In wheat straw: wheat bran of 9:1 with 74% moisture content and pH 4.5-5.5, *A. niger* 38 produced endoglucanase (14.8 units/ml) in 96 h.

Kang *et al* (2004) investigated cellulases and hemicellulases production of *Aspergillus niger* KK2 under solid state fermentation using different ratios of rice straw and wheat bran. *A. niger* KK2 grown on rice straw alone gave maximum filter paper activity (19.5 units/g) in 4 days. Carboxymethylcellulase (129 units/g), β-glucosidase (100 units/g), xylanase (5070 units/g) and β-xylosidase (193 units/g) were concurrently obtained after 5-6 days.

2.4 Raw material for ethanol production: an improved utilization of cassava waste

Srinorakutara *et al* (2004) produced ethanol from cassava waste by 2 steps methods (1) enzymatic hydrolysis, a conversion step of starch and cellulosic materials to fermentable sugar and (2) ethanol fermentation, a conversion step of fermentable sugar to ethanol by *Saccharomyces cerevisiae* TISTR 5596. It was found that cassava waste contained 1% (w/v) of non-water soluble carbohydrate yielded 122.4 g/l of reducing sugar when it was hydrolyzed by mixture of cellulase and pectinase at 28°C for 1 h, then by α-amylase at 100°C for 2 h and finally by glucoamylase at 60°C for 4 h. Pectinase did not directly promote the hydrolysis but helped to reduce viscosity and eased for solid filtration process. Maximum ethanol 3.62% (w/v) was obtained after 24 h when initial reducing sugar used was 89.2 g/l.

A conversion of the cassava waste to ethanol includes two processes: saccharification and fermentation. Enzymatic saccharification of the cassava waste requires high enzyme concentration due to an adsorption of amylase and cellulase to cassava fiber. Therefore, starch solution should be seperated from cellulosic fiber and hydrolysed to fermentable sugar separately.

Enzymatic saccharification of the cellulosic materials is found to be rapid only at the initial stage. The deceleration of the saccharification may be attributed to an inhibition of reaction products such as glucose and cellobiose, and deactivation of enzymes (Huang and Chen, 1988).

In 1997, Takagi *et al* proposed one-stage process for cellulosic ethanol production in which the enzymatic saccharification of cellulose and fermentation of the glucose were conducted simultaneously. This process is referred to as simultaneous saccharification and fermentation (SSF). The advantages of the SSF process are reduction of reactor number and faster saccharification rates. The latter results from removal of glucose from the system as soon as it is formed and its inhibitory effect on the cellulase system is diminished (Ghosh *et al.*, 1982). The alleviation of the end product inhibition consequently results in an increase of cellulose conversion to ethanol compared to separate process of saccharification and fermentation (Huang and Chen, 1988). In addition, the presence of ethanol in the culture medium causes the reaction mixture to be less vulunerable to invasion by undesired microorganisms (Ballesteros *et al*., 2004). However, difficulties associated with the process include different temperature optima for the action of enzymes and ethanol-producing microbes and possible inhibitory effect of ethanol on the cellulase system (Ghost *et al*., 1982).

The critical problem with SSF: the difference in temperature optima of the cellulase and the fermenting microorganism

Thermotolerant ethanol fermenting microorganisms which are able to ferment glucose to ethanol at temperatures above 40°C, which are closer to the optima for the activity of the cellulolytic complex in the range of 40-50°C, is therefore advisable (Ballesteros et al., 2004).

Szcozdrak and Targonaki (1988) screened 58 yeast strains from 12 different genera for their ability to grow and ferment at temperatures above 40ºC. It was reported that some of the *Fabospora* and *Kluyveromyces* strains were able to grow at as high temperature as 46ºC. *Fabospora fragilis* CCY51-1-1 cultivated at 43ºC produced 56 g/l ethanol from 140 g/l glucose, which corresponds to 0.4 g/g (g/ethanol/g glucose) ethanol yield. However, when the cultivation temperature was increased to 46ºC the performance of the strain significantly decreased , and 0.25 g/g ethanol yield was obtained.

Ballesteros *et al* (1991) examined an ability of *Saccharomyces*, *Candida* and *Kluyveromyces* strains to ferment glucose at temperatures above 40ºC. Similarly to the results reported by Szczodrak and Targon (1988), *Candida* and *Saccharomyces* strains were less thermotolerant than *Kluyveromyces* strains. When *Kluyveromyces marxianus* L.G. was cultivated at 42ºC on glucose containing medium, 37.6 g/l ethanol concentration was obtained with an ethanol yield of 0.4 g/g.

Bollók and Réczey (2000) evaluated five different *Kluyveromyces* strains based on the examination of their growth on agar slants and in shake flask cultures at different temperatures. On glucose medium in aerobic cultivation, *K. marxianus* Y01070 proved to be the best thermotolerant strain of all examined strains.

In SSF experiments Ballesteros *et al*. (1991) achieved best conversion of 10 % Solka Floc cellulose substrate to ethanol using both *K. marxianus* L.G. and *K .fragilis* L.G. at temperatures up to 42ºC, where 0.5 and 0.46 ethanol yields were achieved, respectively. The ethanol yields were reduced at 45 ºC because of cell death.

Barron *et al*. (1995) found that the *K. marxianus* IBM3 was capable of producing ethanol at as high temperature as 45ºC using milled paper as substrate. The obtained ethanol yield was 0.11 g/g (21% of the theoretical).

D'Amore *et al* (1989) screened a total of 65 yeast strains from various genera for their ability to grow on glucose and ferment to ethanol at 40°C. They found that *Saccharomyces cesdiastaticus* 62 completely utilized 15% glucose and produced 6.4% (w/v) ethanol.

Beletskaya *et al.,* 1985 found that *Kluyveromyces* strains were more thermotolerant than *Saccharomyces* and *Candida* strains, but *Kluyveromyces* and *Candida* strains were not as ethanol-tolerant as *Saccharomyces* strains.

Varga *et* al (2004) proposed that *Saccharomyces cerevisiae* is the best organism for the fermentation of pretreated lignocellulose substrates.

Temperature compatibility between saccharification and fermentation of SSF is performed by programmed temperature profile.

Huang and Chen (1988) examined SSF technique with temperature profiling using Solka Floc as the substrate. The fermenting microorganism was *Zymomonas mobilis* and culture medium was supplemented with *Trichoderma reesiae* cellulases. Two different strategies were applied: temperature cycling and profiling, to enhance the SSF fermentation. In a cycling study the temperature was changed periodically between the optimal fermenting temperature, 37°C, and the highest tolerable temperature, 40°C, of *Z. mobilis.* In the profiling experiment during the initial phase, the temperature was controlled between 30 and 37°C to allow optimal condition for the propagation of the cells. After the cells entered into their active ethanol production phase, therefore the hydrolysis reaction became the rate limiting step of SSF process, the temperature was increased to 40°C. The results showed that with temperature cycling the ethanol productivity (the final ethanol concentration divided by the reaction time) could be increased from 0.49 g/(1×h)

to 0.62 g/(1×h). However, similar ethanol yield, 0.23 g/g, was obtained as with traditional SSF. In contrary, with temperature profiling the ethanol yield obtained was significantly higher, 0.32 g/g, than that obtained with isothermal SSF. Unfortunately, the productivity, 0.32 g/(1×h), was reduced due to the increased processing time required for the prehydrolysis.

A contrary result was reported by Kadar *et al* (2004). Ethanol was produced from paper sludge in two types of SSF experiments, isothermal SSF and SSF with temperature profiling. SSF resulted in higher ethanol yield (0.31-0.34 g ethanol/g cellulose) compared to SSF with temperature profiling.

The critical problem with SSF: the inhibitory effect of ethanol on cellulase system

Ghost *et al* (1982) showed that hydrolytic activity of *Trichoderma reesei* Qm 9414 cellulase was inhibited by ethanol concentration above 0.75% (v/v).

Ooshima *et al* (1985) concluded that ethanol concentration of 0.9% or greater reduced the enzymatic ability of *Trichoderma viride* cellulase.

Chen and Jin (2006) studied the influence of both ethanol and *Saccharomyces cerevisiae* on cellulase activity of *Penicillium decumbens* in SSF experiment. It was found that ethanol in concentrations between 1% and 7% inhibited the enzymatic hydrolysis of crystalline cellulose but the inhibition was reversible. At ethanol concentration between 1% and 9%, the activity of β-glucosidase increased with increasing ethanol concentration. Yeast had no effect on the enzyme activity.

Most commercially available cellulase enzyme preparations have low β-glucosidase activity. This enzyme is essential, because it converts the cellobiose to glucose, which then can be fermented by the yeast. Supplementing the cellulases with β-glucosidase from external source increases the theoretical ethanol yield from 63 to 84%. (Iynd *et al*., 2001)

Ghosh *et al* (1982) reported that SSF using *Trichoderma* cellulases and *Saccharomyces cerevisiae* enhanced cellulose hydrolysis rate by 13-30% compared with saccharification in the absence of yeast. The requirement for β-D-glucosidase in SSF was lower than in saccharification in the absence of yeast. Maximal ethanol production was attained when the ratio of the activity of β-glucosidase to filter paper activity was ~1.0.

Chen and Jin (2006) suggested to used *Penicillium decumbens* cellulase for lignocellulosic bioconversion. Because there was an abundant of β-glucosidase in the system.

In the present study, starch solution was separated from cassava fiber and hydrolysed to fermentable sugar by commercial α-amylase and glucoamylase. While the cassava fiber was used as substrate for endoglucanase and β-glucosidase production by *Aspergillus niger* and *Aspergillus terreus*, respectively. Then the cellulases produced were used to saccharify cellulosic fiber in SSF experiment. Since *A. niger* and *A. terreus* produce both cellulase and amylase, the starch trapped in the cassava fiber was also be hydrolysed to fermentable sugar. *Saccharomyces cerevisiae* TISTR 5596 which could grow and ferment glucose to ethanol at 40°C was used.

CHAPTER III

MATERIALS AND METHODS

Materials

3.1 Cassava waste

Cassava waste was obtained from Sanguan Wongse Industries Co., Ltd., Nakhonratchasima province in a frozen form (Fig. 3.1). Cassava fiber was prepared from cassava waste by method shown in Fig. 3.2

Fig. 3.1 Cassava waste in a frozen form

3.2 Equipment

3.3.1 Analytical balance : Mettler Toledo, model AG 285, Switzerland.

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

3.3.3 Fermenter : B.E. Marubishi, model 10L, Japan.

3.3.4 Gas chromatography : Shimadzu, model 7AG, Japan.

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

3.3.6 Laminar flow : Lab service Ltd., Clean model V6, Thailand.

3.3.7 Incubator shaker : New Brunswick Scientific, model Innova 2300, USA.

3.3.8 pH meter : Mettler Toledo, model SevenEasy, China.

3.3.9 Precision balance : Mettler Toledo, model PB 3002, Switzerland.

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

3.3.11 Spectrophotometer : Spectronic Instruments, model Spectronic 20, USA.

3.3.12 Water bath : Tolabo, model TW20, Germany.

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

3.3 Chemicals

Chemicals (Analytical grade)	Company
Agar	Becton
Alpha amylase	Spezyme
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
Glucoamylase	Spezyme
Glucose ($C_6H_{12}O_6$)	Sigma
Hydrochloric acid (HCl)	Sigma
Magnesium sulfate heptahydrate ($MgSO_4.7H_2O$)	Merck
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 was obtained from Thailand Institute of Scientific and Technological Research.

3.4.2 *Saccharomyces cerevisiae* TISTR 5596 was obtained from Thailand Institute of Scientific Technological Research.

3.4.3 Fungi (35 isolates) were obtained from Associate Professor Dr.Wichien Kritprechavanich, Department of Microbiology, Faculty of Science, Kasetsart University.

3.5 Culture medium

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

METHODS

3.6 Experiments

Flow diagram of all experiments was shown in Fig. 3.2

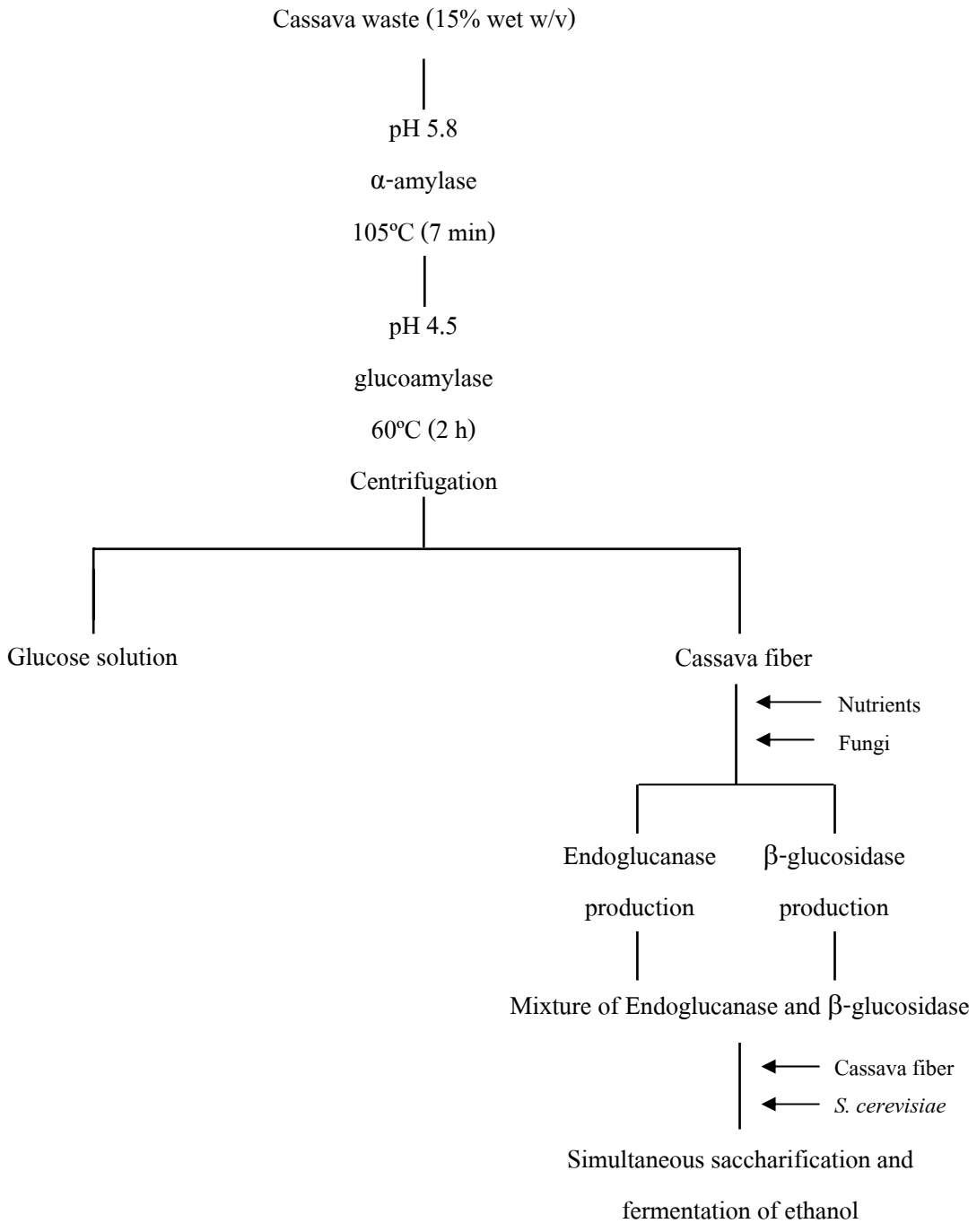


Fig. 3.2 Flow diagram of an experiment

3.7 Microorganism cultivation

3.7.1 Maintainance of microorganisms

Saccharomyces cerevisiae TISTR 5596 and *T. reesei* TISTR 3080 were long-term maintained in liquid nitrogen and freeze-dried form. *S. cerevisiae* grown on yeast peptone dextrose (YPD) agar at 30°C for 2 days was kept at 4°C. *T. reesei* TISTR 3080 and cellulase producing fungi grown on potato dextrose agar (PDA) slant at 30°C for 7 days was kept at 4°C. The above cultures transferred to new fresh medium every week but not more than 4 successions were used for inoculum preparations in routine experiments.

3.7.2 Preparation of inoculum

3.7.2.1 *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 (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.

3.7.2.2 Endoglucanase and β-glucosidase producing fungi

Endoglucanase and β-glucosidase producing fungi were grown on potato dextrose (PDA) agar at 30°C for 7 days. Spores of the cultures were suspended in 0.2% (w/v) Tween 80 and used as an inoculum at final concentration of 10^7 spores/ml.

3.8 Cassava fiber preparation

Cassava waste (15% wet w/v) was digested by α–amylase (0.04% w/w, dry weight basis, DS) at 105°C for 7 min and glucoamylase (0.08% w/w, DS) at 60°C for 2 hrs, pH 4.5 (Spezyme, USA), respectively. Cassava fiber was separated from starch solution by centrifugation at 4°C, 11,500 rpm (9,280 x g) for 20 min. The cassava fiber was used as both carbon source for endoglucanase and β-glucosidase production by *Aspergillus terreus* strain 24 and *A. niger* strain 127, respectively, and substrate for ethanol fermentation by *S. cerevisiae* TISTR 5596 via simultaneous saccharification and fermentation (SSF) method.

3.9 Selection of a high efficient endoglucanase and β-glucosidase producing fungi.

3.9.1 Selection of cellulase producing fungi

Fungi (35 isolates) obtained from Department of Microbiology, Faculty of Science, Kasetsart University were inoculated on carboxymethyl cellulose agar medium (carboxymethyl cellulose (1%), $(NH_4)_2SO_4$ (0.2%), yeast extract (0.1%), agar (1%), pH6), incubated at 30°C for 2 days. The fungal colonies were flooded with 0.1% (w/v) Congo red solution for 1 min and then washed by 0.1 M NaCl. Colonies surrounded by clear zone were selected as cellulase producing fungi.

3.9.2 Quantitative cellulase producing assay

Endoglucanase and β-glucosidase producing fungi which were identified by clear zone producing when grown on carboxymethyl cellulose agar medium were quantitatively determined. Fungal spores were point inoculated at center of 9 cm diameter Petri-dish, incubated at 30°C for 3 days. Peripheral mycelium of the colony was cut as agar block by cork borer (5 mm diameter) and transferred to cassava fiber medium composed 15% wet (w/v) cassava fiber, 0.2% $(NH_4)_2SO_4$, 0.1% KH_2PO_4, 0.025% $MgSO_4{\cdot}7H_2O$, 0.05% yeast extract pH 6.0 and incubated at 30°C (200 rpm) for 5 days. Supernatant obtained after centrifugation at 4°C, 8000 rpm (20,292 x g) for 15 min was used as crude enzyme. Endoglucanase and β-glucosidase activities of the crude enzymes were analysed (see 3.13).

Endoglucanase and β-glucosidase production of the highest endoglucanase and β-glucosidase producing strains were confirmed by using spore suspension of each strain at 10^7spores/ml as inoculum. Endoglucanase and β-glucosidase produced were quantitatively determined as described above.

3.10 Optimization of endoglucanase and β-glucosidase production

Spores of the selected endoglucanase or β-glucosidase producing fungi at final concentration of 10^7spores/ml were inoculated into 15% (wet w/v) cassava fiber medium containing 0.2% $(NH_4)_2SO_4$, 0.1% KH_2PO_4, 0.025% $MgSO_4{\cdot}7H_2O$, 0.05% yeast extract pH 6.0 and incubated at 30°C (200 rpm) for 5 days. The cultures were centrifuged at 4°C, (9,280 x g) for 20 min, and supernatants were used as crude enzyme to quantify endoglucanase and β-glucosidase produced (see 3.13).

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

3.10.1 Endoglucanase and β-glucosidase production profile

The selected fungi was inoculated into 15% (wet w/v) cassava fiber medium containing 0.2% $(NH_4)_2SO_4$, 0.1% KH_2PO_4, 0.025% $MgSO_4.7H_2O$, 0.05% yeast extract pH 6.0 and incubated at 30°C (200 rpm) for 9 days. Endoglucanase and β-glucosidase produced in the culture supernatants were daily analysed.

3.10.2 Effect of nutrients concentration

The selected fungi were inoculated into cassava fiber medium containing 15% (wet w/v) cassava fiber. The nutrients concentration in the cassava fiber medium were step-wise varied. The cultures were incubated at 30°C (200 rpm) and at optimal incubation period (result of 3.10.1). pH 6.0.

Nutrients	**Concentration (% w/v)**
$(NH_4)_2SO_4$	0, 0.2, 0.3, 0.4, 0.6, 0.8 or 1%
KH_2PO_4	0, 0.1, 0.2, 0.4, 0.6 0.8 or 1%
$MgSO_4.7H_2O$	0, 0.025, 0.05, 0.075, 0.1, or 0.125%
yeast extract	0, 0.05, 0.1, 0.15, 0.2, 0.25, 0.3, or 0.35%

Supernatants obtained after centrifugation at 4°C, 9,280 x g (20 min) were used as crude enzyme to quantify endoglucanase and β-glucosidase produced.

3.10.3 Effect of pH

The selected fungi were inoculated into cassava fiber medium containing 15% (wet w/v) cassava fiber, optimal concentration of each nutrients (results of 3.10.2) at various pH (4.5, 5.0, 5.5, 6.0, 6.5 or 7.0), and incubated at the same above conditions. Supernatants obtained after centrifugation at 4°C, 9,280 x g (20 min) were used as crude enzyme to quantify endoglucanase and β-glucosidase produced.

3.10.4 Effect of cassava fiber concentration

The selected fungi were inoculated into the optimized cassava fiber medium (results of 3.10.3) containing various concentration of cassava fiber (10, 15, 20, 25% (wet w/v)) and incubated at the same above conditions. Supernatants obtained after centrifugation at 4°C,

9,280 x g (20 min) were used as crude enzyme to quantify endoglucanase and β-glucosidase produced.

3.10.5 Effect of incubation temperature

The selected fungi were inoculated into the optimized cassava fiber medium (result 3.10.4) and incubated at 30, 35, 40 or 45°C with shaking (200 rpm). Supernatants obtained after centrifugation at 4°C, 9,280 x g (20 min) were used as crude enzyme to quantify endoglucanase and β-glucosidase produced.

3.10.6 Endoglucanase and β-glucosidase production by batch reactor

The selected fungi were cultivated in the optimized cassava fiber medium and incubated at optimized conditions (results of 3.10.5) in 10 liter batch reactor agitation rate (250 rpm) and aeration rate (1 vvm) were used (Fig. 3.3). Supernatants obtained after centrifugation at 4°C, 9,280 x g (20 min) were used as crude enzyme to quantify endoglucanase and β-glucosidase produced.

Fig. 3.3 Endoglucanase and β-glucosidase production in 10 liter batch reactor

3.11 Characterization of endoglucanase and β-glucosidase

Endoglucanase and β-glucosidase of the selected fungi produced at the optimal conditions were characterized.

3.11.1 Effect of temperature on endoglucanase and β-glucosidase activities

Endoglucanase and β-glucosidase activities were assayed by the method described in 3.13, except reaction mixtures were incubated at various temperature (30, 35, 40, 45, 50, 55, 60 or 65°C).

3.11.2 Effect of pH on endoglucanase and β-glucosidase activities

Endoglucanase and β-glucosidase activities were assayed by the method described in 3.13, 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 4.0, 4.5, 5.0, 5.5, 6.0 and 7.0, 7.5 respectively.

3.11.3 Temperature stability

Crude enzyme was pretreated at various temperature for 30 min. Residual endoglucanase and β-glucosidase activities of the pretreated enzymes were analysed (see 3.13). An activity of non-treated enzyme was set as 100%.

3.12 Ethanol fermentation

Ethanol production by simultaneous saccharification and fermentation (SSF) method

Endoglucanase (0.88 Units/ml) was mixed with β-glucosidase (0.62 Units/ml). Ratio of endoglucanase : β-glucosidase in the enzyme mixture was 2:1. *S. cerevisiae* inoculum prepared as describe previously was inoculated at 10% (v/v). The inoculum and cassava fiber were added into the enzyme mixture simultaneously. Endoglucanase and β-glucosidase used to saccharify cassava fiber were 21.43 and 9.88 units per g DS, respectively.

3.12.1 Effect of temperature

Cassava fiber (15% w/v, DS) was simultaneously hydrolysed by the enzyme mixture and fermented to ethanol by *S. cerevisiae* at pH 5. The reaction mixture was incubated without shaking at various temperature (30, 35, 40, 45, 50°C)for 72 h. Ethanol produced in the culture supernatant was analysed by gas chromatography method (see 3.13.3)

3.12.2 Effect of incubation period

Cassava fiber (15% w/v, DS) was simultaneously hydrolysed by the enzyme mixture and fermented to ethanol by *S. cerevisiae* at optimal temperature (result of 3.12.2.1), pH 5 for 96 h without shaking. Ethanol produced in the culture supernatant was analysed daily by gas chromatography method (see 3.13.3)

3.12.3 Effect of endoglucanase/β-glucosidase mixture concentration

Cassava fiber (15% w/v, DS) was simultaneously hydrolysed by various concentration (units/g DS of cassava fiber) of endoglucanase and β-glucosidase, the fermented to ethanol by *S. cerevisiae* at optimal conditions (result of 3.12.2.2), pH 5 without shaking. Ethanol produced in the culture supernatant was analysed by gas chromatography method (see 3.13.3)

3.13 Analytical procedure

3.13.1 Endoglucanase activity assay (Ghose, 1987)

Carboxymethylcellulose (CMC) at 2 % (w/v) in 100 mM citrate buffer pH 6.0 was used as substrate. Reaction mixture consisted of substrate (0.5 ml) and crude enzyme (0.5 ml) was incubated at 30ºc 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_{520nm}.

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

3.13.2 β-glucosidase activity assay (Sternberg, 1977)

Salicin at 0.4 % (w/v) in 100 mM citrate buffer pH 6.0 was used as substrate. Reaction mixture consisted of substrate (0.5 ml) and crude enzyme (0.5 ml) was incubated at 30ºc 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.

3.13.3 Analysis of ethanol by gas chromatography method

Sample (100 μl) was injected to Gas chromatography using Porapak Q column (3.3cm x 2m) equipped with flame ionization detector (model 7AG, Shimadzu, Japan) using

nitrogen as carrier gas at 50 ml/min. Injection and column temperatures were 240°C and 190°C, respectively. Retention time of ethanol was 2 min. Triplicate measurements were performed for each sample.

CHAPTER IV

RESULTS

4.1 Cassava waste

Composition of cassava waste analysed by Kasetsart Agricultural and Agro-Industrial Product Improvement Institute using AOAC method was shown in Table 4.1. Major component was starch (63.44% w/w) followed by cellulosic fiber (14.86% w/w), dry weight basis (DS). Moisture content was 82.37%(w/v) DS.

Table 4.1 Composition of cassava waste

Components	% (w/w)
Starch	63.44
Fiber	14.86
Protein	1.70
Fat	0.20
Ash	1.61

4.2 Cassava waste saccharification

Cassava waste suspended in 100 ml of distilled water (15% wet w/v) was liquefied by α–amylase (0.04% w/w DS of starch) at 105°C, pH 5.8 for 7 min. The liquefied cassava waste was adjusted to pH 4.5 and further hydrolysed by glucoamylase (0.08% w/w DS of starch) at 60°C for 2 hrs. The reaction mixture was centrifuged at 11,500 rpm (9,280 x g) for 10 min. Cassava fiber was separated from the supernatant (cassava waste hydrolysate) and used as substrate for screening of high efficient endoglucanase and β-glucosidase producing fungi, substrate of endoglucanase and β-glucosidase production and substrate for ethanol fermentation by simultaneous saccharification and fermentation (SSF) method.

Saccharified cassava waste, cassava waste hydrolysate and cassava fiber were shown in Fig. 4.1

Fig. 4.1 Saccharified cassava waste (A), cassava waste hydrolysate (B) and cassava fiber (C).

4.3 Selection of a high efficient endoglucanase and β-glucosidase producing fungi

From 35 fungi screened for cellulase production, 19 isolates were cellulase producing fungi as indicated by clear zone surrounded their colonies. The cellulase producing fungi were grown on potato dextrose agar at 30°C for 3 days. Peripheral mycelium of each colony was aseptically cut by 5 mm diameter cork borer, inoculated into 25 ml of 15%(wet w/v) cassava fiber medium (containing 0.2% $(NH_4)_2SO_4$, 0.1% KH_2PO_4, 0.025% $MgSO_4.7H_2O$, 0.05% yeast extract, pH 6.0) in 250 ml flask and incubated at 30°C, 200 rpm for 5 days. Supernatants obtained after centrifugation were analysed for endoglucanase and β-glucosidase activities. As shown in Fig. 4.2A, endoglucanase production was in the range of 0 to 0.9 units/ml. 13 isolates produced 0-0.2 units/ml, 0.2-0.4 units/ml (2 isolates), >0.6-0.9 units/ml (3 isolates) and only 1 isolate produced more than 0.9 units/ml. *Aspergillus terreus* strain 24 produced maximum endoglucanase at 0.93 units/ml. β-glucosidase production of the 19 fungi tested were shown in Fig. 4.2B. *Aspergillus niger* strain 127 produced maximum β-glucosidase (0.019 units/ml). All of the fungi tested produced β-glucosidase less than 0.0098 units/ml. Production of endoglucanase and β-glucosidase of *Aspergillus terreus* strain 24 and *A. niger* strain 127 were confirmed by cultivation at the same above conditions but using spore suspension at final concentration of 10^7 spores/ml as inoculum. The inoculum was inoculated into the cassava fiber medium (25 ml) in 250 ml flask. Endoglucanase and β-glucosidase production of *A. terreus* strain 24 and *A. niger* strain 127 were 0.89, 0.002 and 0.087, 0.01 units/ml, respectively.

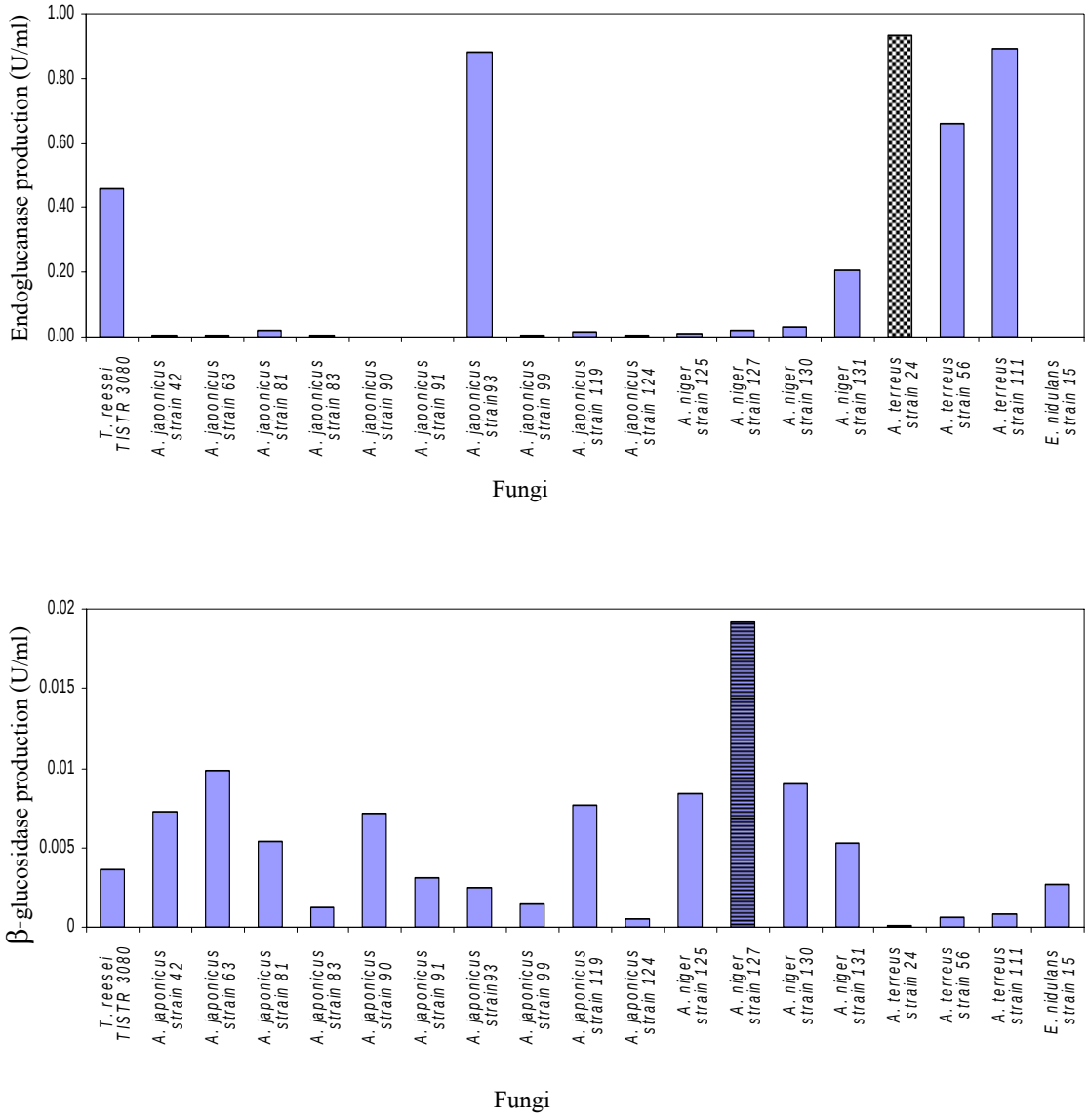


Fig.4.2 Endoglucanase production (A), β-glucosidase production (B) by the 19 cellulase fungi tested. The enzymes produced by *T. reesei* TISTR 3080 were used as reference control**.**

4.4 Optimization of endoglucanase and β-glucosidase production from cassava fiber

Cassava fiber separated from cassava waste saccharified by α-amylase and glucoamylase (Fig. 3.1 and see 3.8) was used as substrate.

4.4.1 Optimization of endoglucanase production by *Aspergillus terreus* strain 24

Aspergillus terreus strain 24 was inoculated into 15% (wet w/v) cassava fiber medium containing 0.2% $(NH_4)_2SO_4$, 0.1% KH_2PO_4, 0.025% $MgSO_4{\cdot}7H_2O$, 0.05% yeast extract pH 6.0 at final concentration of 10^7 spores/ml, and incubated at 30°C (200 rpm) for 5 days. Endoglucanase activity in the culture supernatant obtained after centrifugation at 4°C, 11,500 rpm (9,280 x g) for 20 min was analysed. Concentration of the nutrient ingredients, medium pH and incubation conditions were varied to optimize the endoglucanase production. An optimal condition of prior experiment was used as the basis in the latter experiments.

4.4.1.1 Effect of the incubation period

Aspergillus terreus strain 24 was grown in the same above medium composition but incubated for 9 days. Endoglucanase activity in the culture supernatant was analysed daily. As shown in Fig. 4.3A, maximum endoglucanase was produced at 5 days of incubation.

4.4.1.2 Effect of $(NH_4)_2SO_4$ concentration

Aspergillus terreus strain 24 was grown in the cassava fiber medium containing various concentration of $(NH_4)_2SO_4$ (0, 0.2, 0.3, 0.4, 0.6, 0.8, 1.0% w/v) and incubated at the above condition for 5 days. Maximum endoglucanase produced in 0.6% (w/v) $(NH_4)_2SO_4$ was 1.12 units/ml (Fig. 4.3B).

4.4.1.3 Effect of KH_2PO_4 concentration

Aspergillus terreus strain 24 was grown in the cassava fiber medium containing 0.6% (w/v) $(NH_4)_2SO_4$ but KH_2PO_4 concentration was varied (0, 0.1, 0.2, 0.4, 0.6). The culture was incubated at the above condition for 5 days. Endoglucanase production was maximum (1.18 units/ml) in the medium containing 0.2% KH_2PO_4 (Fig. 4.3C).

4.4.1.4 Effect of $MgSO_4{\cdot}7H_2O$ concentration

Aspergillus terreus strain 24 was grown in the cassava fiber medium containing optimal condition of $(NH_4)_2SO_4$ and KH_2PO_4 but various concentration of $MgSO_4{\bullet}7H_2O$ (0, 0.025, 0.05, 0.075, 0.1, 0.125%w/v). The culture was incubated at the above

condition for 5 days. In the cassava fiber medium containing 0.075% (w/v) $MgSO_4 \cdot 7H_2O$, *Aspergillus terreus* strain 24 gave maximum endoglucanase at 1.23 units/ml (Fig. 4.3D).

4.4.1.5 Effect of yeast extract concentration

Aspergillus terreus strain 24 was grown in the cassava fiber medium containing optimal condition of $(NH_4)_2SO_4$, KH_2PO_4, $MgSO_4 \cdot 7H_2O$ but concentration of yeast extract was varied (0, 0.05, 0.1, 0.15, 0.2, 0.25%w/v). After incubation at the above conditions for 5 days, *Aspergillus terreus* strain 24 was found to produce maximum endoglucanase (1.25 units/ml) in the medium containing 0.1% yeast extract (Fig. 4.3E).

4.4.1.6 Effect of medium pH

Aspergillus terreus strain 24 was grown in the cassava fiber medium containing optimal condition of $(NH_4)_2SO_4$, KH_2PO_4, $MgSO_4 \cdot 7H_2O$, yeast extract but pH of the medium was varied (4.5, 5.0, 5.5, 6.0, 6.5, 7.0). After 5 days of incubation at the above conditions, *Aspergillus terreus* strain 24 produced maximum endoglucanase (1.29 units/ml) in the medium which adjusted pH to 5 (Fig. 4.3F).

4.4.1.7 Effect of cassava fiber concentration

Aspergillus terreus strain 24 was grown in the cassava fiber medium containing optimal condition of $(NH_4)_2SO_4$, KH_2PO_4, $MgSO_4 \cdot 7H_2O$, yeast extract but concentration of the cassava fiber was varied (10, 15, 20, 25% w/v), pH 5 was used. *Aspergillus terreus* strain 24 produced maximum endoglucanase (1.29 units/ml) in the medium containing 15% wet w/v cassava fiber after 5 days of incubation at the above conditions (Fig. 4.3G).

4.4.1.8 Effect of incubation temperature

Aspergillus terreus strain 24 was grown in the optimized cassava fiber medium but incubated at various temperature (30, 35, 40, 45°C). *Aspergillus terreus* strain 24 produced maximum endoglucanase (1.29 units/ml) at 30°C (Fig. 4.3H).

Optimal condition of *Aspergillus terreus* strain 24 for endoglucanase production from cassava fiber was cassava fiber (15% wet w/v), $(NH_4)_2SO_4$ (0.6%), KH_2PO_4 (0.2%), $MgSO_4 \cdot 7H_2O$ (0.075%), yeast extract (0.1%), pH 5 after incubation at 30°C (200 rpm) for 5 days. At this condition, *A. terreus* strain 24 produced endoglucanase 1.29 units/ml and β-glucosidase 0.0016 units/ml.

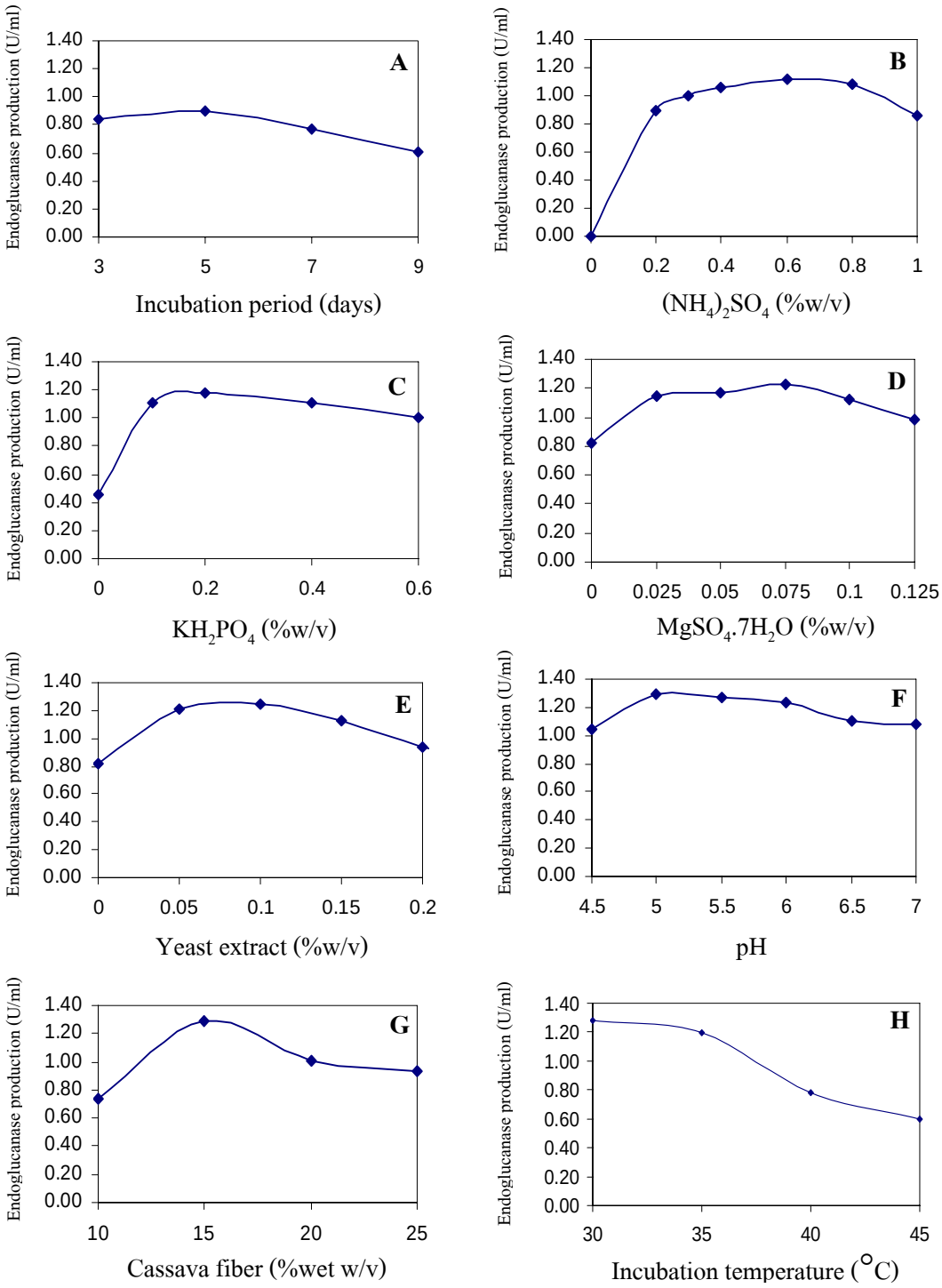


Fig. 4.3 Effect of Incubation period (A), $(NH_4)_2SO_4$ concentration (B), KH_2PO_4 concentration (C), $MgSO_4.7H_2O$ concentration (D), yeast extract concentration (E), pH (F), Cassava fiber concentration (G), Incubation temperature (H) on endoglucanase production from cassava fiber by *Aspergillus terreus* strain 24

4.4.2 Optimization of β-glucosidase production by *Aspergillus niger* strain 127

Aspergillus niger strain 127 was inoculated into 15%(wet w/v) cassava fiber medium containing 0.2% $(NH_4)_2SO_4$, 0.1% KH_2PO_4, 0.025% $MgSO_4 \cdot 7H_2O$, 0.05% yeast extract pH 6.0 at final concentration of 10^7spores/ml, and incubated at 30°C (200 rpm) for 5 days. β-glucosidase activity in the culture supernatant obtained after centrifugation at 4°C, 11,500 rpm (9,280 x g) for 20 min was analysed. Concentration of the nutrient ingredients, medium pH and incubation conditions were varied to optimize the β-glucosidase production. An optimal condition of prior experiment was used as the basis in the latter experiments.

4.4.2.1 Effect of the incubation period

Aspergillus niger strain 127 was grown in the same above medium composition and incubated for 9 days. β-glucosidase activity in the culture supernatant was analysed daily. As shown in Fig. 4.4A, maximum β-glucosidase (0.087 units/ml) was produced at 5 days of incubation.

4.4.2.2 Effect of $(NH_4)_2SO_4$ concentration

Aspergillus niger strain 127 was grown in the cassava fiber medium containing various concentration of $(NH_4)_2SO_4$ (0, 0.2, 0.3, 0.4, 0.6% w/v) and incubated at the above condition for 5 days. Maximum β-glucosidase produced in 0.3% (w/v) $(NH_4)_2SO_4$ was 0.13 units/ml (Fig. 4.4B).

4.4.2.3 Effect of KH_2PO_4 concentration

Aspergillus niger strain 24 was grown in the cassava fiber medium containing 0.3% (w/v) $(NH_4)_2SO_4$ but KH_2PO_4 concentration was varied (0, 0.1, 0.2, 0.4, 0.6, 0.8, 1% w/v). The culture was incubated at the above condition for 5 days. β-glucosidase production was maximum (0.16 units/ml) in the medium containing 0.6% KH_2PO_4 (Fig. 4.4C).

4.4.2.4 Effect of $MgSO_4 \cdot 7H_2O$ concentration

Aspergillus niger strain 127 was grown in the cassava fiber medium containing optimal condition of $(NH_4)_2SO_4$ and KH_2PO_4 but various concentration of $MgSO_4 \cdot 7H_2O$ (0, 0.025, 0.05, 0.075, 0.1, 0.125%w/v). The culture was incubated at the above condition for 5 days. In the cassava fiber medium containing 0.05% (w/v) $MgSO_4 \cdot 7H_2O$, *Aspergillus niger* strain 127 gave maximum β-glucosidase at 0.35 units/ml (Fig. 4.4D).

4.4.2.5 Effect of yeast extract concentration

Aspergillus niger strain 127 was grown in the cassava fiber medium containing optimal condition of $(NH_4)_2SO_4$, KH_2PO_4, $MgSO_4{\cdot}7H_2O$ but concentration of yeast extract was varied (0, 0.05, 0.1, 0.15, 0.2, 0.25. 0.3, 0.35, 0.4% w/v). After incubation at the above conditions for 5 days, *Aspergillus niger* strain 127 was found to produce maximum β-glucosidase (0.89 units/ml) in the medium containing 0.35% yeast extract (Fig. 4.4E).

4.4.2.6 Effect of medium pH

Aspergillus niger strain 127 was grown in the cassava fiber medium containing optimal condition of $(NH_4)_2SO_4$, KH_2PO_4, $MgSO_4{\cdot}7H_2O$, yeast extract but pH of the medium was varied (4.5, 5.0, 5.5, 6.0, 6.5, 7.0). After 5 days of incubation at the above conditions, *Aspergillus niger* strain 127 produced maximum β-glucosidase (0.93 units/ml) in the medium which adjusted pH to 7 (Fig. 4.4F).

4.4.2.7 Effect of cassava fiber concentration

Aspergillus niger strain 127 was grown in the cassava fiber medium containing optimal concentrations of $(NH_4)_2SO_4$, KH_2PO_4, $MgSO_4{\cdot}7H_2O$, yeast extract but concentration of the cassava fiber was varied (10, 15, 20, 25% w/v), pH 7 for 5 days. *Aspergillus niger* strain 127 produced maximum β-glucosidase (0.93 units/ml) in the medium containing 15% (wet w/v) of cassava fiber (Fig. 4.3G).

4.4.2.8 Effect of incubation temperature

Aspergillus niger strain 127 was grown in the optimized cassava but incubated at various temperature (30, 35, 40, 45°C). *Aspergillus niger* strain 127 produced maximum β-glucosidase (0.93 units/ml) at 30°C (Fig. 4.3H).

Optimal condition of *Aspergillus niger* strain 127 for β-glucosidase production from cassava fiber was cassava fiber (15% wet w/v), $(NH_4)_2SO_4$ (0.3%), KH_2PO_4 (0.6%), $MgSO_4{\cdot}7H_2O$ (0.05%), yeast extract (0.35%), pH 7 after incubation at 30°C (200 rpm) for 5 days. At this condition, *A. niger* strain 127 produced β-glucosidase (0.93 units/ml) and endoglucanase (0.042 units/ml).

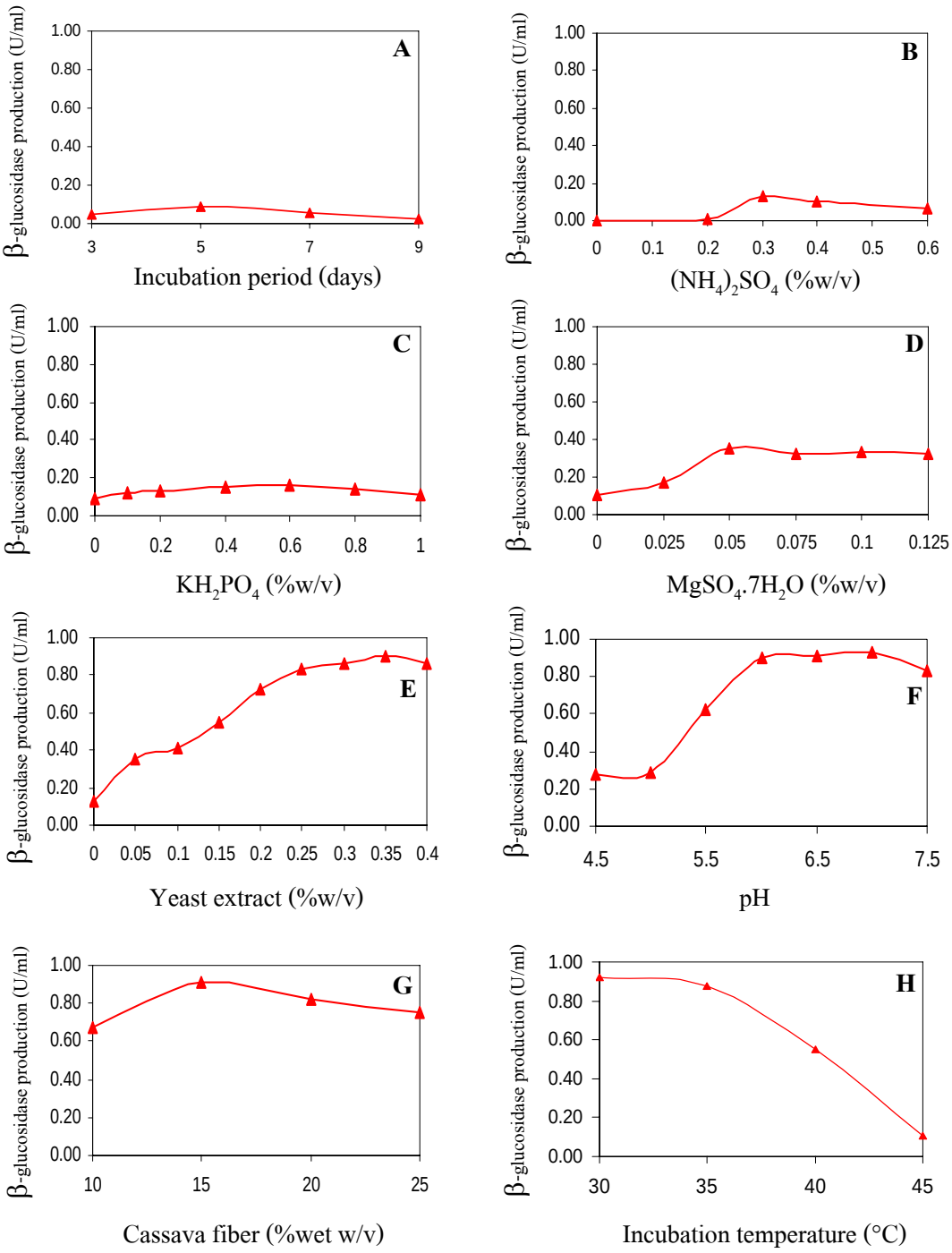


Fig. 4.4 Effect of Incubation period (A), $(NH_4)_2SO_4$ concentration (B), KH_2PO_4 concentration (C), $MgSO_4.7H_2O$ concentration (D), yeast extract concentration (E), pH (F), Cassava fiber concentration (G), Incubation temperature (H) on β-glucosidase production from cassava fiber by *Aspergillus niger* strain 127

4.5 Characterization of *Aspergillus terreus* strain 24 endoglucanase and *Aspergillus niger* strain 127 β-glucosidase

4.5.1 Characterization of *A. terreus* strain 24 endoglucanase

4.5.1.1 Effect of pH on endoglucanase activity

Aspergillus terreus strain 24 endoglucanase was assayed at various pH. Citrate and phosphate buffer (100 mM) at various pH was used for enzyme dilution and substrate preparation. Enzyme reaction mixture was incubated at 30°C for 30 min, and glucose liberated was determined by Somogi-Nelson method. The enzyme showed an optimal pH at 6.0 (Fig. 4.5).

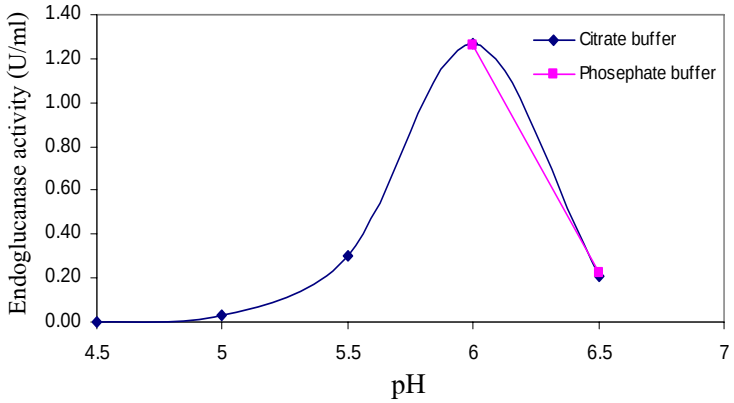


Fig. 4.5 Effect of pH on *Aspergillus terreus* strain 24 endoglucanase activity

4.5.1.2 Effect of temperature on endoglucanase activity

Aspergillus terreus strain 24 endoglucanase was assayed at pH 6 while the enzyme reaction mixtures were incubated at various temperatures for 30 min. As shown in Fig. 4.6, *A. terreus* strain 24 endoglucanase has an optimal temperature at 50°C.

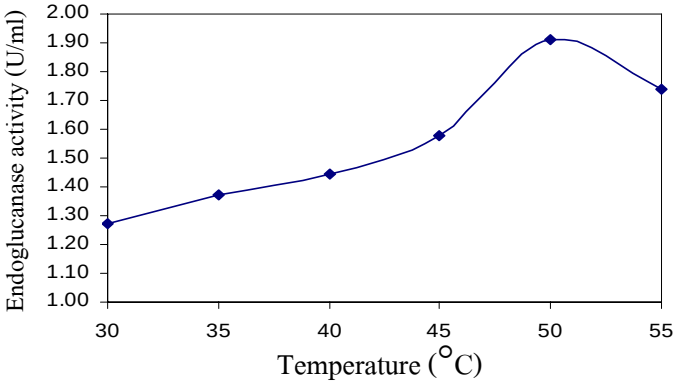


Fig. 4.6 Effect of temperature on *Aspergillus terreus* strain 24 endoglucanase activity

4.5.1.3 Temperature stability of endoglucanase

Aspergillus terreus strain 24 endoglucanase incubated at various temperature for 30 min was assayed for its residual activity at pH 6, 30°C for 30 min. The enzyme was stable up to 45°C. At 40 and 45°C, residual activity was 91.14 and 78.78%, respectively. An activity of non-heated enzyme was set as 100% (Fig. 4.7).

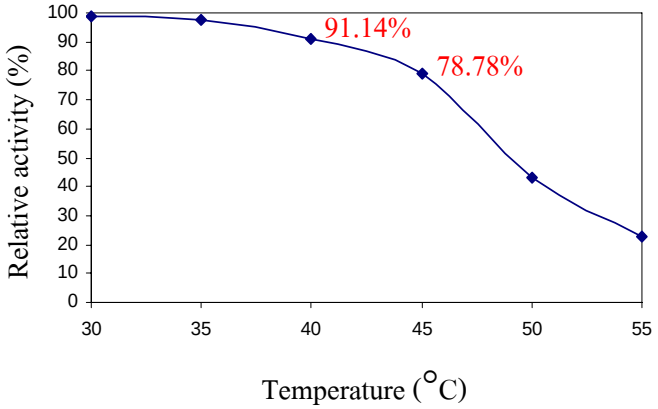


Fig. 4.7 Temperature stability for *Aspergillus terreus* strain 24 endoglucanase activity

4.5.2 Characterization of *A. niger* strain 127 β-glucosidase

4.5.2.1 Effect of pH on β-glucosidase activity

Aspergillus niger strain 127 β-glucosidase was assayed at various pH. Citrate and phosphate buffer (100 mM) at various pH was used for enzyme dilution and substrate preparation. Enzyme reaction mixture was incubated at 30°C for 30 min, and glucose liberated was determined by Somogi-Nelson method. The enzyme showed an optimal pH at 6.0 (Fig. 4.8).

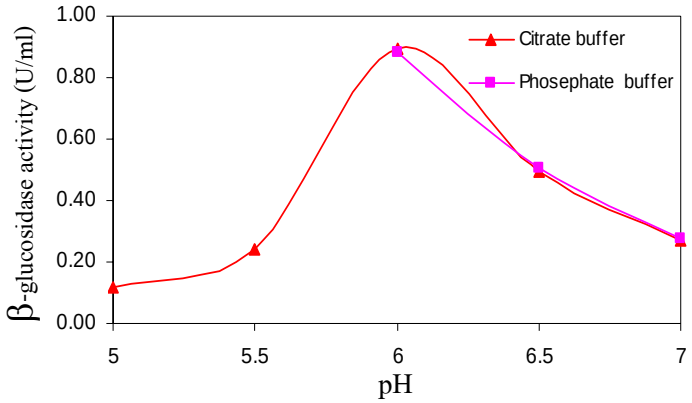


Fig. 4.8 Effect of pH on *Aspergillus niger* strain 127 β-glucosidase activity

4.5.2.2 Effect of temperature on β-glucosidase activity

Aspergillus niger strain 127 β-glucosidase was assayed at pH 6 while the enzyme reaction mixtures were incubated at various temperatures for 30 min. As shown in Fig. 4.9, *A. niger* strain 127 β-glucosidase has an optimal temperature at 55°C.

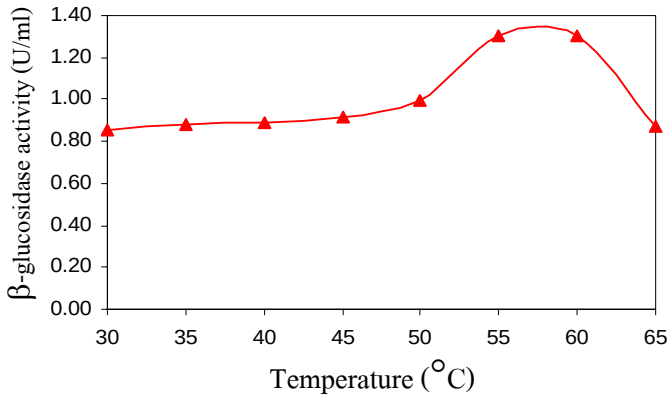


Fig. 4.9 Effect of temperature on *Aspergillus niger* strain 127 β-glucosidase activity

4.5.2.3 Temperature stability of β-glucosidase

Aspergillus niger strain 127 β-glucosidase incubated at various temperature for 30 min was assayed for its residual activity at pH 6, 30°C for 30 min. The enzyme was stable up to 55°C. At 40 and 55°C, residual activity was 93.44 and 82.36%, respectively. An activity of non-heated enzyme was set as 100% (Fig. 4.10).

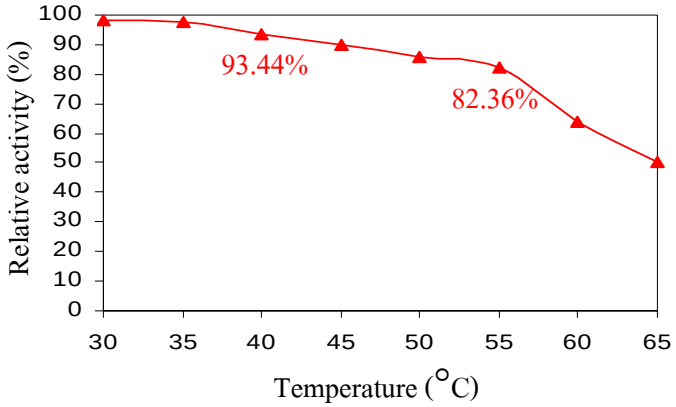


Fig. 4.10 Temperature stability for *Aspergillus niger* strain 127 β-glucosidase activity

4.6 Ethanol production from cassava fiber by simultaneous saccharification and fermentation

4.6.1 Effect of temperature

Cassava fiber obtained after cassava waste was hydrolysed by α-amylase and glucoamylase was used as substrate. The cassava fiber (15% wet w/v) supplemented with 0.2% $(NH_4)_2SO_4$, pH 4.5 was simultaneous saccharified by the enzyme mixture of *A. terreus* strain 24 endoglucanase (endoglucanase (0.85 units/ml), β-glucosidase (0.0016 units/ml)) and *A. niger* strain 127 β-glucosidase (β-glucosidase (0.62 units/ml) and endoglucanase (0.042 units/ml)) and fermented by *S. cerevisiae* at various temperature for 72 h. Endoglucanase: β-glucosidase of the enzyme mixture was 2:1 Endoglucanase and β-glucosidase concentration used to saccharify the cassava fiber was 0.54, 0.25 units/ml or 21.43, 9.88 units/g DS of cassava fiber, respectively. As shown in Fig. 4.11, maximum ethanol (0.64 ± 0.008% v/v) was produced at 40°C.

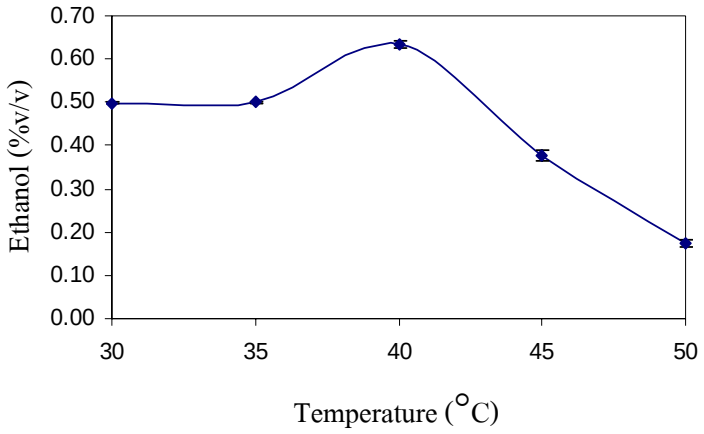


Fig.4.11 Effect of temperature on ethanol production by SSF

4.6.2 Effect of incubation period

In this experiment, the cassava fiber was saccharified at 40°C for 96 h. Every 24 h; ethanol produced was determined. Maximum ethanol (0.64 ± 0.003% v/v) was produced at 72 h of incubation (Fig. 4.12).

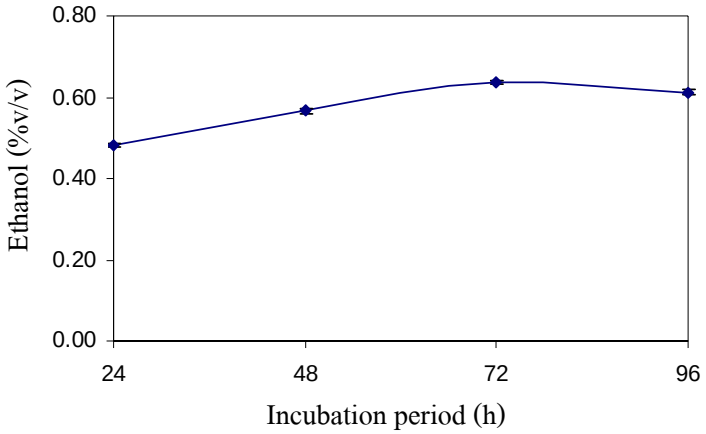


Fig.4.12 Effect of incubation period on ethanol production by SSF

4.6.3 Effect of endoglucanase/β-glucosidase mixture concentration

Various concentration of the cassava fiber was saccharified by the endoglucanase/β-glucosidase mixture at 40°C for 72 h. An optimal enzyme concentration was endoglucanase/β-glucosidase 3.24 units/ 1.5 units per ml or 128.57 units/59.29 units per g DS of cassava fiber. This condition gave 1.07 ± 0.008% v/v of ethanol (Fig. 4.13).

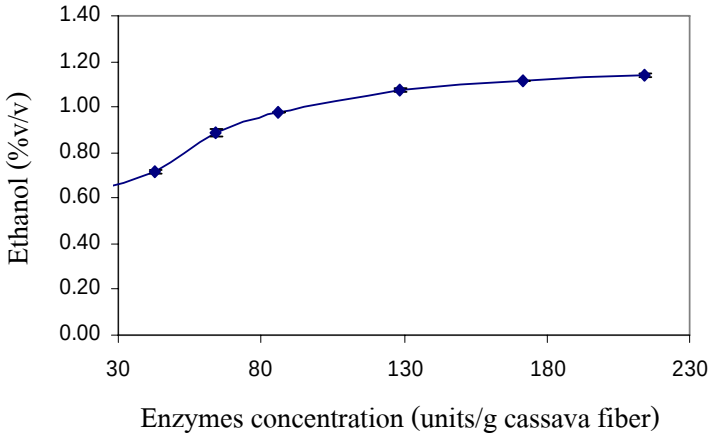


Fig.4.13 Effect of concentration of enzymes mixture on ethanol production by SSF

CHAPTER V

CONCLUSIONS AND DISCUSSIONS

Cassava waste composed of 63% (w/w) starch and 15% (w/w) cellulosic fiber, dry weight basis, was evaluated as substrate for ethanol production. Saccharification of cassava waste (15% w/v, 100 ml) by commercial α-amylase and glucoamylase (Spezyme, USA) using method described by the manufacturer. The hydrolysate was separated from cassava fiber by centrifugation.

The cassava fiber separated was used as carbon source for fungal endoglucanase and β-glucosidase production and as substrate for ethanol production by simultaneous saccharification and fermentation (SSF) method. Thirty-five fungi were screened for cellulase production capability by cultivation on carboxymethyl cellulase agar medium at 30°C for 2 days, then flooded with 1% (w/v) Congo red solution for 1 min and washed by 1N NaCl. Nineteen colonies surrounded by clear zone were selected as cellulase producing fungi. The 19 selected fungi were point inoculated on potato dextrose agar medium and incubated at 30°C for 3 days. Their colonial-peripheral hyphae were cut as agar block, inoculated into cassava fiber medium and incubated at 30°C (200 rpm) for 5 days. Supernatants obtained after centrifugation were quantitatively analyzed for endoglucanase and β-glucosidase. *Aspergillus terreus* strain 24 produced maximum endoglucanase (0.93 units/ml) while *A. niger* strain 127 produced maximum β-glucosidase (0.02 units/ml). Endoglucanase and β-glucosidase productions of the above 2 fungi were confirmed by using their spores at final concentration of 10^7spores/ml as inoculum. *A. terreus* strain 24 produced endoglucanase (0.89 units/ml) and β-glucosidase (0.002 units/ml). *A. niger* strain 127 produced β-glucosidase (0.01 units/ml) and endoglucanase (0.087 units/ml).

Then endoglucanase and β-glucosidase productions of these fungi were optimized by varying concentration of cassava fiber and nutrients, pH, incubation temperature and incubation period. Optimal condition for endoglucanase production of *A. terreus* strain 24 were (15% wet w/v) cassava fiber, 0.6% $(NH_4)_2SO_4$, 0.2% KH_2PO_4, 0.075% $MgSO_4.7H_2O$, 0.1% yeast extract, pH 5 at 30°C (200 rpm) for 5 days. At this condition, *A. terreus* strain 24 produced 1.29 units/ml of endoglucanase and β-glucosidase 0.0016 units/ml β-glucosidase. Optimal condition for β-glucosidase production of *A. niger* strain 127 were (15% wet w/v) cassava fiber, 0.3% $(NH_4)_2SO_4$,

0.6%KH_2PO_4, 0.05% $MgSO_4 \cdot 7H_2O$, 0.35% yeast extract, pH 7 at 30°C (200 rpm) for 5 days. *A. niger* strain 127 produced β-glucosidase (0.93 units/ml) and endoglucanase (0.042 units/ml).

Some properties of the above enzymes were characterized. Optimal pH and temperature of *A. terreus* strain 24-endoglucanase were pH 5 and 30°C. The enzyme was stable up to 40°C. The residual activity at this temperature was 91.14%. Optimal pH and temperature of *A. niger* strain 127-β-glucosidase were pH 6 and 30°C, residual activity of this was 93.44%

Large scale production of *A. terreus* strain 24-endoglucanase and *A. niger* strain 127-β-glucosidase were performed in 10 liter batch reactor using the optimized conditions. The enzymes obtained were mixed to adjust a ratio of endoglucanase and β-glucosidase to 2:1. The enzyme mixture contained endoglucanase (0.54 units/ml or 21.43 units/g DS of cassava fiber) and β-glucosidase (0.25 units/ml or 9.88 units/g DS of cassava fiber) were used to saccharify cassava fiber in SSF ethanol production process.

Simultaneous saccharification and fermentation of cassava fiber to ethanol using *S. cerevisiae* TISTR 5596 was optimized.

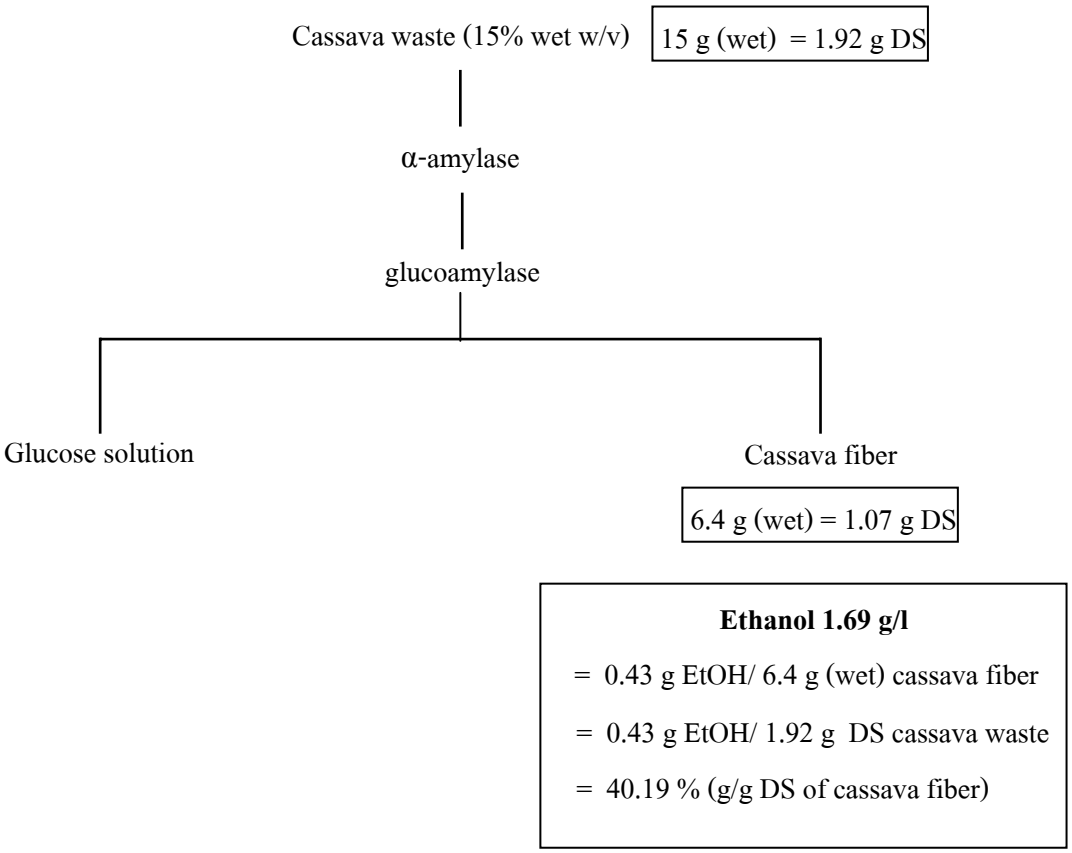
In this work, ethanol 1.07% (v/v) or 40.19% (g/g DS of cassava fiber) was produced from cassava fiber because an advantage of both simultaneous saccharification and fermentation method and a suitable ratio of endoglucanase and β-glucosidase mixture were applied to the system.

Srinorakutara *et al* (2004) produced ethanol 4.58% (v/v) from only starch component of cassava waste (cassava waste hydrolysate). In this experiment ethanol (1.07% v/v) was produced from cassava fiber. Therefore, ethanol production from both cassava fiber and cassava waste hydrolysate is a promising means to increase final ethanol yield from cassava waste by 25%.

Aspergillus niger was found to be a high β-glucosidase producing strain. This result agrees well with Drazic and Noginic (1984) who reported that *Trichoderma reesei* cellulase system was complete but contained low β-glucosidase. *Aspergillus* sp. cellulase system contained high β-glucosidase. Mixture of *T. reesei* and *Aspergillus* sp. cellulase digested corn cob faster than *T. reesei* cellulase or *Aspergillus* sp. cellulase. In 2005, Wen *et al* also reported that mixture of *T. reesei* and *Aspergillus phoenicis* cellulase digested cellulase better than *T. reesei* cellulase alone.

Philippidis (1996) reported that optimal temperature for ethanol production by SSF method was 38°C which was a temperature compromised between optimal temperature of cellulase activity (45-50°C) and *S. cerevisiae* ethanol fermentation (30°C). In general, *S.*

cerevisiae has an optimal temperature for growth and ethanol fermentation at 20-30°C. *S. cerevisiae* TISTR 5596 used in this work was strain improved by TISTR to use in ethanol prototype factory of Thailand. Therefore, it can grow and fermentation ethanol at 40°C. However, there are some reports on *Saccharomyces* that can ferment ethanol at elevated temperature (Ballesteros *et al*., 1991).



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APPENDICES

APPENDIX A

Culture media

1. Potato Dextrose Agar (PDA)

PDA	39	g
DW	1,000	ml

2. Yeast Peptone Dextrose (YPD) Agar

Yeast extract	10	g
Bacto peptone	20	g
Glucose	20	g
Agar	18	g
Distilled water	1000	ml

Adjusted pH 5.0

Sterile by autoclaving at 121ºc, 115 lb/inc ^{2} for 15 min.

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 sugar.

1.1 Somogyi-Nelson Reagent

A. Alkaline Copper Reagent :

- 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 solutions 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 solutions 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 place 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 dilute by adding 5 ml of distilled water.

- Absorbance of samples was measured at 520 nm. Concentrations 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) are prepared in distilled water. Standards of 0, 20, 40, 60, 80, 100, 120, 150, 180 and 200 µg/ml were prepared from glucose solution. The reactions were carried out with the same procedure as described previously. Absorbances were plotted against concentrations of standards.

1.4 Calculation of reducing sugar

Formula Reducing sugar (g/l) = $\frac{A_{520} \text{ x dilution}}{\text{Slope}}$

2. 0.1 M Citrate buffer pH 5.0

Citric acid monohydrate (0.05 M)	10.51	g/l
Trisodium citrate dehydrate (0.05 M)	14.71	g/l

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

3. 0.1 M Citrate buffer pH 6.0

Citric acid monohydrate (0.05 M)	10.51	g/l
Trisodium citrate dehydrate (0.05 M)	14.71	g/l

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

4. 0.1 M Phosphate buffer pH 6.0

di-Sodium hydrogen phosphate (0.05 M)	17.91	g/l
Sodium dihydrogen phosphate (0.05 M)	7.803	g/l

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

5. 0.2% Tween 80

Tween 80	2	g
Distilled water	1000	ml

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

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. 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.

9. 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.

10. Calculation of enzyme unit

One unit of 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

Endoglucanase and β-glucosidase activity **:**

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

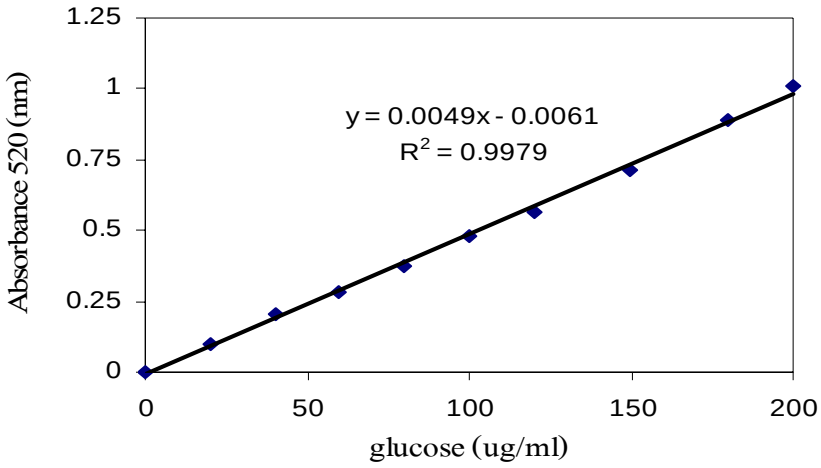
$$\text{Glucose released X mg within 30 min} = \frac{(X) \times 0.185}{0.5} \text{ unit}$$

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

APPENDIX C

Standard curve of glucose

1. Standard curve of glucose



BIOGRAPHY

Miss Woraphan Pim-iam was born in October 26, 1983 in Bangkok, Thailand. She graduated from Department of Microbiology, Faculty of Science, Chulalongkorn University, Thailand with Bachelor Degree of Science since 2005.

Academic presentation :

1. Woraphan Pim-iam, Natchanan Leepipatpiboon, Teerapat Srinarakutara and Ancharida Akaracharanya. 2007. Evaluation of dilute-acid pretreated rice straw as substrate for ethanol production. TSB2007: Biotechnology for Gross National Happiness. October 9-12, 2007 at Thammasat University, Pathumthani.