# ศักยภาพของเซลลูเลสจากราที่แยกได้ในประเทศไทยเพื่อผลิตเอทานอลจากซานอ้อย

นางสาวเบญจพร บัวบาน

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# POTENTIAL OF CELLULASES FROM THAI ISOLATED FUNGI FOR ETHANOL PRODUCTION FROM BAGASSE

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A Dissertation Submitted in Partial Fulfillment of the Requirements
for the Degree of Doctor of Philosophy Program in Biotechnology
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กากชานอ้อยเป็นวัสดุเหลือทิ้งทางการเกษตรที่น่าสนใจในการนำมาใช้เพื่อการผลิตเอทานอล งานวิจัยในครั้งนี้ได้ศึกษาการผลิตเอทานอลจากชานอ้อยโดยใช้วิธีบดด้วยลูกเหล็กเพื่อทำลายโครงสร้างของชานอ้อยเบื้องต้นก่อน จากนั้นนำมาย่อยโดยเอนไซม์จำเพาะและทำการหมักด้วยยีสต์เพื่อให้ได้เอทานอล จากการศึกษาพบว่าการบดชานอ้อยด้วยลูกเหล็กนาน 2 ชั่วโมง สามารถทำลายโครงสร้างของเซลลูโลสในชานอ้อยให้มีโครงสร้างที่ง่ายต่อการย่อยโดยเอนไซม์ได้อย่างมีประสิทธิภาพ ชานอ้อยที่ผ่านการบดด้วยลูกเหล็กถูกนำมาย่อยต่อโดยเอนไซม์ผสมที่ผลิตจากเชื้อรา *Penicillium chrysogenum* BCC 4504 ซึ่งแสดงกิจกรรมของเอนไซม์เซลลูเลส และเชื้อรา *Aspergillus flavus* BCC 7179 ซึ่งแสดงกิจกรรมของเอนไซม์ไซลาเนสและเบต้ากลูโคสิเดส เอนไซม์ผสมดังกล่าวสามารถย่อยชานอ้อยที่ผ่านการบดด้วยลูกเหล็กให้น้ำตาลกลูโคส 84 เปอร์เซนต์ และน้ำตาลไซโลส 70.4 เปอร์เซนต์ ที่อุณหภูมิ 45 องศาเซลเซียส ความเป็นกรดด่าง 5.0 นาน 72 ชั่วโมง ซึ่งปริมาณน้ำตาลที่ได้มีค่าสูงอย่างมีนัยสำคัญกว่าการใช้เอนไซม์ผสมทางการค้าระหว่าง Acremonium cellulase และ Optimash BG จากนั้นนำน้ำตาลที่ได้มาผลิตเอทานอลแบบหมักแยกระบบ (SHF) โดยยีสต์ *Pichia stipitis* BCC 15191 ที่ความเป็นกรดด่าง 5.5 อุณหภูมิ 30 องศาเซลเซียส นาน 24 ชั่วโมง (ระยะเวลารวมทั้งหมด 96 ชั่วโมง) ได้เอทานอล 10.7 กรัมต่อลิตร ซึ่งเทียบได้กับ 0.36 กรัมเอทานอลต่อกรัมน้ำตาลกลูโคสและไซโลส ส่วนการผลิตเอทานอลแบบหมักรวมระบบ (SSF) ได้เอทานอล 10.1 กรัมต่อลิตร เมื่อทำการหมักนาน 72 ชั่วโมง ที่สภาวะการผลิตเดียวกัน งานวิจัยนี้ได้นำเสนอความเป็นไปได้ในการผลิตเอทานอลจากชานอ้อย โดยการทำลายโครงสร้างของชานอ้อยด้วยวิธีทางกลซึ่งเป็นวิธีที่ไม่ใช้สารเคมีและเป็นมิตรสิ่งแวดล้อมร่วมกับการย่อยโดยใช้เอนไซม์ผสมซึ่งผลิตจากเชื้อราที่สามารถใช้วัสดุเหลือทิ้งทางการเกษตรเป็นสับสเตรทในการผลิตเอนไซม์ได้ และทำการหมักเพื่อให้ได้เอทานอลโดยเชื้อยีสต์ที่มีประสิทธิภาพ

สาขาวิชา .....เทคโนโลยีชีวภาพ..... ลายมือชื่อนิสิต.....

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KEYWORDS : ball milling pretreatment / ethanol / lignocellulolytic enzymes / *Pichia stipitis* / sugarcane bagasse

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Sugarcane bagasse is one of the most promising agricultural by-products for conversion to biofuels. Here, ethanol fermentation from bagasse has been achieved using an integrated process combining mechanical pretreatment, enzymatic hydrolysis and fermentation. Pretreatment by ball milling for 2 h was sufficient for nearly complete cellulose structural transformation to an accessible amorphous form. The pretreated cellulosic residues were hydrolyzed by a crude enzyme preparation from *Penicillium chrysogenum* BCC 4504 containing cellulase activity combined with *Aspergillus flavus* BCC 7179 preparation containing complementary β-glucosidase activity. Saccharification yields of 84.0% and 70.4% for glucose and xylose, respectively, were obtained after hydrolysis at 45°C, pH 5 for 72 h, which were significantly greater than those obtained with a commercial enzyme mixture containing Acremonium cellulase and Optimash BG. A high conversion yield of undetoxified pretreated bagasse (5%, w/v) hydrolysate to ethanol was attained on separate hydrolysis and fermentation process using *Pichia stipitis* BCC 15191, at pH 5.5, 30°C for 96 h resulting in an ethanol concentration of 10.7 g/L, corresponding to a conversion yield of 0.36 g ethanol/g available fermentable sugars. Comparable ethanol conversion efficiency was obtained by a simultaneous saccharification and fermentation process which led to production of 10.1 g/L ethanol after 72 h fermentation under the same conditions. This study thus demonstrated the potential use of a simple integrated process with minimal environmental impact with the use of promising alternative on-site enzymes and yeast for the production of ethanol from this potent lignocellulosic biomass.

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| | |
|---|---|
| BCC | BIOTEC Culture Collection |
| °C | degree Celsius |
| CFU | colony forming unit |
| cm | centimeter |
| FPU | filter paper unit |
| GOD | glucose oxidase |
| g | gram |
| h | hour |
| IU | international unit |
| IUPAC | The international Union of Pure and Applied Chemistry |
| kHz | kilohertz |
| kV | kilovolt |
| L | liter |
| M | molar |
| mg | milligram |
| min | minute |
| ml | milliliter |
| mM | millimolar |
| mm | millimeter |
| μm | micrometer |
| μmole | micromole |
| rpm | rounds per minute |
| sec | second |
| sub | substrate |
| TISTR | Thailand Institute of Scientific and Technological Research |
| TSS | total suspended solid |
| w/v | weight by volume |

# CHAPTER I

# INTRODUCTION

Ethanol produced from lignocellulosic biomass has received considerable attention in recent years. Using ethanol as a gasoline fuel additive as well as a transportation fuel helps alleviate global warming and environmental pollutions. In the last decade, most research has tended to focus on developing economical and environmental friendly ethanol production processes, which are based on enzymatic hydrolysis of cellulose and hemicelluloses using abundant renewable lignocellulosic biomass such as industrial wastes, agricultural residues and municipal solid wastes (Sanchez and Cardona, 2008). The enzymatic hydrolysis is mainly carried out with a highly specific cellulase system that avoids degradation product formation and results in potentially high sugar yields at ambient conditions than the traditional diluted or concentrated acid based technology. However, the cost of the cellulases is the most important cost factors in ethanol production processes. In addition, in the enzymatic process, a pretreatment of the lignocellulosic biomass is required to increase the accessibility of cellulose and hemicellulose to the enzymes. Fermentation of the available sugars in lignocellulosic biomass by yeast presents a unique challenge because of the presence of other sugars such as xylose and arabinose (pentose sugars). Toxic wastes generated during pretreatment incur additional problems that are not seen in the current corn to ethanol process.

In order to develop a cost effective process for cellulosic ethanol production, the concept of local community-based ethanol production has been introduced in Thailand. This involves the use of on-site enzyme production using local fungal isolates and fermentation process development with local yeast strains. In this investigation, the assessment of a novel on-site enzyme preparation on ball milled bagasse is reported in combination with the environmental friendly pretreatment process. The enzymatic saccharification process was used in separate hydrolysis and fermentation (SHF) and

simultaneous saccharification and fermentation (SSF) processes for evaluating the convertibility of the cellulose and hemicellulose contained in the substrate to ethanol. The work thus demonstrated the potential of this alternative process for production of cellulosic ethanol from bagasse.

## Objectives

1. To select for fungi producing high yield of cellulases in bagasse medium
2. To optimize the enzyme production by submerged culture fermentation
3. To compare the mechanical pretreatment technologies (ball milling and sonication) for bagasse
4. To determine the most efficient process for ethanol production from bagasse

# CHAPTER II

# LITERATURE REVIEW

## 2.1 Renewable energy

Energy consumption has increased steadily over the last century as the world population has grown and more countries have become industrialized. Crude oil has been the major resource to meet the increased energy demands. Because the economy in most of the countries depends on oil, the consequences of inadequate oil availability could be severe. Therefore, there is a great interest in exploring alternative energy sources. Unlike fossil fuels, ethanol is a renewable energy source produced through fermentation of sugars. Ethanol is widely used as a partial gasoline replacement in the United States. Using ethanol-blended fuel for automobiles can significantly reduce petroleum use and greenhouse gas exhaust. However, the cost of ethanol as an energy source is relatively high compared to fossil fuels. A dramatic increase in ethanol production using the current corn starch-based technology may not be practical because corn production for ethanol will compete for the limited agricultural land needed for food and feed production.

A potential source for low-cost ethanol production is lignocellulosic materials, because they are cheap and available in large quantities. In general, prospective lignocellulosic materials for fuel ethanol production can be divided into six groups: crop residues (cane bagasse, corn stover, wheat straw, rice straw, rice hulls, barley straw, sweet sorghum bagasse, olive stones and pulp), hardwood (aspen, poplar), softwood (pine, spruce), cellulose wastes (newsprint, waste office paper, recycled paper sludge), herbaceous biomass (alfalfa hay, switchgrass, reed canary grass, coastal Bermudagrass, Thimothy grass) and municipal solid wastes (Sanchez and Cardona, 2008). Sugarcane bagasse is one of the largest lignocellulosic agro-industrial by-products. It is a lignocellulosic residue of the sugar industry and is almost completely used up by the sugar factories themselves as fuel for boilers (Pandey *et al*., 2000). In

recent years, there has been an increasing trend towards more efficient utilization of sugarcane bagasse. The various production process used to utilizing bagasse as a substrate include production of chemicals and metabolites such as alcohol and alkaloids, mushroom cultivation, single cell protein and enzymes production.

## 2.2 Lignocellulosic feedstocks

In general, lignocellulosic biomass consists of three major compounds: cellulose, hemicelluloses and lignin, which form a complex structure where the cellulose fibers are embedded in sheaths of hemicelluloses and lignin, and held together by hydrogen and van der Waals bonds (Figure 2.1). The composition and proportions of these compounds vary between plants (Sanchez, 2009) (Table 2.1).

Cellulose is a homopolymer of β-1, 4 linked-glucose units, and its chains constitute various crystal forms by hydrophobic and van der Waals interactions in a cellulose microfibril. When the chains are less ordered, cellulose exists in an amorphous form, and this part of cellulose is more accessible to hydrolytic attack by cellulases. Hemicellulose is a branched chain polysaccharide that consists of the pentose D-xylose and L-arabinose, and the hexose D-glucose, D-mannose, and D-galactose and their acetylated derivatives. Hemicellulose does not form a crystalline region and thus is relatively easy to hydrolyze to sugars. However, the pentose sugars are not easily fermented to ethanol. Lignin is a phenyl-propane polymer with the phenyl propane units held together by ether and carbon-carbon bonds, and it cannot be easily degraded. In addition, lignin prevents accessibility of enzymes to cellulose and absorbs the enzyme components, especially β-glucosidase (Fengel and Wegener, 1984).

**Table 2.1** Composition of some lignocellulosic materials.

| Lignocellulosic residues | Lignin (%) | Hemicellulose (%) | Cellulose (%) | Ash (%) |
|---|---|---|---|---|
| Hardwood stems | 18-25 | 24-40 | 40-55 | Not available |
| Softwood stems | 25-35 | 25-35 | 45-50 | Not available |
| Nut shells | 30-40 | 25-30 | 25-30 | Not available |
| Corn cobs | 15 | 35 | 45 | 1.36 |
| Paper | 0-15 | 0 | 85-99 | 1.1-3.9 |
| Rice straw | 18 | 24 | 32.1 | Not available |
| Sorted refuse | 20 | 20 | 60 | Not available |
| Leaves | 0 | 80-85 | 15-20 | Not available |
| Cotton seeds hairs | 0 | 15-20 | 80-95 | Not available |
| Newspaper | 18-30 | 25-40 | 40-55 | 8.8-1.8 |
| Waste paper from chemical pulps | 5-10 | 10-20 | 60-70 | Not available |
| Primary wastewater solids | 24-29 | Not available | 8-15 | Not available |
| Swine waste | Not available | 28 | 6 | Not available |
| Solid cattle manure | 2.7-5.7 | 1.4-3.5 | 1.6-4.7 | Not available |
| Coastal Bermuda grass | 6.4 | 35.7 | 25 | Not available |
| Switch grass | 12.0 | 31.4 | 45 | Not available |
| S32 rye grass (early leaf) | 2.7 | 15.8 | 21.3 | Not available |
| S32 rye grass (seed setting) | 7.3 | 25.7 | 26.7 | Not available |
| Orchard grass (medium maturity) | 4.7 | 40 | 32 | Not available |
| Grasses (average values for grasses) | 10-30 | 25-50 | 25-40 | 1.5 |
| Sugar cane bagasse | 19-24 | 27-32 | 32-44 | 4.5-9 |
| Wheat straw | 16-21 | 26-32 | 29-35 | Not available |
| Barley straw | 14-15 | 24-29 | 31-34 | 5-7 |
| Oat straw | 16-19 | 27-38 | 31-37 | 6-8 |
| Rye straw | 16-19 | 27-30 | 33-35 | 2-5 |
| Bamboo | 21-31 | 15-26 | 26-43 | 1.7-5 |
| Grass Esparto | 17-19 | 27-32 | 33-38 | 6-8 |
| Grass Sabai | 22.0 | 23.9 | Not available | 6.0 |
| Grass Elephant | 23.9 | 24 | 22 | 6 |
| Leaf Fiber Abaca (Manila) | 8.8 | 17.3 | 60.8 | 1.1 |
| Leaf Fiber Sisal (agave) | 7-9 | 21-24 | 43-56 | 0.6-1.1 |
| Leaf Fiber Henequen | 13.1 | 4-8 | 77.6 | 0.6-1 |
| Coffee pulp | 18.8 | 46.3 | 35 | 8.2 |
| Banana waste | 14 | 14.8 | 13.2 | 11.4 |
| Yuca waste | Not available | Not available | Not available | 4.2 |

**Source:** Sanchez (2009).

Bagasse consists of approximately 50% cellulose and 25% each of hemicellulose and lignin. Chemically, bagasse contains about 50% α-cellulose, 30% pentosans, and 2.4% ash (Pandey *et al*., 2000). Because of its low ash content, bagasse offers numerous advantages in comparison to other crop residues such as rice straw and wheat straw, which have 17.5% and 11.0% ash contents, respectively, for usage in bioconversion processes using microbial cultures. Also, in comparison to other agricultural residues, bagasse can be considered as a rich solar energy reservoir due to its high yields (about 80 t/ha in comparison to about 1, 2, and 20 t/ha for wheat, other grasses and trees, respectively) and annual regeneration capacity (Pandey *et al*., 2000). Naturally, cellulose is associated with hemicellulose and surrounded by lignin seal which is resistant to enzymatic attack. Crystallinity of the cellulose further represents an extra obstacle to hydrolysis. Therefore, the pretreatment process is required to break the lignin seal and disrupt the crystalline structure of cellulose to make cellulose more accessible to the enzymes that convert the carbohydrate polymers into fermentable sugars (Sun and Cheng, 2002).

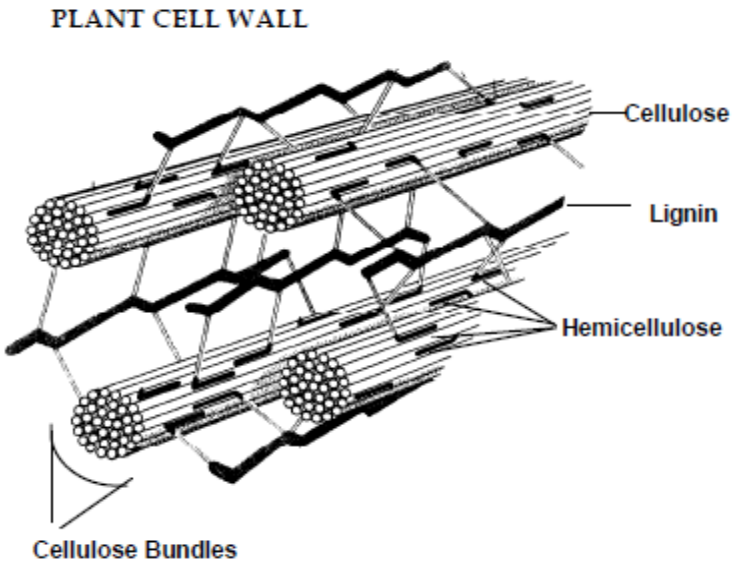



**Figure 2.1** Typically plant cell wall arrangement (Murphy and McCarthy, 2005).

## 2.3 Cellulase production

Commercialization of ethanol production from lignocellulosic biomass is hindered mainly by the prohibitive cost of the currently available cellulase preparation. Reduction in the cost of cellulases can be achieved only by concerted efforts which address several aspects of enzyme production from raw materials used for production to microbial strain improvement. Use of cheaper raw materials can improve the economics of cellulase production. The use of low cost substrates such as agricultural wastes has been suggested as an alternative to reduce the production costs.

Several processes have been reported for the production of enzymes, ethanol and single-cell protein (SCP) using whole-bagasse or treated-bagasse in submerged fermentation (Pandey *et al*., 2000). One of the most widely studied aspects of bagasse application has been on cellulase production. A large number of microorganisms including bacteria, yeasts and fungi have been extensively studied for cellulase production on bagasse. However, fungi are the preferred choice and have been most widely been employed due to their simple aerobic growth conditions and moderate to high production rate. Fungi that have been reported to produce cellulases include *Sclerotium rolfsii*, *Penicillium chrysosporium* and species of *Trichoderma*, *Aspergillus*, *Schizophyllum* and *Penicillium* (Pandey *et al*., 2000). Of all these fungal genera, *Trichoderma* sp. has been most extensively studied for cellulase production (Sun and Cheng, 2002). *Trichoderma reesei* QM-9414 has been employed for cellulase and biomass production from bagasse (Aiello *et al*., 1996).

The operating conditions of fermentation need to be optimized for maximum production in industrial technology. Several approaches have been used for experimental design of process optimization. One-factor-at-a-time (OFAT) method, which involves changing one variable while fixing the others at a certain level (Xu *et al*., 2003) is not relatively widely used. This is because OFAT is rather laborious and time consuming, especially for large number of variables. These limitations of a single factor optimization process can be overcome by using statistical optimization through

fractional factorial design with considering all interactions effects of the factors (Kumar and Satyanarayana, 2007; Khambhatya *et al*., 2007). A two-level fractional factorial design (FFD) was used to determine the effects of six factors, i.e. substrate (domestic wastewater sludge) and co-substrate concentration (wheat flour), temperature, initial pH, inoculum size and agitation rate on the production of cellulase by *Trichoderma harzianum* in liquid state bioconversion. On statistical analysis of the results from the experimental studies, optimum process conditions were found and cellulase activity reached 10.2 FPU/ml at day 3 during the fermentation process which indicated about 1.5-fold increase in production compared to the cellulase activity obtained from the results of design of experiment (Alam *et al*., 2008). This method therefore allows variation of several factors of interest simultaneously and has been used effectively for determination of optimal conditions for enzyme production.

### 2.4 Processes for ethanol production

The process of bioconversion of lignocellulosic biomass to ethanol includes four main steps: pretreatment, enzymatic hydrolysis, fermentation and distillation-rectification-dehydration (Figure 2.2).

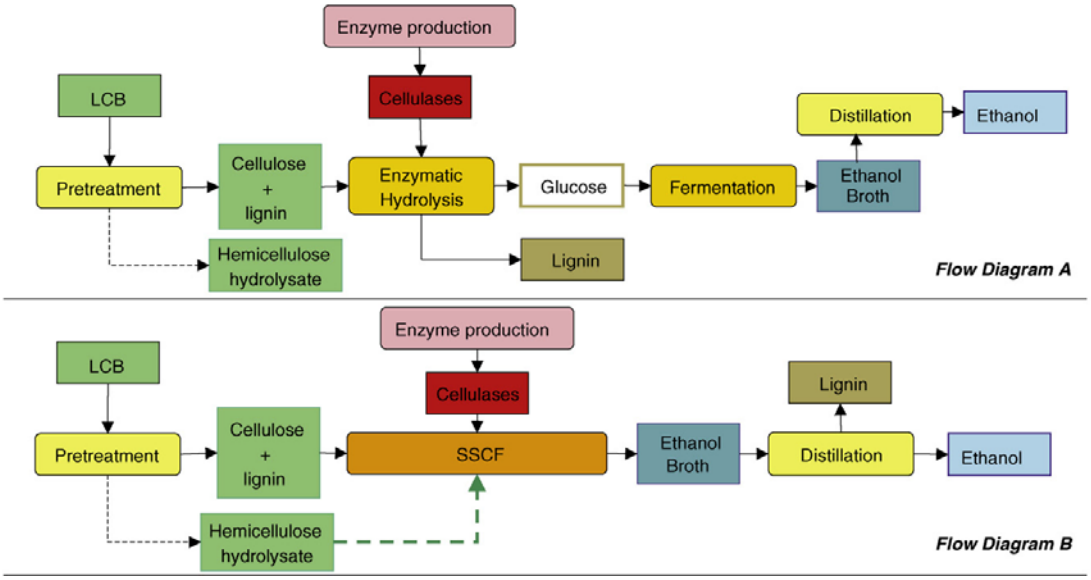



**Figure 2.2** Flow diagram of ethanol production from lignocellulosic biomass (Margeot *et al.*, 2009). Flow diagram A, conventional process (SHF); flow diagram B, simultaneous saccharification and co-fermentation process (SSCF); LCB, lignocellulosic biomass.

### 2.4.1 Pretreatment

Pretreatment has often been found useful to improve accessibility and digestibility of microbial enzyme. The pretreatment resulting in enlargement of the inner surface area of substrate particles is accomplished by partial solubilization and/or degradation of hemicellulose and lignin. This leads to the fractionation of the three components and opening of cellulose structure (Figure 2.3). Several physical and chemical methods are employed for the pretreatment. The choice of pretreatment for lignocellulosic biomass has a major influence on the subsequence enzymatic hydrolysis. Addition of inexpensive chemicals such as acids, bases or organic solvents in pretreatment process is known to have a significant effect on enzymatic hydrolysis. These also give rise to non-sugar composition in the hydrolysate such as furfurals, ferulate and acetate, which may have deleterious effect on fermentative microorganisms (Eggeman and Elander, 2005). These chemicals require corrosion-resistant reactors and must be neutralized or recycled to reduce their negative impacts on the environment (Sun and Cheng, 2002).

The physical pretreatment methods already investigated for bagasse, including steam explosion (Kaar *et al*., 1998) and liquid hot water pretreatment (Laser *et al*., 2002). Ball milling, a mechanical comminution, is regarded as environmentally benign processes because no chemical are used. Furthermore, reduction of crystallinity of cellulose by ball milling or a compression milling treatment has been reported to increase the enzymatic digestibility of various lignocellulosic substrates (Tassinari *et al*., 1980). Vibratory ball milling has been found more effective in breaking down the cellulose crystallinity of spruce and aspen chips and improving the digestibility of the biomass than ordinary ball milling (Millet *et al*., 1979). The maximal sugars yield (more than 80%) of glucose and xylose were achieved from eucalyptus and rice straw after using ball milling pretreatment at the optimal operating time (Inoue *et al*., 2008; Hideno *et al*., 2009).

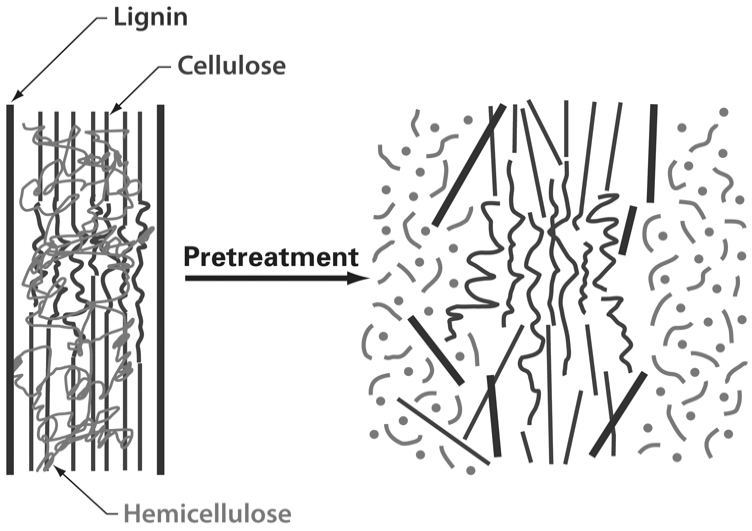



**Figure 2.3** Schematic represent on biomass pretreatment (Mosier et al., 2005).

### 2.4.2 Enzymatic hydrolysis

All major pretreatment methods require a subsequent enzymatic hydrolysis step to achieve high glucose yield for ethanol production. Enzymatic hydrolysis of lignocellulosic biomass is mainly carried out by cellulases. At least three major groups of cellulases are involved in the hydrolysis process: (1) endoglucanase

(endo-1,4-β-D-glucanohydrolase, or EC 3.2.1.4.), which attacks regions of low crystallinity in the cellulose fiber, creating free chain ends; (2) exoglucanase or cellobiohydrolase (EC 3.2.1.91.), which degrades the molecule further by removing cellobiose units from the free chain ends; (3) β-glucosidase (EC 3.2.1.21), which hydrolyzes cellobiose to produce glucose (Coughlan and Ljungdahl, 1988). The mechanism of cellulose hydrolysis is shown in Figure 2.4.

The enzymes occur in multiple forms in enzyme preparation and maximal cellulase activity requires multiple, related enzymes such as endoglucanase, exoglucanase and β-glucosidase to act synergistically for complete conversion of cellulose into glucose. There are also a number of subordinate enzymes that attack hemicellulose, such as xylanase and β-xylosidase whose conjugated action with cellulases results in a higher final sugar production comparing with the use of cellulase alone (Duff and Murray, 1996).

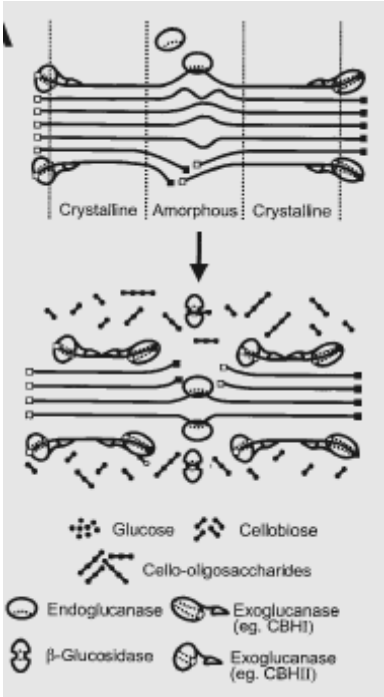



**Figure 2.4** Schematic representation of the hydrolysis of amorphous and microcrystalline cellulose by cellulase systems (Lynd *et al*., 2002).

The cellulases are produced by a large number of microorganisms including bacteria, yeasts and fungi. Among these microorganisms, fungi are the preferred choice for enzyme production and protein enrichment and have most widely been employed. Filamentous fungi, typically *Trichoderma* species, are the preferred source of industrial cellulase preparations because their capacity for extracellular protein production is greater than that of cellulolytic bacteria (Berlin *et al*., 2005b). Most of the commercial cellulases are obtained aerobically from *Trichoderma reesei*, which releases a mixture of cellulases, among which at least two cellobiohydrolases, five endoglucanases, β-glucosidases and hemicellulases can be found (Zhang and Lynd, 2004). A small portion of commercially available cellulases are produced from *Aspergillus niger* (Sanchez and Cardona, 2008).

Although *T. reesei* produces some β-glucosidases, which are responsible for hydrolyzing cellobiose into two molecules of glucose, their activities are not sufficiently high. The remaining cellobiose directly inhibited the activity of cellobiohydrolase. For this reason, β-glucosidase from other sources need to be added in order to complement the action of this fungal cellulases. Use of enzyme mixture containing either cellulase alone or cellulase and other enzymes in the hydrolysis of cellulosic materials has been extensively studied. A mixture of hemicellulases or pectinases with cellulases exhibited a significant increase in the extent of cellulose conversion (Ghose and Bisaria, 1979; Beldman *et al*., 1984). The enzymes mixtures could be customized for individual type of feedstocks and pretreatments in order to optimize hydrolysis (Berlin *et al*., 2005a; Eggeman and Elander, 2005). A cellulose conversion yield of 90% was achieved in the enzymatic saccharification of 8% alkali-treated sugarcane bagasse when a mixture of cellulases (1.0 FPU/g substrate) from *A. ustus* and *T. viride* was used (Mononmani and Sreekantiah, 1987). The ability of a commercial *T. reesei* cellulase preparation (Celluclast 1.5L) to hydrolyze the cellulose and xylan components of dilute acid-pretreated corn stover was significantly improved by supplementation with three types of crude commercial enzyme preparations

nominally enriched in xylanase, pectinase and β-glucosidase activity (Berlin *et al*., 2007).

### 2.4.3 Fermentation process

The classic configuration employed for fermenting biomass hydrolysates involves a sequential process where the hydrolysis of cellulose and the fermentation are carried out in different units. This configuration is known as separate hydrolysis and fermentation (SHF). In the alternative variant, the simultaneous saccharification and fermentation (SSF), the hydrolysis and fermentation are performed in a single unit.

Ethanol production from bagasse has mainly been investigated in SHF set-ups and frequently by fermenting a detoxified hemicellulose hydrolysate (van Zyl *et al*., 1988; Roberto *et al*., 1991; Gong *et al*., 1993; Asghari *et al*., 1996; Martin *et al*., 2002). SSF of bagasse has so-far only been investigated using bacteria (Doran *et al*., 1994) and recombinant xylose fermenting yeast (Rudolf *et al*., 2008).

#### 2.4.3.1 Separate hydrolysis and fermentation (SHF)

When sequential process is utilized, solid fraction of pretreated lignocellulosic material undergoes hydrolysis (saccharification). Once hydrolysis is completed, the resulting cellulose hydrolysate is fermented and converted into ethanol. One of the main features of the SHF process is that each step can be performed at its optimal operating conditions. The most important factors to be taken into account for saccharification step are reaction time, temperature, pH, enzyme dosage and substrate load (Sanchez and Cardona, 2008).

In sugar cane leaves, the best values of all these parameters (reaction time, temperature, pH, enzyme dosage and substrate load) varying in each experimental series which the value of one of the factors fixing the other ones. Approximately, 65–70% cellulose conversion was achieved at 50°C and pH of 4.5. Although enzyme doses 100 FPU/g cellulose caused almost a 100% hydrolysis, this

amount of cellulases is not economically justifiable. Hence, 40 FPU/g cellulose dosage was proposed obtaining only 13% reduction in conversion. Regarding the substrate concentration, solids loads of 10% was defined as the most adequate considering arising mixing difficulties and accumulation of inhibitors in the reaction medium (Krishna *et al*., 1998). The monomeric sugars were obtained using an enzymatic cocktail of the cellulase mixture Celluclast 2L (75 FPU/g and 12 Cellobiose IU/g) and the β-glucosidase preparation Novozyme 188 (392 Cellobiose IU/g) for saccharification of sugarcane bagasse pretreated by steam explosion. An ethanol yield of the detoxified hydrolysate 0.18 g/g dry bagasse was achieved in the subsequent fermentation using a xylose-utilising *Saccharomyces cerevisiae* (Martin *et al*., 2002).

#### 2.4.3.2 Simultaneous saccharification and fermentation (SSF)

The SSF process shows more attractive indexes than the SHF because of its higher ethanol yields and less energetic consumption. In this case, the cellulases and microorganisms are added to the same processing unit allowing the glucose formed during the enzymatic hydrolysis of cellulose to be immediately consumed by the microbial cells and is converted into ethanol. Thus, the inhibition effect caused by the sugars over the cellulases is neutralized. However, the need of employing more dilute media to reach suitable rheological properties makes the final product concentration diminished. In addition, this process operates at non-optimal conditions for hydrolysis and therefore requires higher enzyme dosage. This positively influences on substrate conversion, but negatively on process costs (Sanchez and Cardona, 2008).

It was reported that the optimal conditions of the SSF of sugar cane leaves, as it was for the SHF, were 40°C and pH 5.1 for 3-d cultivation, resulting 31 g/L of ethanol from an initial substrate load as high as 15% (Krishna *et al*., 1998). Nevertheless, the enzyme dosage was quite high (100 FPU/g cellulose). The resulting slurry of the steam pretreatment of sugarcane bagasse in SSF tests using recombinant *S. cerevisiae* was conducted and the best initial load of substrate was 5% (w/w) at pH

5.0, yielding 0.35 g/g fermentable sugars at the start of the process. The cellulase mixture load was Celluclast 1.5L with cellulase activity of 12 FPU/g of substrate and Novozyme 188 with β-glucosidase activity of 12 IU/g substrate for saccharification of bagasse (Rudolf *et al*., 2008).

#### 2.4.3.3 Fermentation of pentose sugar

One of the main problems in ethanol production from lignocellulosic is that *S. cerevisiae* can ferment only certain mono- and disaccharides such as glucose, fructose, maltose and sucrose. In addition, pentoses obtained during hemicellulose hydrolysis (mainly xylose) cannot be assimilated by this yeast.

One approach to solve this is the use of naturally-occurring pentose fermenting microorganisms as found in some species of yeasts and bacteria. Yeasts such as *Pichia stipitis*, *Candida shehatae* and *Pachysolen tannophilus* can assimilate pentose (Skoog and Hahn-Hagerdal, 1988). *P. stipitis* has shown to be the most promising strain for industrial application, because it ferments xylose with a high ethanol yield and apparently produces no xylitol. Furthermore, it has no vitamin requirements for xylose fermentation and is able to ferment a wide range of sugars, including cellobiose (Nigam, 2001). The total theoretically ethanol yield could be increased by 25% through the use of an efficient xylose-fermenting yeast that could convert both hexoses and pentoses to ethanol (Bjorling and Lindman, 1989). *Pichia stipitis* belongs to a group of yeasts isolated from decaying wood and the larvae of wood inhabiting insects (Toiviola *et al*., 1984). The ecological niche of this yeast provides it with capabilities to utilize most of the sugars present in wood. *P. stipitis* has developed various cellulases and hemicellulases to break down wood into monomeric sugars (Jeffries *et al*., 2007). One of those enzymes is β-glucosidase which breaks down cellobiose into glucose monomers; therefore *P. stipitis* has the ability to ferment cellobiose.

In yeasts, such as *S. cerevisiae*, ethanol is produced when sugar concentrations are relatively high, even under aerobic conditions. This phenomenon is known as the Crabtree effect. Unlike *S. cerevisiae*, *P. stipitis* is a respiratory yeast, which does not produce ethanol under aerobic conditions, even in the presence of excess sugars (Klinner *et al*., 2005). The choice to produce ethanol or cell mass by *P. stipitis* depends on the $O_2$ supply to the cells. At high aeration rates, only cell mass is produced and at low aeration rates, ethanol is produced (du Preez, 1994).

*P. stipitis* is able to ferment glucose, xylose, mannose, galactose and cellobiose (Parekh and Wayman, 1986). It also has the ability to produce cell mass from L-arabinose but not ethanol (Nigam, 2002). *P. stipitis* exhibits both low-affinity and high-affinity proton symport systems that operate simultaneously (Kilian and Uden, 1988). The low-affinity transport system is shared between glucose and xylose for sugar transport. Glucose inhibits xylose transport by noncompetitive inhibition in the low-affinity proton symport system (Kilian and Uden, 1988). The low-affinity transport is used when sugar concentrations are high and the high affinity systems are used when sugar concentrations are low. Repression of xylose uptake occurs in fermentation media containing glucose and xylose. Therefore, glucose is the preferred sugar by *P. stipitis* in ethanol production. The rate of glucose consumption is higher than xylose under similar growth conditions (Agbogbo *et al*., 2006). The transport of sugars into the cells is the rate limiting step in the utilization of sugars for ethanol production in *P. stipitis* (Legthelm *et al*., 1988).

The optimal temperature for P. stipitis fermentation is between 25 and 33$^{o}$C and the optimal pH is 4.5–5.5 (du Preez *et al*., 1986). Oxygen plays an important role in cell growth, redox balance, functioning of the mitochondria and generation of energy for xylose transport in *P. stipitis* (Skoog and Hahn-Hagerdahl, 1990). Fermentation in *P. stipitis* is not induced by high sugar concentrations, but inactivated by aerobic conditions (Passoth *et al*., 1996). The investigation by Passoth et al. revealed that the branching point between respirative and fermentative metabolism

which includes enzymes such as pyruvate decarboxylase, alcohol dehydrogenase and aldehyde dehydrogenase were induced by a reduction in oxygen tension (Passoth *et al*., 1996). Some studies have shown that *P. stipitis* produces ethanol under anaerobic conditions (Delgenes *et al*., 1986), but microaerobic conditions are optimal for ethanol production (Grootjen *et al*., 1990).

*P. stipitis* has been used to ferment various types of pretreated biomass such as red oaks, wheat straw, sugarcane bagasse, rice straw, corn cob, corn stover, aspen wood, pine wood and poplar wood. The ethanol produced ranged from 6 to 41 g/L, at a yield of 0.31–0.48 g ethanol/g sugars consumed. The percentage of sugar consumed was in the range of 78–100% and fermentation times between 5 and 100 h using detoxified pretreated substrate (Agbogbo and Coward-Kelly, 2008). In most of these studies, the pretreated substrate was detoxified by methods such as steam stripping (Parekh *et al*., 1987), overliming (Amartey and Jeffries, 1994; Eken-Saracoglu and Arslan, 2000), molecular sieves and mixed bed of resins (Tran and Chambers, 1986). In other cases, the hydrolysate was used without detoxification by adjusting the pH to around 6.0 (van Zyl *et al*. 1988; Agbogbo and Wenger, 2007).

In order to develop a cost effective process for cellulosic ethanol production, the concept of local community-based bioethanol production has been introduced in Thailand. This involves the use of on-site enzyme production using local fungal isolates and fermentation process development with local yeast strains. In this investigation, the assessment of a novel on-site enzyme preparation on ball milled bagasse is reported in combination with the environmental friendly pretreatment process. The enzymatic saccharification process was used in separate hydrolysis and fermentation (SHF) and simultaneous saccharification and fermentation (SSF) processes for evaluating the convertibility of the cellulose and hemicellulose contained in the substrate to ethanol. The work thus demonstrated the potential of this alternative process for production of cellulosic ethanol from bagasse.

# CHAPTER III

# MATERIALS AND METHODS

## 3.1 Materials

### 3.1.1 Microorganisms

Fungal strains for cellulases production were obtained from BIOTEC Culture Collection. They produced high level of cellulase and xylanase activity in wheat bran soybean medium. Stock cultures were maintained on PDA plate. After 7 days of incubation at 25°C, the agar plates were stored at 4°C. All yeast strains (Appendix C) used for glucose and xylose fermentation for ethanol production were also obtained from BIOTEC Culture Collection. *Saccharomyces cerevisiae, Kluveromyces marxianus* and *Candida shehatae* were obtained from TISTR. They were all grown at 30°C and maintained at 4°C on YPD agar plate.

### 3.1.2 Materials

Sugarcane bagasse was obtained from Mitr Phol Sugar Factory and utilized as a substrate for both ethanol and enzyme production. The biomass waste was dried in an oven at 45°C for 24 h, coarsely cut by cutter milling (model FRITCH, German), passed through a 1 mm screening sieve and stored at room temperature. The cellulose, hemicelluloses and lignin fractions of bagasse were determined according to Tappi test method (Tappi, 1992) (α-cellulose-TAPPI Method T203 om-83; Klason lignin-TAPPI Method T222 om-83; Pentosans-TAPPI Method T223 hm-84 and Ash-TAPPI Method T211 om-93). The monomeric sugar composition was analyzed by acid hydrolysis of polysaccharides using the standard method with slight modifications (Hideno *et al*., 2009). The commercial enzymes used in comparison for bagasse hydrolysis were Acremonium cellulase from *Acremonium cellulolyticus* (Meiji Seika Co., Tokyo, Japan) and Optimash BG (Genecor International, Palo Alto, California, USA).

### 3.1.3 Culture media for fungi and yeast cultivation

The formula for culture media was shown in Appendix A. Potato dextrose agar (PDA), yeast extract-peptone-dextrose (YPD) agar were culture medium for cultivation of fungi and yeast. Bagasse medium (Mandels and Weber, 1969) was used for screening fungal cellulase production. Xylose medium (Toivola *et al*., 1984) was used for screening xylose fermentation yeast and yeast extract-peptone-xylose (YPX) medium (Nigam, 1999) was used for yeast inoculum preparation in ethanol fermentation. The basal medium for fermentation (Marques *et al*., 2008; Ohgren *et al*., 2007) was used during SHF and SSF processes.

## 3.2 Methods

### 3.2.1 Mechanical pretreatment

#### 3.2.1.1 Sonication pretreatment

The cut-milled bagasse was sonicated by ultrasonic processor (model Vibra-Cell VCX 600, Sonics & Materials Inc., USA) with maximum power output of 600 watts and operates at a constant frequency of 20 kHz. Sonication tests were conducted using 10 ml cut-milled bagasse slurries with 2.5% (w/v) TS. The slurry samples were treated in a batch-mode at four different amplitude (power) levels, 40, 50, 60 and 70 without temperature control. The slurry samples were sonicated at each power level for 1-minute treating and 1-minute pausing interval for 10 min. Temperature increase was observed during sonication. The sonicated bagasse was dried and used for subsequent on enzymatic hydrolysis.

#### 3.2.1.2 Ball milling pretreatment

The cut-milled bagasse was dried at 45$^{o}$C for 3 days before milling and then subjected to a planetary ball milling (model TI-300 system, CMT Co., Saitama, Japan). An amount of 100 g (50 g x 2) of cut-milled bagasse was added to a 500-ml milling cup containing 90 stainless steel balls (diameter 2 cm) and milled at 250 rpm. Milling time was varied for 1, 2 and 4 h at room temperature with a 10-minute

milling and 10-minute pausing interval. After which, size distribution of dried pretreated bagasse was analyzed using scanning electron microscope (SEM) analysis. In addition, the crystallinity of cellulose was determined using X-Ray diffraction analysis. The ball milled bagasse was used for all subsequent experiment on enzymatic hydrolysis and fermentation.

### 3.2.2 Chemical pretreatment

#### 3.2.2.1 Acid pretreatment

To analyze the composition of bagasse, acid pretreatment method was performed (Hideno *et al*., 2009). Briefly, the cut-milled bagasse sample that passed through sieves with 250-125 μm screens was oven dried at 105°C for 18 h. The oven dried sample (30 mg) was hydrolyzed with 72% sulfuric acid (0.3 ml) at 30°C for 1 h. The acid was diluted to final concentration of 4% by adding 8.4 ml distilled water. The mixture was heated at 121°C for 1 h. The monomeric sugars in the soluble fraction were neutralized with barium hydroxide and analyzed by high-performance liquid chromatography.

### 3.2.3 Fungal strain selection

Submerged culture was carried out in 250 ml Erlenmeyer flasks, which contained 1 g of bagasse as a carbon source in 50 ml minimal medium (0.1% (w/v) yeast extract, 0.02% (w/v) $MgSO_4.7H_2O$, 0.04% (w/v) $KH_2PO_4$, 0.02% (w/v) KCl, 0.5% (w/v) $NH_4NO_3$, 0.001% (w/v) $FeSO_4.7H_2O$, 0.001% (w/v) $ZnSO_4$ and 0.001% (w/v) $MnSO_4$, pH 5.8). The medium was sterilized at 121°C for 15 min. Four pieces of agar (diameter 1 cm) containing fungal culture were inoculated into bagasse medium and incubated in a rotary shaker (model Innova 4230, New Brunswick Scientific, USA) at agitation speeds of 200 rpm at 25°C for 7 days. After incubation, the suspended materials and fungal biomass were separated by centrifugation (8,000 rpm for 20 min). The clarified supernatant was used for enzyme activity assay and enzymatic hydrolysis determination.

### 3.2.4 Experimental procedure for cellulase production

To determine factors responsible for high level of enzyme production, small scale experiment was carried out in 250 ml Erlenmeyer flasks containing 1.5-2.5% (w/v) bagasse in 50 ml of minimal medium containing 0.02% (w/v) $MgSO_4.7H_2O$, 0.02% (w/v) KCl, 0.001% (w/v) $FeSO_4.7H_2O$, 0.001% (w/v) $ZnSO_4$ and 0.001% (w/v) $MnSO_4$. The yeast extract used as a nitrogen source was varied from 0.5-1.5% (w/v) and the medium was supplement with 0-0.5% (w/v) of each wheat bran and soybean meal, 0.1-0.5% (w/v) of ammonium nitrate and 0.2-0.6% (w/v) of potassium hydrogen phosphate. The initial pH of bagasse mixture medium was varied from 5-6. The sample was sterilized at 121$^{o}$C for 15 min. Four pieces of agar (diameter 1 cm) containing fungal culture were inoculated into the bagasse mixture medium and incubated in the rotary shaker at agitation speeds of 150-200 rpm. The temperature and incubation time were varied from 25-30$^{o}$C for 7-10 days. The suspended materials and fungal biomass were separated by centrifugation at 8,000 rpm, 4$^{o}$C for 20 min. The supernatant was used for the assay of cellulase activities (Filter paper activity; FPase, endoglucanase activity; CMCase and β-glucosidase activity).

### 3.2.5 Design of experiment by Plackett Burman Design

Plackett Burman Design was employed in order to determine which experimental parameters would influence the production of cellulases. Ten experimental parameters (factors), i.e. bagasse concentration (A), yeast extract concentration (B), initial pH (C), temperature (D), agitation speed (E) and incubation time (F), wheat bran concentration (G), soybean meal concentration (H), ammonium nitrate concentration (I) and potassium hydrogen phosphate concentration (J) with the ranges of minimum (-1), maximum (+1) and central point (0) were selected. The levels of parameters for experimental design that would be responsible for enzyme production were varied as shown in Table 3.1. The total 14 experiments were generated using the statistical software Design-Expert 7 P based on minimum design. The cellulase activity (FPase) was taken as the response in the experimental design.

**Table 3.1** Levels of parameters for experimental design.

| Factor | Low (-1) | Center point (0) | High(+1) |
|---|---|---|---|
| Bagasse (w/v); A | 1.5% | 2% | 2.5% |
| Yeast extract (w/v); B | 0.1% | 0.3% | 0.5% |
| Initial pH; C | 5 | 5.5 | 6 |
| Temperature ($^{o}$C); D | 25 | 27.5 | 30 |
| Agitation speed (rpm); E | 150 | 175 | 200 |
| Incubation time (day); F | 7 | 8.5 | 10 |
| Wheat bran (w/v); G | 0% | 0.25% | 0.5% |
| Soybean meal (w/v); H | 0% | 0.25% | 0.5% |
| Ammonium nitrate (w/v); I | 0.1% | 0.3% | 0.5% |
| Potassium hydrogen phosphate (w/v); J | 0.2% | 0.4% | 0.6% |

### 3.2.6 Analytical methods

#### 3.2.6.1 Enzyme activity analysis

All enzymes in this study were determined according to the standard procedure recommended by the Commission on Biotechnology, IUPAC (Wood and Bhat, 1988) with further details as follows. For filter paper activity (FPase), the reaction mixture was consisted of 1 ml of 50 mM acetate buffer pH 5.0, Whatman filter paper No. 1 (1 cm x 6 cm) and 0.5 ml of enzyme solution. The assay mixture was incubated at 50$^{o}$C for 60 min. The released reducing sugars were measured by the DNS method (Miller, 1959). One unit of enzyme activity (FPU) was defined as amount of enzyme that produces 1 μmole reducing sugar as glucose per minute under the assay condition. For endoglucanase activity (CMCase), the total reaction of 1 ml containing 0.5 ml of 2% (w/v) carboxymethyl cellulose (CMC) in 50 mM acetate buffer pH 5.0 and 0.5

ml of enzyme solution was incubated at 50°C for 30 min. The released reducing sugars were measured by the DNS method (Miller, 1959). One unit of enzyme activity was defined as amount of enzyme that produces 1 μmole reducing sugar as glucose per minute under the assay condition. Xylanase activity was determined under similar conditions as described above, except 2% (w/v) birchwood xylan was used as the substrate instead of CMC. One unit of enzyme activity was defined as amount of enzyme that produces 1 μmole reducing sugar as xylose per minute under the assay condition. For β-glucosidase activity, the total reaction of 1 ml containing 0.2 ml of 50 mM acetate buffer pH 5.0, 0.1 ml of 10 mM *p*–nitrophenyl-β-D-glucopyranoside (PNPG), 0.65 ml of distilled water and 0.05 ml of enzyme solution was incubated at 50°C for 10 min, the reaction was stopped by adding 0.5 ml of 1 M $Na_2CO_3$ and the color that developed as a result of *p*-nitrophenolate liberation was measured at 405 nm using UV-VIS Spectrophotometer (model UV-2550, Shimadzu Co., Japan). One unit of enzyme activity was defined as amount of enzyme that produces 1 μmole *p*-nitrophenol per minute under the assay condition. The protein concentration of culture filtrate was determined using bovine serum albumin as a standard (Lowry *et al*., 1951). All analyses were performed in duplicate.

#### 3.2.6.2 Physical characterization of ball milled bagasse

Untreated and pretreated bagasse samples were characterized using scanning electron microscopy and X-ray diffraction analysis. The samples were sputtered with Pt-Pd for 100 sec (Iron Sputter; Hitachi, Tokyo, Japan) and examined by Field Emission Scanning Electron Microscope (model S-3400N, Tokyo, Japan) at 2 kV. The crystallinities of samples were performed using an X-ray diffractometer (model Rigaku RINT-TTR3, Rigaku co., Tokyo, Japan) with nickel filtered Cu Kα radiation (λ= 0.1542 nm) at 50 kV and 30 mA. Samples were scanned over the range of $2\theta$ = 2-60° at a rate of 2°/minute. The crytallinity index (CI) could be calculated from the crystallinity patterns (Teramoto *et al*., 2008). The CI values were expressed by the following equation:

$$CI = \left[ I_{001} - I_{001B} \right] / I_{001}$$

where $I_{001}$ and $I_{001B}$ were the intensity at peak $2\theta = 16.3^{\circ}$ and that of the slope line at the same peak, respectively; the slope line was drawn as shown in Figure 4.3.

#### 3.2.6.3 Sugar and ethanol concentration analysis

Monosaccharide sugars and ethanol were analyzed using a high performance liquid chromatography system (Jasco Co., Tokyo, Japan) equipped with a refractive index detector (model RI-2031 Plus, Jasco Co., Tokyo, Japan). Glucose and xylose were analyzed using Aminex HPX-87P column (Bio Rad, USA) with a Carbo-P micro-guard cartridge (Bio Rad, USA). The column temperature was set at 80°C. Samples were eluted at flow rate of 1 ml/min with doubly deionized water. Ethanol and remaining sugars after fermentation were analyzed using Aminex HPX-87H column (BioRad, USA) with a Cation H micro-guard cartridge (BioRad, USA). The column temperature was set at 65°C. Samples were eluted at flow rate of 0.6 ml/min with 5 mM $H_2SO_4$ in doubly deionized water. All samples were filtered through 0.2 μm filters before analysis. Reducing sugars and glucose after enzymatic hydrolysis of raw bagasse were analyzed using DNS method (Miller, 1959) and a GOD-mutarotase reagent kit (Glucose CII Test Wako; Wako Pure Chemicals, Osaka). All analyses were performed in duplicate.

### 3.2.7 Enzymatic saccharification

Enzymatic hydrolysis was performed using a standard assay. Bagasse hydrolysis reaction of 1-ml total volume contained 5% (w/v) of pretreated bagasse in 50 mM sodium acetate buffer, pH 5.0 with 5 FPU/g substrate of Acremonium cellulase and 20 U/g substrate of xylanase from Optimash BG or an appropriate dilution of the BIOTEC enzyme cocktail preparations. The reaction mixture was incubated at 45°C for 72 h and mixed with a horizontal rotator (model 2210, Wakenyaku Co., Japan) (Inoue *et al.*, 2008). Samples were withdrawn at various intervals, centrifuged and stored at -20°C. The monosaccharide and profiles were determined using HPLC.

### 3.2.8 Ethanol fermentation

#### 3.2.8.1 Yeast screening for xylose fermentation

Type strain of 150 yeast species listed in BIOTEC culture collection as both glucose and xylose assimilation were examined. From YPD agar slant, one full loop of yeast was inoculated into 5 ml of YPD culture broth and incubated for 24 h at 30°C with shaking 200 rpm. 5% (v/v) of yeast culture was transferred to a culture tube containing 10 ml of xylose medium. All yeasts were grown after incubation for 72 h at 30°C with shaking 200 rpm. At the end of incubation, all cultures were centrifuged at 8,000 rpm, 4°C for 20 min and the supernatants were stored at -20°C until analysis of ethanol production by HPLC.

#### 3.2.8.2 Yeast inoculum preparation

Experiment was performed in a 250 ml Erlenmeyer flask containing 50 ml of YPX culture medium. Cultivation was carried out at 30°C with shaking 200 rpm for 24 h. The SHF and SSF process were performed as described previously (Marques *et al*., 2008; Ohgren *et al*., 2007) with slight modifications described below.

#### 3.2.8.3 Separate hydrolysis and fermentation (SHF)

The bagasse hydrolysis reaction of 8 ml total volume contained 0.5 g pretreated bagasse with an enzyme cocktail preparation in 50 mM sodium acetate buffer, pH 5.0. The reaction was incubated at 45°C with continuous mixing for 72 h. The hydrolysate was then adjusted to pH 5.5 and sterilized at 121°C for 15 min. Fermentation was performed in a 20 ml glass bottle with a rubber seal stopper in a total reaction volume of 10 ml containing 8 ml of the hydrolysate, 1 ml of 10x basal medium for fermentation and 1 ml of yeast cell suspension ($5.5 \times 10^9$ CFU/ml). The mixture was incubated at 30°C with continuous mixing for 120 h. Controls containing no bagasse hydrolysate were also included to determine the background ethanol production from other existing carbon sources in the medium. Samples were withdrawn at time intervals and concentration of sugars and ethanol were determined by HPLC.

#### 3.2.8.4 Simultaneous saccharification and fermentation (SSF)

For SSF experiments, the fermentation mixture of 10 ml final volume were prepared in 20-ml glass bottles with rubber seal stoppers containing 5% (w/v) pretreated bagasse in 50 mM sodium acetate buffer, pH 5.5, and 1x basal medium for fermentation which were sterilized at 121$^{o}$C for 15 min prior to addition of enzyme cocktail preparation to initiate saccharification. The reaction was performed for 1 h at 30$^{o}$C before the addition of 1 ml of yeast cell suspension (5.5x10$^{9}$ CFU/ml). Fermentation conditions, sampling and analysis were performed as described above for SHF. A saccharification control containing no yeast inoculum was also included under the same conditions.

# CHAPTER IV

# RESULTS AND DISCUSSION

## 4.1 Selection of cellulolytic enzymes producing fungi

A group of 766 fungal strains from BIOTEC Culture Collection (BCC) was screened for ability to produce high level of cellulase, xylanase, amylase and β-glucosidase using screening media, wheat bran soybean medium (Appendix A) at 200 rpm, 30°C. Four fungal strains with high level of cellulase and xylanase activities at wide range pH and temperature were selected. They were subsequently cultured in minimal medium containing raw bagasse as a sole carbon source. Shaking and static conditions were examined for enzymes production. The cellulase and xylanase activities produced from selected fungal isolates are shown in Table 4.1-4.2, respectively.

**Table 4.1** Enzyme activities produced from BIOTEC fungal isolated in submerged fermentation [a].

| BCC | Fungi | Enzyme activity (U/ml) [b] | | | |
|---|---|---|---|---|---|
| | | FPase (Filter paper) | CMCase (CMC) | β-glucosidase (PNPG) | Xylanase (Birchwood xylan) |
| 4504 | *Penicillium chrysogenum* | 0.04 | 0.32 | 0.19 | 5.34 |
| 7179 | *Aspergillus flavus* | 0.02 | 0.32 | 0.52 | 13.48 |
| 5744 | *Pithomyces maydicus* | 0.03 | 0.26 | 0.01 | 9.93 |
| 4603 | *Aspergillus niger* | 0.02 | 0.22 | 0.16 | 2.69 |

[a] enzyme activities were produced in raw bagasse medium under submerged condition (25°C and 200 rpm)

[b] enzyme activities were determined at 50°C

**Table 4.2** Enzyme activities produced from BIOTEC fungal isolated in solid-state fermentation [a].

| BCC | Fungi | Enzyme activity (U/ml) [b] | | | |
|---|---|---|---|---|---|
| | | FPase (Filter paper) | CMCase (CMC) | β-glucosidase (PNPG) | Xylanase (Birchwood xylan) |
| 4504 | *Penicillium chrysogenum* | 0.01 | 0.11 | 0.01 | 0.46 |
| 7179 | *Aspergillus flavus* | 0.01 | 0.29 | 0.19 | 6.59 |
| 5744 | *Pithomyces maydicus* | 0.02 | 0.18 | 0.01 | 1.42 |
| 4603 | *Aspergillus niger* | 0.02 | 0.28 | 0.29 | 5.10 |

[a] enzyme activities were produced in raw bagasse medium under solid-state condition (25°C)

[b] enzyme activities were determined at 50°C

Culture supernatants produced from submerged and solid-state conditions were examined for enzymatic hydrolysis at 30°C and 45°C for 72 h. Glucose and reducing sugar (Table 4.3 and 4.4) were determined after enzymatic hydrolysis using GOD-mutarotase reagent kit (Glucose CII Test Wako) and DNS method.

**Table 4.3** Sugar yields after enzymatic hydrolysis of raw bagasse at 30°C.

| Fungi | Submerged fermentation | | Solid-state fermentation | |
|---|---|---|---|---|
| | Reducing sugar [a] (mg/g substrate) | Glucose [b] (mg/g substrate) | Reducing sugar [a] (mg/g substrate) | Glucose [b] (mg/g substrate) |
| *Penicillium chrysogenum* | 51.05 | 19.17 | 22.94 | 2.15 |
| *Aspergillus flavus* | 56.56 | 12.78 | 47.85 | 12.47 |
| *Pithomyces maydicus* | 47.06 | 11.55 | 25.91 | 4.13 |
| *Aspergillus niger* | 21.72 | 5.36 | 35.89 | 16.21 |

[a] determined by DNS method

[b] determined by GOD-mutarotase reagent kit (Glucose CII Test Wako)

**Table 4.4** Sugar yields after enzymatic hydrolysis of raw bagasse at 45°C.

| Fungi | Submerged fermentation | | Solid-state fermentation | |
|---|---|---|---|---|
| | Reducing sugar [a] (mg/g substrate) | Glucose [b] (mg/g substrate) | Reducing sugar [a] (mg/g substrate) | Glucose [b] (mg/g substrate) |
| *Penicillium chrysogenum* | 94.61 | 10.16 | 38.75 | 0.42 |
| *Aspergillus flavus* | 109.34 | 1.72 | 71.78 | 0.30 |
| *Pithomyces maydicus* | 138.40 | 3.13 | 81.92 | 4.02 |
| *Aspergillus niger* | 21.68 | ND [c] | 47.02 | 2.83 |

[a] determined by DNS method

[b] determined by GOD-mutarotase reagent kit (Glucose CII Test Wako)

[c] not detected

Among these four selected fungal strains, *Penicillium chrysogenum* BCC 4504 produced the highest level of cellulase in bagasse containing medium. In addition, it produced higher level of cellulase activity in submerged fermentation (Table 4.1 and 4.2). In fact, crude cellulase production from BCC 4504 had appreciable level of FPase (0.04 U/ml or 0.4 U/mg) and CMCase (0.32 U/ml or 3.58 U/mg) activity but low level of β-glucosidase (0.19 U/ml or 2.11 U/mg) activity in submerged fermentation using raw bagasse medium (Table 4.1). The amount of sugars after enzymatic hydrolysis at 30°C and 45°C for 72 h were also obtained (Table 4.3 and 4.4). The FPase, CMCase and β-glucosidase activities were quite low when compared to the results of previous groups (Oguntimein *et al*., 1992; Aiello *et al*., 1996; Kang *et al*., 2004). The optimize culture conditions of cellulase production were therefore required to increase the cellulases yield.

Interestingly, among four selected strains, *Aspergillus flavus* BCC 7179 produced higher level of β-glucosidase and xylanase activity in submerged fermentation than solid state fermentation (Table 4.1 and 4.2). Crude enzyme preparation had appreciable level of β-glucosidase (0.52 U/ml or 5.17 U/mg) and xylanase (13.48 U/ml or 134.78 U/mg) activity in raw bagasse medium. The efficient saccharification of lignocellulosic biomass requires the cooperative action of cellulase and xylanase enzyme activities for complete conversion of cellulose and hemicelluloses to obtain a higher final sugar production. The BCC 7179 was therefore selected as β-glucosidase producing fungi to complement the hydrolysis of bagasse with the BCC 4504. Combination of BCC 4504 cellulase and BCC 7179 β-glucosidase were used as a potential enzyme mixture for low cost lignocellulosic biomass conversion.

Several studies for cellulase production by using different substrates and microorganisms have been found with different yield. This is because various natural substrates are able to induce secreted enzymes which are favorable to degrade particular combinations of polysaccharides and chemicals found in the carbon source. Crude cellulase and xylanase preparations used in this study were produced by

submerged fermentation using raw bagasse as a substrate. The production of cellulase and xylanase had been shown to be inducible and were affected by the nature of the substrate used in production. Therefore, sugarcane bagasse served as a potential substrate for inducing the enzyme production.

### 4.2 Optimization of cellulase production

This part of work aimed to screen for important medium components with respect to the main effects for cellulase (FPase) production by Plackett-Burman design. The Plackett-Burman experimental design is a two factorial design, which identifies the critical physic-chemical parameters required for elevated cellulase production by screening *n* variables in *n* + 1 experiments. The variables (*n* variables in *n*+1 experiments) chosen for the present study were bagasse concentration (%, w/v), yeast extract concentration (%, w/v), initial pH (C), temperature ($^{o}$C), agitation speed (rpm) and incubation time (day), wheat bran concentration (%, w/v), soybean meal concentration (%, w/v), ammonium nitrate concentration (%, w/v) and potassium hydrogen phosphate concentration (%, w/v). All the variables were denoted as numerical factors and investigated at two widely spaced intervals designated as -1 (low level) and +1 (high level). The variables were chosen based on the medium described by Mandels and Weber (1969) and physical characteristics of fungal cellulase (Ahamed and Vermette, 2008). The experiment was conducted in 14 runs to study the effect of the selected variable. Table 4.5 represents the results of the screening experiments using Plackett-Burman design. Statistical analysis of the responses were performed which is presented in Table 4.6.

**Table 4.5** Plackett-Burman experimental design for screening of variables affecting cellulase (FPase) production [a].

| Run | Experimental factors | | | | | | | | | | Enzyme activity (U/ml) | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | A: bagasse | B: yeast extract | C: pH | D: temperature | E: speed | F: time | G: wheat bran | H: soybean meal | I: $NH_4NO_3$ | J: $KH_2PO_4$ | FPase | CMCase | β-glucosidase |
| 1 | 2.5 | 0.1 | 5 | 25 | 200 | 7 | 0.5 | 0.5 | 0.1 | 0.6 | 0.026 | 0.504 | 0.565 |
| 2 | 2.5 | 0.5 | 5 | 25 | 150 | 10 | 0 | 0.5 | 0.5 | 0.2 | 0.025 | 0.529 | 0.568 |
| 3 | 1.5 | 0.1 | 5 | 25 | 150 | 7 | 0 | 0 | 0.1 | 0.2 | 0.004 | 0.342 | 0.035 |
| 4 | 1.5 | 0.1 | 5 | 30 | 150 | 10 | 0.5 | 0 | 0.5 | 0.6 | 0.028 | 0.505 | 0.568 |
| 5 | 1.5 | 0.1 | 6 | 25 | 200 | 10 | 0 | 0.5 | 0.5 | 0.6 | 0.018 | 0.49 | 0.551 |
| 6 | 2.5 | 0.5 | 6 | 25 | 150 | 7 | 0.5 | 0 | 0.5 | 0.6 | 0.033 | 0.499 | 0.571 |
| 7 | 1.5 | 0.5 | 5 | 30 | 200 | 7 | 0.5 | 0.5 | 0.5 | 0.2 | 0.059 | 0.525 | 0.57 |
| 8 | 2.5 | 0.1 | 6 | 30 | 200 | 7 | 0 | 0 | 0.5 | 0.2 | 0.005 | 0.412 | 0.564 |
| 9 | 1.5 | 0.5 | 6 | 30 | 150 | 7 | 0 | 0.5 | 0.1 | 0.2 | 0.064 | 0.488 | 0.566 |
| 10 | 1.5 | 0.5 | 6 | 25 | 200 | 10 | 0.5 | 0 | 0.1 | 0.2 | 0.047 | 0.497 | 0.563 |
| 11 | 2 | 0.3 | 5.5 | 27.5 | 175 | 8.5 | 0.25 | 0.25 | 0.3 | 0.4 | 0.043 | 0.516 | 0.569 |
| 12 | 2 | 0.3 | 5.5 | 27.5 | 175 | 8.5 | 0.25 | 0.25 | 0.3 | 0.4 | 0.042 | 0.519 | 0.567 |
| 13 | 2.5 | 0.1 | 6 | 30 | 150 | 10 | 0.5 | 0.5 | 0.1 | 0.2 | 0.062 | 0.534 | 0.571 |
| 14 | 2.5 | 0.5 | 5 | 30 | 200 | 10 | 0 | 0 | 0.1 | 0.6 | 0.022 | 0.472 | 0.568 |
| control | 2 | - | 5.5 | 25 | 200 | 7 | - | - | - | - | 0.025 | 0.404 | 0.541 |

[a] all experiments were performed in duplicate

**Table 4.6** Statistical analysis of the model [a].

| Factors | Coefficient | F-value | *p*-value |
|---|---|---|---|
| Model | | 869.50 | |
| Intercept | 0.03275 | | |
| A-bagasse | -0.00392 | 368.1667 | 0.0331 |
| B-yeast extract | 0.008917 | 1908.167 | 0.0146 |
| C-initial pH | 0.005417 | 704.1667 | 0.0240 |
| D-temperature | 0.00725 | 1261.50 | 0.0179 |
| E-agitation rate | -0.00325 | 253.50 | 0.0399 |
| F-duration | 0.000917 | 20.16667 | 0.1395 |
| G-wheat bran | 0.00975 | 2281.50 | 0.0133 |
| H-soybean meal | 0.009583 | 2204.167 | 0.0136 |
| I-ammonium nitrate | -0.00475 | 541.50 | 0.0273 |
| J-potassium phosphate | -0.00092 | 20.16667 | 0.1395 |

[a] analysis by ANOVA one way

The F-value and probability value (*p*-value) are tools for evaluating the significance and contribution of each of the parameters to the statistical model. The pattern of interactions between the variables is indicated by these coefficients. The large magnitude of F-value and the smaller the *p*-value are an indication of high significance of the corresponding coefficient. The *p*-value closes to 0.00 indicated model terms are significant and contribute to the model, while others can be neglected and eliminated from the model. The model F-value of 869.50 implied that the model was significant. The *p*-value suggested that bagasse, yeast extract, initial pH, temperature, agitation rate, wheat bran, soybean meal and potassium phosphate were significant model terms. The magnitude of the effects indicated that the level of significance of the variable on cellulase (FPase) production from BCC 4504 is shown in Figure 4.1

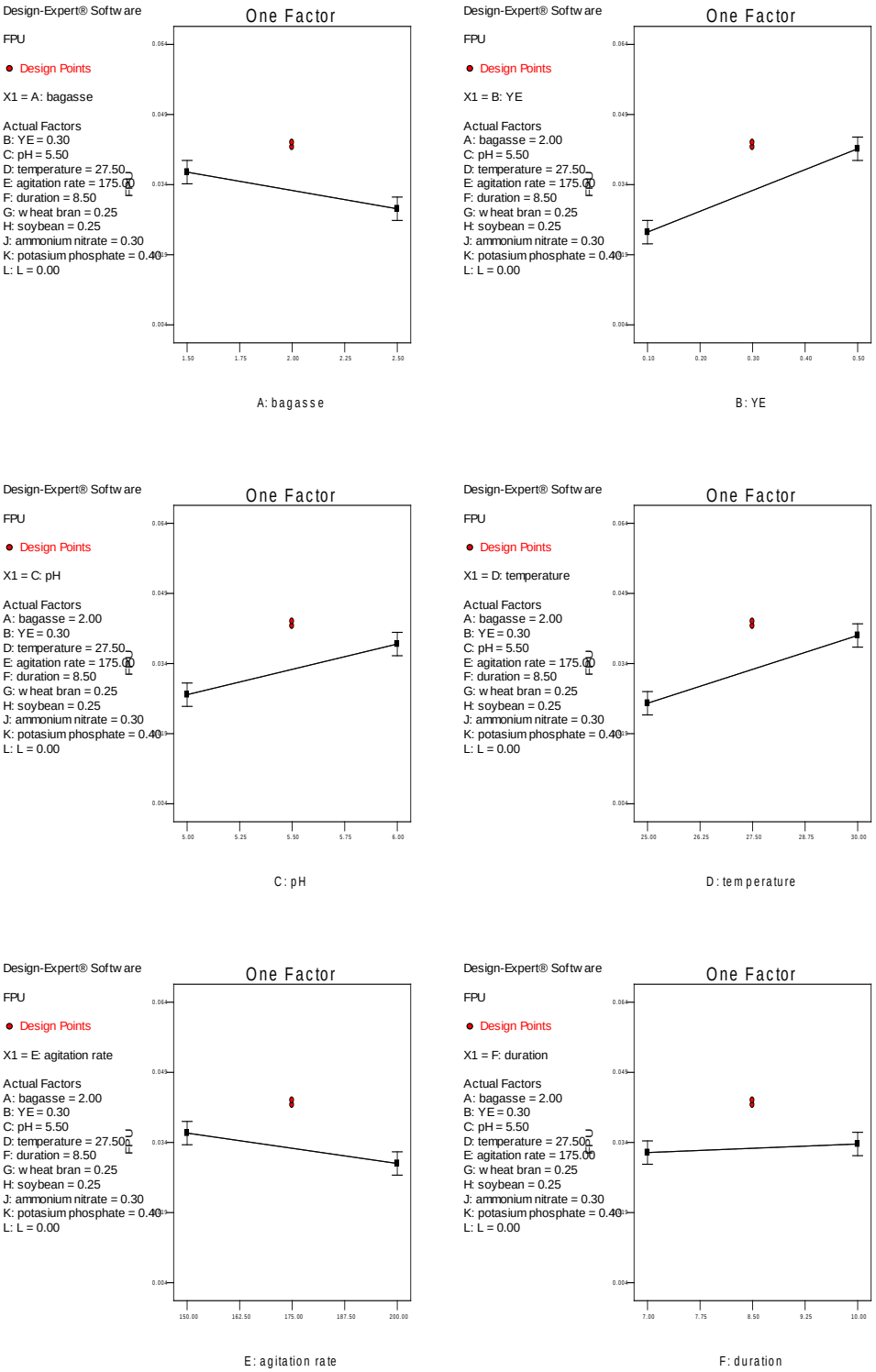



**Figure 4.1** Effect of various factors on cellulase (FPase) production.

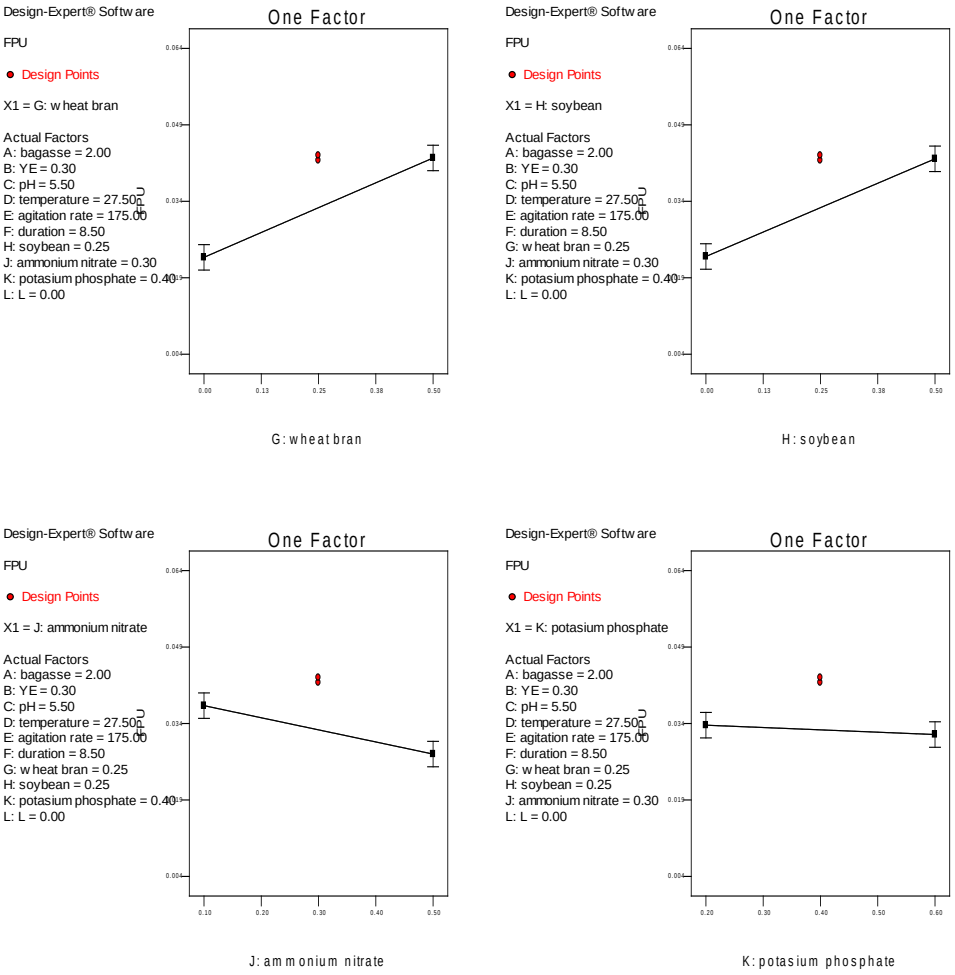



**Figure 4.1** (continued).

Among the variables studied, yeast extract, initial pH, temperature, wheat bran and soybean meal were identified as positive significant variables influencing cellulase production. Bagasse, agitation rate and ammonium nitrate were identified as negative effect on cellulase production indicating the interactions were not favorable to the response. It was interesting that production of cellulase increased as slight decrease in the bagasse concentration. This is in agreement with the results reported by Alam *et al*. (2008), showed a decrease in cellulase (FPase) production at increase domestic wastewater sludge over 1% total solid contents of substrate. The decrease in cellulase

(FPase) activity may be due to the accumulative effect of enzymatic by-products especially glucose and cellobiose which are known to inhibit both endoglucanase and exoglucanase. According to agitation rate, it has been observed that high speed of agitation causes higher sheer forces which might cause the damage of the filamentous mycelia of the fungi as the result the growth and product formation of this process may be reduced (Feng *et al*., 2003; Techapun *et al*., 2003)

The results showed that the organic nitrogen source (yeast extract, wheat bran and soybean meal) had positive effect over inorganic one (ammonium nitrate). Furthermore, the study revealed that an increase of organic nitrogen source lead to an enhancement of cellulase production. These corroborate the results of Kammoun *et al*. (2008) which indicated that amylase yield could be increased by using organic nitrogen source in the submerged fermentation medium. These results might due to the organic nitrogen source using in this study are also carbon source and contain trace of minerals and ions that could enhance growth and resulting in an increase of the enzyme production. According to the results, increase initial pH (from 5.0 to 6.0) and temperature (from 25$^{o}$C to 30$^{o}$C) showed positive effect on the cellulase production. Chahal *et al*. (1992) had demonstrated that the higher pH (6.0) was more preferable for cellulase activity on lignocellulosic substrate than on pure cellulose. It was observed that optimum pH and temperature for cellulase production depend on the characteristic of each microorganism. Most of the filamentous fungi especially *Trichoderma* have been found to be able to grow and metabolize within a range of initial pH 4-6 and temperature 25-35$^{o}$C (Latifian *et al*. 2007).

The pattern of interactions (3D) between the variables was indicated (Appendix B). Most of the interactions were found to be as significant except for the bagasse and agitation rate, bagasse and duration, bagasse and ammonium nitrate, bagasse and potassium phosphate, pH and agitation rate, pH and ammonium nitrate, pH and potassium phosphate, agitation rate and duration, agitation rate and ammonium nitrate and agitation rate and potassium phosphate. These indicated that the interactions were

not favorable to the response. The pattern of interactions (3D) assist to identify the optimum process conditions with maximum response for the levels of parameters (factors) tested in the design of experiments. The maximum response is referred by the surface confined in the smallest ellipse in the contour plot. The perfect interaction between the independent variables can be shown when elliptical contours are obtained (Muralidhar *et al*., 2001). From the results, the optimal level of the response was not shown by the pattern of interaction (3D). Thus the significant variables (bagasse, yeast extract, initial pH, temperature, agitation rate, wheat bran, soybean meal and potassium phosphate) were selected and their optimal levels were identified using response surface methodology.

It can be seen from Table 4.5 that experiment No.9 showed the highest cellulase (FPase) yield (0.064 U/ml). The results gave about 3-fold increase in cellulase (FPase) activity compared to that obtained from control (0.025 U/ml). The best combination condition obtained by the statistical analysis were bagasse 1.5% (w/v), yeast extract 0.5% (w/v), soybean meal 0.5% (w/v), $NH_4NO_3$ 0.1% (w/v), $KH_2PO_4$ 0.2% (w/v), pH 6.0, temperature 30$^{o}$C, 150 rpm and 7 days. Thus this combination conditions was further used for cellulase production from BCC 4504.

## 4.3 Mechanical pretreatment of bagasse

### 4.3.1 Bagasse

Sugarcane bagasse was obtained from Mitr Phol Sugar Factory and utilized as a substrate for both ethanol and enzyme production. The cellulose, hemicelluloses and lignin fraction of bagasse were determined according to TAPPI test method (Tappi, 1992). According to chemical composition analysis, the bagasse used in this study contained 44.1% cellulose, 27.7% hemicellulose, 22.5% lignin and 1.8% ash. The compositions were measured within the normal range for sugarcane bagasse (Pandey *et al*., 2000). The monomeric sugar compositions were analyzed by acid hydrolysis of polysaccharides using the standard method with slight modifications

(Material and method section 3.2.2.1). The monomeric sugar compositions of raw bagasse are shown in Table 4.7. The sugar composition was used for all subsequent calculation of saccharification yields.

**Table 4.7** Monomeric sugars composition of sugarcane bagasse on dry solid basis.

| Component | mg/g bagasse |
|---|---|
| Glucose | 355.7 |
| Xylose | 229.2 |
| Arabinose | 29.8 |
| Galactose | 6.4 |

### 4.3.2 Effect of mechanical pretreatment on enzymatic hydrolysis of bagasse

Bagasse samples were pretreated by either sonication and/or ball milling prior to enzymatic hydrolysis. The pretreated samples were subsequently examined for their rendering ability to the enzymatic hydrolysis. Glucose achieved after enzymatic hydrolysis from various conditions of pretreated bagasse is shown in Table 4.8

**Table 4.8** Sugar yields after enzymatic hydrolysis of various conditions of pretreated bagasse [a].

| Pretreatment | | Glucose [b] (mg/g substrate) | Hydrolysis [c] (%) |
|---|---|---|---|
| Grinding | | 64.19 | 18.03 |
| Sonication | 40 amplitude | 74.96 | 20.49 |
| | 50 amplitude | 74.04 | 20.80 |
| | 60 amplitude | 76.69 | 21.54 |
| | 70 amplitude | 78.44 | 22.03 |
| Ball milling | | 183.33 | 51.50 |
| Ball milling + Sonication | 40 amplitude | 179.03 | 50.30 |
| | 50 amplitude | 180.13 | 50.60 |
| | 60 amplitude | 182.18 | 51.17 |
| | 70 amplitude | 182.94 | 51.39 |

[a] crude enzyme were produced from BCC 4504 under the optimized conditions of submerged fermentation (0.064 U/ml of FPase, 0.488 U/ml of CMCase and 0.566 U/ml of β-glucosidase).

[b] determined by GOD-mutarotase reagent kit (Glucose CII Test Wako)

[c] calculated base on total glucose composition in raw bagasse

Crude enzyme from BCC 4504 was observed to hydrolyze various conditions of pretreated bagasse at 30°C for 72 h. The results showed that ball milling pretreatment gave higher level of glucose than sonication. The glucose yield after enzymatic hydrolysis of ball milling bagasse was 51.50%. Sonication treatment of ball milling bagasse showed the same level of glucose yield as ball milling bagasse. This indicated that sonication was not effective in elevating the glucose digestibility on ball milling treated sample. Nitayavardhana *et al*. (2008) reported that ultrasound

pretreatment facilitated the release of starch granules from cassava fiber and thus enhanced the reducing sugar yields. This is due to the different compositions and structure in the substrate. Ball milling was the most efficient and effective pretreatment for enzymatic hydrolysis comparing to the other two methods. The ball milled bagasse was therefore used for all subsequent experiments on enzymatic saccharification and fermentation.

## 4.4 Ball milling pretreatment of bagasse

### 4.4.1 Physical characterization of ball milled bagasse

Raw bagasse was milled for the various length of time (1, 2 and 4 h). After which, SEM and X-ray diffraction analysis were examined as shown in Figure 4.2 and 4.3.

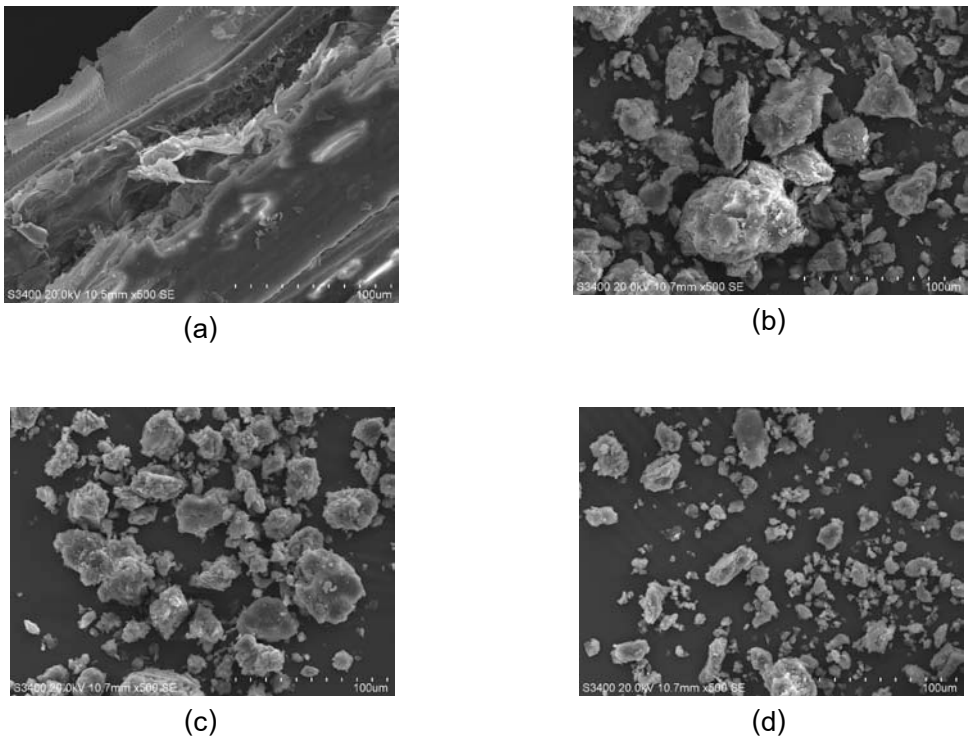
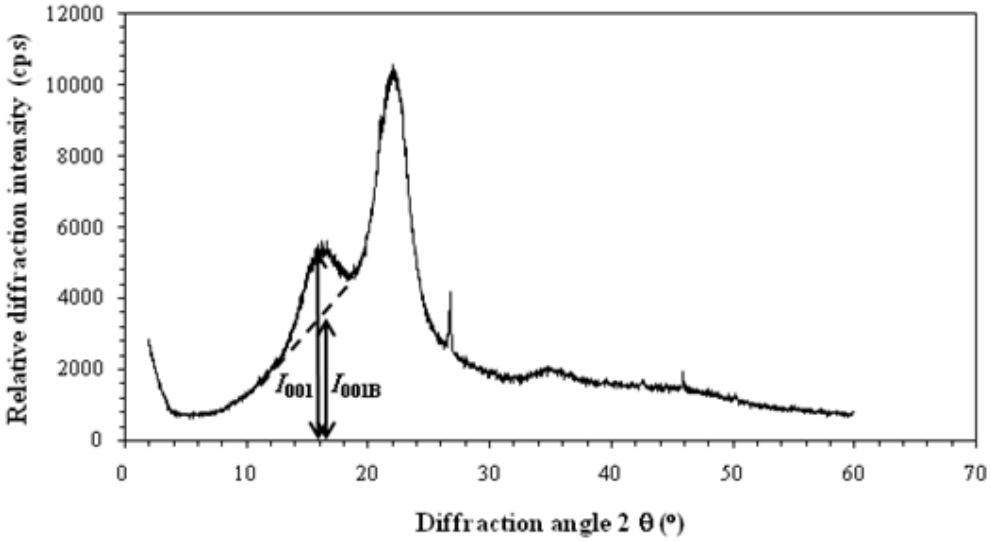



**Figure 4.2** Scanning electron microscopy (SEM) analysis of bagasse. (a) Raw bagasse; (b) Milling for 1 h; (c) Milling for 2 h; (d) Milling for 4 h.

(a)




(b)

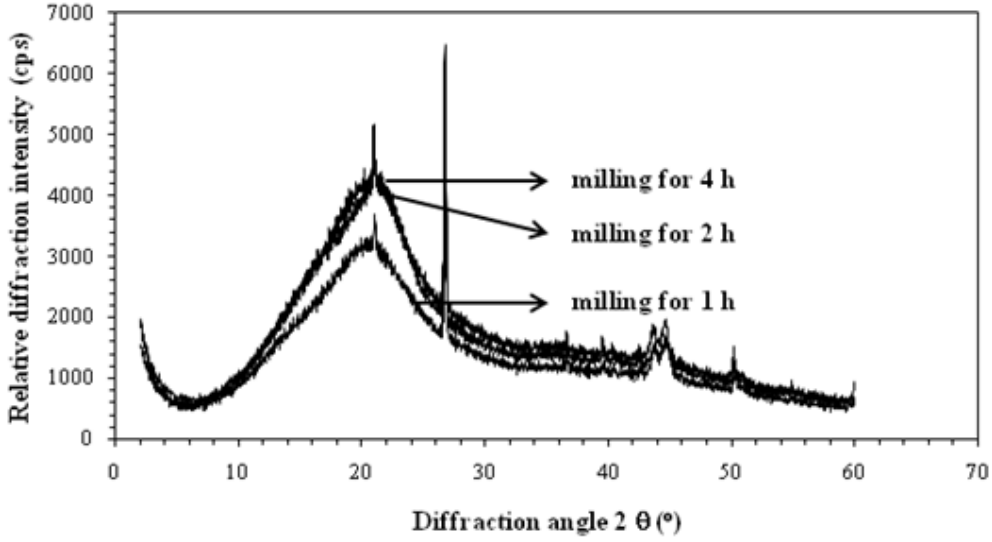



**Figure 4.3** X-ray diffraction analysis of cellulose structure in bagasse. (a) Pattern of crystalline cellulose in raw bagasse; (b) Pattern of amorphous cellulose in pretreated bagasse after milling for 1, 2 and 4 h.

In this research, conditions for mechanical ball milling pretreatment were optimized to increase the hydrolysis efficiency of the enzymes on the substrate. The effects of milling time (1, 2 and 4 h) on the pretreatment of bagasse were evaluated at room temperature. Figure 4.2 and 4.3 show the structural analysis of the pretreated bagasse at various milling times. As revealed by X-ray diffractometry, the crystalline structure of cellulose was present in raw bagasse, while it was completely disrupted yielding the amorphous form of cellulose after pretreatment of 1, 2 and 4 h. The CI value of raw bagasse was 0.31 and the CI of ball milling-treated samples was reduced to zero over the milling time. Based on the CI values, the crystalline cellulose was disrupted after ball milling for 1 h yielding the amorphous form of cellulose. SEM analysis showed that the starting raw material contained large fibers, which was then deformed into remarkably smaller fragments after milling, which was in accordance with the data from X-ray diffractometry.

### 4.4.2 Effect of milling time on enzymatic hydrolysis of pretreated bagasse

Effect of milling time of ball milling pretreatment on enzymatic hydrolysis of pretreated bagasse was evaluated using commercial cellulase and xylanase cocktail. The amount of glucose and xylose liberated is shown in Figure 4.4.

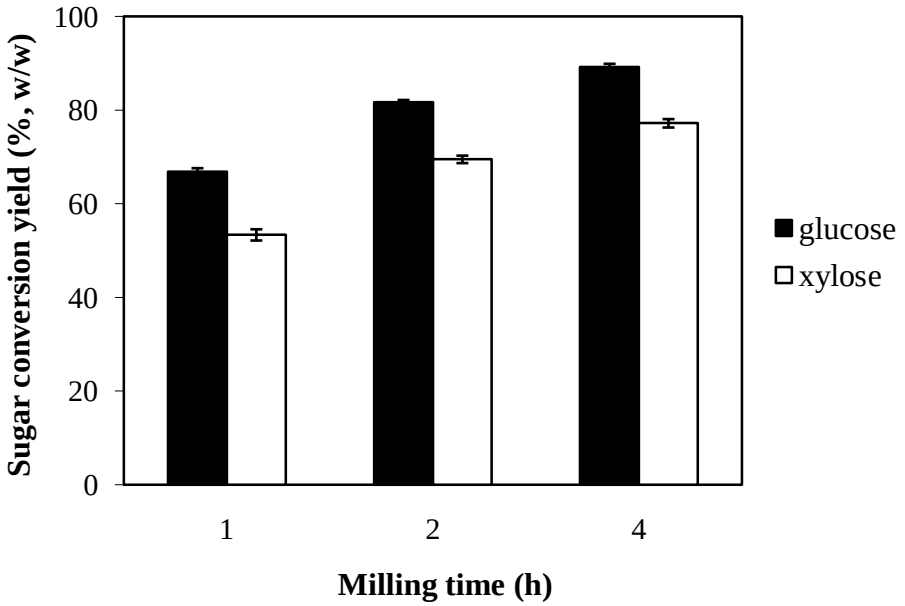



**Figure 4.4** Effect of ball milling time on enzymatic hydrolysis of pretreated bagasse. Bagasse samples (5%, w/v) were hydrolyzed with a commercial enzyme containing 5 FPU/g substrate of cellulase from Acremonium cellulase and 20 U/g substrate of xylanase from Optimash BG at 45°C, pH 5.0 for 72 h.

Commercial cellulase used in this study was Acremonium cellulase which was produced from *Acremonium cellulolyticus* (Yamanobe *et al*., 1990). Acremonium cellulase is previously shown to be more efficient at hydrolyzing eucalyptus (Inoue *et al*., 2008) and rice straw (Hideno *et al*., 2009) than conventional cellulase from *Trichoderma* sp. due to the presence of higher β-glucosidase activity in the crude enzyme. Optimash BG is a commercial xylanase (Genecor International, Palo Alto, California, USA) preparation which was produced from *Trichoderma reesei*.

The yield of glucose and xylose after enzymatic hydrolysis using the commercial enzyme formulation based on Inoue *et al*., (2008) (Acremonium cellulase at 5 FPU/g substrate of cellulase and 20 U/g substrate of xylanase from Optimash BG at 45°C, pH 5.0 for 72 h) were increased with the milling time, with the greatest yield of 89.2% and 77.2% for glucose and xylose, respectively after 4 h of milling (Figure 4.4).

The saccharification yield thus corresponded with the effectiveness of transformation of crystalline cellulose to amorphous form during milling pretreatment. From the result, it suggested that 4 h of milling produced the highest glucose and xylose during enzymatic hydrolysis. However, 2 h of milling time was more suitable, as it gave similar yields of glucose and xylose, which using less time, consequently conserving less energy. This is in agreement with previous studies on eucalyptus and rice straw in which the monomeric sugar yields after enzymatic hydrolysis increased with milling time and the optimal milling time depended on the biomass structure and composition (Inoue *et al*., 2008; Hideno *et al*., 2009). The SEM and X-ray diffraction analysis results of pretreated bagasse are in agreement with other studies showing that ball milling pretreatment leads to the reduction of particle size and crystallinity of cellulose, thus improving substrate digestibility by increasing enzyme accessibility to the amorphous cellulose (Tassinari *et al*., 1980; Ago *et al*., 2004). For further study, 2 h of milling pretreatment was selected for pretreatment condition.

### 4.4.3 Effect of cellulase loading on enzymatic hydrolysis of pretreated bagasse

Effect of cellulase loading on the enzymatic hydrolysis of pretreated bagasse was examined. Commercial cellulase (Acremonium cellulase) concentration was varied from 0, 5, 10 and 40 FPU/g substrate with the fixed amount of Optimash BG (20 U/g substrate of xylanase). The relationship between cellulase loading and the digestibility of pretreated bagasse is shown in Figure 4.5.

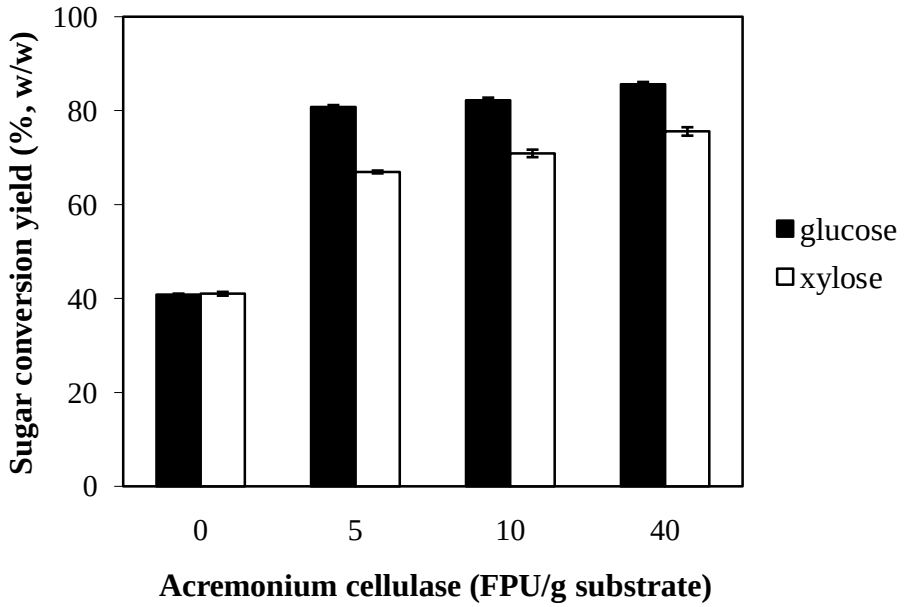



**Figure 4.5** Effect of cellulase loading on enzymatic hydrolysis of pretreated bagasse. Bagasse samples (5%, w/v) were hydrolyzed using various commercial cellulase loading from Acremonium cellulase with fixed 20 U/g substrate of xylanase from Optimash BG at 45°C, pH 5.0 for 72 h.

In this study, the concentration of cellulase that gave the highest sugar yield was selected. The result showed that 5 FPU/g substrate of cellulase was the lowest loading that still gave high sugar yield. Increasing amount of the Acremonium cellulase in combination with the fixed amount of Optimash BG (20 U/g substrate of xylanase) did not lead to any significant improvement of saccharification yield.

### 4.5 Hydrolysis of pretreated bagasse using the optimal BCC fungal enzyme cocktail

In most studies of lignocellulosic substrate hydrolysis, cellulase from *Trichoderma* sp. has been used. However, it contains only a small amount of β-glucosidase activity which restricts the conversion of cellobiose to glucose, resulting in inhibition of the upstream endo-glucanase and exo-glucanase activities and thus leading to lower saccharification efficiency (Zaldivar *et al*., 2001). Supplementation with β-glucosidase has been reported to increase the overall saccharification efficiency

resulting in an improved sugar yield (Zhou *et al*., 2009). In this study, *P. chrysogenum* BCC 4504 and *A. flavus* BCC 7179 were selected based on their ability to produce key lignocellulolytic enzymes from the extensive screening of fungi in the BIOTEC Culture Collection. The activities of various lignocellulolytic enzymes including FPase, CMCase, β-glucosidase, xylanase and β-xylosidase from both fungal strains after downstream processing are presented in Table 4.9 and Figure 4.6 in comparison to those of commercial enzymes.

**Table 4.9** Enzyme activity analysis of the commercial and BCC fungal enzyme preparations used for hydrolysis of the pretreated bagasse.

| Enzyme | Enzyme activity (U/ml) | | | | |
|---|---|---|---|---|---|
| | FPase (Filter paper) | CMCase (CMC) | β-glucosidase (PNPG) | Xylanase (Birchwood xylan) | β-xylosidase (PNPX) |
| Acremonium cellulase | 24.8 | 205.8 | 103.4 | 309.6 | 0.5 |
| Optimash BG | ND [a] | 4.2 | 84.5 | 485.8 | 31.5 |
| *Pennicillium chrysogenum* BCC 4504 | 0.9 | 12.6 | 0.1 | 145.9 | 0.03 |
| *Aspergillus flavus* BCC 7179 | 0.3 | 0.2 | 1.5 | 109.1 | 0.9 |

[a] not detected

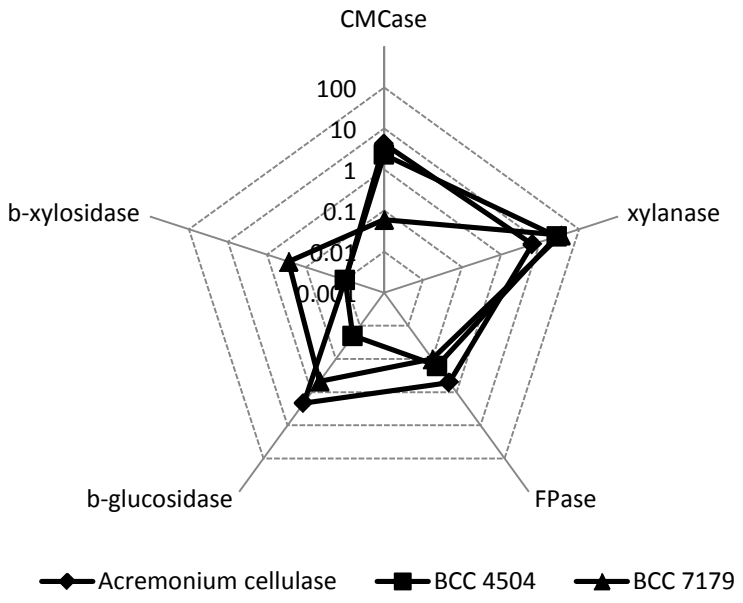



**Figure 4.6** Enzyme activity pattern of the commercial and BCC fungal enzyme preparations used for hydrolysis of the pretreated bagasse.

The crude enzyme from *P. chrysogenum* BCC 4504 contained endo-glucanase and endo-xylanase as the major activities while the crude enzyme from *A. flavus* BCC 7179 contained relatively low endo-acting activity on cellulose but possessed high downstream activities acting on small substrates, in addition to its high endo-xylanase activity. Since the enzyme preparations from these two fungal strains had complementary enzyme activities, they would be expected to show cooperative action on hydrolysis of lignocellulosic substrates. Sugar profile and yields from hydrolysis of the pretreated bagasse with different enzyme preparations were compared as shown in Figure 4.7 and 4.8.

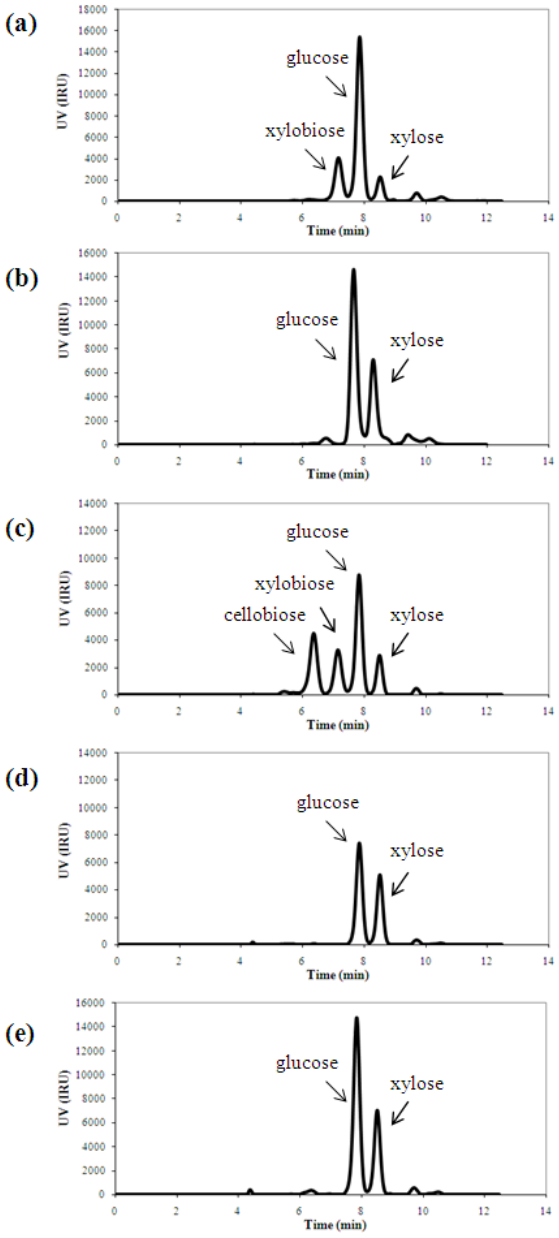
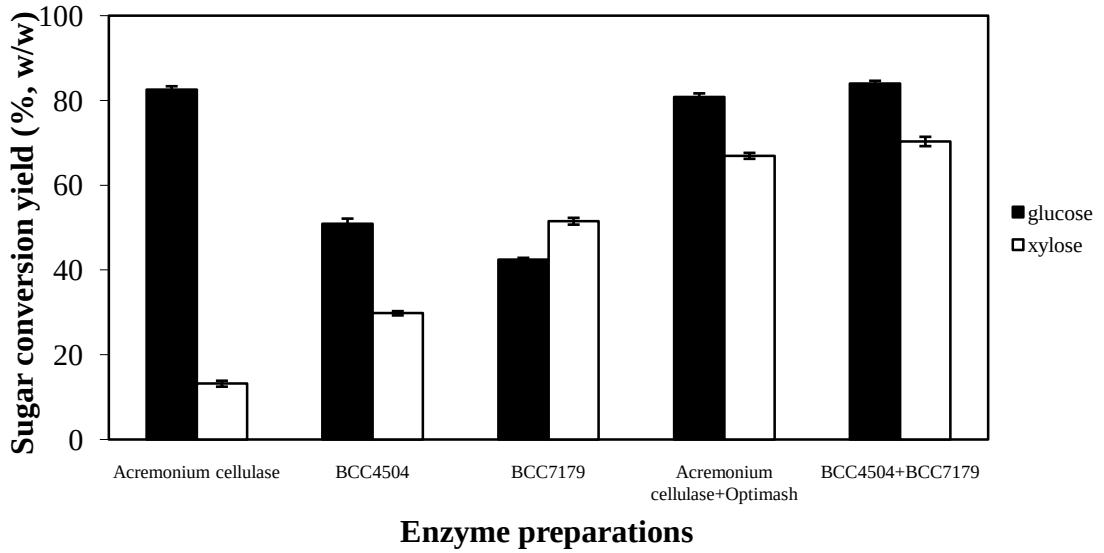



**Figure 4.7** Sugar profiles from enzymatic hydrolysis of the pretreated bagasse (5% w/v) with different enzyme preparations. (a) 5 FPU/g substrate of Acremonium cellulase; (b) 5 FPU/g substrate of Acremonium cellulase mixed with 20 U (as xylanase activity)/g substrate of Optimash BG; (c) 5 FPU/g substrate of cellulase from *P. chrysogenum* BCC 4504; (d) 10 U (as β-glucosidase activity)/g substrate of enzyme from *A. flavus* BCC 7179; (e) 5 FPU/g substrate of cellulase from *P. chrysogenum* BCC 4504 mixed with 10 U (as β-glucosidase activity)/g substrate of enzyme from *A. flavus* BCC 7179.

(a)




(b)

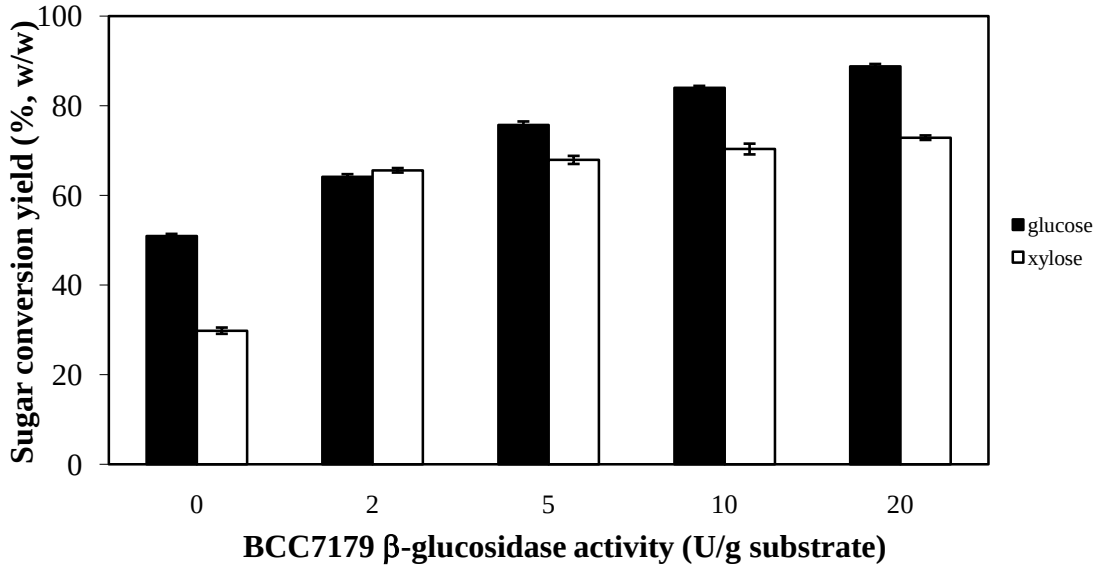



**Figure 4.8** Effect of enzyme mixtures on the release of sugars from 5% (w/v) pretreated bagasse. (a) Enzymes used included: 5 FPU/g substrate of Acremonium cellulase, 20 U (as xylanase activity)/g substrate of Optimash BG, 5 FPU/g substrate of cellulase from *P. chrysogenum* BCC 4504, and 10 U (as β-glucosidase activity)/g substrate of enzyme from *A. flavus* BCC 7179. (b) 5 FPU/g substrate of cellulase from BCC 4504 with varying β-glucosidase units of BCC 7179.

The results showed that the use of commercial Acremonium cellulase preparation alone (5 FPU/g substrate and 21 U/g substrate of β-glucosidase) resulted in high glucose but low xylose yields as a significant xylobiose was detected (Figure 4.7a), most likely due to the inadequate xylanase and β-xylosidase activities (equivalent to 62 and 0.1 U/g substrate, respectively). Optimash BG, source of hemicellulase enzyme activities, was then mixed (20 U/g substrate based on xylanase activity at which contained 0.13 U/g substrate of β-xylosidase) with Acremonium cellulase preparation to increase xylose yield (Figure 4.7b) and led to an improvement of saccharification yield to 80.8% glucose and 67.0% xylose (Figure 4.8a). Based on the same FPase unit to the commercial cellulase (5 FPU/g substrate), the use of *P. chrysogenum* BCC 4504 enzyme preparation alone resulted in lower glucose and xylose yields than the commercial Acremonium cellulase preparation while cellobiose and xylobiose were present (Figure 4.7c). This was mainly due to the low β-glucosidase and β-xylosidase activities in the BCC 4504 enzyme preparation (equivalent to 0.8 and 0.1 U/g substrate, respectively).

Combination of the *P. chrysogenum* BCC 4504 cellulase (5 FPU/g substrate) with the *A. flavus* BCC 7179 enzyme preparation containing high β-glucosidase and xylanase activities at varying concentration led to a markedly improved saccharification yield over the BCC 4504 or BCC 7179 enzymes used alone (Figure 4.7c, 4.7d, 4.7e and 4.8a). The use of BCC 7179 at 10 U/g substrate based on β-glucosidase activity was found to be optimal (84% glucose and 70.4% xylose yields) as higher concentration did not lead to a significant corresponding increase in xylose yield, though slightly increase in glucose yield was observed (Figure 4.8b). However, based on economic basis, 10 U/g substrate of β-glucosidase from BCC 7179 was selected as the optimal amount of enzymes used.

The saccharification yields using the optimal ratio of BCC 4504 / BCC 7179 cocktail (total activity unit: 7 U/g substrate of FPase, 73 U/g substrate of CMCase, 11 U/g substrate of β-glucosidase, 1,560 U/g substrate of xylanase and 6 U/g substrate of β-xylosidase) were significantly higher or at least comparable to the laboratory's

optimized commercial enzyme formulation containing Acremonium cellulase and Optimash BG (total activity: 5 U/g substrate of FPase, 42 U/g substrate of CMCase, 24 U/g substrate of β-glucosidase, 83 U/g substrate of xylanase, and 1.5 U/g substrate of β-xylosidase) (Figure 4.8a). For direct comparison based on the total FPase activity, increasing enzyme loading of the Acremonium cellulase to 10 and 40 FPU/g substrate in combination with the fixed amount of Optimash BG (20 U/g substrate of xylanase) did not lead to any significant improvement in saccharification yield (Figure 4.5).

The difference between the sugar yields would be partially due to some higher composite enzyme activities (especially for the strong xylanase activity) in the on-site produced BCC enzyme cocktail in comparison to the optimized commercial enzyme mixture. The synergistic action of the BCC 4504/BCC 7179 mixture showed superior effect on improving saccharification yield compared to the use BCC 4504 or BCC 7179 alone. This might be due to the activity complementation by β-glucosidase activity in BCC 7179 which reduces end-product inhibition by cellobiose, while the high xylanase activity in BCC 7179 increases hydrolysis of xylan, thus improving the accessibility of cellulose by BCC 4504 cellulase activity. The use of different enzyme preparations with complementary activities to increase saccharification efficiency has been reported elsewhere for other substrates (Berlin *et al*., 2005b; Tabka *et al*., 2006; Berlin *et al*., 2007). From preliminary cost estimation, the use of on-site enzyme production provided a promising alternative enzyme formulation for efficient saccharification of the pretreated substrates at relatively low cost. The distinct properties of the on-site enzyme were the broad spectrum of enzyme activities produced when agricultural waste was used as the carbon source and their specificities on saccharification screened specifically on target local biomass substrates. These were significant advantages from the viewpoint of practical biomass hydrolysis for local ethanol production facilities. The combination of these two sources of hydrolytic enzymes with complementary activities was therefore applied for saccharification of the pretreated bagasse for fermentation.

### 4.6 Yeast screening for ethanol production

The ethanol production from lignocellulosic hydrolysate by yeast requires strain that can ferment both hexose and pentose sugars in the hydrolysate with high ethanol yield. In addition, the yeast strain which able to ferment sugars to ethanol at high temperature is desirable when employing to SSF process. One hundred and fifty xylose utilizing yeast strains from BIOTEC Culture Collection were primarily screened for xylose fermentation using 2% (w/v) xylose medium at 30°C. Ethanol yield was determined by HPLC. 33 yeast strains were selected based on their ability to produce high level of ethanol after fermentation for 72 h (data not shown) and subsequently secondary screened for glucose and xylose fermentation at 30, 40 and 45°C in 2% (w/v) glucose and 2% (w/v) xylose medium. The remaining glucose and xylose and ethanol yield of selected yeast strains were determined after fermentation for 96 h compared with reference yeast strains as shown in Table 4.10-4.12. Fermentation parameters at 30, 40 and 45°C were evaluated as shown in Table 4.13-4.15.

**Table 4.10** Selected yeast strain for ethanol production in 2% (w/v) glucose and 2% (w/v) xylose medium at 30°C. The remaining glucose and xylose were determined.

| Yeast | Name | Concentration (g/L) [a] | | |
|---|---|---|---|---|
| | | Glucose | Xylose | Ethanol |
| BCC 15191 | *Pichia stipitis* | ND [b] | 3.91 | 18.41 |
| Ref. 5057 | *Kluyveromyces marxianus* | ND [b] | 17.03 | 9.05 |
| Ref. 5339 | *Sacharomyces cerevisiae* | ND [b] | 17.68 | 9.78 |
| Ref. 5843 | *Candida shehatae* | ND [b] | 9.70 | 15.73 |

[a] determined after fermentation for 96 h

[b] not detected

**Table 4.11** Selected yeast strain for ethanol production in 2% (w/v) glucose and 2% (w/v) xylose medium at 40°C. The remaining glucose and xylose were determined.

| Yeast | Name | Concentration (g/L) [a] | | |
|---|---|---|---|---|
| | | Glucose | Xylose | Ethanol |
| BCC 7755 | *Candida tropicalis* | ND [b] | 10.49 | 12.39 |
| Ref. 5057 | *Kluyveromyces marxianus* | ND [b] | 16.92 | 9.56 |
| Ref. 5339 | *Sacharomyces cerevisiae* | ND [b] | 16.27 | 10.11 |
| Ref. 5843 | *Candida shehatae* | ND [b] | 18.03 | 11.41 |

[a] determined after fermentation for 96 h

[b] not detected

**Table 4.12** Selected yeast strain for ethanol production in 2% (w/v) glucose and 2% (w/v) xylose medium at 45°C. The remaining glucose and xylose were determined.

| Yeast | Name | Concentration (g/L) [a] | | |
|---|---|---|---|---|
| | | Glucose | Xylose | Ethanol |
| BCC 7755 | *Candida tropicalis* | ND [b] | 11.51 | 15.30 |
| Ref. 5057 | *Kluyveromyces marxianus* | ND [b] | 18.10 | 9.14 |
| Ref. 5339 | *Sacharomyces cerevisiae* | 7.68 | 18.03 | 5.61 |
| Ref. 5843 | *Candida shehatae* | 19.23 | 19.46 | ND [b] |

[a] determined after fermentation for 96 h

[b] not detected

Fermentation parameters of the best selected yeast strain at 30, 40 and 45°C are shown in Table 4.13-4.15 compared with reference strains.

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**Table 4.13** Fermentation parameters of ethanol production from selected yeast strain at 30°C.

| Yeast | Ethanol (g/L) | Sugar utilized (g/L) | Ethanol production rate (g/L/h) | Sugar consumption rate (g/L/h) | Yp/s [a] | Ethanol yield [b] (%) |
|---|---|---|---|---|---|---|
| BCC 15191 | 18.41 | 36.09 | 0.19 | 0.38 | 0.51 | 100 |
| *Kluyveromyces marxianus* | 9.05 | 22.97 | 0.09 | 0.24 | 0.39 | 76.47 |
| *Sacharomyces cerevisiae* | 9.78 | 22.32 | 0.10 | 0.23 | 0.44 | 86.27 |
| *Candida shehatae* | 15.73 | 30.30 | 0.16 | 0.32 | 0.52 | 101.96 |

[a] Yield product/substrate = g ethanol/g fermentable sugars consumed

[b] as percentage of maximum theoretical ethanol yield 0.51 g ethanol/g glucose and xylose

**Table 4.14** Fermentation parameters of ethanol production from selected yeast strain at 40°C.

| Yeast | Ethanol (g/L) | Sugar utilized (g/L) | Ethanol production rate (g/L/h) | Sugar consumption rate (g/L/h) | Yp/s [a] | Ethanol yield [b] (%) |
|---|---|---|---|---|---|---|
| BCC 7755 | 12.39 | 29.51 | 0.13 | 0.31 | 0.42 | 82.35 |
| *Kluyveromyces marxianus* | 9.56 | 23.08 | 0.10 | 0.24 | 0.41 | 80.39 |
| *Sacharomyces cerevisiae* | 10.11 | 23.73 | 0.11 | 0.25 | 0.43 | 84.31 |
| *Candida shehatae* | 11.41 | 21.97 | 0.12 | 0.23 | 0.52 | 101.96 |

[a] Yield product/substrate = g ethanol/g fermentable sugars consumed

[b] as percentage of maximum theoretical ethanol yield 0.51 g ethanol/g glucose and xylose

**Table 4.15** Fermentation parameters of ethanol production from selected yeast strain at 45°C.

| Yeast | Ethanol (g/L) | Sugar utilized (g/L) | Ethanol production rate (g/L/h) | Sugar consumption rate (g/L/h) | Yp/s [a] | Ethanol yield [b] (%) |
|---|---|---|---|---|---|---|
| BCC 7755 | 15.30 | 28.49 | 0.16 | 0.30 | 0.54 | 74.51 |
| *Kluyveromyces marxianus* | 9.14 | 21.90 | 0.10 | 0.23 | 0.42 | 41.10 |
| *Sacharomyces cerevisiae* | 5.61 | 21.97 | 0.06 | 0.23 | 0.26 | 27.45 |
| *Candida shehatae* | ND [c] | 20.54 | ND [c] | 0.21 | ND [c] | ND [c] |

[a] Yield product/substrate = g ethanol/g fermentable sugars consumed

[b] as percentage of maximum theoretical ethanol yield 0.51 g ethanol/g glucose and xylose

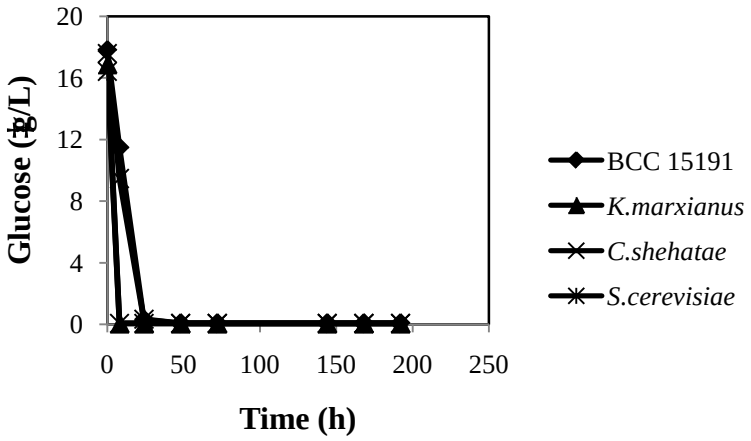
[c] not detected

The resulted showed that BCC 15191 was selected as it is efficient strain in producing ethanol from glucose and xylose at 30°C compared to the reference yeast strains. Glucose was completely consumed and xylose consumption rate was 0.17 g/L/h. The total sugar consumption rate of 0.38 g/L/h resulted in 0.51 g ethanol/g fermentable sugars consumed (glucose and xylose) of ethanol yield corresponding to 100% of the maximum theoretical value. BCC 7755 was selected as it is efficient strain in fermenting glucose and xylose at 40 and 45°C compared to the reference yeast strains. Glucose was completely consumed but xylose consumption rate was lower than BCC 15191 (0.09-0.10 g/L/h). Total sugars (glucose and xylose) consumption rate approximately 0.3 g/L/h resulted in 0.42-0.54 g ethanol/g fermentable sugars consumed (glucose and xylose) of ethanol yield at 40 and 45°C, respectively. The main propose of this study was to find the suitable yeast which could ferment glucose and xylose at high temperature. The results indicated that *Pichia stipitis* BCC 15191 had high ability to ferment both glucose and xylose only at low temperature (30°C). *Candida tropicalis*

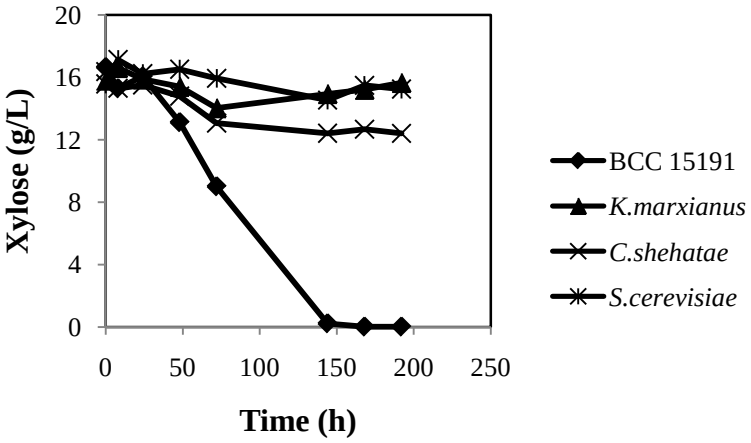
BCC 7755 had high ability to ferment glucose but low ability to ferment xylose at high temperature (40-45°C) resulting lower ethanol yield.

The profile of sugars consumption and ethanol production in sugar mixture of *P. stipitis* BCC 15191 at 30°C was examined and compared with the reference yeast strains (Figure 4.9). The glucose was completely consumed after 24 h and followed by xylose fermentation. All of xylose was consumed within 144 h with the ethanol concentration and yield of 18.29 g/L and 0.54 g ethanol/g fermentable sugars consumed (glucose and xylose), respectively, corresponding to 105.88% of the maximum theoretical value. Xylose was fermented after which glucose was completely consumed and at a slower rate than that of glucose assimilation. This is in agreement with previous reports that *P. stipitis* grown in glucose/xylose mixtures ferments preferentially glucose, since xylose assimilation is competitively inhibited by glucose (Agbogbo *et al*., 2006). Another isolate of *P. stipitis* has also been reported which shows the same advantage over *Saccharomyces cerevisiae*, namely its ability to ferment glucose, xylose, mannose, galactose and cellobiose present in lignocellulosic biomass hydrolysate resulting in high ethanol yield (van Zyl *et al*., 1991). *P. stipitis* BCC 15191 was selected to the most potential yeast strain to produce ethanol from lignocellulosic hydrolysate at 30°C regard to its ability to produce high ethanol yield from sugar (glucose and xylose) mixture.

(a)




(b)




(c)

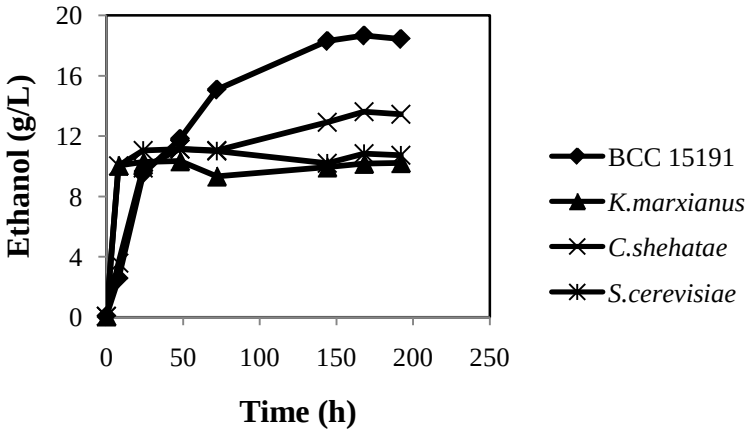



**Figure 4.9** Time course for ethanol production from selected yeasts in 2% (w/v) glucose and 2% (w/v) xylose mixture at 30°C. (a) Glucose consumption; (b) Xylose consumption; (c) Ethanol production.

## 4.7 Ethanol Fermentation by *Pichia stipitis*

### 4.7.1 Separate hydrolysis and fermentation (SHF) of ball milling pretreated bagasse

For the SHF process, 5% (w/v) ball milling pretreated bagasse was hydrolyzed by BCC enzyme cocktail containing 5 FPU/g substrate of cellulase from BCC 4504 and 10 U/g substrate of β-glucosidase from BCC 7179 at 45°C for 72 h. Enzymatic hydrolysate obtained from pretreated bagasse was used as a fermentation medium, with nutritional supplementation, to produce ethanol using *P. stipitis* BCC 15191. Time course of ethanol production is shown in Figure 4.10 and the summary of the fermentation results are in Table 4.16.

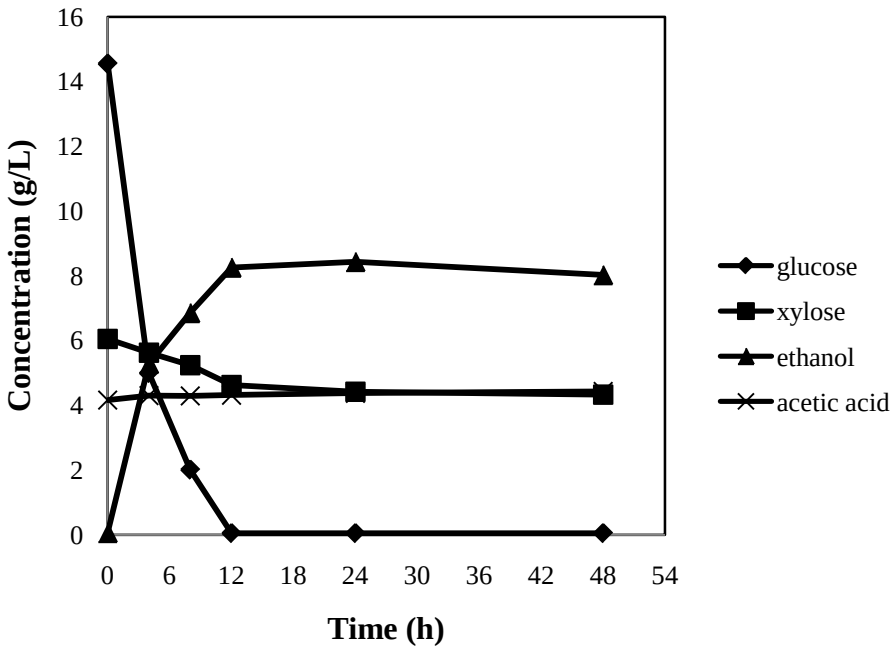


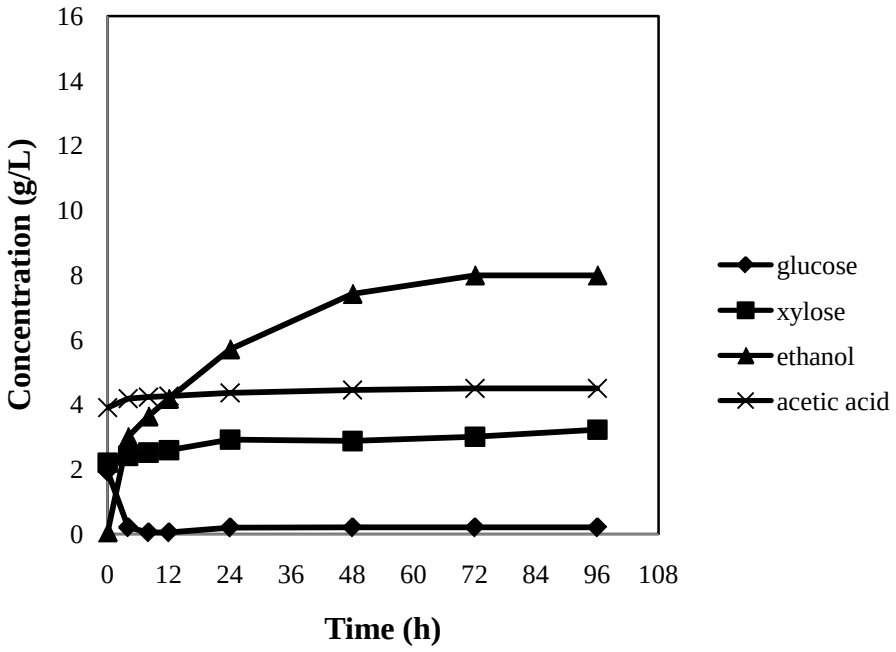

**Figure 4.10** Time course of ethanol production by *Pichia stipitis* BCC 15191 from 5% (w/v) pretreated bagasse hydrolysate at pH 5.5 and 30°C using separate hydrolysis and fermentation process (SHF).

The fermentation results showed that xylose was also fermented with glucose, but at a slower rate. The slow rate of xylose assimilation is probably due to repression of xylose uptake when glucose is present, as glucose is the preferred sugar, (Panchal *et al*., 1988; Nigam, 2001; Agbogbo *et al*., 2006). The results also showed that xylose was not completely consumed and residual xylose (4.4 g/L) remained in the broth after 120 h fermentation, whereas all the glucose was assimilated within 12 h of fermentation. The slow xylose consumption during fermentation may also be due to the presence of toxic compounds such as acetic acid which inhibited the growth and fermentation activity of the yeast (van Zyl *et al*., 1988; Roberto *et al*., 1991). After 24 h, the yield of ethanol production was 0.52 g ethanol/g fermentable sugars consumed (glucose and xylose) corresponded to a volumetric productivity rate of 0.35 g ethanol/L/h with the maximal ethanol concentration of 8.43 g/L (Table 4.16). No significant level of ethanol production was observed with the basal medium in the absence of biomass hydrolysate. The ethanol yield achieved in this study is higher than those obtained from other studies using various lignocellulosic biomass hydrolysate fermented by wild type strains of *P. stipitis.* Their productivities were in the range of 0.03-0.57 g/L/h with ethanol yields of 0.24-0.46 g/g obtained (van zyl *et al*., 1988; Roberto *et al*., 1991; Nigam, 2001; Okur-Telli and Saracoglu-Eken, 2008).

### 4.7.2 Simultaneous saccharification and fermentation (SSF) of ball milling pretreated bagasse

Enzymatic hydrolysis and fermentation process was simultaneously performed (SSF) using 5% (w/v) ball milling pretreated bagasse with the same ratio of enzyme mixture on SHF process mentioned earlier. Time course of ethanol production is shown in Figure 4.11 and the summary of the fermentation results are shown in Table 4.16.

(a)




(b)

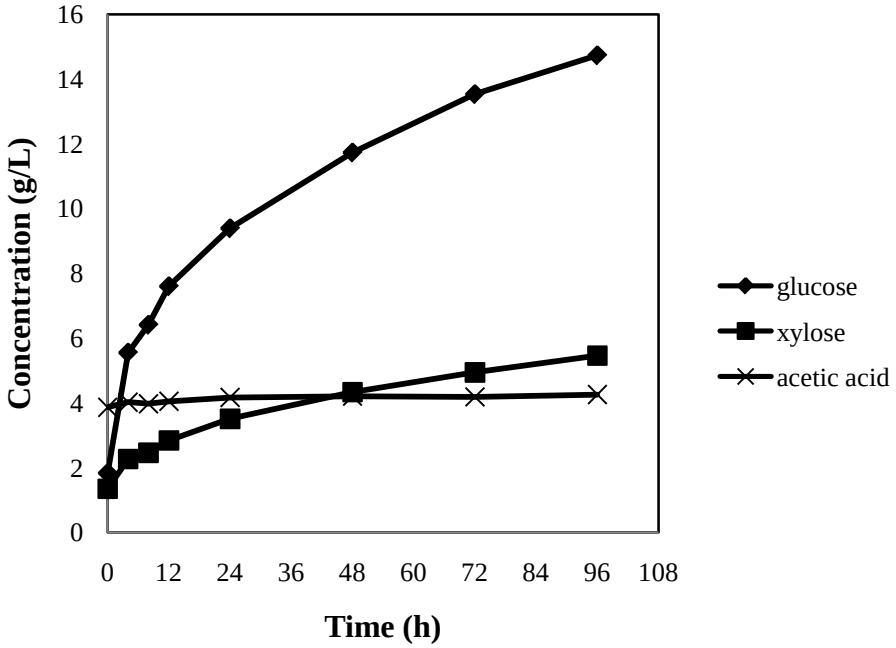



**Figure 4.11** Time course of ethanol production by *Pichia stipitis* BCC15191 from 5% (w/v) pretreated bagasse at pH 5.5 and 30°C using simultaneous saccharification and fermentation process (SSF). (a) Ethanol fermentation; (b) Saccharification control.

The fermentation results showed that glucose accumulated during the first few hours of cultivation, after which no further accumulation was detected. This indicated that yeast cells were metabolically active during the entire course of the fermentation. This also means that enzymatic hydrolysis was the rate-limiting step for ethanol production in the early fermentation phase (Kadar *et al*., 2004). Xylose assimilation in SSF was more efficient than that in SHF (40.1% vs. 27.6% after 120 h fermentation). However, higher concentration of by-product, xylitol was observed in SSF in comparison to SHF (1.3 g/L vs. 0.2 g/L after 120 h fermentation) (data not shown). The maximum ethanol concentration was 7.99 g/L after 72 h of incubation (Figure 4.11), corresponding to an ethanol production yield of 0.52 g ethanol/g fermentable sugars consumed (glucose and xylose) (Table 4.16). The ethanol yield in this study is higher than those obtained previously from a comparable simultaneous fermentation process using steam-pretreated sugarcane bagasse hydrolysate fermented by *P. stipitis* CBS6054 from which 0.22 g ethanol/g fermentable sugars were obtained (Rudolf *et al*., 2008). The ethanol yield reported here is also higher than other studies employing various substrates and fermentation processes, indicating the high efficiency of the developed integrated process (van zyl *et al*., 1988; Roberto *et al*., 1991; Nigam, 2001; Okur-Telli and Saracoglu-Eken, 2008). The ethanol yield coefficient from SSF in this study was closely to SHF (Table 4.16). However, the fermentation step on SHF was preceded by a prolonged period of 72 h of enzymatic hydrolysis which must be accounted for the total residence time (84 h), whereas the SSF process was completed after 72 h of incubation. Therefore, SSF is considered advantageous over SHF since the total time of conversion was shorter and the overall ethanol production process is simpler (Olofsson *et al*., 2008).

**Table 4.16** Ethanol production from ball milling pretreated bagasse by *Pichia stipitis* BCC 15191 at 30°C.

| Process | Ethanol (g/L) | Sugar consumption (g/L) | Ethanol production rate (g/L/h) | Sugar consumption rate (g/L/h) | Yp/s [a] | Theoretical ethanol yield (%) | Total process time (h) |
|---|---|---|---|---|---|---|---|
| SHF | 8.43 | 16.14 | 0.35 | 0.67 | 0.52 | 101.96 | 84 |
| SSF | 7.99 | 15.25 | 0.11 | 0.21 | 0.52 | 101.96 | 72 |

[a] ethanol yield coefficient (g ethanol/g fermentable sugars consumed), was calculated as the final ethanol concentration divided by sugars consumption (glucose and xylose).

Pentose fermenting yeasts require a careful control for maintaining low oxygen levels in the culture medium needed for their oxidative metabolism. Additionally, these yeasts successfully ferment pure xylose but not the aqueous hemicellulose streams generated during the biomass pretreatment, probably due to the presence of different inhibitors (Chandrakant and Bisaria, 1998). For pentose utilizing microorganisms, the hexoses are definitely the easier and faster assimilable substrate for the conversion to ethanol. This derives in a diauxic growth. If fermentation time is not sufficiently long, pentose remained in the medium will decrease the utilization rates of the lignocellulosic complex (Sanchez and Cardona, 2008). As a rule, microorganisms prefer glucose over galactose followed by xylose and arabinose (Gong *et al.*, 1999). This is explained by the catabolic repression that glucose exerts on the uptake rates of xylose and other pentoses.

In both fermentation processes (SHF and SSF), acetic acid (3.8-4.5 g/L) was detected by HPLC analysis (Figure 4.10 and 4.11), indicating the formation of acetic acid in the pretreatment process. Formation of inhibitory compounds including aliphatic acids, *e.g.* acetic, formic and levulinic acid, furan derivatives, *e.g.* furfural and 5-hydroxy-methyl-furfural (HMF) and phenolic compounds have been reported under

most pretreatment conditions, which can inhibit the subsequent fermentation by yeast strains (Mussatto and Roberto, 2004). In other studies using various other pretreatment methods, including, acid hydrolysis, steam and wet oxidation pretreatment, acetic acid were also detected. However, the acetic acid released after ball milling pretreatment of bagasse is lower than that from acid hydrolysis (Chandel *et al*., 2007), and steam pretreatment (Martin *et al*., 2002), and comparable to that from wet oxidation (Martin *et al*., 2007). Acetic acid is inhibitory to yeasts in general and concentrations of about 2-5 g/L have been reported to be inhibitory to *P. stipitis.* The inhibitory effect increases with a decrease in pH as the number of undissociated molecules is higher at low pH. Therefore, most fermentation studies of the hydrolysate have been conducted at a pH about 2 units higher than p*K*a value (4.8, 25$^{o}$C) to minimize the inhibitory effect of acetic acid (van Zyl *et al*., 1991). Removal or dilution of this compound could lead to improvement of the fermentability of lignocellulosic hydrolysate (Larsson *et al*., 1999). In this study, extensive detoxification step should be avoided in order to achieve for the process with overall economic efficiency (von Sivers *et al*., 1994). This can be achieved by further optimization of the pretreatment conditions to reduce the formation of inhibitory products *e.g.* by combination of more than one pretreatment methods under milder conditions (Inoue *et al*., 2008) or the use an additional simple detoxification step such as neutralization, over-liming with calcium hydroxide, activated charcoal, ion exchange resins and enzymatic detoxification using laccase (Chandel *et al*., 2007).

# CHAPTER V

# CONCLUSION

The production of cellulases by *P. chrysogenum* BCC 4504 in submerged fermentation was investigated. *P. chrysogenum* BCC 4504 is a potential fungal for production of cellulases from agro-industrial waste. The incorporation of sugarcane bagasse into media should contribute to a decrease in the cost of the production of enzyme complexes, thus contributing to the economic production of bioethanol.

In this study, an alternative process for ethanol fermentation of bagasse was demonstrated. High ethanol yields were obtained from a simple process employing mechanical ball milling pretreatment, enzymatic hydrolysis and co-fermentation of glucose and xylose. The optimized combination of enzyme mixtures from *P. chrysogenum* BCC 4504 and *A. flavus* BCC 7179 were shown to hydrolyze pretreated bagasse efficiently and the hydrolysate can be fermented to high ethanol yield using *P. stipitis* BCC 15191. The use of local microbial strains for the on-site production of crude enzymes and development of fermentation process has been considered a platform for promising utilization of biodiversity resources for establishment of sustainable biotechnology-based industry. Further improvement should include the reduction of acetic acid generation during the pretreatment step by simple detoxification and also the optimization of the fermentation conditions to improve xylose utilization, which would lead to the increase on ethanol production efficiency from this potentially valuable lignocellulosic agricultural residue.

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# APPENDICES

# Appendix A

## Media

All of media were add distilled $H_2O$ to final volume of 1 L and sterilized at 121°C for 15 min. The pH was adjusted with NaOH or HCl before addition of agar and sterilization.

1. **Potato dextrose agar (PDA)**

   Suspended 39.0 g powder in 1 L of distilled $H_2O$

2. **Wheat bran soybean medium**

| | | |
|---|---|---|
| Wheat bran | 30.0 | g |
| Soybean medium | 20.0 | g |

3. **Yeast extract-peptone-dextrose (YPD) agar**

| | | |
|---|---|---|
| Yeast extracts | 10.0 | g |
| Peptone | 20.0 | g |
| Glucose | 20.0 | g |
| Agar | 20.0 | g |
| Adjusted pH 5.0 | | |

4. **Bagasse medium**

| | | |
|---|---|---|
| Bagasse | 20.0 | g |
| Yeast extracts | 1.0 | g |
| $MgSO_4.7H_2O$ | 0.2 | g |
| $KH_2PO_4$ | 0.4 | g |
| KCl | 0.2 | g |
| $NH_4NO_3$ | 5.0 | g |
| $FeSO_4.7H_2O$ | 0.01 | g |
| $ZnSO_4$ | 0.01 | g |
| $MnSO_4$ | 0.01 | g |
| Adjusted pH 5.8 | | |

5. **Xylose medium**

| | | |
|---|---|---|
| Yeast extracts | 5.0 | g |
| Xylose | 20.0 | g |

6. **Yeast extract-peptone-xylose (YPX) medium**

| | | |
|---|---|---|
| Yeast extracts | 10.0 | g |
| Peptone | 20.0 | g |
| Xylose | 50.0 | g |
| Adjusted pH 5.0 | | |

7. **Basal medium for fermentation**

| | | |
|---|---|---|
| Yeast extracts | 1.0 | g |
| $(NH_4)_2SO4$ | 5.0 | g |
| $MgSO_4.7H_2O$ | 0.025 | g |

# Appendix B

## Raw data

**Table B1** Specific enzyme activities produced from BIOTEC fungal isolated in submerged fermentation.

| BCC | Fungi | Specific enzyme activity (U/mg protein) | | | |
|---|---|---|---|---|---|
| | | FPase (Filter paper) | CMCase (CMC) | β-glucosidase (PNPG) | Xylanase (Birchwood xylan) |
| 4504 | *Penicillium chrysogenum* | 0.40 | 3.58 | 2.11 | 59.28 |
| 7179 | *Aspergillus flavus* | 0.18 | 3.21 | 5.17 | 134.78 |
| 5744 | *Pithomyces mydecus* | 0.26 | 2.57 | 0.07 | 99.26 |
| 4603 | *Aspergillus niger* | 0.20 | 2.44 | 1.72 | 29.91 |

All analyses were performed in duplicate.

**Table B2** Specific enzyme activities produced from BIOTEC fungal isolated in solid-state fermentation.

| BCC | Fungi | Specific enzyme activity (U/mg protein) | | | |
|---|---|---|---|---|---|
| | | FPase (Filter paper) | CMCase (CMC) | β-glucosidase (PNPG) | Xylanase (Birchwood xylan) |
| 4504 | *Penicillium chrysogenum* | 0.15 | 1.80 | 0.07 | 7.58 |
| 7179 | *Aspergillus flavus* | 0.13 | 3.66 | 2.31 | 82.36 |
| 5744 | *Pithomyces mydecus* | 0.25 | 3.02 | 0.05 | 23.62 |
| 4603 | *Aspergillus niger* | 0.30 | 3.45 | 3.68 | 63.71 |

All analyses were performed in duplicate.

**Table B3** Effect of ball milling time on enzymatic hydrolysis of pretreated bagasse.

| Milling time (h) | Glucose | | Xylose | |
|---|---|---|---|---|
| | mg/g substrate | % yield | mg/g substrate | % yield |
| 1 | 237.71 | 66.83 | 122.34 | 53.38 |
| 2 | 290.59 | 81.70 | 159.31 | 69.51 |
| 4 | 317.36 | 89.22 | 176.97 | 77.21 |

All analyses were performed in duplicate.

**Table B4** Effect of cellulase loading on enzymatic hydrolysis of pretreated bagasse.

| Acremonium cellulse (FPU/g substrate) | Glucose | | Xylose | |
|---|---|---|---|---|
| | mg/g substrate | % yield | mg/g substrate | % yield |
| 0 | 145.26 | 40.84 | 94.00 | 41.01 |
| 5 | 287.38 | 80.79 | 153.45 | 66.95 |
| 10 | 292.30 | 82.18 | 162.52 | 70.91 |
| 40 | 304.54 | 85.62 | 173.23 | 75.58 |

All analyses were performed in duplicate.

**Table B5** Specific enzyme activity analysis of the commercial and BCC fungal enzyme preparations used for hydrolysis of the pretreated bagasse.

| Enzyme | Specific enzyme activity (U/mg protein) | | | | |
|---|---|---|---|---|---|
| | FPase (Filter paper) | CMCase (CMC) | β-glucosidase (PNPG) | Xylanase (Birchwood xylan) | β-xylosidase (PNPX) |
| Acremonium cellulase | 0.51 | 4.24 | 2.13 | 6.37 | 0.01 |
| Optimash BG | ND [a] | 0.31 | 0.16 | 34.99 | 0.23 |
| *Pennicillium chrysogenum* BCC 4504 | 0.16 | 0.23 | 0.02 | 26.81 | 0.01 |
| *Aspergillus flavus* BCC 7179 | 0.10 | 0.06 | 0.47 | 33.99 | 0.28 |

[a] not detected

All analyses were performed in duplicate.

**Table B6** Effect of enzyme mixtures on the release of sugars from 5% (w/v) pretreated bagasse in 50 mM sodium acetate buffer, pH 5.0 for 72 h at 45°C; (a).

| Enzyme preparation | Glucose | | Xylose | |
|---|---|---|---|---|
| | mg/g substrate | % yield | mg/g substrate | % yield |
| Acremonium cellulase | 296.96 | 82.57 | 30.20 | 13.18 |
| BCC 4504 | 181.35 | 50.94 | 68.31 | 29.80 |
| BCC 7179 | 151.10 | 42.48 | 118.08 | 51.52 |
| Acremonium cellulase + Optimash | 287.38 | 80.79 | 153.45 | 66.95 |
| BCC 4504 + BCC 7179 | 298.89 | 83.96 | 161.21 | 70.34 |

All analyses were performed in duplicate.

**Table B7** Effect of enzyme mixtures on the release of sugars from 5% (w/v) pretreated bagasse in 50 mM sodium acetate buffer, pH 5.0 for 72 h at 45°C; (b).

| BCC 7179 β-glucosidase activity (U/g substrate) | Glucose | | Xylose | |
|---|---|---|---|---|
| | mg/g substrate | % yield | mg/g substrate | % yield |
| 0 | 181.35 | 50.94 | 68.31 | 29.80 |
| 2 | 228.10 | 64.13 | 150.33 | 65.59 |
| 5 | 269.23 | 75.69 | 155.65 | 67.91 |
| 10 | 298.89 | 84.03 | 161.21 | 70.34 |
| 20 | 315.93 | 88.82 | 167.02 | 72.82 |

All analyses were performed in duplicate.

**Table B8** Time course for glucose fermentation from selected yeasts in 2% (w/v) glucose and 2% (w/v) xylose mixture at 30°C; (a).

| Time (h) | Glucose (g/L) | | | |
|---|---|---|---|---|
| | BCC 15191 | *K. marxianus* | *C. shehatae* | *S. cerevisiae* |
| 0 | 17.79 | 16.89 | 17.57 | 16.42 |
| 8 | 11.45 | 0.06 | 9.46 | 0.06 |
| 24 | 0.28 | 0.06 | 0.32 | 0.06 |
| 48 | 0.06 | 0.06 | 0.06 | 0.06 |
| 72 | 0.06 | 0.06 | 0.06 | 0.06 |
| 144 | 0.06 | 0.06 | 0.06 | 0.06 |
| 168 | 0.06 | 0.06 | 0.06 | 0.06 |
| 192 | 0.06 | 0.06 | 0.06 | 0.06 |

All analyses were performed in duplicate.

**Table B9** Time course for xylose fermentation from selected yeasts in 2% (w/v) glucose and 2% (w/v) xylose mixture at 30°C; (b).

| Time (h) | Xylose (g/L) | | | |
|---|---|---|---|---|
| | BCC 15191 | *K. marxianus* | *C. shehatae* | *S. cerevisiae* |
| 0 | 16.64 | 15.8 | 16.35 | 15.57 |
| 8 | 15.28 | 16.61 | 15.29 | 17.13 |
| 24 | 16.04 | 15.87 | 15.51 | 16.22 |
| 48 | 13.11 | 15.4 | 14.79 | 16.51 |
| 72 | 9 | 14.03 | 13.06 | 15.93 |
| 144 | 0.22 | 14.94 | 12.42 | 14.54 |
| 168 | 0.01 | 15.21 | 12.67 | 15.48 |
| 192 | 0.01 | 15.65 | 12.41 | 15.24 |

All analyses were performed in duplicate.

**Table B10** Time course for ethanol production from selected yeasts in 2% (w/v) glucose and 2% (w/v) xylose mixture at 30°C; (c).

| Time (h) | Ethanol (g/L) | | | |
|---|---|---|---|---|
| | BCC 15191 | *K. marxianus* | *C. shehatae* | *S. cerevisiae* |
| 0 | 0.08 | 0.08 | 0.08 | 0.08 |
| 8 | 2.56 | 10.05 | 3.59 | 10.04 |
| 24 | 9.5 | 10.29 | 9.89 | 11.05 |
| 48 | 11.81 | 10.35 | 11.15 | 11.14 |
| 72 | 15.07 | 9.34 | 11.05 | 11.01 |
| 144 | 18.29 | 9.94 | 12.92 | 10.21 |
| 168 | 18.65 | 10.18 | 13.61 | 10.85 |
| 192 | 18.42 | 10.23 | 13.45 | 10.74 |

All analyses were performed in duplicate.

**Table B11** Time course of ethanol production by *Pichia stipitis* BCC15191 from 5% (w/v) pretreated bagasse hydrolysate at pH 5.5 and 30°C using separate hydrolysis and fermentation process (SHF).

| Time (h) | Concentration (g/L) | | | |
|---|---|---|---|---|
| | Glucose | Xylose | Ethanol | Acetic acid |
| 0 | 14.56 | 6.05 | 0.08 | 4.16 |
| 4 | 5.00 | 5.63 | 6.68 | 4.30 |
| 8 | 2.02 | 5.24 | 8.66 | 4.29 |
| 12 | 0.06 | 4.62 | 10.43 | 4.32 |
| 24 | 0.06 | 4.41 | 10.65 | 4.37 |
| 48 | 0.06 | 4.33 | 10.13 | 4.43 |
| 72 | 0.06 | 4.31 | 9.99 | 4.44 |
| 96 | 0.06 | 4.33 | 10.03 | 4.42 |
| 120 | 0.06 | 4.38 | 10.10 | 4.45 |

All analyses were performed in duplicate.

**Table B12** Time course of ethanol production by *Pichia stipitis* BCC15191 from 5% (w/v) pretreated bagasse at pH 5.5 and 30°C using simultaneous saccharification and fermentation process (SSF), (a).

| Time (h) | Concentration (g/L) | | | |
|---|---|---|---|---|
| | Glucose | Xylose | Ethanol | Acetic acid |
| 0 | 1.93 | 2.21 | 0.08 | 3.91 |
| 4 | 0.21 | 2.41 | 3.80 | 4.19 |
| 8 | 0.06 | 2.52 | 4.60 | 4.23 |
| 12 | 0.06 | 2.59 | 5.28 | 4.26 |
| 24 | 0.21 | 2.92 | 7.21 | 4.36 |
| 48 | 0.21 | 2.87 | 9.37 | 4.45 |
| 72 | 0.21 | 3.01 | 10.09 | 4.50 |
| 96 | 0.21 | 3.22 | 10.09 | 4.50 |
| 120 | 0.21 | 3.48 | 10.20 | 4.54 |

All analyses were performed in duplicate.

**Table B13** Time course of ethanol production by *Pichia stipitis* BCC15191 from 5% (w/v) pretreated bagasse at pH 5.5 and 30°C using simultaneous saccharification and fermentation process (SSF), (b).

| Time (h) | Concentration (g/L) | | | |
|---|---|---|---|---|
| | Glucose | Xylose | Ethanol | Acetic acid |
| 0 | 1.83 | 1.34 | - | 3.86 |
| 4 | 5.55 | 2.26 | - | 4.02 |
| 8 | 6.41 | 2.46 | - | 3.97 |
| 12 | 7.60 | 2.84 | - | 4.05 |
| 24 | 9.39 | 3.51 | - | 4.17 |
| 48 | 11.73 | 4.33 | - | 4.20 |
| 72 | 13.53 | 4.94 | - | 4.18 |
| 96 | 14.73 | 5.46 | - | 4.26 |
| 120 | 15.30 | 5.81 | - | 4.20 |

All analyses were performed in duplicate.

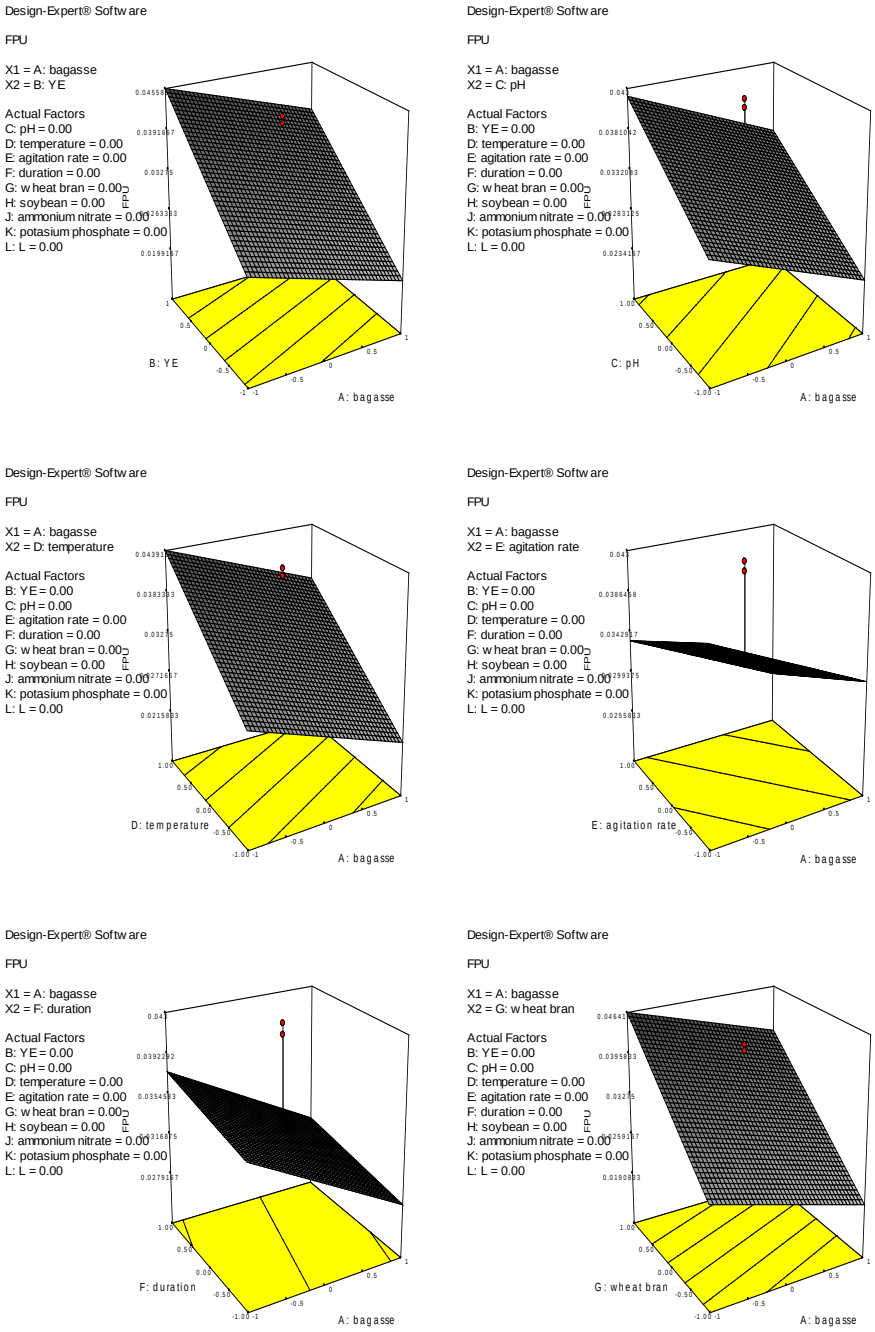


**Figure B1** The pattern of interactions (3D) between the variables on cellulase (FPase) production.

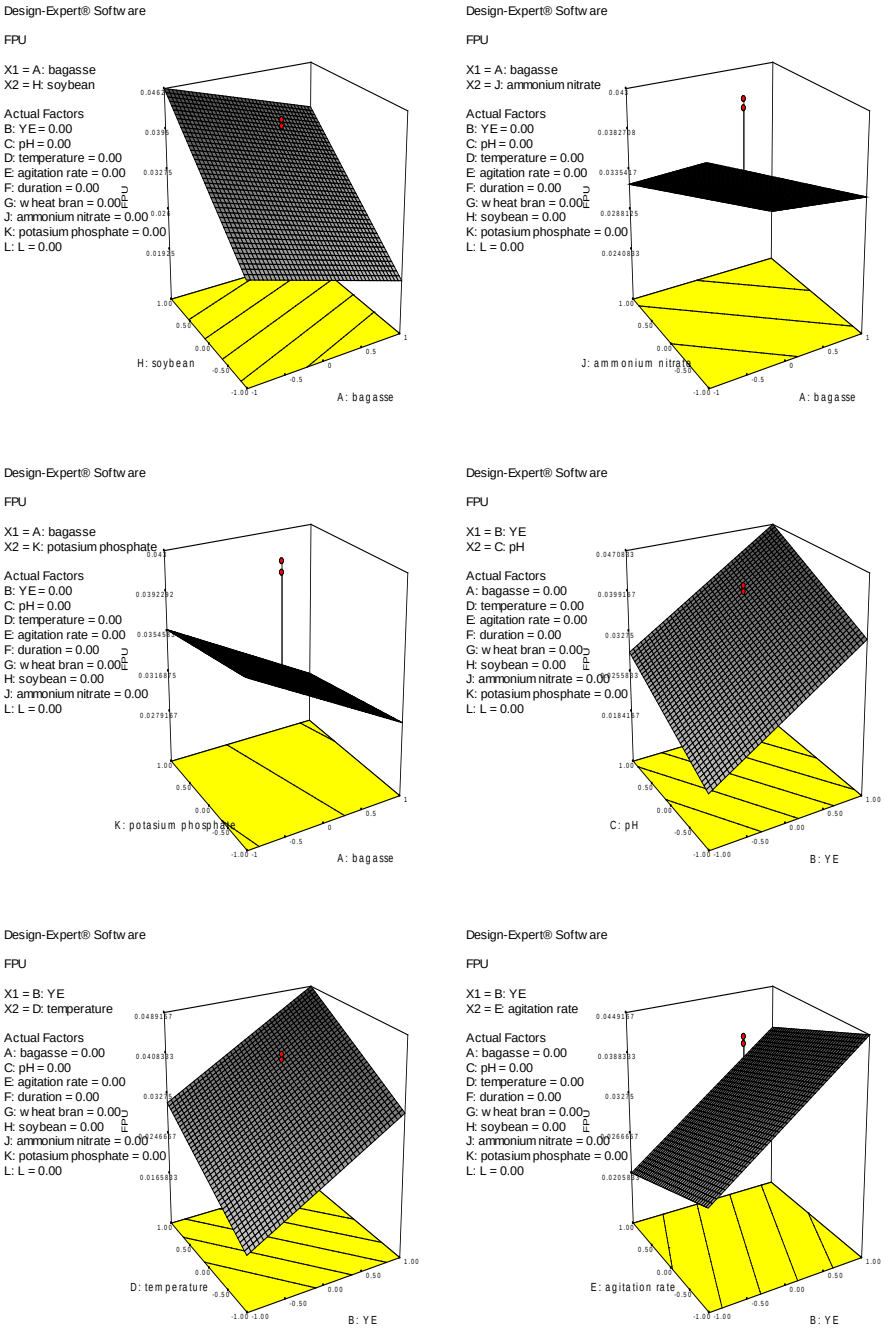


**Figure B1** (continued).

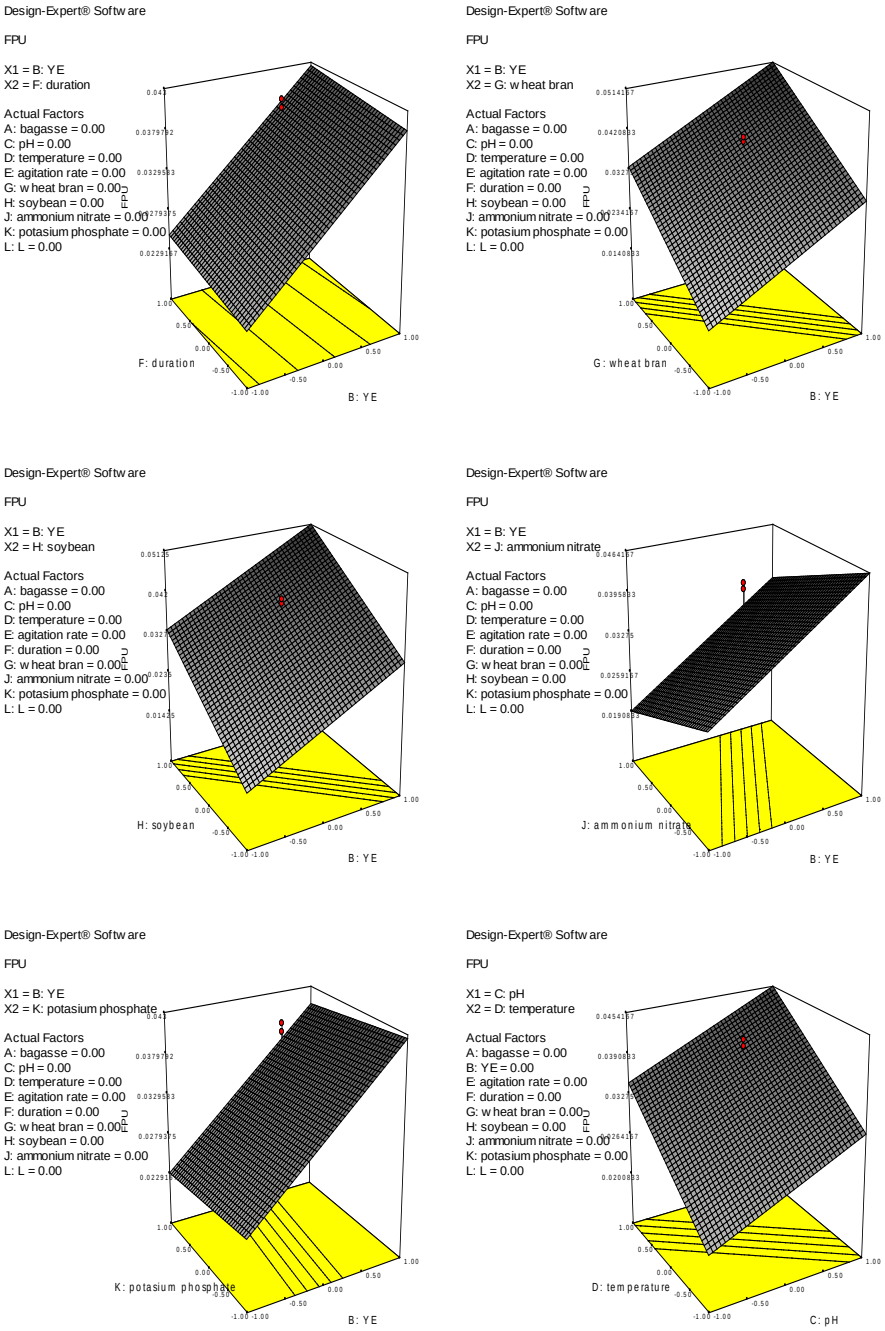


Figure B1 (continued).

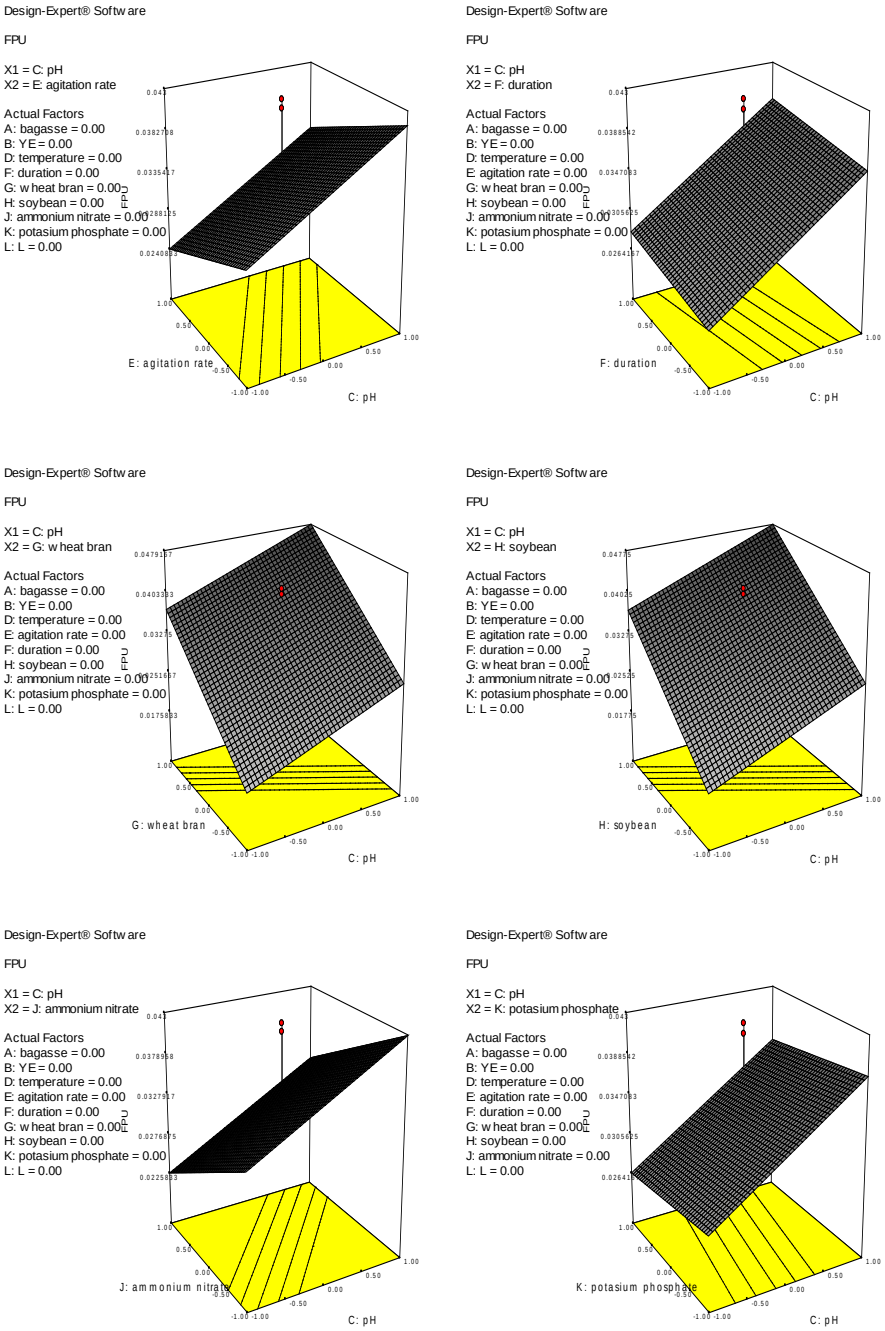


Figure B1 (continued).

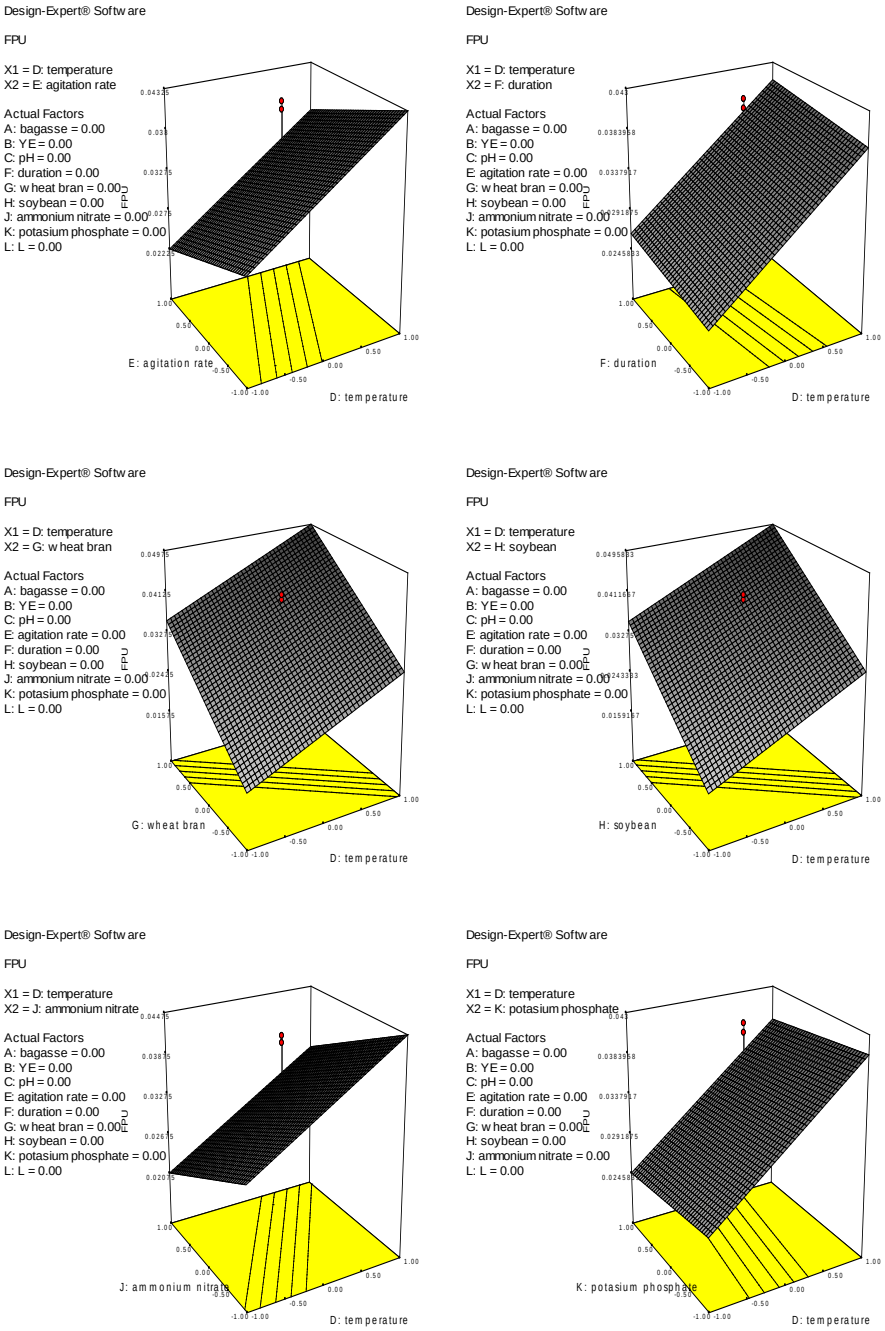


Figure B1 (continued).

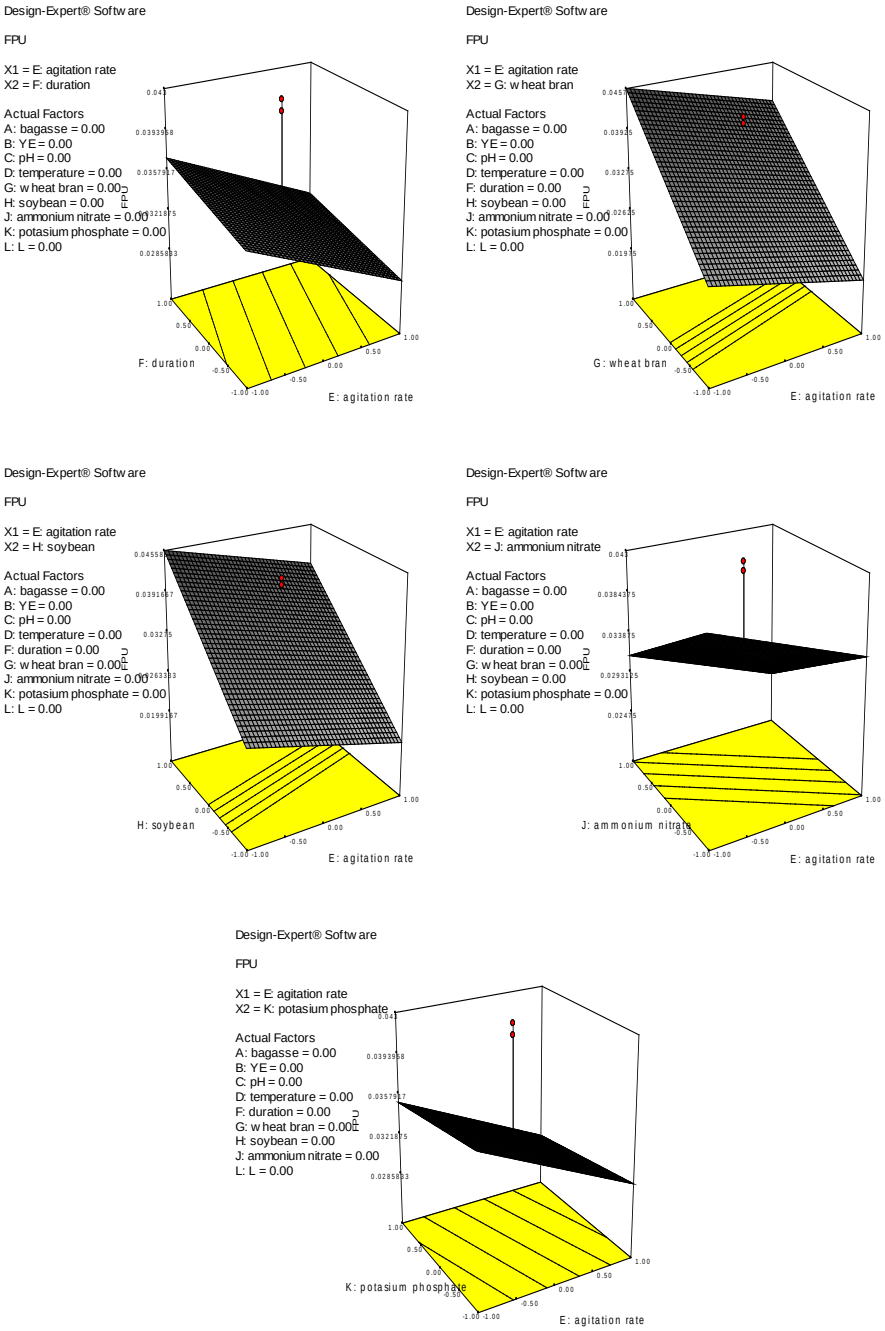


Figure B1 (continued).

# Appendix C

## List of yeast strains

**Table C1** List of xylose-utilizing yeast strains.

| Strain No. | BCC No. | Name |
|---|---|---|
| ST-1 | 7701 | *Cryptococcus humicola* |
| ST-3 | 7701 | *Pichia* sp. |
| ST-4 | 7701 | *Pichia* sp. |
| ST-7 | 7707 | *Pichia* sp. |
| ST-10 | 7710 | *Unidentified* |
| ST-11 | 7711 | *Sporidiobolus ruineniae* var. *ruineniae* |
| ST-12 | 7712 | *Sporidiobolus ruineniae* var. *ruineniae* |
| ST-15 | 7715 | *Candida* sp. UWO(PS)00-147.*3* |
| ST-16 | 7716 | *Unidentified* |
| ST-17 | 7717 | *Candida thailandica* |
| ST-18 | 7718 | *Candida* sp. |
| ST-19 | 7719 | *Candida* sp. |
| ST-20 | 7720 | *Candida parapsilosis* |
| ST-21 | 7721 | *Aureobasidium pullulans* |
| ST-24 | 7724 | *Candida gotoi* |
| ST-25 | 7725 | *Unidentified* |
| ST-27 | 7727 | *Candida leandrae* |
| ST-28 | 7728 | *Metschnikowia koreensis* |
| ST-30 | 7730 | *Pichia* sp. |
| ST-31 | 7731 | *Williopsis saturnus* var. *markii* |
| ST-33 | 7733 | *Candida* sp. |
| ST-34 | 7734 | *Stephanoascus smithiae* |
| ST-35 | 7735 | *Candida diversa* |
| ST-36 | 7736 | *Galactomyces* sp. |
| ST-37 | 7737 | *Pichia* sp. |
| ST-38 | 7738 | *Pichia* sp. |
| ST-39 | 7739 | *Candida* sp. |
| ST-40 | 7740 | *Candida rancensis* |

Table C1 (continued).

| Strain No. | BCC No. | Name |
|---|---|---|
| ST-41 | 7741 | *Pichia sydowiorum* |
| ST-42 | 7742 | *Unidentified* |
| ST-43 | 7743 | *Candida* sp. |
| ST-44 | 7744 | *Unidentified* |
| ST-45 | 7745 | *Unidentified* |
| ST-46 | 7746 | *Debaryomyces sp. NRRL Y-7804* |
| ST-47 | 7747 | *Unidentified* |
| ST-48 | 7748 | *Candida tropicalis* |
| ST-49 | 7749 | *Candida* sp. |
| ST-50 | 7750 | *Candida* sp. |
| ST-51 | 7751 | *Unidentified* |
| ST-52 | 7752 | *Unidentified* |
| ST-55 | 7755 | *Candida tropicalis* |
| ST-56 | 7756 | *Unidentified* |
| ST-57 | 7757 | *Metschnikowia* sp. |
| ST-58 | 7758 | *Unidentified* |
| ST-59 | 7759 | *Trichosporon* sp. |
| ST-60 | 7760 | *Candida* sp. |
| ST-61 | 7761 | *Candida tropicalis* |
| ST-71 | 8307 | *Cryptococcus* sp. |
| ST-73 | 8309 | *Cryptococcus* sp. |
| ST-78 | 8314 | *Candida* sp. |
| ST-79 | 8315 | *Candida* sp. |
| ST-87 | 8323 | *Sporidiobolus* sp. |
| ST-90 | 8326 | *Sporidiobolus* sp. |
| ST-91 | 8327 | *Sporidiobolus* sp. |
| ST-92 | 8328 | *Sporidiobolus* sp. |
| ST-93 | 8329 | *Sporobolomyces* sp. |

Table C1 (continued).

| Strain No. | BCC No. | Name |
|---|---|---|
| ST-94 | 8330 | *Sporobolomyces* sp. |
| ST-95 | 8331 | *Candida* sp. |
| ST-98 | 8334 | *Sporidiobolus* sp. |
| ST-100 | 8336 | *Sporobolomyces* sp. |
| ST-101 | 8337 | *Telletiopsis* sp. |
| ST-102 | 8338 | *Sporidiobolus* sp. |
| ST-103 | 8339 | *Unidentified* |
| ST-107 | 8343 | *Sporobolomyces* sp. |
| ST-110 | 8346 | *Unidentified* |
| ST-111 | 8347 | *Cryptococcus* sp. |
| ST-112 | 8348 | *Unidentified* |
| ST-115 | 8351 | *Sporidiobolus* sp. |
| ST-119 | 8355 | *Sporobolomyces* sp. |
| ST-122 | 8358 | *Wickerhamia* sp. |
| ST-123 | 8359 | *Sporobolomyces* sp. |
| ST-128 | 8364 | *Sporidiobolus* sp. |
| ST-139 | 8375 | *Unidentified* |
| ST-144 | 8380 | *Bullera* sp. |
| ST-145 | 8381 | *Cryptotrichosporon* sp. |
| ST-150 | 8386 | *Bullera sinensis* |
| ST-159 | 8395 | *Sporobolomyces* sp. |
| ST-164 | 8400 | *Candida* sp. |
| ST-166 | 14948 | *Exobasidium vexans* |
| ST-172 | 14954 | *Bullera* sp. |
| ST-174 | 14956 | *Sporobolomyces odoratus* |
| ST-176 | 14958 | *Bullera sinensis* |
| ST-181 | 14963 | *Bullera sinensis* |
| ST-182 | 14964 | *Exobasidium vexans* |

Table C1 (continued).

| Strain No. | BCC No. | Name |
|---|---|---|
| ST-184 | 14945 | *Sporobolomyces* sp. |
| ST-186 | 14947 | *Bullera* sp. |
| ST-192 | 14972 | *Sporobolomyces* sp. |
| ST-194 | 11752 | *Candida sithepensis* |
| ST-195 | 14974 | *Sporidiobolus* sp. |
| ST-198 | 14946 | *Sporobolomyces* sp. |
| ST-201 | 14979 | *Cryptococcus* sp. |
| ST-204 | 14982 | *Sporobolomyces odoratus* |
| ST-208 | 14986 | *Sporobolomyces odoratus* |
| ST-211 | 11755 | *Candida* sp. |
| ST-213 | 14988 | *Exobasidium* sp. |
| ST-219 | 14993 | *Unidentified* |
| ST-221 | 14995 | *Unidentified* |
| ST-224 | 11758 | *Candida* sp. |
| ST-225 | 11759 | *Candida easanensis* |
| ST-226 | 14997 | *Sporobolomyces* sp. |
| ST-228 | 11760 | *Candida easanensis* |
| ST-229 | 11761 | *Candida easanensis* |
| ST-236 | 11768 | *Pichia* sp. |
| ST-237 | 11769 | *Pichia* sp. |
| ST-246 | 15003 | *Candida* sp. |
| ST-248 | 11774 | *Candida* sp. |
| ST-249 | 11775 | *Candida* sp. |
| ST-252 | 11778 | *Candida gotoi* |
| ST-253 | 15005 | *Candida* sp. |
| ST-267 | 15019 | *Candida palmae* |
| ST-269 | 15021 | *Schizoblastosporion* sp. |
| ST-299 | 11782 | *Trichosporon* sp. |

Table C1 (continued).

| Strain No. | BCC No. | Name |
|---|---|---|
| ST-300 | 11783 | *Candida* sp. |
| ST-311 | 11790 | *Candida pattaniensis* |
| ST-315 | 11794 | *Candida* sp. |
| ST-318 | 11797 | *Trichosporon* sp. |
| ST-320 | 11799 | *Pichia* sp. |
| ST-328 | 11804 | *Candida* sp. |
| ST-329 | 11805 | *Candida* sp. |
| ST-331 | 11807 | *Candida* sp. |
| ST-333 | 11809 | *Candida* sp. |
| ST-334 | 11810 | *Pichia* sp. |
| ST-335 | 11811 | *Pichia* sp. |
| ST-337 | 11813 | *Candida* sp. |
| ST-358 | 15070 | *Candida* sp. |
| ST-360 | 15072 | *Unidentified* |
| ST-365 | 15077 | *Candida* sp. |
| ST-366 | 15078 | *Candida* sp. |
| ST-370 | 15082 | *Candida* sp. |
| ST-377 | 15089 | *Candida* sp. |
| ST-431 | 15128 | *Candida* sp. |
| ST-433 | 15130 | *Pichia* sp. |
| ST-441 | 15137 | *Candida* sp. |
| ST-445 | 15138 | *Pichia* sp. |
| ST-451 | 15142 | *Candida* sp. |
| ST-492 | 15178 | *Unidentified* |
| ST-506 | 15191 | *Unidentified* |
| ST-522 | 15206 | *Unidentified* |
| ST-523 | 15207 | *Unidentified* |
| ST-528 | 15212 | *Unidentified* |

**Table C1** (continued).

| Strain No. | BCC No. | Name |
|---|---|---|
| ST-537 | 15221 | *Unidentified* |
| ST-545 | 15229 | *Unidentified* |
| ST-546 | 15230 | *Unidentified* |
| ST-547 | 15231 | *Unidentified* |
| ST-577 | 15261 | *Unidentified* |
| ST-584 | 15268 | *Unidentified* |
| ST-585 | 15269 | *Unidentified* |
| ST-587 | 15271 | *Unidentified* |
| ST-590 | 15274 | *Unidentified* |
| ST-591 | 15275 | *Unidentified* |

# Appendix D

## Calculation

1. **Enzyme activity**

   U/ml = (μmole sugar X dilution factor) / (volume X incubation time)

2. **Sugar conversion yield**

   % (w/w) = (mg sugar/g substrate) X 100 / (total mg sugar/g substrate)

3. **Y product / substrate**

   Y p/s = (g/L ethanol) / (g/L total sugar consumed)

4. **Theoretical ethanol yield**

   % = (Y p/s) X 100 / 0.51

# BIOGRAPHY

Ms. Benchaporn Buaban was born on April 26, 1972 in Nakornsawan, Thailand. She was graduated with a Bachelor Degree (Microbiology) from the faculty of Science, King Mongkut's University of Technology Thonburi in 1994. In 1999 she was graduated with a Master Degree (Biotechnology) from the Graduate School, Kasetsart University. She has been studying for a Degree of Doctoral Phylosophy of Science in Biotechnology, the Faculty of Science, Chulalongkorn University since 2005. At present she is an assistant researcher in Ethanol and Bioconversion Team, Biomass Technology Research Center AIST Chugoku, Hiroshima, Japan.