CELLULASE PRODUCTION BY *TRICHODERMA REESEI* RUT C30

A Dissertation

Presented to

The Graduate Faculty of The University of Akron

In Partial Fulfillment

of the Requirements for the Degree

Doctor of Philosophy

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August, 2008

CELLULASE PRODUCTION BY *TRICHODERMA REESEI* RUT C30

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Dissertation

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ABSTRACT

As the price of oil continues to increase and the concern over global warming heightens, finding alternative renewable sources of energy becomes more and more imperative. Considering the abundance of lignocellulosic biomass, the potential significance of its conversion to fuel such as ethanol has long been recognized. Ethanol production from lignocellulosic biomass requires the breakdown of the solid material to simple sugars that can be consumed by microorganisms. The breakdown of lignocelluloses includes the important step of hydrolyzing cellulose, which are β-1, 4 linked polymers of glucose, by a group of enzymes collectively termed cellulase. The cost of cellulase production profoundly influences the economics of the entire ethanol production process. *Trichoderma reesei* Rut C-30 is the most commonly used fungal strain for industrial cellulase production. With an overall goal of decreasing the cellulase production cost, this thesis work was focused on two topics: (1) investigation of the performance of different cellulase-inducing substrates in *T. reesei* Rut C-30 fermentation and (2) development of a more advanced model to describe the culture behaviors of *T. reesei* Rut C-30 grown on lactose-based media. The

cellulase-inducing substrates investigated in this work included the acid-treated hydrolysate, lactose, and the sophorolipids produced by the yeast *Candida bombicola*. In addition to the above focused topics, the effects of culture conditions on cellulase production were also investigated.

The acid-treated hardwood hydrolysates used in the study were prepared by a two-stage process, with different durations of boiling and acid concentrations. The results indicated that the inducing ability of the hydrolysates decreased with increasing boiling time (less oligomers). The observation was attributed to the lower amounts of inducing oligomers remaining after the longer boiling in acid. When compared with cultures growing on mixed carbon substrates of cellulose and glucose, the culture growing on hydrolysates showed a longer lag phase of about 24 hours (before the active cell growth began), but produced adequately comparable final cellulase activity.

The study with lactose as an inducing substrate was conducted in both batch and continuous culture systems with lactose and other relevant substrates (glycerol, glucose and galactose) as the carbon source. Instead of direct ingestion, lactose is believed to be hydrolyzed by extracellular enzymes to glucose and galactose, which are then taken up by the cells. The study results indicated that glucose strongly represses the galactose metabolism: Cells started to consume galactose only after the

glucose had been depleted. A mathematical model incorporating all important metabolic activities was developed to describe the culture behaviors. All of the experimental results obtained were used in model fitting to generate a set of best-fit model parameters. The study provided significant conceptual and quantitative insights to the lactose metabolism and cellulase production by *T. reesei* Rut C-30.

This study was also the first to hypothesize and demonstrate the use of sophorolipids as the inducing substrate for cellulase production. A unique process for cellulase production using a mixed culture of *T. reesei* Rut C30 and *Candida Bombicola* growing on glycerol-based media was investigated. Hypothetically, the sophorolipids produced by *C. bombicola* were hydrolyzed to form sophorose, which then served as the inducer for cellulase production by *T. reesei*. Further study to optimize the sophorolipid-induced cellulase production process is recommended.

ACKNOWLEDGEMENTS

I would like to express my appreciation to my advisor, Dr. Lu-Kwang Ju, for his valuable suggestions and ideas in helping me to finish my Ph.D program. With his profound knowledge and rich research experience in bioprocess engineering, he not only inspires me how to conduct the research and solve problems in a systematic scientific way, but also presents a role model to enlighten my research ability through this applied and fundamental research topic. I would like to give a special thanks to Dr. Patrick Lee for preparing the dilute acid-pretreated hydrolysate applied in the evaluation of feasibility using hydrolysate. In addition, I would like to thank my committee, Dr. Amy Milsted, Dr. Stephanie Lopina, Dr. Ping Wang and Dr. Wei-Ping Zheng for their valuable criticisms. I sincerely appreciate the cooperation and assistance from my groupmates and several undergraduate students.

I am deeply indebted to my wife, Qin Zhang, also as a friend and colleague, to support me and my family, in Taiwan, who always worries about me during my research work. May joy and health be with my family always, especially my son, Matthew.

TABLE OF CONTENTS

Page

LIST OF TABLES

LIST OF FIGURES

CHAPTER I

INTRODUCTION

1.1 Nature and Significance

For many years, researchers have been trying to find the alternative energy sources for the transportation fuel because fossil energy is limited. Lately, the focus has been changed to renewable bioenergy. According to a recent report from DOE, the bioenergy accounts for three percent of the primary energy production in the United States (Energy Information Administration Posted: August 15, 2005). Currently, the largest manufactured bioenergy is bioethanol. Traditionally, bioethanol production was used in the wine industry, but in the last years or so, bioethanol has been used more in the fuel industry. In 2006, fuel ethanol use was up to 269,000 barrels/day (The United States International Trade Commission (Commission) 2006 HTSA -- Effective January 1, 2006). To obtain low-cost bioethanol, using lignocellulosic materials is one of the options. Lignocellulosic materials contain abundant polysaccharides. Pretreatment can help

polysaccharides and sugars to be liberated. The cellulooligomers, known as polysaccharides with enormous molecular weight, not only require cellulase to perform the hydrolysis of polysaccharides but also induce cellulase. Therefore, the low-cost bioethanol is achievable with an economic cellulase production process by optimizing the factors of culture condition and using lignocellulosic hydrolysate.

1.1.1 Lignocellulosic material

Plants can cleanse carbon dioxide in atmosphere. The photosynthesis requires carbon dioxide and then liberates oxygen when plants grow. This represents a form of clean energy when plants or agriculture products, known as lignocellulosic materials, are used for a source of fuel, such as bioethanol or combustion. The bioethanol production requires lignocellulosic materials as substrates in the process. The composition of substrates treated from lignocellulosic materials in the process affects the bioethanol production.

Lignocellulosic materials consist of cellulose, hemicellulose, lignin, and some extractives. The composition is different from species to species. For example, there were about 50% cellulose, 22% lignin, 23% hemicellulose and 5% extractives in hardwoods.

In comparison, there were 45% cellulose, 30% hemicellulose, 15% lignin and 10% extractives in herbaceous energy crops (McMillan 1994b). Furthermore, the detailed structures of these substances, in particularly cellulose and hemicellulose, determine the composition of fermentable sugars in the hydrolysates.

Cellulose is a polysaccharide consisting of β-1, 4-linked glucose units, which can be hydrolyzed by cellulase for glucose. Hemicellulose consists of xylan polysaccharides and xyloglucan with a backbone of β-1, 4 linked xylopyranosyl units substituted with three 4-O-methyl glucuronic acid per 97 xylopyranosyl units (Mazumder et al. 2005) and is responsible for the pentoses, generally, xylose. Lignin is a complex that is formed by ether linkage within bulk lignin and also by ester linkage between the cell wall and cell tissues (Oliveira et al. 2006). The practical uses of lignocellulosic materials depend on the hydrolysates after enzymatic hydrolysis.

1.1.2 Cellulase

Cellulose conversion is accomplished by the concerted action of a group of enzymes, collectively termed cellulases. Three distinct enzymatic activities are required: a β-1, 4-glucan glucoanohydrolyase that has endocellulase activity, a β-1, 4-glucan

cellobiohydrolyase that has exocellulase activity, and a β-glucosidase that cleaves cellobiose to glucose. The hydrolysis of cellulose begins with a random internal hydrolysis by the endoglucanase to disrupt the crosslinking and to create new polymer ends accessible to the exocellulase. This also solubilizes the substrate by reducing intrachain hydrogen bonding. The cellobiohydrolase hydrolyses the non-reducing end of cellulose and generates cellobiose with some larger oligosaccharides. Finally, the β-glucosidase completes the breakdown process by generating glucose from cellobiose. The mechanism of induction and repression with respect to cellulase, stimulated by these hydrolysates of cellulose influences cellulase production.

In most cases, the production and/or activity of cellulase components are repressed or inhibited by the hydrolysis products. We have clearly demonstrated this in a previous experiment using resting cells of *T. reesei* RUT C-30 (Ju and Afolabi 1999). The cells harvested at the exponential-growth phase were suspended in N-free media containing 5 g/L of Avicel (cellulose, Sigma) and different glucose concentrations, i.e., 1, 5, 10, and 20 g/L. The production of cellulase began only when the glucose was nearly depleted in all cases, and the highest production was obtained in the system with only 1 g/L of glucose.

The feedback regulation allows the organisms to conserve energy when sufficient glucose is present. The repression by the end-product glucose is especially difficult to control in conventional batch fermentation. The glucose concentration depends on the dynamic balance between the rates of glucose generation (by cellulose hydrolysis) and consumption (by microbial uptake). At low concentrations of cellulase and/or cellulose, the glucose generation may be too slow to meet the need of active cell growth and function. On the other hand, cellulase synthesis can be halted by glucose repression when glucose accumulates because of its faster generation than consumption. For batch fermentation using solid substrates, the slower glucose generation can lead to a prolonged lag phase initially while the glucose repression limits the cellulase production period. The glucose repression may be reduced by fed-batch operations, which have been shown to improve the cellulase productivity (Taherzadeh et al. 2000).

The mechanism of cellulase induction is not clear and controversial to date. Cellulase is not only induced by cellulose and its hydrolysates, cellulooligomers, but also lactose and sophorose, low molecular weight sugars. Cellulose and cellulooligomers cannot penetrate cell membrane and so does lactose. Furthermore, none of their final products, generally glucose or galactose, shows induction capability (Ju and Afolabi 1999;

Fekete et al. 2007). The induction could sheer happen *in vivo*. Therefore, researchers demonstrated the transglycosylation from glucose to sophorose was the most possible mechanism to cellulase induction (Vaheri et al. 1979).

1.1.3 Pretreated hydrolysate

The economic production of cellulase is important in the effective conversion of cellulose to sugar for ethanol production. In the commercial facility, it will be more economical in using the sugar solution (hydrolysate)/pretreated material produced from biomass for cellulase production than synthetic sugar/biomass medium. This pretreated material is generally made of chemically or physically treated raw materials such as hardwood, softwood, and other crops. It is capable of cellulase induction and has rich mixture of fermentable sugars. Therefore, pretreated material and hydrolysate will be produced using TVA (Tennessee Valley Authority) hydrolysis facility and dilute acid pretreatment to study the cellulase production.

The process of dilute acid pretreatment was changed at the last step in order to obtain different ratios of cellulooligomers to sugars (mono-saccharides). There are six-batch hydrolysates in this study. According to TVA, mixed hardwood sawdust was

first obtained from a local sawmill. Concentrated sulfuric acid (80 wt%) was added slowly to the mixed biomass. Significant reduction in volume resulted from the mixing. The final "gel" was dark and viscous. At the end of mixing, distilled water was added to the "gel", to dilute the acid to 20%. The acid solution was boiled gently, with water addition to compensate for vaporization loss, for a total of 120 min. During the boiling process, acid solution was harvested for different batch at 15, 30, 45, 60, 90, and 120 min. The composition of pretreated hydrolysate varies with boiling time.

Although ample fermentable sugars and cellulooligomers present in the lignocellulosic hydrolysate benefit cellulase production, the by-products from pretreatment process also has been produced. The by-products are toxic such as furfural, acetic acid, levulinic acid, hydroxymethylfurfural (HMF). By-products have tremendous impact on cell growth and cellulase production. For instance, the hydrolysate containing by-products have demonstrated that the high concentration of HMF, furfural or acetic acid caused close to a cessation of cell growth and low cellulase production (McMillan 1994b; Palmqvist et al. 1997; Lasson et al. 1999). Once the by-products were removed from the hydrolysate, the cells began to utilize hydrolysate and cellulase production was significantly recovered (Lo et al. 2005).

1.1.4 Factors of culture condition affecting cellulase production

The factors of culture condition have different characters of influence to affect cellulase production. The intrinsic difference between factors, in this study, pH, dissolved oxygen, and the state of substrates is inherent in processes.

First, the state of substrates changes the property of solution. Substrates could be solid or liquid form when applied in the process. For example, solid cellulosic materials were commonly used as both the carbon/energy substrate and the source of inducers for the fermentation process (Yu et al. 1998; Sethi et al. 1999; Zhang et al. 2007). The use of solid substrates, however, causes certain problems to the operation and productivity in cellulase production process. In particular, the solids increase the burden on agitation, power input or energy cost, and viscosity. Due to high viscosity, using solids in the process is detrimental to the efficiency of oxygen supply in the bioreactors. The compromised oxygen supply, in turn, reduces the employable cell concentration and the attainable productivity. Cellulase production increased by reducing the solid substrate concentrations when dissolved oxygen was inadequately (Szengyel et al. 1997). On the other hand, soluble substrates and inducers can efficiently benefit cellulase production when compared with solids. They can provide a better culture condition with respect to

DO (dissolved oxygen), and viscosity. Besides, cellulase synthesis in *Trichoderma* is subject to induction-repression mechanism, including induction by the intermediates during cellulose hydrolysis, and glucose repression by glucose simultaneously (Ilmen et al. 1997; Ju and Afolabi 1999; Manabu and Fusao 2001). The soluble substitutes must still be able to induce the cellulase synthesis and have least repression effect, owing to easier degradation than solids. Furthermore, among the several pure saccharides studied, sophorose was the most powerful soluble inducer for *Trichoderma* (Mandels et al. 1962; Nisizawa et al. 1971; Sternberg and Mandels 1982; Jeong et al. 1985). Nevertheless, sophorose was expensive and still less potent in induction ability when compared to solid cellulose (Sternberg and Mandels 1979). Bailey and Taehtiharju (2003) have recently used the cheaper lactose as the sole inducing C-source for cellulase production plus the base addition for pH control as the guiding factor to regulate the substrate feeding. Also, the soluble enzymatic hydrolysates of pure cellulose or complex cellulosic materials such as wastepaper have been demonstrated to have induction ability in cellulase synthesis (Allen and Mortensen 1981; Ju and Afolabi 1999).

Dissolved oxygen is another key element to cellulase production. Cells, the most productive strain *Trichoderma reesei* Rut C-30 (Mandels et al. 1962), produce cellulase

under aerobic condition (Emerson and Natvig 1981). When the oxygen limitation happens, cells stop growing and producing cellulase. Subsequently, cells suffocate and die rapidly. In addition, the morphology changes when the DO changes. For example, At low DO condition (typically less than 30%), cells will also change their morphology to pellet form, which has poor cellulase production (Cui et al. 1997). Under the anaerobic condition, oxygen deficiency could also cause sporulation. This is an adverse condition to cellulase production. Therefore, DO is unlikely to be changed up and down in the process.

Besides substrates and DO, cellulase production requires a constant and suitable pH. Cells produce organic acids while doing aerobic carbon catabolism. Without pH control, pH decreases as the process lasts. The operational pH range is pH 3.0 - 6.0, for *T. reesei* RUT C30 (Ryu and Mandels 1980; Juhasz et al. 2004). Once the pH is controlled at outside of proper range or with sensible pH variation, the change of proton concentration may affect cytoplasmic pH, which could cause intracellular proteins, function at stable pH, to be denatured. Furthermore, in order to have extracellular enzymes function well, a proper pH is required for the optimal enzyme activity. Cellulase production generally involves the hydrolysis reaction between substrate and enzymes. Cellulase, induced by

cellulose, is produced after cellulose has been hydrolyzed. The chosen pH affects the rate of cellulose hydrolysis and thus, cellulase production.

To conclude, pretreated hydrolysate provides an approach to achieve a low-cost biomass based bioethanol and an efficient cellulase production process needs an optimization of the factors affecting culture condition. An expensive cellulase production in the fermentation process is going to determine how effective the process can be utilized for further applications by using renewable cellulosic materials and wastes (Palmer 1975; Shin et al. 2000). Therefore, the induction ability of different carbon substrates, including lactose, dilute acid-treated hydrolysate, and sophorolipids, on cellulase production by *T. reesei* Rut C-30 was evaluated in this thesis work. A mechanistic model was also developed to incorporate the important mechanisms that control cellulase synthesis, and to predict the change of productivity as the cell metabolism changes. The cellulase induction ability by sophorolipids was hypothesized and confirmed in this work. Some work was done to investigate the issues associated with the future usage of sophorolipids in cellulase production.

1.2 Scope of research

The synthesis of cellulase involves complicated metabolism occurring inside the cells of *T. reesei* Rut C30. For bioprocess engineering, the observation and manipulation of outside environmental conditions of cells from macroscopic viewpoints are critical. Therefore, the scope of research here falls into the following three subjects: (1) examination of the factors affecting cellulase production, (2) examination of the cellulase production using dilute acid-treated hydrolysate, and (3) examination of the metabolic changes of cells grown on lactose in a continuous culture system.

1. Factors: Environment change of culture condition affects cell growth and cellulase production. The culture condition is controlled by several operational factors. The effect of these operational factors on cellulase production was observed and examined by using batch systems.
2. Dilute acid treated hydrolysate: The sawdust collected from sawmill was treated with dilute acid pretreatment method. After being treated, the pre-treated hydrolysates are used as substrates for cell growth and cellulase production. The effect on cell growth behavior and cellulase production is evaluated by using pre-treated hydrolysates in both batch and continuous systems.

3. Cell metabolism using lactose: Lactose is a cellulase inducer and carbon source for cell growth. A series of experiments have been done in batch and continuous systems to study its effects on cell metabolism and cellulase production. In order to further understand the metabolic changes, a mechanistic model is built and examined.

1.3 Research objectives

The specific objectives in this project are to

(1) Determine the effects of different limiting nutrients on prolonged cell survival and cellulase synthesis during the stationary phase

(2) Determine the effect of dilute acid pretreated hydrolysate on the cellulase production

(3) Establish a mathematical model to describe the culture behaviors of cellulase-producing *Trichoderma reesei* Rut C-30

(4) Evaluate the feasibility of using sophorolipids as an inducer in cellulase fermentation process.

1.4 Structure of the dissertation

This dissertation consists of six chapters. Chapter I is a brief introduction on the background and objectives of the research conducted in this thesis work. In Chapter II, the fundamental principle and the knowledge and findings reported in the literature for each subject related to this research are presented. Chapter III presents all the analytical methods throughout the thesis. Reported in Chapters IV to VII are the experimental results obtained in this thesis work. The focus of Chapter IV is the effects of each critical factor of culture conditions on cell growth and cellulase production. In Chapter V the results of cellulase production using pretreated dilute acid hydrolysates as substrate are presented. An evaluation on the performance in continuous culture of a dilute acid pretreated hydrolysate is also reported in Chapter VI. A mechanistic model is described in Chapter VI. The model was developed to describe the behaviors of *T. reesei* Rut C-30 in the continuous culture system with a lactose-based medium. In Chapter VII, a newly designed process for sophorolipid-induced cellulase production using a mixed culture of *T. reesei* Rut C30 and *C. bombicola* is described. In Chapter VIII, the conclusions with respect to each objective are addressed and also the recommendations for the future study are proposed.

CHAPTER II

LITERATURE REVIEW

2.1 Origin of *Trichoderma reesei*

Cellulase has attracted significant attention since the researchers discovered its ability to hydrolyze cellulose. The good cellulase-producing strains were soon isolated, including the strain *Trichoderma viride* QM6a first selected from a soil sample at Bougainville Island. *T. viride* QM6a was subjected to many genetic improvement for cellulase production. *T. reesei* Rut C30 was a hyperproducing strain selected after UV light treatment (Montenecourt and Eveleigh 1979). *Trichoderma* strains have been used in cellulase production for two decades.

The differentiation of taxa of the cellulase producing strains in the *Trichoderma* genus is difficult and confusing. Fungi belong to one of the five kingdoms in ecosystem, which was defined by Robert Whittaker based on the way of taking nutrients into the cells (Whittaker 1978). Fungi include yeasts, molds, and mushrooms. Within the

kingdom of fungi, there are two large phyla, basidiomycota and ascomycota. Both phyla have mycelium structure, except for yeasts. Besides the morphological difference, basidiomycota (mushroom shape) and ascomycota (mycelium), another difference between these two phyla is the way of sexual reproduction. Basidiomycota have club-shaped ends called basidia with external spores called basidiospores, while ascomycota have many hyphae containing ascospores in sexual spore-bearing cells, asci. However, some of the species in these two big phyla, which are asexually reproduced (anamorph state), are classified into deuteromycota, also called molds. Therefore, for example, *Trichoderma reesei*, which has mycelial morphology and asexual reproduction, is a mold. For those most commonly used cellulase producing species, *Hypocrea jecorina* (*Trichoderma reesei*), is an ascomycete (Whittaker 1978).

Trichoderma species have been popular industrial strains in many applications. Viridiol, a dihydro-derivative of viridian and high herbicidal activity, was produced by *Trichoderma* species (Jones and Hancock 1987). *Trichoderma* species also have been used for plant growth promotion, to increase germination rates and dry weights (Harman et al. 1989). The conidia of *Trichoderma* have been used for propagules in the biocontrol program (Elad et al. 1993). Cellulase enzymes have been used for many applications as

well, such as detergent, starch, brewing, animal feed, and ethanol production. For example, it has found more and more use in the textile industry. Cellulase breaks off the small cotton fiber ends on the yarn surface and helps to create more gentle and comfortable textile than the stone-washing machines does, which is called biopolishing or biofinishing (Galante et al. 1998).

2.2 Functions of enzyme cellulase

Trichoderma reesei can grow on a variety of carbon sources such as glycerol, glucose, sophorose, xylose, cellulose, hemicellulose, and lactose. Small molecules like glucose and glycerol can easily get through the cell membrane and be directly metabolized as an energy source. Other disaccharides and polysaccharides cannot be taken directly by cells without being altered or broken down by enzymes into small molecules, like glucose or galactose. Cellulase is the only enzyme that can break down cellulose, a β-1, 4-linkage polysaccharide, into glucose. However cellulase induction happens *in vivo* and glucose or galactose cannot induce cellulase. Researchers have suggested that the induction of cellulose could come from the sophorose provided by the transglycosylation of either cellobiose or glucose, driven by β-glucosidase (Gritzali and

brown 1979; Vaheri et al. 1972)(Saloheimo et al. 2002). Almost all the genes for cellulase enzymes have already been identified, including those for cellobiohydrolases (exoglucanases) (*cbh*1, *cbh*2), endoglucanases (*egl*1, *egl*2, *egl*3, *egl*4, and *egl*5), and β-glucosidase (*bgl*1, *bgl*2). Some of them may be responsible to the occurrence of transglycosylation.

Endoglucanases (EC 3.2.1.4, EG I, EG II, EG III, EG IV, and EG V), which cleave a long chain of β-1, 4-glycosidic bonds randomly, are the first enzyme so attack the cellulosic materials, making a long cellulose chain into shorter chains. It is a wide-spectrum enzyme, which can hydrolyze a wide range of different cellulosic materials such as cellulose, hemicellulose, and carboxylmethyl cellulose (CMC). Although EGs have silimar structure to exoglucanases (CBHs), EGs, with more than one open catalytic sites, can act on the different places of long chain cellulose and easier when compared to CBHs, only one tunnel shape catalytic site. Also, EG I is not found in conidia (Messner et al. 1991) and EG II is found not responsible for the transglycosylation (Suominen et al. 1993). With the deletion of *egl*1 gene, it reduces all levels of the cellulases expression but CBH I and CBH II (Seiboth et al. 1997). EG III and BGL I, among all cellulases, do not have a modular structure and a cellulose binding

site (Penttila et al. 1986). Only EG III and EG V have lower molecular weights, 25 and 23 kDa respectively, and they could hydrolyze filter paper and carboxylmethyl cellulose but not Avicel, a commercialized cellulose made by Sigma. EG III had the lowest activity among EGs (Karlsson et al. 2002). EG IV, 56 kDa, is regulated by CRE1, a repressor protein and like CBH I, EG I, and EG V, it has two Cellulase Binding Domains in C-terminus (Saloheimo et al. 1997).

Exoglucanases (EC 3.2.1.91), also known as cellobiohydrolases (CBH, CBH I and CBH II), attack the two ends of each shorter polysaccharide chain. The end product would be further hydrolyzed to cellobiose, a dimer of glucose. It typically accounts for 80% of the amount of enzyme cellulases produced in *Trichoderma* fermentation. The structure of exoglucanases has two functional domains, catalytic domain and cellulose-binding domain, which are connected by a linker peptide. There are two types of CBHs, CBH I and CBH II. CBH I attacks the reducing ends of cellulose, and CBH II the non-reducing ends. There are two activator proteins found in *T. reesei*, ACEI and ACEII, which activated the process of cellulase transcription. ACEII, a cellulase activator protein involving glutamine-proline rich activation domain, contains a zinc binuclear cluster DNA-binding motif bound to the 5'GGCTAATAA sequence, which commonly

exists in cellulase genes. The 5'GGCTAATAA sequence is found in *cbh*1, behind an ATG initiator -779 bp, and *cbh*2 promoters. On the other hand, ACEI which is transcribed from an *ace*1 gene encoding a zinc finger, bound to eight sites within a *cbh*1 promoter containing 5'AGGCAAA sites (Aro 2003). In addition, the deletion of *cbh*1 makes the levels of mRNA of *cbh*2 increase and with both deletion of *cbh*1 and *cbh*2, it stops cell growth and cellulase induction in a cellulose-based medium (Suominen et al. 1993). When *cbh*2 gene is deleted, it does not influence cell growth and cellulase induction at all (Seiboth et al. 1992).

The last enzyme is β-glucosidase (EC 3.2.1.21, BGL I and BGL II). β-glucosidase has been found in three places, cell membrane (Umile and Kubicek 1986), intracellular (Inglin et al. 1980), and extracellular (Chririco and Brown 1987). One of BDLs is BGL I, an extracellular protein and the gene of BGL I has been isolated (Barmett et al. 1991). Also, having multiple copies of *bgl*1 gene in transformant of *T. reesei* Rut C30 increases the rate of cellulose hydrolysis and this implies that the rate limiting step of cellulose hydrolysis is limited by the small amount of BGL I protein (Barmett et al. 1991). The deletion of *bgl*1 hinders the induction of other cellulase enzymes by cellulose, but it does not affect the induction by sophorose. Besides, the culture still shows β-glucosidase

activity, most likely from BGL II, after the deletion of *bgl*1 gene (Fowler and Brown 1992). The gene of *bgl*2 has been found (Takashima et al. 1999) and characterized as an intracellular (but not cell-membrane bound) protein of about 50 kDa (Saloheimo et al. 2002). The CREI repressor protein, induced by glucose (detailed in next section), still regulates the expression of *bgl*2 and the CREI binding site is also found in the *bgl*2 promoter region. In addition, *bgl*2 still can be expressed in glucose-based medium when using *T. reesei* Rut C30. Like BGL I, BGL II showed significant transglycosylation activity when using either 40% of glucose or 20% of cellubiose (equal molar concentration). Sophorose could be hydrolyzed by BGL II, but BGL II could not hydrolyze lactose (Saloheimo et al. 2002).

2.3 Carbon catabolite repression.

Over the past 50 years, the regulation of expression of cellulase enzymes has been extensively studied. These studies have mostly been done on the most productive species *T. reesei* (Johnston et al. 1994; Ilmen et al. 1996; Ilmen 1997; Takashima et al. 1998; Strauss et al. 1999). The expression of cellulase enzymes is coded by a series of genes for cellobiohydrolases (exoglucanases), endoglucanases and β-glucosidase. The synthesis of

these enzymes is mainly controlled by the regulation of end-product repression, namely the level of glucose concentration (Figure 2.1).

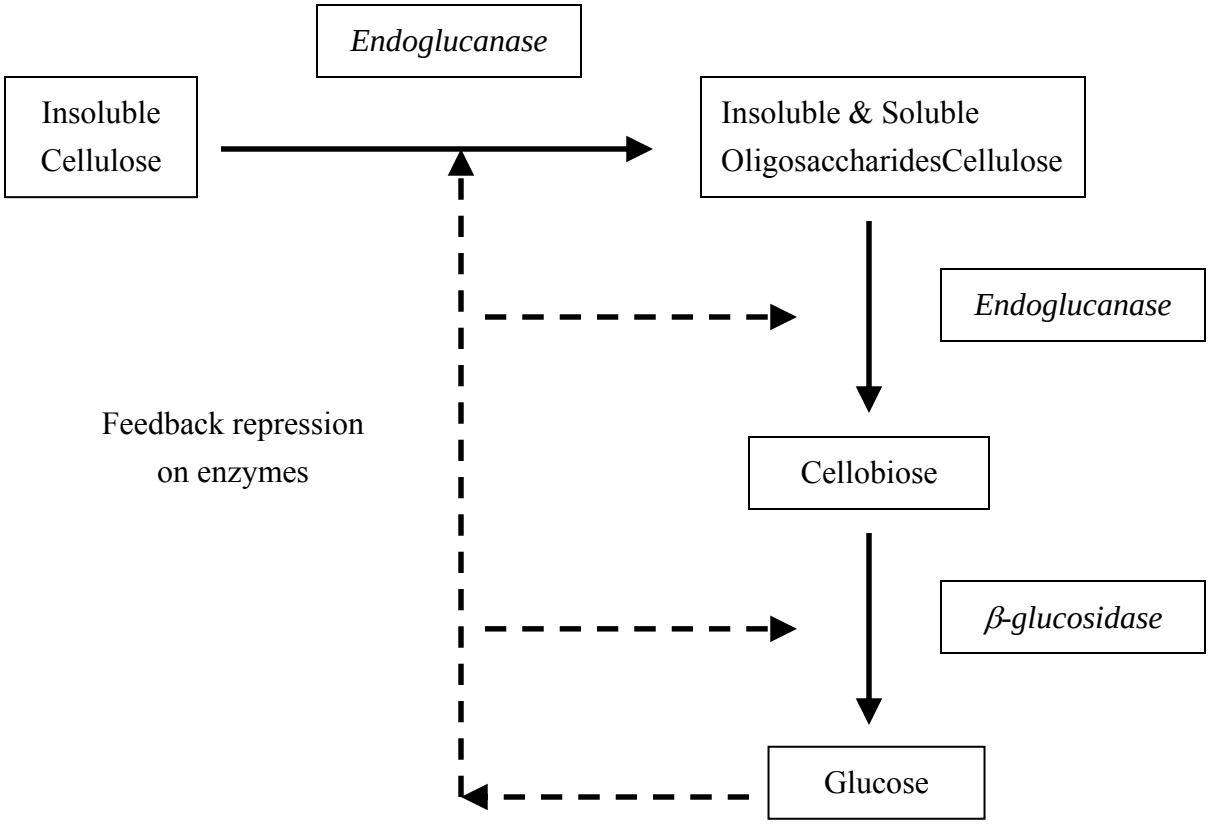

Figure 2.1 Carbon catabolite repression on cellulase synthesis (Bailey and Ollis 1944)

The function of cellulase is primarily used for the hydrolysis of cellulosic materials such as cellulose, and hemicellulose. Cellulase consists of three types of enzymes, endoglucanases, cellobiohydrolases (exoglucanases), and β-glucosidase. The rate of synthesis of cellulase is controlled by the concentration of each end product, catalyzed by each enzyme. For example, at the condition of high concentration of glucose, it would not only repress the further synthesis of β-glucosidases, also the synthesis of endoglucanases. Therefore, when the rate of hydrolysis is fast which would make the instant accumulation of end products higher than the threshold for each enzyme, cells would stop producing cellulases. The mechanism of the end-product repression has been well reviewed by (Ilmen 1997).

The mechanism of carbon catabolite repression has been found in both prokaryotes and eukaryotes. The mechanism of gene regulation in eukaryotes is different from that in prokaryotes. This mechanism in both is controlled by the level of glucose concentration. From another viewpoint, glucose induces the expression of repressor proteins. In *E. coli.*, for example, cyclic AMP-binding protein (CAP) was found as an activator of the catabolite-repressed genes and its activity varies with different glucose concentrations. The concentration of CAP, binding cyclic AMP (cAMP), is also reduced as glucose

concentration increases. The level of complex of cAMP and CAP controls the transcription of the catabolite-repressed genes. With excessive supply of cAMP, carbon catabolite repression could be de-repressed (Yamakawa and Kuno 1983). In eukaryotes, a great diversity of proteins act on catabolite-repressed genes, differing from species to species. DNA-binding repressor proteins are found in *Saccharomyces cerevisiae* with MIG 1 (Johnston et al. 1994; Treitel and Carlson 1995), *Aspergillus nidulans* with CRE A (Strauss et al. 1999), and *T. reesei* and *harziamum* with CRE I (Ilmen 1997). Nonetheless, the action to each protein is different. MIG 1 would bind to target sequence in the promoters with help of the complex, SSN6 (CYC8)-TUP1 (Treitel and Carlson 1995). Besides, MIG 1 represses other genes when using other sugars for growth, such as, galactose and sucrose. For instance, the promoters of *SUC*2 gene, encoding invertase (Nehlin and Ronne 1990), and GAL 1 (Johnston et al. 1994), encoding galactokinase are directly bound by MIG 1. CRE A repression happens at post-transcriptional level and formed the complex of CRE A-DNA binding. CRE A is less or degraded when concentrations of glucose and ethanol are lower. Cellulase gene and alcohol dehydrogenase gene, *alc*A, expressions are both repressed by CRE A (Strauss et al. 1999). Cellulase promoters are bound by CRE I proteins. The levels of *Cre*1 mRNA increase as

glucose concentration increases. CRE I, found in *T. reesei* Rut C30, has higher level of mRNA than the level in QM9414 and the length and pattern of CRE I are different. This smaller CRE I protein, produced by Rut C30 only, could partially repress *cbh*1 repression (Ilmen et al. 1996).

The structural configurations of proteins cause the carbon catabolite repression to take place. *T. reesei* QM 9414 has *cre*1 gene with 402 amino acids, open reading frame (ORF) of 1206 nucleotides, and *T. hazianum* gene has 409 amino acids, an ORF of 1227 nucleotides. The cDNA of *cre*1 gene has 1946 bp with a 5' end at 288 bp of upstream of the predicted initiator ATG followed by two polyadenylation regions at about 280 and 450 bp before the stop codon in downstream of the translation (Ilmen et al. 1996; Takashima et al. 1998). The two zinc finger regions of CRE I are highly homologous to the same zinc fingers of MIG 1 protein in yeast and CRE A protein in *A. nidulans*. The CRE I promoters have exact same sequence with the sequence of binding site on CRE A, the two zinc finger regions, Cys_2-His_2-class zinc finger regions. Therefore, both CRE A and CRE I should share the same target sequence, the consensus sequence 5'-SYGGRG-3' located in the upstream region of cellulase genes. However, *T. reesei* Rut C30 has shortened CRE I protein and misses one of the two zinc fingers, found in normal

CRE I. This partial repressor protein might be the reason that makes the *T. reesei* Rut C30 high tolerance of glucose carbon catabolite repression. In addition, the binding action occurred in the regulation of transcription. It has been found whether the inducers of cellulase are present in culture broth or not, CRE I would be translated as long as the presence of glucose in broth (Ilmen et al. 1996). Vautard-Mey et al.(1999) reported that the location of CRE I protein changed from nucli to cytosol fractions in *Sclerotinia sclerotiorum* when glucose was depleted in culture medium. Therefore, the cellulase gene is derepressed when CRE I are all in cytosol fractions. This behavior has also been found in *A. nidulans* (Vautard-Mey et al. 1999).

2.4 The function of galactosidase

Enzymes as biological catalysts involve the mechanisms of induction and degradation. Generally, they react with a specific configuration within a molecule, not an entire structure. For example, cellulases are induced by β-1,4 linkage between two glucoses and they break down the linkage afterwards. Galactosidase is an enzyme to hydrolyze substrates like raffinose and lactose, into sucrose and α-D-galactose, and glucose and β-D-galactose, respectively. There are two types of galactosidases which

work on different specific structures and they both could be induced by pretreated hydrolysates.

α-Galactosidase (AGL, α-D-galactoside galactohydrolase, EC3.2.1.22; from genes *agl*1, *agl*2, and *agl*3) is an intracellular enzyme present both in the cytoplasm and on the cell membrane (Thornton 2005). It is an enzyme specifically working on the α-1,6 linkage, such as that in raffinose, PNP-Galactopyranoside, and 4-methylumbelliferyl α-D-galactopyranoside (Shabalin et al. 2002). It can also hydrolyze α-1,6-linked D-galactopyranose units which animals and human cannot digest. In addition, α–galactosidase synthesis can be induced by the hydrolysate of the hemicellulose found in softwood containing galactomannanes and galactoglcomannanes, and then be used for converting the hydrolysate to single sugars (Clarke et al. 2000). AGL I shows the highest activity among the three AGLs and *T. hamatum* could only produce AGL I (Thornton 2005).

β-Galactosidase (EC 3.2.1.23, BGA I) specifically hydrolyzes non-reducing β-D-galactose ends of β-D-galactosides, for example, lactose. BGA I is only found extracellularly and it is induced strongly by L-arabinose, L-arabinitol, and weakly by lactose and galactose (Seiboth B. 2005; Fekete et al. 2007). After the hydrolysis of

lactose, glucose goes through the Embden-Meyerhof-Parnas (EMP) pathway and galactose goes through the Leloir pathway. CRE I repressor protein still partially regulates the expression of *bga*1 when using *T. reesei* Rut C30 (Saloheimo et al. 2002). The optimal condition for β-galactosidase activity is at pH 5.0 and 60 ^{o}C (Gamauf et al. 2007).

2.5 Different inducers of cellulase

The filamentous fungus, *Trichoderma reesei*, is well recognized as a most productive strain for producing cellulase enzymes. The research regarding cellulase production is generally done by using *Trichoderma* species, for example, the effect of different inducers on cellulase production or catabolism in *Trichoderma* species. Cellulase enzymes are partially secondary metabolites and they require inducers to activate the processes of protein expression. Therefore, to understand how the synthesis of these cellulase enzymes triggered by different substrates, at genetic level, and protein expression, is the most important fundamental question for the cellulase production. Cellulose is a natural inducer. Some other inducers are also known, such as lactose, sophorose and dilute acid-treated hydrolysate.

2.5.1 Cellulose

Cellulose, a water insoluble polysaccharide of β-1, 4 glucosidic bonds $(C_6H_{10}O_5)_n$, is the most abundant biomass in the world. Because of its availability and accessibility, lignocellulosic material has been considered as a main source of ethanol production. It also has been considered as a natural inducer of cellulase. However, the mechanism of induction by cellulose is still not clear because being large insoluble macromolecules, cellulose cannot go through the plasma membrane directly. Cellulose is degraded by cellulase into oligosaccharides, cellobiose, and glucose sequentially. It has been proved that the synthesis of cellulase was regulated by the end-product, glucose, at the transcriptional level. The mRNA of cellulase genes was expressed when the glucose concentration was low or the cells were transferred to a glucose free system containing cellulose (Margolles-Clark et al. 1997; Kubicek and Penttila 1998). Although high contents of solid cellulose cause agitation and oxygen transfer problems in the fermentors, they often allowed the production of higher enzyme activities when compared with the use of other weak inducers like lactose and xylose (Muthuvelayudham and Viruthagiri 2006).

2.5.2 Hemicellulose

Hemicellulose, amorphous cellulose, is an easier target for hydrolysis process. It is also a polymer of mainly pentoses with hexoses and sugar acids. Hemicellulose generally presents in a form of xylan which is a β-D-xylose units linked by a 1,4-glycsidic bond. For complete hydrolysis of xylan, it requires several types of hydrolytic enzymes and xylanase is the most important for breaking down long chain back bone to xylooligosacharides or xylose dimers followed by the hydrolysis of xylooligosaccharides by β-D-xylosidase. Most of hemicellulose, above 80%, enzyme degraded to form CO_2, H_2O and when used sulfuric acid, it generated furfural and 5-hydroxy-methl-furfural (HMF) by hydrolyzing pentoses and hexoses, which inhibited cellulase synthesis (Lasson et al. 1999).

However, like cellulase, the induction-repression mechanism controlled the efficiency and production of xylanase. Xylanase synthesis is also affected by glucose concentration, known as glucose repression. Xylan and various xylooiligomers have been reported to have induction ability to xylanase. Other sugars, such as xylose, lactose, sorbose, and cellobiose may have induction or repression effect depending on the type of species. For example, the 48-hour old resting cells of *T. reesei* PC-3-7 and QM9414 were

used for induction ability study on testing different substrates, xylose, sorbose, xylooligomers, and sophorose at 28 ^{o}C, 200 rpm and pH 4.0 (Xu et al. 1998).

2.5.3 Hydrolysates

Effective cellulase production is important for the utilization of renewable cellulosic materials and wastes (Ju and Afolabi 1999). Batch or fed-batch fermentations of the fungi *Trichoderma* were typically used for cellulase production (Czajkowska et al. 1992). Cellulase synthesis in *Trichoderma* is subject to induction by the intermediates formed during the cellulose hydrolysis (Sternberg and Mandels 1979; Ladisch et al. 1981). Solid cellulosic materials were therefore commonly used in the fermentation as both the carbon/energy substrate and the source of cellulase inducers, e.g., cellulose (Grethlein 1985; Perminova et al. 1985). The use of solid substrates, however, causes problems to the fermentation operation and productivity. In particular, the solids increase the burden on agitation and lower the oxygen supply efficiency of the bioreactors, which in turn reduces the employable cell concentration and the attainable productivity. It has been shown that lower solid concentrations yielded higher cellulase production (Szengyel et al. 1997).

More recently, we have been investigating the feasibility of combining *in situ* foam fractionation with cellulase fermentation (Zhang et al. 2007). Potentially the coupled process may improve cellulase production by minimizing catabolite (glucose) repression and reducing cellulase degradation by proteases. When examining the foaming behaviors of fermentation broths of *Trichoderma reesei* Rut C-30, the solid cellulose (Avicel) powders were found carried out by the foam although increasing its concentration led to less foamate generation. To prevent or minimize the substrate loss, it is desirable not to use the solid cellulose as the substrate in the coupled foaming fermentation process. On the other hand, the soluble substitute should still be able to fully induce the cellulase synthesis. Among several pure saccharides studied, sophorose was the most powerful soluble inducer for *Trichoderma* (Lee 1982; Sternberg and Mandels 1982; Jeong et al. 1985). Nevertheless, besides being expensive, sophorose was still considered less potent than cellulose for cellulase induction (Mandels et al. 1962). The induction by sophorose was also reported to be less complete: some components of the cellulase (exo- and some endo-glucanases) were absent in sophorose-induced systems (Mandels et al. 1962). Lactose has been successfully used in continuous culture recently, as sole carbon source and obtained high cell concentration and high enzyme productivity with stable cell

morphology by using pH control indicating cell physiology (Bailey and Tahtiharju 2003). However, the mechanism of induction of lactose remains unclear to date.

The enzymatic hydrolysate of pure cellulose and that of complex cellulosic materials such as wastepaper have been shown more effective for cellulase induction than simple saccharides (Szengyel et al. 1997; Ju and Afolabi 1999). The last is often found with complex hydrolysate and has been attributed to some by-products generated during the hydrolysis processes. These include furfural, acetic acid, levulinic acid, hydroxymethylfurfural, and some other organic compounds (McMillan 1994b; Palmqvist et al. 1997; Lasson et al. 1999). TVA's process improved the conversion of cellulosic material to hydrolysate by harmonizing operation condition with minimal biohazard concentration. In addition, the residue of process could be used as a substitute for coal in combustion process. Overliming has been reported to reduce the inhibitory effect on cellulase production (Ranatunga et al. 2000; Martinez et al. 2001), in addition to its essential role of neutralizing pH for the hydrolysate produced by acid-based processes. Once calcium sulfate generated by reaction of sulfuric acid and calcium hydroxide, it can be easily removed by centrifugation.

It should be noted that each cellulosic material, like wheat straw, hardwood or softwood, has its own specific composition. It has been proven that hardwood can be treated easier than softwood (Brownell and Saddler 1984; Schwald et al. 1989; McMillan 1994b). The characteristics of cellulosic material are important for cellulase induction such as crystallinity (Lee 1983; Bertran and Dale 1985) and specific surface area (Grethlein 1985; Converse et al. 1990), both affecting the degradation of cellulose. Specific surface area is related to the shape and size of pore and particle of cellulosic material (Cowling and Brown 1969; Stone 1969; Ladisch et al. 1981). Crystalline region is more difficult to be hydrolyzed than the amorphous region and the rate of overall cellulose hydrolysis is controlled by how much it exists in the cellulose solids (Huang 1975; Ladisch 1983; Coughlan 1985). These two specific characteristics are also critical for cellulase production and it is difficult to find substitute that mimics their effects (Mattinen et al. 1995). Therefore, by using chemical or physical method, increasing the specific surface area and reducing the level of crystallinity has been considered beneficial to increase cellulase production (Gharpuray 1983; Grous 1986; Hans and Alvin 1991).

Pretreatment is the way to make raw materials, such as hardwood and softwood, to be more easily degradable and accessible for enzymatic hydrolysis. McMillan (1994a)

has reviewed all the pretreatment methods reported before 1994. One of the methods, dilute acid pretreatment, was considered more efficient, lower-cost and easier to make commercial quantity of hydrolysate. However, some undesirable by-products would be produced by this method, namely, furfural, acetic acid, levulinic acid, hydroxymethylfurfural (HMF) and some other organic compounds (McMillan 1994b; Palmqvist et al. 1997; Lasson et al. 1999). In order to remove those toxic or inhibitory to cells, the hydrolysis is followed by an overliming process, which uses lime for detoxification. The detoxified hydrolysate can then be used as substrate for cell growth and cellulase production.

2.5.4 Lactose

Lactose is a byproduct of production and of lactose-free milk. It is also an economical water soluble inducer for cellulase production. Lactose is a disaccharide consisting of β-D-galactose and β-D-glucose molecules bonded with a β-1, 4 glycosidic linkage. Lactose can induces lactase (β-1, 4 galactosidase) and cellulases when using *T. reesei* (Castillo et al. 1984; Collen et al. 2004). β-Galactosidase can hydrolyse the lactose into glucose and galactose. Still, like cellulose, the induction mechanism of lactose on

cellulase synthesis remains unclear. Researchers have suggested that the slower digestion of lactose can prevent the metabolites repression by glucose (Merivuori et al. 1984; Messner and Kubicek 1991). After being generated from the lactose hydrolysis, glucose goes through the Embden-Meyerhof-Parnas (EMP) pathway and galactose goes through the Leloir pathway (Frey 1996). Because of its low cost, lactose has so far been a good choice to be used inducing substrate for cellulase production.

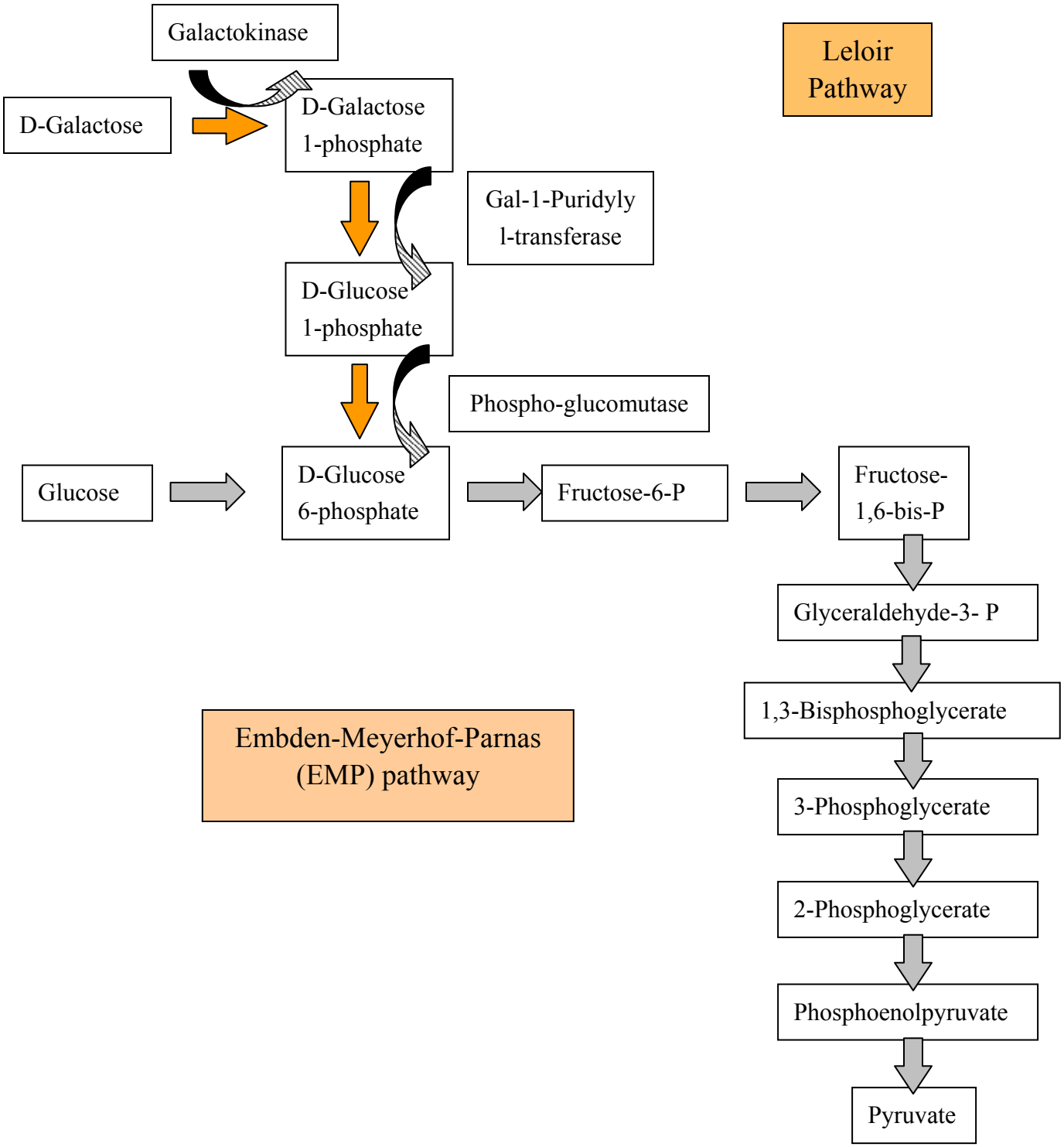

Figure 2.2 Metabolic pathway of glucose (EMP) pathway (Ritter 1996) and galactose (Leloir Pathway) (Frey 1996)

2.5.5 Sophorose

Sophorose (a dimer of two β-1, 2-linked glucose units) has been recognized as a powerful inducer in terms of the amount required for the cellulase activity induced (Mandels et al. 1962). Although sophorose is the best inducer for *T. reesei*, it did not induce the synthesis of cellulase in several other cellulase-producing species, such as *Phanerochaete janthinellum* (Mernitz et al. 1996) and *Aspergillus purpurogenum* (Gielkens et al. 1999). It was also a poor inducer for β-glucosidase (Vaheri et al. 1979). Vaheri et al. (1979) also showed that sophorose could be formed in the homogenized cell suspension prepared from cells pre-grown cultured with cellobiose as an inducer and glycerol as a carbon source. The sophorose formation was hypothesized as a result of transglycosylation by β-glucosidase or endoglucanase I from either cellobiose or glucose. However, the high cost of sophorose makes the utilization in industrial processes not economical.

2.6 Factors affecting cellulase production

Cellulase production using *T. reesei* Rut C30 fermentation process is a complex system. Many factors affect cellulase productivity, including pH, dissolved oxygen

concentration, and agitation speed. An acute change of culture condition could cause an irreversible damage on cell or enzyme activities. The importance of each factor varies with different function or purpose of cultivation. The fundamental understanding on how each factor is going to affect cells and cellulase production is essential for the further studies.

2.6.1 Effect of pH

pH is the proton [H^+] concentration and the change of acid-base reactions. The determination of pH is based on the potential given by Nernst equation, Eq. 2.6.1.1 (Haber and Klemensiewicz 1909), the difference of proton concentration between inside and outside of glass pH electrode. Pure water has pH at 7, neutral condition with disassociated proton and hydroxide ion at concentration of 7 x 10^7, respectively. pH has been known to affect enzymes and cells' metabolism tremendously. To optimize culture condition, it is best to understand the interaction between cell's metabolism and pH.

$$E = E_0 + 0.0591 \log\left(\frac{H^+_{inside}}{H^+_{outside}}\right) \qquad \text{(Eq. 2.6.1.1)}$$

Because of the sensitivity of intracellular enzymes on pH, having a constant intracellular pH is a must for cell to function properly in terms of reactions catalyzed by

enzymes, DNA and RNA replications, and ATP generation from respiration. An unstable intracellular pH could cause the change of activities of pH sensitive proteins, and the rate of synthesis of proteins, DNA and RNA. The proton gradient across the plasma membrane is, therefore, essential for the growth of all microorganisms. However, all the reactions are operated at relatively constant intracellular pH. Therefore, to control the change of external pH is essential in terms of concentrations of charged substance and proton present in aqueous phase.

The change of concentration of proton affects not only pH value, but also cell's metabolism. The difference of proton concentration can be indicated by pH difference, since the technology is limited in directly measuring the proton concentration. The difference of proton concentration also forms the electrochemical gradient, proton motive force, providing membrane potential in the range of –170 to –230 mV (Bailey and Ollis 1944). This potential is the driving force for ATP generation in prokaryotes. The same mechanism is responsible for energy generation in eukaryotes, but it happens in the mitochondria. In addition, prokaryotes and eukaryotes respond differently to the change of proton concentration. For prokaryotes, since the change of extracellular pH, within certain extent varying from species to species, did not make the intracellular pH change

at all, the effect of pH is not immediate but would have indirect impact on cell membrane and other compounds such as membrane bound enzymes, and enzymes for respiration (Russell 1987). For fungi, the cytoplasmic pH is typically close to pH 7.0 ± 0.5, varying with species. To maintain a constant intracellular pH, it relies on the removal of excessive proton across cytoplasmic membrane. When the environmental pH is over the operational pH, typically pH 2.0 to pH 7.0, the intracellular pH becomes unstable (Bracey et al. 1998).

The function of enzymes was influenced by pH as well. The changes of pH could cause two types of effects in biological system such as charge and proton concentration. When the acid or base is added in solution, the dissociation of solute will release the proton [H^+] for acid, or hydroxide ion [OH^-] for base. According to Henderson-Hasselbalch equation, the change of pH can affect the ratio of conjugate base and weak acid. This also affects the charges present in the solution. The charges also play an important role on the biological activity of molecule. Such as charge-charge interaction (charges repel, opposite charges attract) can affect the shapes, chemical characteristics, and the physical properties of compounds (Ritter 1996).

Many researchers have studied the effect of pH on cellulase production (Brown and Zainudeen 1977; Mukhopadhyay and Malik 1980; Ryu and Mandels 1980; Kansoh et al. 1998). Brown and Zainudeen (1977) reported maximum specific cell growth rate increased as pH increased when cultured *T. viride* QM 9123 at pH of 2.5, 3.0, and 4.0 in continuous cultivation. Kansoh et al. (1998) tested different initial pH effect, maintained by buffer solution, on cellulase production using *T. reesei* and hydrolysate make of sugar cane bagasse, and found the optimal pH range was from 5.0 to 6.0. However, Ryu and Mandels (1980) reported that the best induction condition of using cellulose was at range from pH 3.0 to 4.0. On the other hand, Mukhopadhyay and Malik (1980) used pH cycling method to obtain high cellulase productivity from 25.0 to 38.75 IU/L/h when compared with control using 3% cellulose and *T. reesei* QM 9414. In addition, cellulase produced by *T. reesei* have been found to have the highest catalytic capability at pH 2.5, 60 ^{o}C and the most stable condition was between pH 3.5 to 6.0 and lower than 50 ^{o}C (Sakamoto et al. 1984). The properties of cellulases produced by different species may differ in the optimal condition for their performance. For example, optimal condition for cellulase produced by *T. koningii* was at pH 6.0 (Halliwell and Griffin 1978) and that by *Clostridium thermocellum* was at pH 5.7 to 6.1 and 70 ^{o}C (Johnson et al. 1982).

2.6.2 Effect of dissolved oxygen concentration

Oxygen is required for cell growth of most eukaryotes. It is an electron acceptor for respiration when using sugars. Therefore, it is good to understand how the dissolved oxygen affects the cell growth and respiration.

Dissolved oxygen is a critical factor in generating energy. In general, oxygen first diffuses across cell membrane and this could be a rate-limiting step from bulk liquid to surface of cells because of the cell size or morphology of cells. Because of the structural difference between prokaryotes and eukaryotes, the ways of oxygen consumed by them are different too. For eukaryotes, cells consume oxygen in vivo, mitochondria, where the respiration happens after across cell membrane. However, prokaryotes consume the majority of oxygen, diffused from bulk liquid for ATP generation, in between the cell membrane, embedded proteins for respiration such as electron transfer oxidases, oxygen transferases, and mixed function oxidases. The respiration involved oxygen, oxidative phosphorylation or aerobic respiration, generates 1270 kj of energy each time. Oxygen is also a terminal electron accepter. In the end of respiration, oxygen will merge with two protons to become a water molecule. With sufficient supply of oxygen, cells will have best efficiency on generating energy and growth (Bailey and Ollis 1944).

Cell morphology is affected by DO. When the DO is low, cells somehow manage their defense mechanism to form pellets (Lejeune and Baron 1995). Forming pellets will lower the viscosity of broth and limit the maximum cell growth, because of the change of morphology and the limitation of oxygen mass transfer rate to the core of pellet, respectively. Besides, cellulase production decreases when pellets form (Domingues et al. 2000). Since pellets typically form in adverse condition, pellets themselves may not be solely responsible for a poor cellulase production. The effect of dissolved oxygen on cellulase production of different morphologies may vary since there are several forms of fungi such as mycelia, clumps, and pellets. On the other hand, supplying excessive oxygen could also cause hyperoxidant state, which has too much oxygen radicals or oxygen species, for example, O_2^-, HO_2, H_2O_2 and HO

Oxygen transfer limitation often happens at filamentous culture. The high biomass concentration causes high viscosity and low gas hold-up, which both are not beneficial to pass the threshold of critical oxygen. The value of mass transfer coefficient (K_La) indicates how good or efficient the oxygen distributed in reactor. K_La is determined by the equation (Eq. 2.6.2.1) of oxygen transfer rates (OTR), which is driven by the difference of oxygen concentrations between equilibrium with the bulk gas phase and

final output, very small. In order to have high K_La, increasing agitation rate is the most effective way. However, considering balance of shear strength and good oxygen transfer rate, sometimes pure oxygen may be a good alternative for supporting high cell concentration.

$$OTR = K_La\ (C^*-C) \Rightarrow K_La \cdot C^* \qquad \text{(Eq. 2.6.2.1)}$$

2.6.3 Effect of agitation

Mechanical mixing of broth in bioreactor is necessary for cellulase production with *T. reesei* Rut C30. Due to requirement of oxygen and evenly distributed nutrients, cellulase production and cell growth are affected by agitation tremendously.

With agitation in reactor, it provides several benefits. One benefit is distribution of nutrients than in a none-mixing system. With same chance of getting on nutrients to each cell, there is no dead space or segregated cells in reactor. This will have a good control in surrounding environment to make each cell has same cellulase production rate and growth rate. Second, strong shear strength, generated by agitation, also provides good breakdown on bubbles, which increases oxygen mass transfer rate. Smaller bubble size has larger interfacial. For cellulase production, the percentage of dissolved oxygen is

typically maintained above 30% (Allen and Mortensen 1981; Mukataka et al. 1988). Besides, agitation traps bubbles to have longer retention time, which is also an important factor for oxygen mass transfer, especially when having surface aeration instead of sparging air in the reactor. Next, in the case involving solids or powder mixing, solids increase viscosity and power for staying on same required speed. For example, supplement of Avicel in bioreactor, caused oxygen limitation at 130 rpm. However, they also reported it somehow enhanced the oxygen transfer rate into the pellets with presence of Avicel (Lejeune and Baron 1995). Also, baffle creates turbulence and shortens the time to achieve maximum mixing, which usually needs to be taken into consideration when designing a bioreactor.

In contrast to an optimal agitation rate, there are some detrimental situations needed to be avoided. First, high agitation always comes with high shear stress and shear strength. High shear stress and strength can cause the problem, especially to filamentous cells. Filamentous cells reproduce in a way of extending filaments to certain length. This behavior causes high viscosity as cell concentration gets higher. In order to keep at same agitation rate, power input must be increased and the shear strength as well. Therefore, filaments could be split and the cellulase production could be reduced. Second, when the

gas hold-up is too much, the liquid volume increases and sometime comes with foaming problem. With supplement of antifoam reagent, it increases the complexity of downstream separation. In addition, high shear strength could cause the denaturation of cellulase. Meurisse et al.(1993) reported the cellulase enzyme activity was affected by agitation when the system operated with pure enzyme solution but the β-glucosidase activity remained the same. On the other hand, low agitation rate causes problem, too. Low agitation will have larger bubble size and less gas hold-up, which means smaller interfacial area and less oxygen mass transfer rate. Cells die when oxygen is not enough and they stop growing afterwards. In the worst case, portion of space in reaction could be stagnant.

In summary, increasing agitation speed can reduce bubble size and increase interfacial area. It also affects the morphology of fungus and cellulase production. Hence, it is not always good to push agitation speed too high.

CHAPTER III

EXPERIMENTAL APPRATUS AND PROCEDURE

3.1 Preparation of inoculum

The *Trichoderma reesei* Rut C30 (ATCC no.56765) was received from USDA's NRRL culture collection, (United States Department of Agriculture, Agricultural Research Service Culture Collection, Peoria, IL). The sealed and lyophilized cell powders were contained in a glass tube and preserved in a - 10 ^{o}C freezer. After cutting off the top of glass tube, we mixed the cell powders with 50 ml sterilized potato dextrose rich medium (Fisher Scientific, Fair Lawn, NJ) in a 250 ml flask and incubated the culture under magnetic agitation at room temperature for 48 hours. The culture was then used to prepare agar plates for culture maintenance, as described in the following: An inoculation loop was sterilized and dipped in the broth. The loop was then spread on the pre-prepared agar plates containing potato dextrose agar (Sigma, St. Louis, MO). The streaked plates were incubated for 24 hours at 28 ^{o}C temperature and then sealed with tapes for preventing contamination. After writing down the date and strain name, plates were stored in a 4 ^{o}C refrigerator.

In order to keep cells active, cells were transferred to new plates monthly.

To prepare the inoculum for experiments, the streak plates were incubated for 24 hours before transferred to a 50 ml potato dextrose rich medium in a 250 ml flask. It required at least 48 hours under gentle agitation for the cells to fully develop. The 50 ml broth was then either directly transferred to the 1 L working volume of production medium in a 2 L flask or bioreactor, or, if the volume of final production medium was larger than 1 L, transferred to a 200 ml potato dextrose rich medium in a 500 ml flask before being used as the inoculum for the production medium.

The following medium composition was used for the batch and continuous systems throughout the thesis work (Table 3.1). The carbon source may change according to the purpose of the particular experiment.

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Table 3.1. Medium used in the fermentation based on 10g Lactose/L

Nutrient	Carbon Limit	Nitrogen Limit	Phosphorus Limit	Agitation study
Carbon source	Lactose 10 g/L	Lactose 10 g/L	Lactose 10 g/L	Lactose 5 g/L Cellulose 5 g/L
KH_2PO_4	2.0 g/L	2.0 g/L	0.44 g/L	2.0 g/L
$(NH_4)_2SO_4$	1.4 g/L	0.7 g/L	1.4 g/L	1.4 g/L
Peptone	1.0 g/L	1.0 g/L	1.0 g/L	1.0 g/L
Urea	0.3 g/L	0.3 g/L	0.3 g/L	0.3 g/L
KCl	---	---	0.895 g/L	---
$MgSO_4 \cdot 7H_2O$	0.3 g/L	0.3 g/L	0.3 g/L	0.3 g/L
$CaCl_2$	0.3 g/L	0.3 g/L	0.3 g/L	0.3 g/L
Tween 80	0.2 mL/L	0.2 mL/L	0.2 mL/L	0.2 mL/L
$FeSO_4 \cdot 7H_2O$	5.0 mg/L	5.0 mg/L	5.0 mg/L	5.0 mg/L
$CoCl_2$	2.0 mg/L	2.0 mg/L	2.0 mg/L	2.0 mg/L
$MnSO_4 \cdot H_2O$	1.6 mg/L	1.6 mg/L	1.6 mg/L	1.6 mg/L
$ZnSO_4 \cdot 7H_2O$	1.4 mg/L	1.4 mg/L	1.4 mg/L	1.4 mg/L

3.2 Hydrolysate preparation

The hydrolysate used in this study was prepared by Dr. Patrick K.-C. Lee at Tennessee Valley Authority (Muscle Shoals, AL) using a two-stage process developed by TVA. The preparation procedure is included below for reference purpose.

Mixed hardwood sawdust was obtained from a local sawmill at Muscle Shoals, AL and sieved through a 20-mesh screen. It had a moisture content of 9.3%, measured by a moisture analyzer (Ohaus MB200). The biomass (220.5 g) was put in

a 6-quart bowl of a KitchenAid dough mixer equipped with the dough hook. Concentrated sulfuric acid (80 wt%) was added slowly to the mixed biomass. A total of 210.0 g of acid was added so that a solid to acid ratio of 1.05 was obtained. The acid-biomass "gel" was kneaded for 30 minutes starting at acid addition. Significant reduction in volume resulted from the mixing. The final "gel" was dark and viscous.

At the end of mixing, 609.5 g of distilled water was added to the "gel", to dilute the acid to 20%. The dough hook was replaced with the wire whip and the mixture was stirred for 15 minutes in order to dissolve all clumps. The unreacted solids were removed by vacuum-filtering the mixture through a plastic sheet (ca. 20 mesh). The filtrate was boiled gently, with water addition to compensate for vaporization loss, for a total of 120 min. During the boiling, samples of about 100 mL each were removed at 15, 30, 45, 60, 90, and 120 min. The boiled filtrate samples were cooled by immersing in an ice/water bath. The cooled samples had pH of about 1.8. Calcium hydroxide was then added to adjust the sample pH to 9.0. The "overliming" precipitated out the excess sulfate and was reported to remove/detoxify certain inhibitory compounds generated in the hydrolysis process (Larsson et al. 1999). The overlimed samples were centrifuged to remove the solid precipitates. The supernatants were then adjusted for pH to 7, using 1 N HCl, and used to prepare the media for the cellulase production study.

3.3 Analytical methods:

All the analytical methods for this research work are described in this section. The methods are following instruction of manual provided by the companies or the previous studies.

3.3.1 Total reducing sugar analysis (DNS method):

The reducing sugar concentration was measured by the non-specific dinitrosalicylic acid (DNS) method, based on the color formation of DNS reagent when heated with reducing sugars (Miller et al. 1988). The DNS reagent was prepared by dissolving 10 g of 3, 5-dinitrosalicylic acid in 400 ml deionized water, along with addition of 200 ml of 2 M NaOH, and then adding more deionized water to bring the total volume to 1 liter. This method could be used for measuring sugars which contained reducing ends, such as glucose, lactose, galactose, and xylose. The standard deviation associated with this method was determined to be about 0.001 g/L by measuring three samples with the same concentration.

The measurement was done by adding 1 ml of standard solution or sample supernatant into 3 ml of DNS reagent and boiled for 5 min in test tubes. Boiled test tubes were removed and supplemented with DI water to make the total volume of 25 ml. After gentle but thorough mixing, about 2 ml of the solution were transferred to

a 3 ml plastic cuvette and read at 550 nm with a spectrophotometer (Model UV-1601, SHIMADZU Corporation, Columbia, MD).

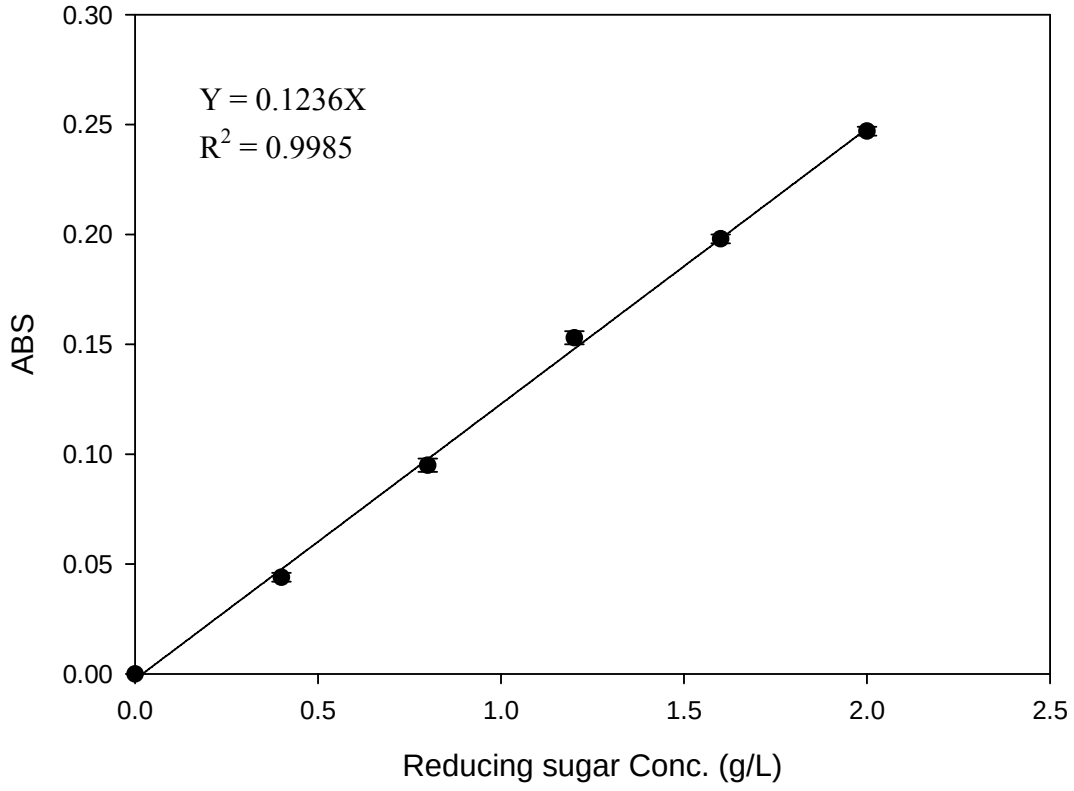

Figure 3.1 Standard curve of DNS method using glucose

3.3.2 Glucose analysis using enzymatic bioanalysis kit

Residual glucose concentration was measured by using enzymatic glucose assay kit (Sigma P7119, procedure No. 510). The method was based on the following enzymatic reactions: Glucose was first catalyzed by glucose oxidase to

gluconic acid and H_2O_2 (Reaction 3.1). The produced H_2O_2 along with o-dianisidine (colorless) were then reacted with peroxidase to oxidize the colorless o-dianisidine to the brown o-dianisidine (Reaction 3.2). The light absorbance was detected at 450 nm with a UV/VIS spectrophotometer (Model UV-1601, SHIMADZU Corporation, Columbia, MD). The intensity of brown color was expected to be proportional to the glucose concentration.

$$\text{Glucose} + 2H_2O + O_2 \xrightarrow{\text{Glucose Oxidase}} \text{Gluconic Acid} + 2H_2O_2 \qquad (3.1)$$

$$H_2O_2 + \text{o-Dianisidine} \xrightarrow{\text{Peroxidase}} \text{Oxidized o-Dianisidine (Brown)} \qquad (3.2)$$

Samples, collected form culture broth, were centrifuged in order to separate the cells from the supernatant. 0.5 ml of the supernatant was mixed with 5.0 ml of pre-prepared PGO enzyme solution (Peroxidase/ Glucose Oxidase), a mixture of 1 capsule of PGO enzymes and 100 ml of deionized water. The test tube with solution was incubated for 30 min at 37 ^{o}C. After the color developed, the test tube was taken for measuring the absorbance.

3.3.3 Lactose and Galactose analyses using enzymatic bioanalysis kit

The analysis of residual lactose and galactose used an enzymatic bioanalysis kit (Boehringer Mannheim/R-biopharm 176 303). The lactose concentration was determined by two consecutive reactions. Lactose was first hydrolyzed by

β-galactosidase to galactose and glucose (Reaction 3.3). The galactose and NAD^+ (nicotinamide-adenine dinucleotide), as the proton acceptor, were then reacted to form D-galactonic acid and NADH, catalyzed by β-galactose dehydrogenase (Reaction 3.4). The amount of NADH was determined by the intensity of light absorbance at 340 nm. The following equation was verified with standard lactose solutions.

$$\text{Lactose} + H_2O \xrightarrow{\beta\text{-Galactosidase}} \text{D-galactose} + \text{D-glucose} \qquad (3.3)$$

$$\text{D-galactose} + NAD^+ \xrightarrow{\text{Gal-DH}} \text{D-galactonic acid} + NADH + H^+ \qquad (3.4)$$

The reading was converted to lactose concentration (g/L) according to the following equation:

$$C = \frac{V \times MW}{\varepsilon \times d \times v \times 1000} \times \Delta A \qquad \text{(Eq. 3.1)}$$

where

V = final volume [ml] (= 3.3 ml used in this study)

v = sample volume [ml] (= 0.1 ml in this study)

MW = molecular weight of the substance to be assayed [g/mol] (= 360.32 g/mol for lactose)

d = light path [cm] (= 1.0 cm in our procedure)

ε = extinction coefficient of NADH at 340 nm = 6.3 L/mmol-cm, and

ΔA = difference between two readings at 340 nm, as described in more details below together with the measurement procedure.

There were five bottles in the commercial test kit. Bottle 1 contained 600 mg of a lyophilizate, made of citrate buffer with pH at 6.6, 35 mg NAD, and magnesium sulfate, dissolved in 7 ml deionized water. Bottle 2 contained 1.7 ml of a β-galactosidase solution with about 100 Units of the enzyme activity. Bottle 3 contained 34 ml of a potassium diphosphate buffer solution with pH at 8.6. Bottle 4 contained 1.7 ml of a galactose dehydrogenase solution with 40 U of the enzyme activity. Bottle 5 was a lactose assay standard solution, 0.504 g/L.

The measurement of lactose concentration was done with sample supernatant collected after centrifugation. 0.1 ml of the supernatant was mixed with 0.2 ml of Bottle 1 solution and 0.05 ml of Bottle 2 (β-galactosidase) solution, transferred to a 5 ml cuvette, and incubated for 20 min at room temperature. After the incubation, 1 ml of Bottle 3 solution and 1.9 ml of deionized water were added into the cuvette, mixed, and let stand for 2 min before the cuvette content was read with the spectrophotometer at 340 nm. After the first reading (A1), 0.05 ml of Bottle 4 (galactose dehydrogenase) solution was added to the cuvette (making the final total volume of 3.3 ml) and incubated again for 30 min at room temperature. The

incubated cuvette content was then measured at 340 nm again for the second reading A2. The lactose concentration could then be calculated by Equation 3.1 with $\Delta A = A2 - A1$.

Similar principle and procedure were used for the galactose concentration measurement, except that the analysis involved only Reaction 3.4 and the MW used was 180.16 g/mol in the calculation with Equation 3.1**.** The standard deviation associated with this analysis was 0.0001 g/L by measuring three samples with the same concentration, for both lactose and galactose.

3.3.4 Intracellular protein analysis

The intracellular protein was determined by using Bradford protein assay kit. The method was based on the Bradford dye-binding procedure (Bradford 1976) which used Coomassie Brilliant Blue G-250 dye to bind the protein containing basic and aromatic amino acid residues. It was suitable for the proteins or polypeptides with molecular weights larger than 3000. The linear range for the protein assay was 1.25 – 25 μg/ml proteins and the standard deviation was 0.001 g/L, determined by measuring three samples with the same concentration. The bovine serum albumin was used to prepare standard solutions for establishing the calibration curve.

By centrifugation, the cells in samples were collected and washed twice with deionized water. The cells were then lysed in 5 mL of 0.2 N NaOH, at 100^{o}C for 20 min. The protein concentration of the lysate was then measured by the standard Bradford assay. The 5 ml of dye reagent and 100μl was mixed in a 25 ml vial. After 5 min waiting, the blue color completely developed. The absorbance at 595 nm was measured with a UV/VIS spectrophotometer (Model UV-1601, SHIMADZU Corporation, Columbia, MD).

3.3.5 Cell dry weight

The cells in 5-ml broth were collected by centrifugation at 8,000 rpm (9,300 *g*) for 10 min using a Sorvall RC 5C Plus Superspeed Centrifuge (Sorvall, Newtown, CT). The cell pellet was washed by 5-ml DI water and centrifuged again for another 10 min. This procedure was repeated twice before the washed cells were transferred to an aluminum pan. Each pan was written down the date and weight for later data collection and calculation before dried in a 120 ^{o}C oven for 24 hours. The final cell concentration was calculated by subtracting the weight of empty pan from the weight of the pan with dried biomass, and then multiplied by 200 to account for the ratio between 1 L and the 5 ml of sample volume.

3.3.6 Cellulase analysis (Filter Paper Unit method)

The total activity of cellulase was measured by the standard filter paper assay method (Mandels et al. 1976). Cellulase hydrolyzed the filter papers (Whatman #1, 1 x 6 cm^2) consisting of cellulose to the final product, glucose. The amount of glucose released by the hydrolysis represented the cellulase activity in the solution.

The analysis was again done on the sample supernatant collected by centrifugation. 0.5 ml of supernatant was mixed with 1 ml of buffer solution, i.e., 0.05 M sodium citrate pH 4.8, in a 25 ml graduated test tube. A piece of Whatman filter paper (1 x 6 cm^2) was placed and submerged in the solution on the bottom of test tube. The test tube was incubated in a hot water bath for 60 min at 50 ^oC. After the incubation, the total reducing sugar analysis was performed by the DNS method, as described earlier in this Chapter, for measuring the glucose concentration. Once the glucose concentration was known, the cellulase activity could be obtained by using Eq. 3.2.

The cellulase activity was determined by following calculation:

1 U = 1 μmol of Glucose/min = 0.18 mg Glucose/min

0.18 μmol of Glucose/ml · min = 1 mg of glucose x $(0.18)^{-1}$ $(Wt/Mw)^{-1}$ x $(0.5)^{-1}$ ml^{-1} x $(60)^{-1}$ min^{-1} (Eq. 3.2)

When 1 mg of Glucose released from the filter paper with condition at 50 ^oC

and incubated for 60 min, the cellulase activity could be calculated as 1mg of glucose x $(0.18)^{-1}$ $(Wt/Mw)^{-1}$ x $(0.5)^{-1}$ ml^{-1} x $(60)^{-1}$ min^{-1} , which was equal to 0.185 μmol of Glucose/ml · min. Generally, the cellulase activity was presented as FPU/ml, also known as U/ml.

3.3.7 Lactase (β-galactosidase) analysis

The lactase analysis method reported by Castillo et al. (1984) was adopted in this study. Lactase activity was determined by mixing 1 ml of 100 mM o-nitrophenyl-galactopyranoside (ONPG, Sigma) in deionized water with 1 ml of supernatant collected from culture broth. The test tube was placed in water bath for 10 min at 50 ^{o}C to release the reaction product, o-nitrophenol (ONP). 1 ml of 5 % Na_2CO_3 was then added to stop the reaction and waited for 1 min. When the color was fully developed, the test tube was read for the absorbance at 400 nm. The intensity of absorbance was proportional to the o-nitrophenol concentration and the activity of lactase was determined according to the Eq. 3.3. O-nitrophenol (MW 139.11, Fluka Inc., 88-75-5) was also used to prepare the standard solutions for calibration purpose.

One unit of lactase activity (U) was defined by measuring the amount of product (ONP) being released from the hydrolysis reaction: 1 U = 1 μmol of

ONP/min = 0.13911 mg ONP/min

1 x $(0.139)^{-1}$ x 1 ml x $(10)^{-1}$ min = 0.71942 μmol of ONP/ml · min (U/ml) (Eq. 3.3)

When 1 mg of ONP released from the ONPG with condition at 50 ^{o}C and incubated for 10 min, the cellulase activity could be calculated as 1mg of ONP x $(0.139)^{-1}$ $(Wt/Mw)^{-1}$ x $(1)^{-1}$ ml^{-1} x $(10)^{-1}$ min^{-1} , which was equal to 0.71942 μmol of ONP/ml · min. Generally, the cellulase activity was presented as U/ml.

3.3.8 Sophorolipids analysis (Anthrone method)

The quantitative analysis of sophorolipids was done by anthrone method. Samples, 5 ml each, were collected from the bioreactor and then centrifuged at 8000 rpm. The pH of 5 ml of supernatant was adjusted to pH 2 and then extracted by 20 ml ethyl acetate for 24 hours. 0.5 ml of solution from the organic phase (upper solution) was placed in a vial and air dried till complete evaporation of the solvent. 1.7 ml of 1 M $NaHCO_3$ and 3.3 ml of anthrone reagent were added into the vial kept in an iced water batch. The vial, afterwards, was placed in a thermal block for 16 min at 95 ^{o}C. The final color should be green when sophorolipds were present and the reading from UV/VIS at 625 nm (SHIMADZU UV-1601) was recorded.

The anthrone reagent was prepared with 0.2 g of anthrone powder dissolved in 100 ml of H_2SO_4 (95.8%). Sophorose was used to prepare standard solutions for

establishing the calibration curve. The above method actually measured the sophorose concentration in the sample. A conversion factor, 2.04 based on the ratio between the molecular weight of sophorose and the average MW of the two main SLs, was multiplied to convert the sophorose concentration to sophorolipid concentration, taking into account the ratio between the average molecular weight of the major sophorolipids and that of sophorose (Hu and Ju 2001).

3.3.9 Glycerol analysis

One ml of collected supernatant from culture broth was transferred into a test tube, mixed with 1 ml of periodate reagent, and let react for 5 min at room temperature. The mixture was then mixed with 2.5 ml of acetylacetone reagent and heated for 20 min at 50 ^{o}C. Afterwards the sample was measured at 410 nm. The calibration curve was established with standard solutions of 1mg to 20 mg/L of glycerol. The error bars on glycerol concentration in all studies indicated that the standard deviation was calculated from three samples taken at the same time.

The periodate reagent was prepared by mixing two solutions: one solution contained 65 mg of $NaIO_4$ dissolved in 90 ml of water; the other solution contained 7.7 g of ammonium acetate in 10 ml of acetic acid. The acetylacetone reagent was prepared by mixing 2.5 ml of acetylacetone with 247.5 ml of isopropanol, and was

stored in the dark space covered by aluminum foil. Both reagents were kept in refrigerator.

3.4 Crude sophorolipids and purification

Sophorolipids (SLs) were obtained from *Candida bombicola* fermentation following Hu's procedures (Hu 2000). The production medium contained 100 g/L of glucose and 100 g/L of soybean oil in a culture flask with 1 L working volume. The other necessary nutrients in the medium are given in the following: sodium citrate 5.0 g/L, yeast extract 5.0 g/L, $(NH_4)_2SO_4$ 2.0 g/L, KH_2PO_4 1.0 g/L, $MgSO_4$ 4.0 g/L, $FeSO_4 \cdot H_2O$ 0.1 g/L, $CaCl_2$ 0.1 g/L, and NaCl 0.1 g/L. The fermentation broth was harvested when the yeast had stopped growing for 72 hours and the soybean oil layer was gone. The whole broth was centrifuged at 6000 rpm for 15 min. The cells were discarded and the supernatant was collected into a 3 L flask. Supernatant was extracted twice with equal volume of ethyl acetate, each with 24 hours of extraction time. The crude SLs collected from the ethylacetate extract were used in the experiments for studying the feasibility of using sophorolipids as inducers to cellulase synthesis.

CHAPTER IV

FACTORS AFFECTING CELLULASE PRODUCTION

4.1 Abstract

Trichoderma reesei is known of their capability of cellulase production. The cellulase production is affected by culture conditions such as agitation rate, nutritional factors, and type of inducer. The effect of these factors on cellulase production was studied by using *Trichoderma reesei* Rut C30 in a BioFlo 110 bioreactor. The following conclusions could be drawn: First, the cellulase activity produced in the fermentation was found very low, around 0.18 FPU/ml, at a high agitation rate of 1000 rpm, but was much better, around 1.6 FPU/mL, at 500 rpm. Second, the carbon-limited medium gave best results among the media limited by different nutrients. Third, the acetic acid present in the dilute-acid pretreated hard wood hydrolysate indeed affected the survival rate of *T. reesei* when cultured in Petri dishes, containing the mixture of hydrolysate and potato dextrose agar. With low concentrations of acetic acid, the cells had better survival rates.

Fourth, a newly designed pH-based feed control strategy was our first attempt to achieve long-term high productivity of cellulase. The control strategy was based on the true cell metabolism reflected by the different slopes of pH change. The experimental results indicated a potential of controlling the culture at the optimal metabolic condition for cellulase production, instead of by the constant feeding in continuous culture (e.g., at the dilution rate of 0.03 (1/h)).

4.2 Introduction

Cellulase is widely used in textile and detergent industries. It is more and more important to the companies which use lignocellulosic material as substrate in bioethanol industry nowadays. Cellulase production requires specific culture conditions. The process using *Trichoderma reesei* Rut C30 requires an inducer to initiate cellulase synthesis and suitable culture condition for cell growth.

The culture condition is a conjunction of several operational factors. One of the factors is agitation. Agitation provides mixing of all elements in the reactor. By doing so, it also generates shear that breaks large bubbles into small ones to improve the oxygen transfer rate. The equation, $k_l = 0.13\ (\alpha^3\ \mu_l(P/V)/\rho_l D)^{1/4} Sc^{-2/3}$, showed the relationship of power input (P) with oxygen mass transfer coefficient (k_l). The symbols in the equation includes (α) a constant, (μ_l) liquid viscosity, (ρ_l) liquid density, (Sc) Schmidt number, and (V) liquid volume. The oxygen mass transfer coefficient is proportional to power input (Bailey and Ollis 1944). Stronger shear strength also results in better oxygen mass transfer. In addition, agitation also affects the cell morphology (Cui et al. 1997; Domingues et al. 2000). *T. reesei* Rut C30 is a filamentous fungus. As the cells grow, the filaments extend. The cell entanglement causes high broth viscosity. The power input increases as the liquid viscosity (μ_l) increases at a fixed agitation rate, in order to maintain same oxygen mass transfer and good mixing. Still, higher power input results in higher shear strength. Higher shear strength causes shorter filament lengths than the lower (Galindo et al. 2004). In a study by Lejeune and Baron (1995), the cellulase production was found to be the same at 400 rpm and 130 rpm agitation rates; however, it was attributed to different limiting factors: 130 rpm caused oxygen limitation while 400

rpm could cause too much damage to cells due to high shear stress. The 300 rpm had the best cellulase production when using the soluble inducer, lactose (Lejeune and Baron 1995).

Nutritional factors are essential to the growth of *T. reesei* Rut C30 in cellulase production process. To grow and produce cellulase, cells require various elements in the media, including carbon, nitrogen, phosphorus, and other macro- and micronutrients. Among them, carbon, nitrogen, and phosphorus affect cell metabolism the most. Carbon is the main component of cellular constituents. Nitrogen is the major component for the amino acids and nucleic acids. Membrane lipids, nucleic acids, and ATP contain significant amount of phosphorus. *T. reesei* cannot grow without any of these essential elements. Also, cellulase is associated with the nutritional factors. The excessive amount of glucose repressed the synthesis of cellulase. Once glucose concentration is lower than the threshold, cellulase can be synthesized with the presence of inducers, such as lactose and cellulose. Generally speaking, Cells at higher concentrations can produce higher total amount of cellulase when the nutrients are sufficient.

Cellulase is a secondary metabolite which is produced at stationary phase of *T. reesei*. Excessive glucose concentration present in culture media represses the induction of cellulase. When the glucose concentration is getting lower, the rate of cell growth slows down and eventually stops. This behavior can be viewed as the metabolic change during the fermentation. Cells consume the carbon source through catabolism. By doing so, they generate organic acids when glucose is oxidized. The more organic acids they generate, the lower pH in the process they have. Therefore, the rate of producing organic acids or pH decreasing can indicate the rate of cell growth and carbon source

consumption. With this knowledge, the process can be automated by controlling the pH to a desired cell metabolism in a continuous culture.

The dilute acid-pretreated hydrolysate provided by TVA contains a variety of cellulooligomers and sugars. It can be used for an inducer and carbon/energy source in cellulase production. However, it also contains other toxic substances which inhibit the cell growth, such as furfural, 5-hydroxy-methl-furfural (HMF) and acetic acid. The majority of furfural and HMF can be removed by overliming process which adds calcium chlorides to react with. With acetic acid, it becomes volatile when pH is low. Acetic acid has a pKa of 4.8 at 25 °C. At pH 5, less than half of the acetate was present as the volatile acetic acid, whereas almost all would be in the acid form at pH < 3.8. As a result, by blowing fresh air through hydrolysate solution, this should reduce the concentration of acetic acid in hydrolysate. Once these three major toxic substances are removed, *T. reesei* should be able to use dilute acid-pretreated hydrolysate for growth and cellulase production.

This study tends to analyze the factors which affect cell growth and cellulase production. We investigated the effects of agitation, nutritional factors on cellulase production and the toxicity of hydrolysate on cell growth. By using pH control strategy with lactose-based medium, we performed the attempt of finding the optimal cell metabolism of producing cellulase. This is an essential work for the next step to the optimization of cellulase production process.

4.3 Materials and Methods

Due to the different apparatus we used from other studies, the procedure described below may differ from others.

4.3.1 Agitation study:

There were two experiments carried out in the same condition with two different agitation rates, 500 and 1000 rpm in Bioflo 110 bioreactor. Cellulase was synthesized as long as the inducer existed. Cells could use lactose as an energy source and inducer at exponential growth stage in order to avoid consuming cellulose as food directly. The experimental design was to add 5.0 g/L of cellulose in the reactor when the reducing sugar (5.0 g/L lactose) was consumed completely. In addition, by changing agitation rate, the change of cellulase production was recorded. Cells were grown in a Bioflo 110 bioreactor with same culture conditions at beginning, 25 ^{o}C, pH 5.0, besides two different agitation rates which operated separately. pH was controlled by the addition of 2N NaOH. Therefore, the time adding approximately 5 g/L of cellulose into bioreactor was rather different, both between 40-45 hours (at the time having low reducing sugar concentration).

4.3.2 Nutritional factors study:

Three nutritional factors were tested for influence of cellulase production. 10 g/L of lactose was used as an inducer and energy source for this study. The experiments were executed in 2L flasks. The preparation of cell culture followed the preparation of inoculum described in Chapter 3. The inoculum was transferred to flasks containing the

production medium, 500 ml each. The complete medium formula (C-limited, N-limited, and P-limited) were given in Chapter 3, Table 3.1. Each experimental data point was an averaged value from three samples taken at the same time.

4.3.3.1 Preparation of phosphorus-limited medium:

The amount of phosphorus must be reduced for the study. The concentration of potassium phosphate (KH_2PO_4) was 2.0 g/L originally. According to the composition of cells, phosphorus accounts for 2% of cell body. The final cell concentration was set at 5.0 g/L for comparison between experiments. The required phosphorus was supposed to be 5.0 g-Cell × 2% = 0.1 g required Phosphorus. Therefore, the required potassium phosphate was 0.1/(31/136) = 0.44 g/L for the growth of 5.0 g/L cells. Additional KCl was included in the medium to compensate for the amount of potassium reduced with the lowered potassium phosphate. The amount of reduced potassium was (2 – 0.44) × 39/136 = 0.45 g-K. Therefore, 0.895 g/L of KCl, the sum of 0.45/(39/74.5), was added for compensation.

4.3.3.2 Preparation of nitrogen-limited medium:

The Mandels et al.'s medium (1962), originally designed for carbon limitation, was tested for nitrogen limited condition previously (data not showed) and the final cell concentration was around 6 g/L of biomass. The amount of ammonia sulfate was reduced in half for approximately 5 g/L cells. Detailed formula listed in Chapter 3, table 3.1.

4.3.4 Correlation of cell dry weight and intracellular protein concentration

	Run 1	Run 2	Run 3
Carbon source	glucose 10 g/L	galactose 10 g/L	glucose 5 g/L galactose 5 g/L

Cell preparation followed same procedure described above. The three runs were done in Bioflo 110 bioreactor at different time with basic Mandels et al.'s medium (C-limited, table 3.1) accompanying with different carbon sources. The culture conditions were same in all three runs, with a controlled pH at 5.0 ± 0.2 by addition of 2 N NaOH, room temperature, 500 rpm agitation, 1.5 L working volume, and DO above 70%.

4.3.5 Preparation of dilute acid-pretreated hydrolysate

The procedure of preparation of dilute acid-pretreated hydrolysate was described in Chapter 3. In order to evaluate the toxicity of acetic acid present in overlimed hydrolysate, three different hydrolysate concentrations, 50%, 70%, and 100%, were tested for the toxicity of hydrolysate which was aerated by fresh air for 24 hours. The pure hydrolysate was used as solvent according to the percentage to dissolve potato dextrose agar powder for a final 39 g/L agar solution, total volume 60 ml in a flask. Later, the solution was distributed evenly into six Petri dishes. Each concentration had two Petri dishes for testing.

4.3.6 pH-based feed control for continuous cellulase production

The culture medium used was the same as that in Chapter 3 with C-limited medium, except using 10 g/L of lactose as carbon source and cellulase inducer instead of glucose. The feed medium used in the continuous-culture phase contained 20 g/L of lactose but the same concentrations for all other medium components. The culture volume was approximately 1 L, maintained with an overflow opening on the side of the fermentor wall. The medium was first inoculated with 50 mL of seed culture prepared in the rich Potato Dextrose Broth, and allowed to grow as batch fermentation to late exponential-growth phase before the continuous feeding was implemented.

The pH-based control algorithm was constructed and implemented using Labview 7.1 (National Instruments, Austin, TX). The pH was monitored using a set of pH probe (Mettler Toledo, Columbus, OH) and controller (Model 5656, Cole Parmer, Vernon Hills, IL). An analog-to-digital signal converter (NI PCI-6024E, National Instruments, Austin, TX) passed the pH signal into a computer.

4.3.7 Analytic methods

The analytic methods regarding cellulase activity, cell concentration, and lactose concentration have been described in Chapter 3. The error bars regarding the cellulase analysis, in this chapter were determined by the standard deviation of cellulase activity derived from same sample which measured three times at different time.

4.4 Results

The preliminary study on the factors affecting cellulase production was carried out by using *T. reesei* Rut C30 in a series of experiments.

4.4.1 The influence of agitation rate

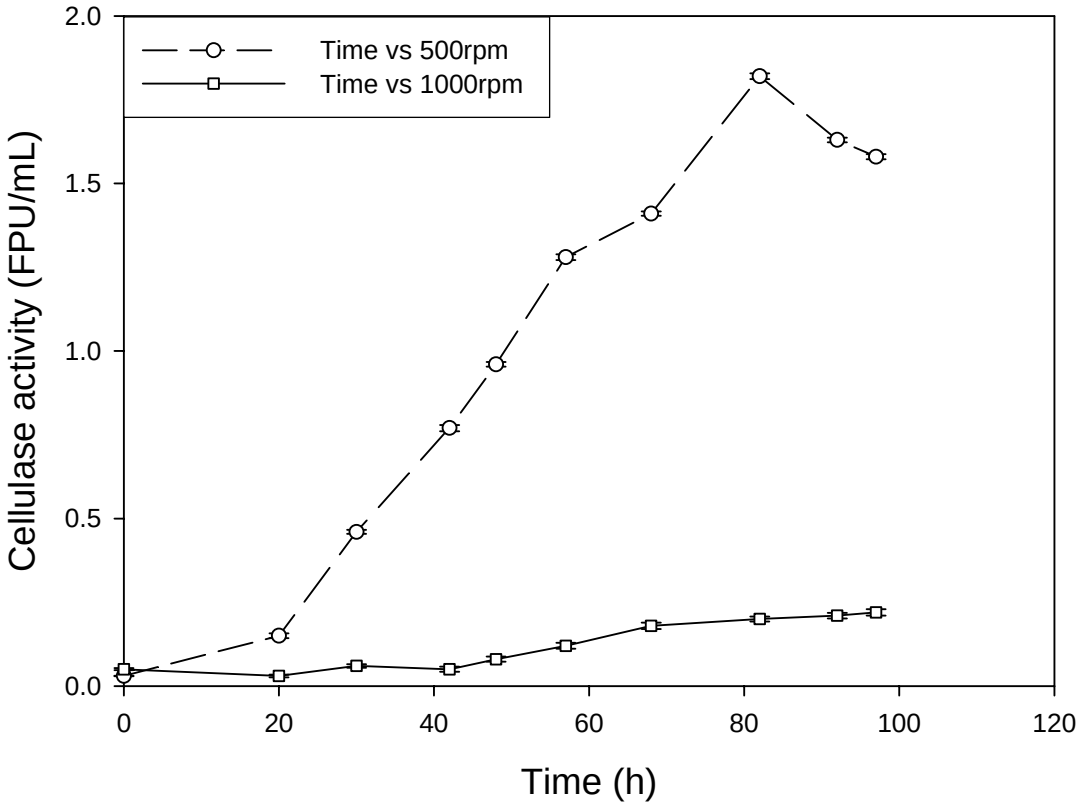

Figure 4.1 Cellulase production affected by different agitation rates

The cellulase production was investigated at two different agitation speeds, 500 rpm and 1000 rpm, in a Bioflo 110 bioreactor. Two batch cultures were done separately and sampled at the same time intervals. 5 g/L of lactose was introduced at 0 hour and another

5 g/L of cellulose was added after 45 hours, when lactose was close to be completely consumed (lactose concentration was tested afterwards). The results are shown in Figure 4.1. Agitation at 500 rpm had high cellulase activity, 1.9 FPU. This was the best cellulase activity ever in our previous test runs. Besides cellulase production and lower final cell concentration in the experiment at 1000 rpm, there was no significant difference from other measurable data between two agitation rates (data not shown). Nonetheless, at high agitation 1000 rpm, the mechanical forces could damage the mechanism of cells to produce cellulase, which merely had cellulase activity around 0.1 FPU.

4.4.2 Study on effect of nutrient-limited system

Three nutrient-limited media were studied, namely, carbon-limited, nitrogen-limited and phosphorus-limited. The experiments were carried out with 500 ml working volume in 2 L flasks, with same initial condition in all 3 experiments as described in the Material and Method section. The results were shown in Figure 4.2. The results showed that the highest cellulase production was obtained in the culture with the carbon-limited medium and the others had about the same level. Compared with the nitrogen- and phosphorus-limited media that showed early sporulation around 45 to 50 hours, spores did not appear in the C-limited culture till around 70 hours. Partial pellet formation was also observed in the P-limited culture. This study was made in Erlenmeyer flasks agitated with magnetic stirring. Cultures in such systems were found to give lower FPU than the cultures grown in the Bioflo 110 bioreactor with turbine impeller for agitation.

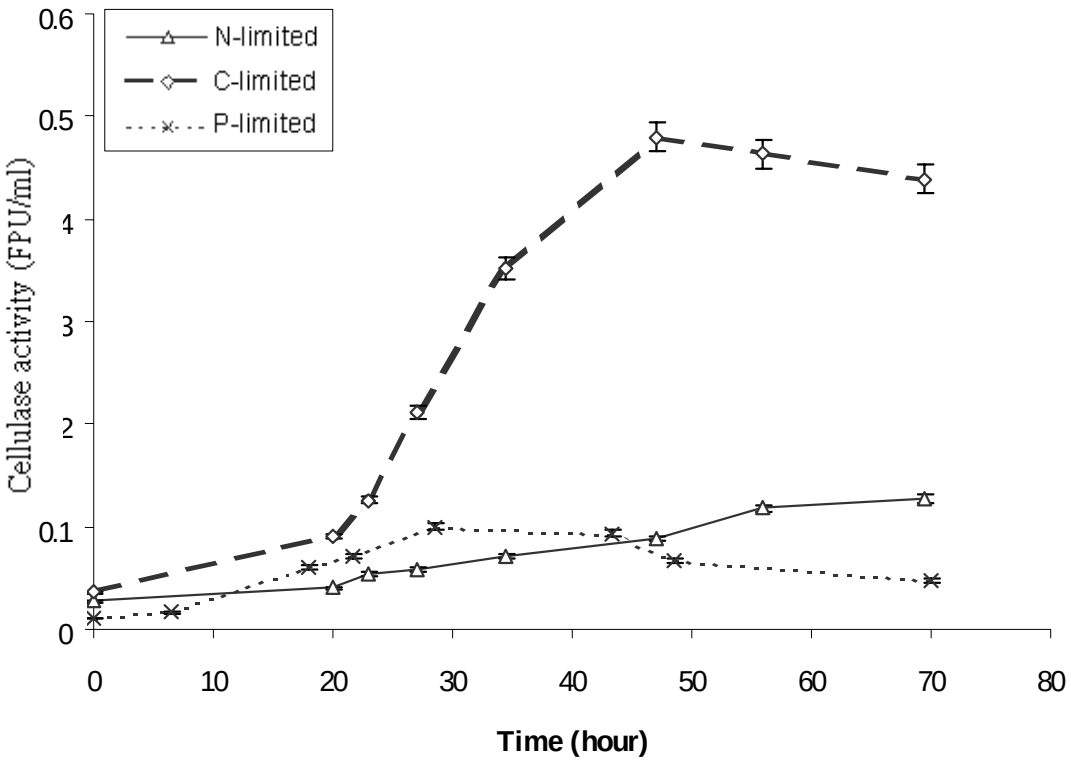

Figure 4.2 The comparison of cellulase production using different nutrient-limited mediums

4.4.3 Cell concentration

The entire set of data collected from three batches with different carbon sources was presented in Figure 4.3. The measurement of cell concentration by both cell dry-weight (CDW) and intracellular-protein (IP) concentrations has also been described in our

previous publication (Zhang et al. 2007), where a linear correlation between the two concentrations was employed. The current work was much more extensive. Many samples from the batch fermentations were subjected to both measurements of dry-weight and intracellular-protein concentrations. These are summarized in Figure 4.3. The correlation was apparently nonlinear, with lower intracellular protein contents (per unit dry weight) observed in the samples of higher cell concentrations. The best-fit second-order correlation is

$$CDW = 7.14\ (IP)^2 + 6.87\ (IP), \qquad r^2 = 0.9183$$

The cause of the nonlinear relationship is unknown. Nonetheless, the samples of higher cell concentrations corresponded to the cultures closer to the stationary phase. Presumably the lower protein contents in such systems reflected the slower metabolism (requiring less intracellular enzyme activities) and/or the exhaustion of peptide nutrients (peptone) in the medium.

As intracellular protein analysis requires smaller sample volumes, IP analysis was done on many samples in this study without the simultaneous cell dry weight measurement. For these samples, the above equation was used to estimate their cell dry weight concentrations. With this new relationship, the cell yield was averagely close to 0.6 g dry-cell/ g sugar and at the later stage, intracellular protein content decreased because of lack of sufficient amount of nutrient and slowdown of cell growth. This result accorded with Pakula et al.'s (2005) result, maximum growth yield of lactose about 0.6 g Cell/g.

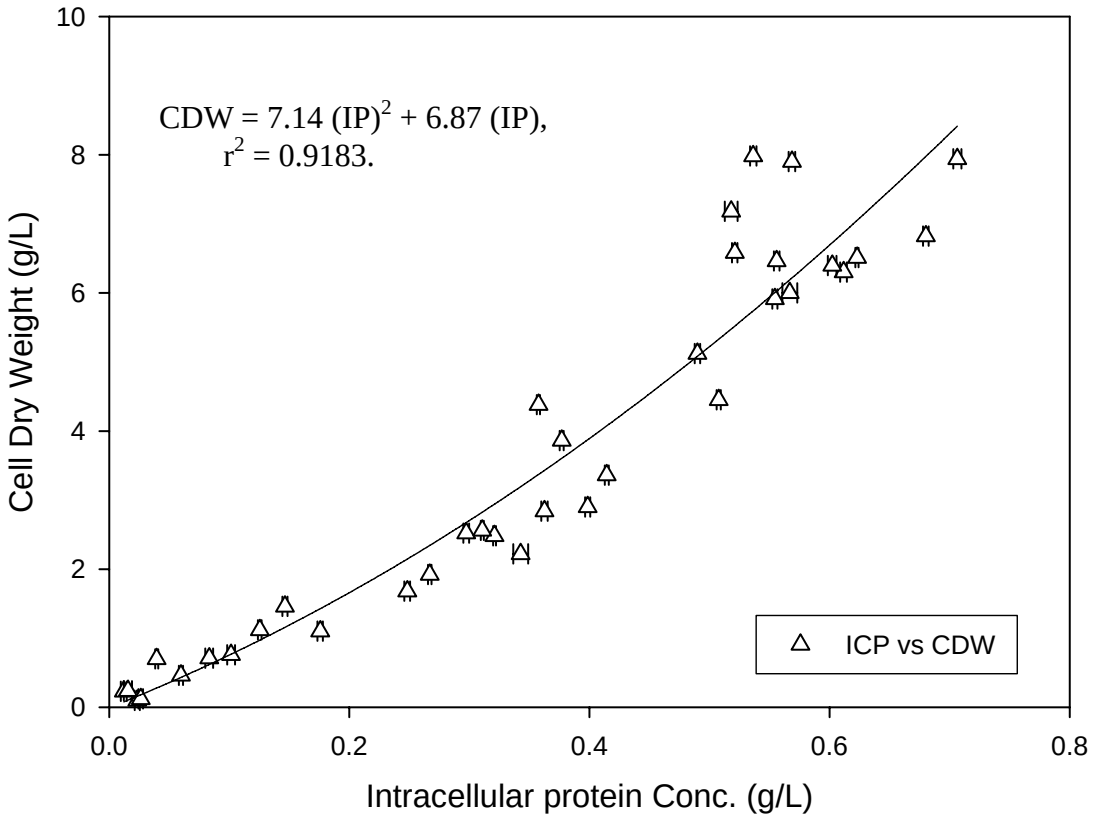

Figure 4.3. Correlation between Cell Dry Weight (CDW) concentrations and intracellular protein concentrations.

4.4.4 Toxicity evaluation on aerated hydrolysate

When the medium prepared from this hydrolysate was added as the continuous feed, the cells gradually turned to spores. In another set of experiment, the lower sugar content hydrolysate from different batch was used in the fermentation process as the only carbon source and inducer; as a result there was almost no cell growth in the system.

An attempt was therefore made to remove the volatile acetic acid by simply blowing filter-sterilized air into the acidified hydrolysate (at pH 5). After 3, 5, and 7 days of aeration, the hydrolysate was supplemented with other medium components and used to

prepare agar plates containing 50%, 75% and 100% of hydrolysate, respectively. After spreading 1 mL of inoculum on these plates, the cell growth, indicated as colony formation, was observed. The results obtained are shown in Table 4.1. Clearly, aerating the acidified hydrolysate helped to remove some of the inhibitory compounds. The detoxification efficiency may be significantly improved by lowering the pH of the acidified hydrolysate.

Table 4.1. Colony growth of *T. reesei* Rut C30 on agar plates with acid hydrolysate of hardwood sawdust.

Duration of Aeration for Inhibitor Removal	**Hydrolysate Strength**		
	50%	70%	100%
3 days	–	–	–
5 days	+	+	–
7 days	++	+	–

NOTE: "—": No apparent growth; "+": Slow growth; "++": Moderate growth

4.4.5 pH-Based Feed Control for Continuous Cellulase Production

As a reference, one experiment was carried out with a constant feed rate to give D = 0.03 (1/h). Other experiments were conducted with the feed rates computer-controlled to give the slope of pH decrease in the continuous-culture phase at different percentages of the maximum slope observed during the batch culture.

The pH set point was 5.0. Once the pH dropped to the set point, the computer activated a peristaltic pump for a ½-turn to add a fixed amount (~ 1.25 mL) of base (1N NaOH) into the broth. pH jumped up and then started to decrease gradually again. The slope of this decrease was calculated for each base addition cycle (i.e., between two consecutive base additions). The maximum slope was identified during the batch cultivation, typically occurring at the end of exponential-growth phase. A specific percentage of the maximum slope was then calculated and set as the reference for comparison, to guide the adjustment of feed rate as described below. The continuous feeding typically started at a rate corresponding to a dilution rate of 0.0165 (1/h). The resultant slope of pH decrease was then compared with the set percentage. The logical decisions used in the control algorithm are summarized in Figure 4.5. For example, if the measured slope was smaller than the set percentage, the feed rate was increased by 0.11 mL/min, corresponding to an increase in dilution rate of 0.0066 (1/h). If the feed-rate increase did not result in an increasing pH slope in the subsequent cycle (which was extremely rare), the feed rate would be increased again. In the typical case, the feed rate increase would lead to an increasing pH slope, and the new feed rate would be maintained in the subsequent cycles as long as the pH slope was still increasing, even if the resultant pH slope was still smaller than the set slope. The above strategy was taken to avoid divergent fluctuations caused by over-adjustments. Only when the pH slope was no longer increasing, the final slope would be compared with the set slope to decide if further increase in feed rate was necessary. Similar approaches were used in the algorithm to respond to other possible outcomes in the pH-based control, as summarized in Figure 4.5.

For comparison, the concentration profiles of cell dry weight, FPU, lactose (measured as reducing sugars), and extracellular proteins observed in two continuous culture systems are shown in Figure 4.4: (A) with the conventional, constant dilution rate of 0.03 (1/h) and (B) with the feed rate controlled by the pH-based strategy, for maintaining the slope of pH decrease in the continuous-culture phase at 20 % of the maximum slope observed during the batch cultivation. In the fixed dilution rate system, lactose accumulated during the continuous-culture phase, which in turn led to cell growth and negligible FPU production. On the other hand, in the system under pH-based feed control, the lactose concentration was maintained at negligible concentration, except during the initial transition period immediately following the onset of continuous feed. Cell concentration was kept practically constant. More importantly, a continuous cellulase production was obtained.

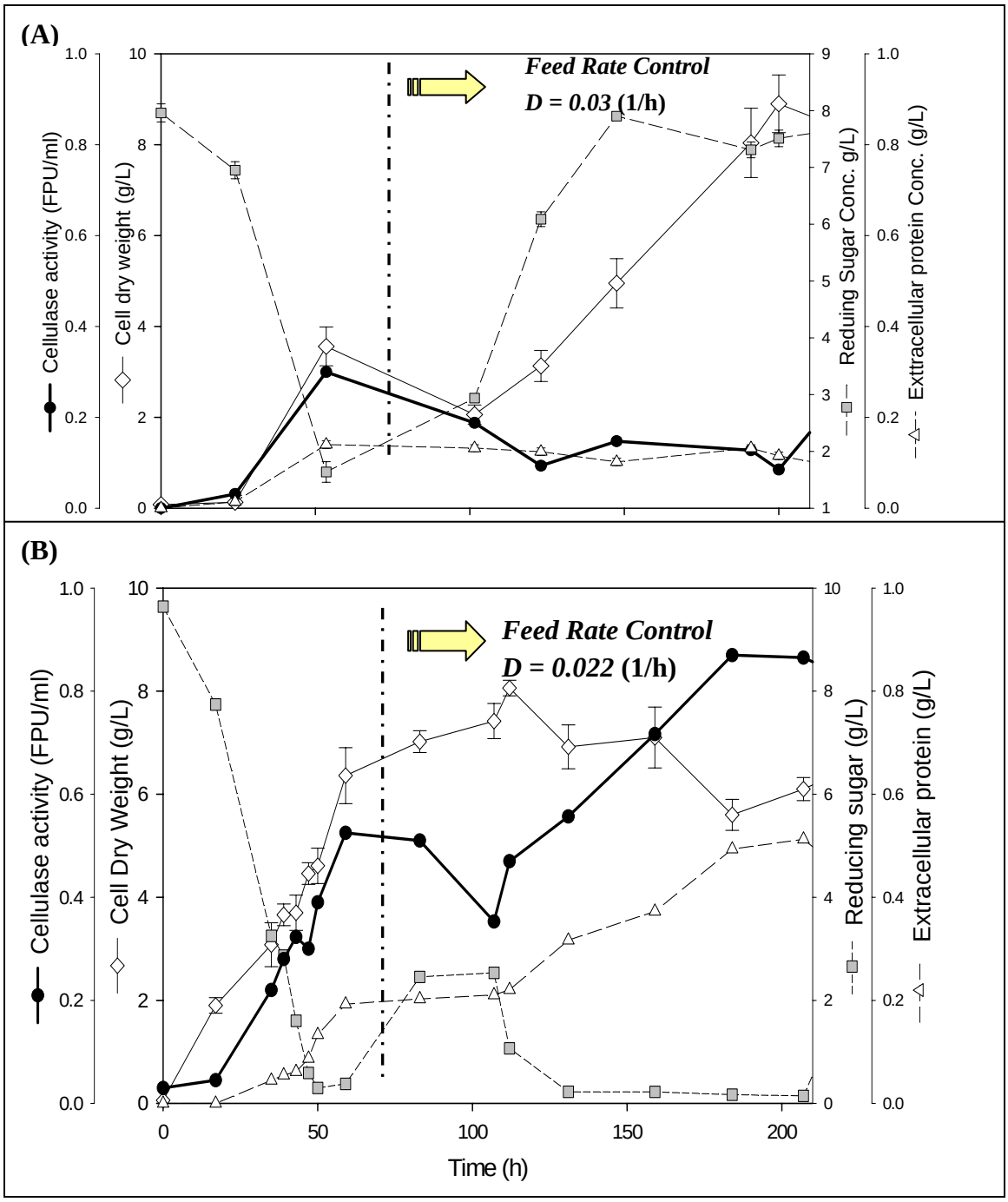

Figure 4.4 : (A) with the conventional, constant dilution rate of 0.03 (1/h) and (B) with the feed rate controlled by the pH-based strategy, for maintaining the slope of pH decrease in the continuous-culture phase at 20 % of the maximum slope observed during the batch cultivation.

4.5 Discussion

A preliminary study on factors affecting cellulase production was carried out to evaluate the cellulase production under different culture conditions. The study factors were agitation, and nutritional factors. The ratio of intracellular protein and cell dry weight was also studied for the use of determination of biomass concentration.

Only two agitation speeds were tested at 500 and 1000 rpm. The highest cellulase activity, 1.9 FPU/ml, was obtained at 500 rpm. Lejeune and Baron (1995) have done four lower agitation speeds, 130, 200, 300 and 400 rpm, for testing mass transfer and rheology in a 50 g/L cellulose culture condition. Their results showed 300 rpm agitation had 60 % higher cellulase production than that of having oxygen limitation at 130 rpm agitation rate. Hence, higher agitation rate had good oxygen transfer rate and bubble distribution. However, most filamentous fungi were sensitive to shear strength (Maria 2004). In our case, 500 rpm provided the good mixing and accessible DO. Apparently, 1000 rpm was too high to benefit cellulase production using *T. reesei* Rut C30. The breakage of cell body and shorter filaments were also observed at 1000 rpm.

The nutritional factors were investigated under same induction ability, 10 g/L of lactose, and close to same cell concentration. C-limited showed best cellulase activity among these three test runs. Different nitrogen content was also tested, and reduction of the amount of ammonia sulfate had better cellulase activity when compared to lack of urea in N-limited medium (data not shown). N- and P-limited mediums both showed early sporulation and pellet formation, which became less productive then normal filaments. This also indicated the synthesis of cellulase required sufficient N and P

elements. Besides, Ilmen et al. (1997) reported the synthesis of cellulase was not caused by starvation in either carbon or nitrogen-limited conditions.

Acetate, formed by deprotonation of acetic acid (HAc), was known as a main inhibitory compound in dilute acid-pretreated hydrolysate. By removal of acetic acid, a new equilibrium could be reached by inversing the course of reaction: acetate + H^+ -> HAc. Therefore, aerating hydrolysate removed not only acetic acid, but also acetate. Lowering pH could also make the removal process more efficient. After 7 days continuously blowing air through prepared hydrolysate, the lowest hydrolysate concentration in agar plate showed best survival rate, which indicated acetic acid and acetate could not be removed completely. A better design with improved gas-liquid interfacial mass transfer would also prove beneficial to the detoxification efficiency. However, there would be a huge problem if this treatment applied in a large scale. So later on, the disaccharide lactose, a cheap diary byproduct, has been used in the fermentation process.

While the pH-based feed control strategy has not been fully optimized, the feasibility of its implementation for favorable continuous cellulase production has been clearly demonstrated. It is interesting to note the roughly parallel profiles of FPU and extracellular proteins in both systems shown in Figure 4.4. Cellulase fermentation with the lactose-based media was shown to produce less non-cellulase proteins, when compared to that with media containing either hardwood hydrolysate or glucose plus Avicel (Zhang et al. 2007). This property is important to the subsequent product separation and purification by foam fractionation or other methods.

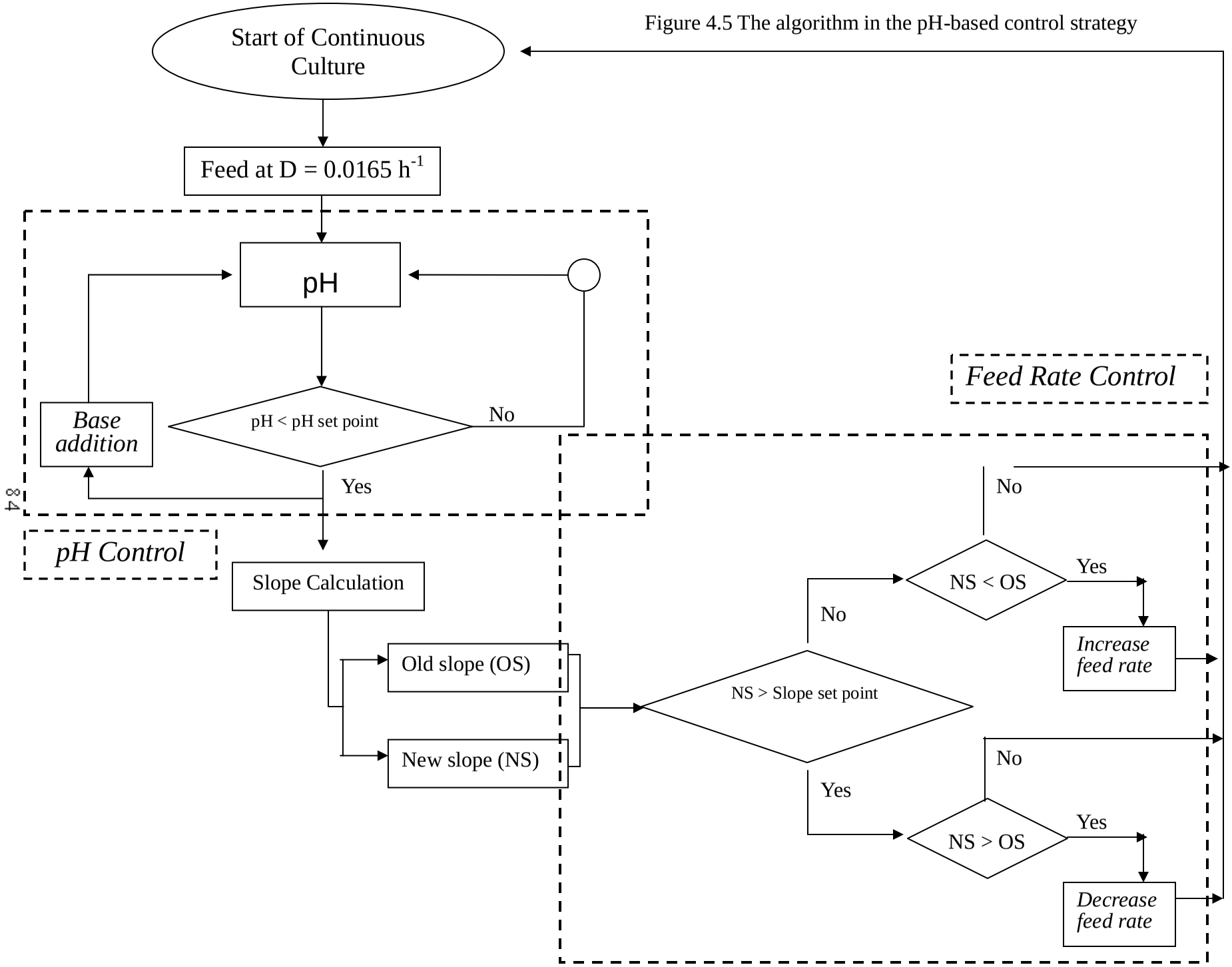

Figure 4.5 The algorithm in the pH-based control strategy

4.6 Conclusion

The cellulase production process was complex, involving culture conditions and control strategy. The main function for agitation was to provide good mixing. This included the good oxygen mass transfer rate and good distribution of chemicals and biomass, and 500 rpm agitation speed had both. In addition, the shear strength at 1000 rpm caused severe damage on cellulase production and cells. Detoxification of hydrolysate, made by hard wood saw dust, by stripping off acetic acid showed the possibility of using dilute acid-pretreated hydrolysate for cell growth and cellulase production. This also provided the opportunity to use the same method on other types of renewable biomass for further biofuel utilization.

pH-based feed control strategy was our first attempt on long term high productivity of enzyme cellulase process. The control strategy was based on true cell metabolism, indicated by the change of pH slope. For seeking a best metabolic stage of cellulase production in *T. reesei* Rut C30 cultivation, it has shown the better results than that in constant feeding (dilution rate at D = 0.03) at low dilution rate. Although the control mechanism was empirical and dynamic, it presented a way for monitoring cells metabolism and sugar consumption.

CHAPTER V

CELLULASE PRODUCTION BY *TRICHODERMA REESEI* USING SAWDUST HYDROLYSATE

5.1 Abstract

Sawdust hydrolysates were investigated for their ability to support cell growth and cellulase production, and for potential inhibition to *Trichoderma reesei* Rut C30. Simultaneous fermentations were conducted to compare the hydrolysate-based media with the controls having equivalent concentrations of glucose and Avicel cellulose. Six hydrolysates differing in the boiling durations in the hydrolysis procedure were evaluated. The hydrolysates were found to support cell growth and induce active cellulase synthesis. The maximum specific cellulase production rate was 0.046 FPU/(g-CDW-h) in the hydrolysate-based systems, much higher than that (0.017 FPU/(g-CDW-h)) in the controls. The hydrolysate was also evaluated for its induction capability in cellulase production in chemostat culture, with the dilution rate ranging from 0.03 to 0.08 (1/h).

production in chemostat culture, with the dilution rate ranging from 0.03 to 0.08 (1/h). The cell yield, $Y_{X/Hydrolysate}$, and the maintenance constant were 0.61 g-CDW/g-hydrolysate and 0.0069 g-CDW/(g-hydrolysate-h), respectively. The highest cellulase activity, 1,320 Unit/L, was observed at D = 0.03 (1/h). The averaged specific cellulase production rate at all dilution rates examined was 8.88 Unit/(g-CDW-h).

5.2 Introduction

Effective cellulase production is important for the utilization of renewable cellulosic materials and wastes (Araujo and D'Souza 1980; Lynd L R et al. 2002). Batch or fed-batch fermentations of the fungus *Trichoderma* are often used for cellulase production (Mohagheghi et al. 1988; Shin et al. 2000). Cellulase synthesis in *Trichoderma* is subject to induction, the natural inducers being the intermediates formed during the cellulose hydrolysis (Huang 1975; Ladisch et al. 1981). Solid cellulosic materials were, therefore, commonly used as both the carbon/energy substrate and the source of inducers in the fermentation (Huang 1975; Lee 1982; Lee 1983). The use of solid substrates, however, causes certain problems to the fermentation's operation and productivity. In particular, the solids increase the burden on agitation and lower the oxygen supply efficiency of the bioreactors. The compromised oxygen supply, in turn, reduces the employable cell concentration and the attainable productivity. Lower solid concentrations have been shown to yield higher cellulase production (Szengyel et al. 1997).

Recently we have been investigating the feasibility of combining *in situ* foam fractionation with cellulase fermentation (Zhang et al. 2007). The coupled process has the

potential of improving cellulase production by minimizing catabolite (glucose) repression and reducing cellulase degradation by proteases. During the study on foaming behaviors of fermentation broths of *Trichoderma reesei* Rut C-30, the foams were found to also remove the solid cellulose (Avicel) present. To minimize the substrate loss, it is desirable to use soluble substrates in the coupled foam-fermentation process. The soluble substitutes must still be able to induce the cellulase synthesis. Among several pure saccharides studied, sophorose was the most powerful soluble inducer for *Trichoderma* (Mandels et al. 1962; Nisizawa et al. 1971; Sternberg and Mandels 1982; Jeong et al. 1985). Nevertheless, sophorose was expensive and still less potent in induction when compared to cellulose (Sternberg and Mandels 1979). Bailey and Taehtiharju (Bailey and Taehtiharju 2003) have recently used the cheaper lactose as the sole inducing C-source for cellulase production, using the base addition for pH control as the guiding factor to regulate the substrate feeding. Also, the enzymatic hydrolysate of pure cellulose or complex cellulosic materials such as wastepaper has been shown effective in cellulase induction (Allen and Mortensen 1981; Ju and Afolabi 1999b; Yasar et al. 2001).

In this study, the hydrolysate generated from a process developed by Tennessee Valley Authority (TVA) (Lee and Moore 2002) was investigated for its ability to support cell growth and cellulase production, and for its potential inhibition to microorganisms. The latter was often found with complex hydrolysate and attributed to acid hydrolysis byproducts such as furfural, acetic acid, levulinic acid, and hydroxymethylfurfural (HMF) (McMillan 1994a; Palmqvist et al. 1997; Lasson et al. 1999). Overliming has been reported to reduce the inhibitory effects (Ranatunga et al. 2000), in addition to neutralizing the acid used in hydrolysis.

In this study, simultaneous fermentations were conducted to compare the hydrolysate-based media with their equivalent controls of mixed glucose and Avicel cellulose. The hydrolysates prepared with different procedures could therefore be evaluated for their efficacy in cellulase production. A chemostat culture study was also conducted using the hydrolysate as the carbon source in the feed medium.

5.3 Materials and methods

The procedure of preparation in batch and continuous fermentation processes for this study was described in this section.

5.3.1 Hydrolysate Preparation

The hydrolysis essentially involves two steps: First, the sawdust was mixed with concentrated sulfuric acid (80 wt %) at a solid to acid ratio of 1.05, to form a viscous “gel”. Second, the gel was diluted with water to 20 % acid and filtered to remove the inert solids. The filtrate was then boiled for 15 minutes. The quickly cooled hydrolysate was then over-limed and centrifuged to remove the precipitate. The supernatant collected was adjusted to pH 7 and used to prepare the culture medium. To raise the sugar concentration in the hydrolysate, for use as the continuous feed in the fermentation, the hydrolysis procedure was modified at the beginning of the second step, i.e., the acid was diluted to 40 %, instead of 20 %, before the filtration and subsequent boiling.

5.3.2 Microorganism, Media and Fermentation

Trichoderma reesei Rut C30 (NRRL 11460) was maintained at 4 °C on slants of Potato Dextrose Agar (Sigma; 39 g/L, as recommended) and subcultured every 3 to 4 weeks. The inocula were prepared by transferring three loops of cells from an agar slant to 50 ml of Potato Dextrose Broth (Sigma) in a 250-ml flask. After grown for 4 days in a shaker (Model 4703, Queue Orbital shaker, Parkersburg, WV) at 200 rpm and 25 °C, 1 ml of the culture was inoculated to each system. The medium composition was essentially the same as that of Mandels (1976) except that the 10 g/L of cellulose was replaced by different inducing C-substrates. For the hydrolysate-based systems, 10 g/L of combined cellulosic oligomers and glucose was used. For the controls, cellulose powders and glucose were added at the same amounts as the oligomers and glucose, respectively, in the corresponding hydrolysates. Twelve fermentations were conducted: six hydrolysates and six corresponding controls. The concentrations of glucose and cellulose/oligomers in the studied systems are summarized in Table 5.1. The hydrolysates differed in the boiling durations as described earlier. The cultures were grown for 5 days in shake flasks containing 55 ml of medium each, under the aforementioned conditions for inoculum preparation. Periodical samples were taken and analyzed.

Table 5.1. Concentrations of glucose and cellulose/oligomers in hydrolysate-based media and corresponding controls

Batch	Hydrolysate-Based Media					Controls			
	Before Autoclaving			After Autoclaving		Before Autoclaving		After Autoclaving	
	Glucose	Total Sugars	Oligomers	Red. Sugars	Oligomers	Glucose	Cellulose	Red. Sugars	Cellulose
1	2.1	6.2	7.2	10.4	3.6	2.1	7.2	2.6	6.8
2	3.1	8.3	5.9	13.0	2.8	3.1	5.9	3.5	5.5
3	4.0	9.9	4.7	14.2	2.3	4.1	4.7	4.3	4.5
4	5.3	11.6	3.5	15.0	1.9	5.3	3.5	5.9	3.0
5	7.4	14.1	1.7	16.7	0.7	7.4	1.7	8.5	0.6
6	9.0	16.1	0.5	16.2	1.5	9.0	0.5	9.3	0.3

The units for all concentrations are (g/L).

The remaining concentrations of oligomers and cellulose after autoclaving are estimated using equations similar to eq. (1).

5.3.3 Continuous culture

The preparation of inoculum followed the same procedure as that mentioned above. The continuous culture study was carried out in a bioreactor (Newbrunswick Bioflo 110) with 1 L of medium. The medium was carbon-limited and the composition was modified from that described by Mandels (Mandels 1976). The composition of feed medium was exactly the same as culture medium. The carbon source of the medium was prepared from the dilute acid-treated hydrolysate, which had been determined to contain about 20 g/L of total reducing sugars including 4.6 g/L of glucose. 4.5 L of the overliming-detoxified hydrolysate was used to make up 9 L of the fresh feed medium, giving about 10 g/L of total reducing sugars. The continuous culture conditions were set at pH 5.0 ± 0.2 (by the addition of 2 N NaOH), dissolved oxygen concentrations (DO) above 70% air saturation (by using mixtures of pure oxygen and air as the influent gas, automatically controlled using the Labview 7.1 software), 500 rpm mechanical agitation, and temperature at 25 ± 1 ^{o}C. Olive oil was added automatically as the anti-foam reagent. pH was set at 5.0 and controlled by base addition. Liquid level was maintained by using Labview program to control two pumps for periodically removal of broth.

Continuous culture was carried out in 1L working volume Bioflo 110 bioreactor when the batch culture reached maximum cell concentration. The steady state was determined by the measurement of intracellular protein, sugar concentration and cellulase enzyme activity after three to five residence times. The control of removal of waste was performed by pumping out medium every 15 min at lower dilution rate and 10min at higher dilution rate instead of using overflow. The detailed analytical methods, regarding cell concentration, sugar concentration, and enzyme activities, are described in Chapter 3.

The calculated cell dry weight (CDW) was based on the relationship established in Chapter 4.

5.4 Analytical Methods

The hydrolysate analyses were done after neutralization with calcium carbonate and removal of solids by centrifugation. The supernatants were measured for glucose concentration by YSI 2700 Select Biochemistry Analyzer. Sugars and degradation products were analyzed using an HPLC system (Thermal Separation Products Spectra System) with an RI detector. The columns used were Bio-Rad Aminex HPX-87P (85 ^{o}C) and -87H (65 ^{o}C) for sugar and organic acid analysis, respectively. The mobile phases were water and 0.01 N sulfuric acid, respectively; both at 0.6 ml/min.

The fermentation samples were analyzed for reducing sugar concentrations, cell concentrations, and cellulase activities. The methods are described in more detail in chapter 3 (Wu and Ju 1998; Zhang et al. 2007). Briefly, the reducing sugar concentration was measured by the non-specific dinitrosalicylic acid (DNS) method. The cell dry-weight concentrations could not be measured directly in the controls containing solid cellulose. All samples were therefore analyzed for intracellular protein concentrations and the corresponding cell dry-weight concentrations estimated using a pre-established correlation: [Cell Concentration] (g/L) = [Intracellular Protein Concentration] (g/L) × 8.0 (± 0.5). The total cellulase activity was measured by the standard filter paper assay (Margollas-Clark 1996).

5.5 RESULTS

The results of using dilute acid pretreated hydrolysates in batch and continuous culture were presented in this section.

5.5.1 Hydrolysis

Table 5.2 summarizes the concentrations of various sugars and degradation products present in the overlimed hydrolysates with different boiling times. A hydrolysate sample was autoclaved at 120 °C for 2 h to give the maximum glucose concentration, [G_{max}], attainable. Also included are the oligomer concentrations estimated using the following equation:

$$[Oligomers] = \left([G_{\max}] - \left([G_1] + [G_2] \cdot \left(\frac{360}{342} \right) \right) \right) \cdot \left(\frac{165}{180} \right) \quad (1)$$

[G_1] and [G_2] are the concentrations of glucose and cellobiose, respectively. The ratio of 360/342 is used to calculate the glucose concentration from cellobiose hydrolysis; 342 is the molecular weight of cellobiose, and 360 (= 2 × 180) accounts for the 2 glucose molecules from hydrolysis of each cellobiose molecule. The glucose concentrations from glucose and cellobiose are thus first subtracted from [G_{max}] to obtain the remainder that should have come from oligomer hydrolysis. The oligomer concentration is then estimated by multiplying the remainder by the ratio of 165/180, assuming that the oligomers have an average degree of polymerization (DP) of 6 (i.e., 165 = (180 × 6 – (18 × 5))/6.). The ratio would not change much with different DPs, e.g., 168/180 for DP = 3 and 163.8/180 for DP = 10.

Table 5.2. Sugars, degradation products, and cellulosic oligomers in hydrolysates with different durations of boiling

Hydrolysate Boiling Duration (min)	Concentration (g/L)										
	Sugars						Degradation Products				Oligomers
	Glucose	Cellobiose	Xylose	Galactose	Arabinose	Mannose	Acetic Acid	Levulinic Acid	HMF	Furfural	
15	2.10	0.83	3.76	<0.10	0.15	0.23	1.61	<0.10	<0.10	<0.10	7.25
30	3.06	1.31	4.44	<0.10	0.38	0.44	1.58	<0.10	<0.10	<0.10	5.91
45	4.05	1.57	4.94	<0.10	0.41	0.54	1.51	<0.10	<0.10	<0.10	4.74
60	5.27	1.70	5.32	<0.10	0.41	0.58	1.48	<0.10	<0.10	<0.10	3.51
90	7.39	1.59	5.59	<0.10	0.42	0.69	1.29	<0.10	<0.10	<0.10	1.66
120	9.04	1.28	5.81	<0.10	0.44	0.79	1.21	<0.10	<0.10	<0.10	0.46
Autoclaved	10.88	<0.10	4.73	<0.10	0.35	0.86	1.64	<0.10	0.09	0.69	--

Note:

1. **The autoclaved sample was autoclaved at 120 °C for 2 h, to give the maximum glucose obtainable from the hydrolysis.**
2. **HMF – hydroxymethylfurfural.**
3. **The oligomer concentrations were calculated according to eq. (1), as described in the text.**

As shown in Table 5.2, the hydrolysates had similar cellobiose concentrations (0.83-1.70 g/L) but notably different oligomer concentrations, which decreased from 7.25 g/L after 15 min of boiling to 0.46 g/L after 120 min of boiling. None of the hydrolysates contained excessive amounts of potentially inhibitory levulinic acid, HMF or furfural.

5.5.2 Batch Fermentation

The profiles of cell growth, decreasing in reducing-sugar concentration, and cellulase (FPU) production are shown in Figures 5.4 and 5.5 for the control and hydrolysate systems, respectively.

Cell Growth: Distinctly longer lag phases, ~ 1 day, were observed for cell growth in all of the hydrolysate systems, presumably because of the presence of hydrolysis by-products (McMillan 1994a; McMillan 1994b; Palmqvist et al. 1997; Lasson et al. 1999). Nonetheless, the cells proliferated rapidly after the lag phase and reached the stationary phase in one day.

As shown in Figure 5.4-a, glucose supported higher cell growth than cellulose, presumably because of the diffusion limitation of the insoluble cellulose (Marten et al. 1996) and/or the additional resources and energy spent on producing the cellulase required for cellulose hydrolysis (as indicated in Figure 5.4-c). On the other hand, the maximum cell concentrations were approximately the same in all of the hydrolysate systems (Figure 5.5-a), indicating the easier assimilation of hydrolysates than the insoluble cellulose. The maximum cell concentrations in the hydrolysate systems were however lower than those in the controls (see Discussion).

Reducing-Sugar Consumption: In all systems, the profiles of decrease in reducing-sugar

concentration mirrored those of cell growth. Stationary phase was reached at depletion of the sugars, confirming that the systems were C source-limited. Reducing-sugar concentrations were higher in the hydrolysate systems than in the controls, because the former contained sugars other than glucose (Table 5.1) and because large fractions of the hydrolysates' oligomers were hydrolyzed to sugars during the steam sterilization (Table 5.2). The higher sugar concentrations in the hydrolysates did not yield more cells, although the sugars were all depleted (Figure 5.5-b).

Cellulase Production**:** For the controls (Figure 5.4-c), the cellulase production clearly increased with increasing cellulose contents. The effect was particularly significant when cellulose was 20%-60% of the total C substrates (glucose plus cellulose). Below 20%, the cellulose content was too low to provide sustained induction, with the cellulose being hydrolyzed while the glucose concentration was still high enough to repress active cellulase synthesis. At cellulose content > 60%, the cellulase production appeared to reach a plateau. Similar but less pronounced effects were also observed in the hydrolysate systems (Figure 5.5-c). Note that the hydrolysates with lower than 20% of oligomers (H5 and H6) supported active cellulase production while the corresponding controls (C5 and C6) did not. Xylose is known to induce cellulase synthesis, although not nearly as effective as cellulose (TAO et al. 2001). It is likely that the non-glucose sugars present in the hydrolysates facilitated the induction for cellulase synthesis. The cellulase concentrations in the hydrolysate systems leveled off or started to decrease after 90 h, suggesting the depletion of inducing oligomers and sugars. Most importantly, the effectiveness of the hydrolysates as soluble cellulase inducers is clearly demonstrated. To be more quantitative, the maximum specific cellulase production rate in each system

was calculated for the most productive 1-day period, i.e., 89-113 h for the controls and 65.5-89 h for the hydrolysate-based systems. The rates were then averaged for all productive systems, which for the controls included the 4 systems with high enough cellulose contents (C1-C4) and for the hydrolysate-based systems included all 6 systems. The averaged maximum specific rate was 0.046 FPU/g cells-h for the hydrolysate-based systems, much higher than that (0.017 FPU/g cells-h) for the controls.

5.5.3 Continuous Culture

The results of cell concentration, FPU activity, and residual reducing sugar concentration obtained in the continuous culture study at the dilution rate (D) of 0.03 to 0.08 (1/h) are shown in Figure 5.1.

Reducing Sugar Concentration

The residual reducing sugar concentrations were constant, at 1.185 (± 0.007) g/L, at all but the highest D examined. The residual sugar concentration was slightly higher (1.46 g/L) at D = 0.08 (1/h). The non-zero reducing sugar concentrations at low D indicated that some of the “reducing sugars” in the acid hydrolysate, detected by the DNS method, were not degradable by *T. reesei*. With 13.5 g/L of total reducing sugars in the feed, the above value implied that about 8.8 % of the reducing sugars in the acid hydrolysate were not degradable.

Following the simple unstructured model for continuous culture systems (Szengyel et al. 1997), at steady state,

$$\mu = \frac{\bar{\mu} S}{K + S} = D + k_d \qquad (2)$$

where μ is specific cell growth rate (1/h), $\bar{\mu}$ is maximum specific cell growth rate, K is Monod (half-maximum) constant for the substrate (i.e., the "degradable" portion of the reducing sugars), and k_d is the specific cell death rate (1/h). Equation (2) can be rearranged to

$$S = \frac{K\left(D + k_d\right)}{\bar{\mu} - D - k_d} \qquad (3)$$

The value of k_d was determined to be 0.016 (1/h), as described in the next section for Cell Concentrations. With Equation (3) and the experimental results of S at different D, the values for $\bar{\mu}$ and K were estimated to be 0.096 (1/h) and 0.00057 g/L, respectively, using the built-in Solver program in Microsoft Excel to minimize the sum of square of the errors between the experimental and calculated values for S.

The best-fit value for $\bar{\mu}$ (=0.096 (1/h)) agreed well with the previous results of batch fermentation (Lo et al. 2005) where $\bar{\mu}$ was observed to be about 0.1 (1/h). This growth rate was lower than that obtained with glucose as the substrate (0.13 (1/h)) (Lo et al. 2008), consistent with the observation of some inhibitory effects of the hydrolysate. Nonetheless, the cell growth rate on the hydrolysate was still appreciably higher than that on galactose (0.06 (1/h)) (Lo et al. 2008).

Cell Concentration:

The cell concentrations increased slightly as D increased from 0.03 (1/h) to 0.05 (1/h) and then remained relatively constant at 6.64 ± 0.19 g/L at higher D. Similarly, the

"apparent" cell yield ($Y_{X/S}^{AP}$) from the (degradable) reducing sugars in the hydrolysate increased from 44 % at D = 0.03 (1/h) to 49 % at D = 0.042 (1/h) and eventually to 57 % at D = 0.08 (1/h). The significantly different apparent cell yields indicated relatively substantial endogenous metabolism or cell death in the hydrolysate-based medium. This observation is consistent with the findings in the previous batch fermentation study (Lo et al. 2005) that the growth of *T. reesei* Rut C30 in the acid-hydrolysate-based medium required much longer lag phases.

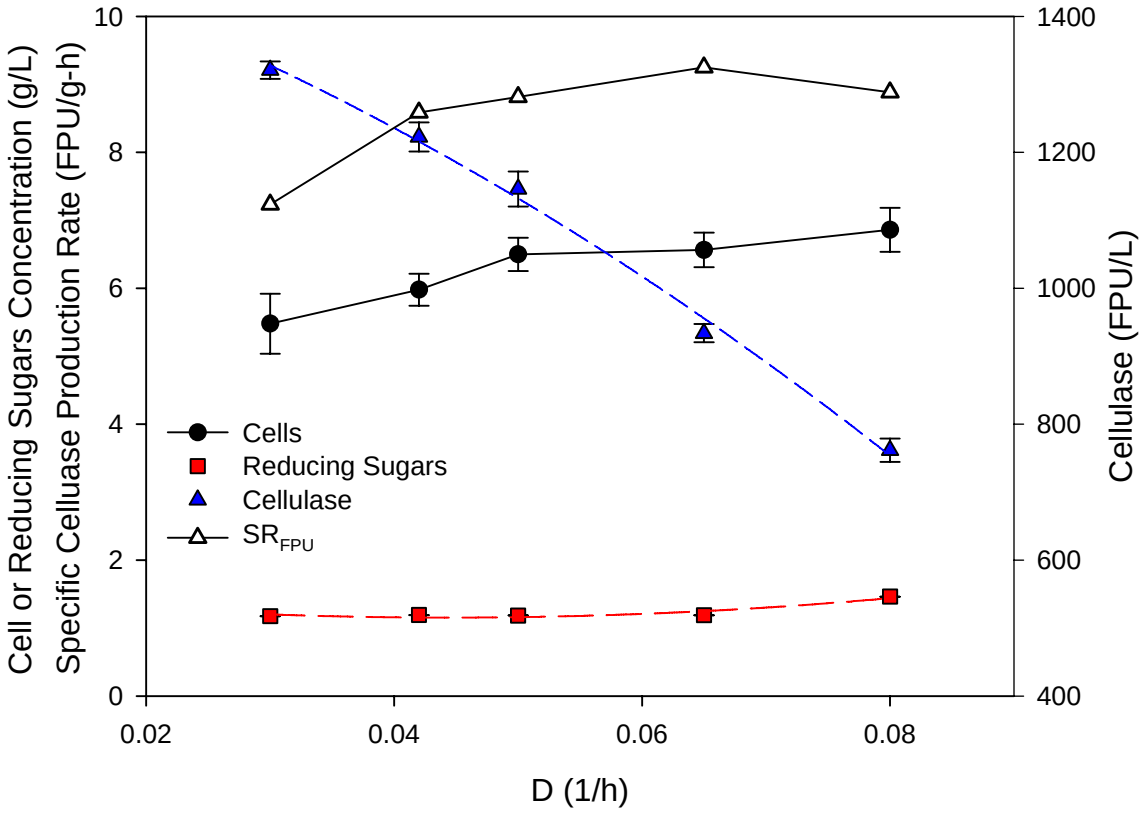

Figure 5.1 The continuous culture of *T. reesei* Rut C30 with dilute acid hydrolysate as substrate

The maintenance coefficient (i.e., specific substrate consumption rate by endogenous metabolism), m_s, can be determined using the following equation (Shuler and Kargi 1992):

$$\frac{1}{Y_{X/S}^{AP}} = \frac{1}{Y_{X/S}^{M}} + \frac{m_s}{D} \qquad (4)$$

where $Y_{X/S}^{M}$ is the maximum cell yield (without endogenous metabolism or maintenance energy) and m_s can be related to the cell death rate k_d (1/h) by the following equation:

$$m_s = \frac{k_d}{Y_{X/S}^{M}}. \qquad (5)$$

The values of $1/Y_{X/S}^{AP}$ were therefore plotted against (1/D) in Figure 5.2. Accordingly, m_s was determined to be 0.023 g sugars/g CDW-h, $Y_{X/S}^{M}$ was 67.6%, and k_d was about 0.016 h^{-1}.

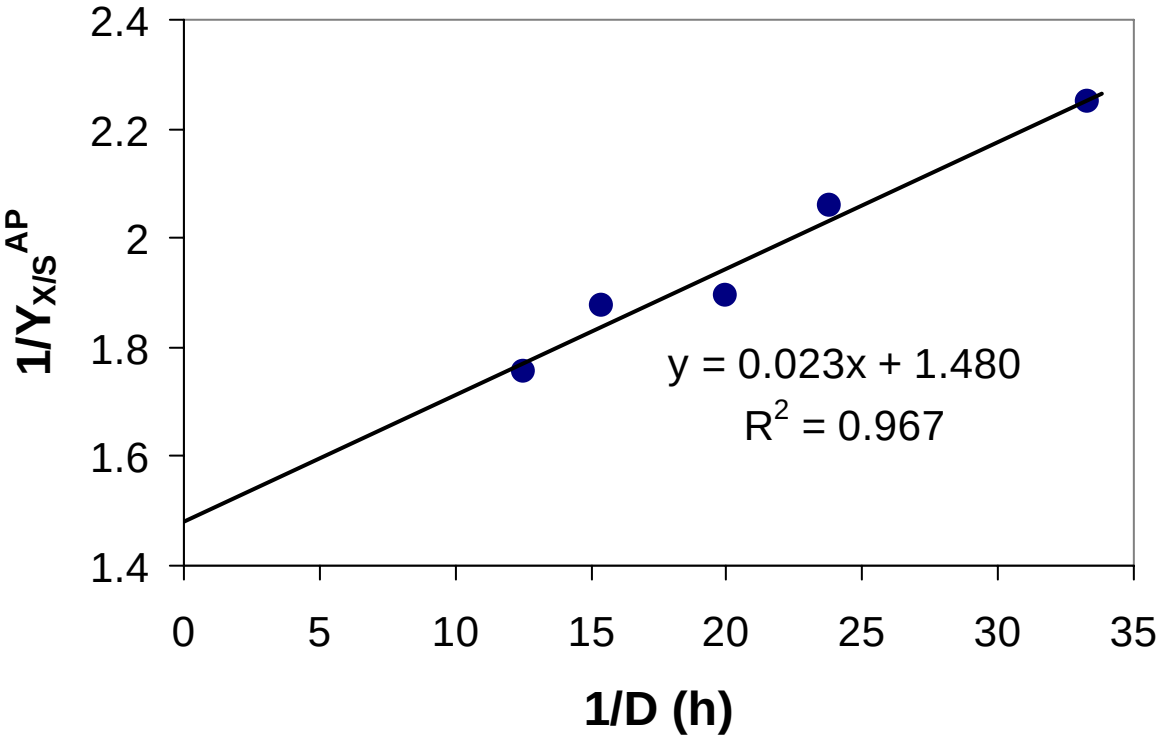

Figure 5.2 The estimation of $Y_{X/S}^{M}$, maximum cell yield, and maintenance coefficient, $m_{s,}$ via plotting $1/Y_{X/S}^{AP}$ Vs (1/D)

Cellulase Activity:

As the dilution rate was increased from 0.03 to 0.08 (1/h), the cellulase (FPU) activity declined almost linearly from 1320 FPU/L to 760 FPU/L (Figure 5.1). The specific cellulase production rates SR_{FPU} were however relatively constant, i.e., 8.88 ± 0.27 (FPU/g CDW-h) at D = 0.042 – 0.08 (1/h), and only slightly lower, i.e., 7.23 (FPU/g CDW-h) at D = 0.03 (1/h). The decrease of FPU with increasing D was therefore not

because of slower FPU production but largely reflected the faster dilution (or removal from the bioreactor) at higher D.

A similar FPU profile was observed in another continuous culture study recently conducted in this laboratory, using lactose as the inducing substrate (Lo et al. 2008). The maximum FPU observed in that study was about 1200 FPU/L and the specific FPU production rate was also approximately constant (except at the lower D, similar to this study), at about 8.2 FPU/g CDW-h. On the other hand, in an earlier continuous culture study using a wastepaper hydrolysate prepared with a different procedure that involved acid pretreatment followed by 4-day enzymatic hydrolysis, the specific FPU production rate increased significantly from 2.2 FPU/g CDW-h at D = 0.012 (1/h) to 12.2 FPU/g CDW-h at D = 0.12 (1/h) (Ju and Afolabi 1999). The much lower SR_{FPU} found at lower D was attributed to insufficient residual concentrations of the inducing oligomers, due to more thorough degradation in these systems of longer retention times. Given the observations of these two other studies, the finding of relatively constant SR_{FPU} in the current study implied that the acid hydrolysate prepared by the particular procedure indeed retained sufficiently high oligomer contents for inducing cellulase production in the continuous culture of *T. reesei*.

5.6 DISCUSSION

Szengyel et al. (1997) found that *T. reesei* would consume soluble sugars after all furfural in the hydrolysate of steam-pretreated willow was consumed. Palmqvist et al. (1997) reported that *T. reesei* could digest most of the compounds in the hydrolysate including the putative toxic compounds such as furfural, acetic acid and HMF. In

addition, glucose and xylose were found consumed simultaneously. Although the cell growth profiles were not shown in either study, the existence of lag phase could be inferred from the profiles of sugar consumption in their experiments. The observation of longer lag phases in the hydrolysate-based systems in this work was therefore consistent with the literature reports.

Table 5.3. Cell yields in controls and hydrolysate-based systems

Batch	Controls			Hydrolysate-Based Systems		
	X (g/L)	Y_{Total}	Y (Best fit)	X (g/L)	Y_{Total}	Y (Best fit)
1	3.0	0.32	$Y_{G1} = 0.43$	2.6	0.14	$Y_{G1+G2} = 0.21$
2	2.8	0.32		2.6	0.13	
3	2.9	0.33		2.6	0.14	$Y_{X+A+M} = 0.08$
4	3.2	0.36	$Y_{Gn} = 0.25$	2.9	0.16	
5	3.6	0.40		2.7	0.14	$Y_{Gn} = 0.21$
6	4.3	0.46		2.8	0.17	

(1) X: maximum cell concentration, Y: apparent cell yield (g dry cells/g substrate), Y_{Total}: Y based on total C substrates (all sugars, oligomers, and cellulose), G_1: glucose, G_2: cellobiose, G_n: oligomers/cellulose, X+A+M: xylose, arabinose, and mannose.

(2) Y (Best fit): best-fit Y values calculated using Solver in Microsoft Excel (see text).

As summarized in Table 5.3, the apparent cell yields from all of the C sources (including sugars and oligomers/cellulose), Y_{Total}, ranged 0.32-0.46 in the controls but only 0.13-0.17 in the hydrolysate-based media. The varying Y_{Total} in the controls were due to their different fractions of glucose and cellulose. Using the Solver program in Microsoft Excel, the separate cell yields from glucose (Y_{G1}) and cellulose (Y_{Gn}) were estimated from the equation: [cell concentration (X)] = Y_{G1} [G_1] + Y_{Gn} [G_n]. The best-fit yields were Y_{G1} = 0.43 and Y_{Gn} = 0.25. As the experiments were done without pH control, these yields were significantly lower than those reported in the literature under well-controlled pH conditions. For example, the cell yield was 0.76 from the reducing sugars in an enzymatic hydrolysate of wastepaper at pH 5.0 and 25 °C (Ju and Afolabi 1999). Nonetheless, Y_{Gn} = 0.25 was close to the value of 0.29 observed by Schaffner and Toledo (1991) in similar batch fermentations.

Similarly the cell yields in the hydrolysate-based systems were estimated as Y_{G1+G2} = 0.21, Y_{Gn} = 0.21, and Y_{X+A+M} (cell yield from combined xylose, arabinose, and mannose) = 0.08. The outcome of equal Y_{G1+G2} and Y_{Gn} was somewhat artificial because the fitting was done with a reasonable restriction of $Y_{G1+G2} \geq Y_{Gn}$. Nonetheless, the two similar yields confirmed the easy assimilation of oligomers. Y_{G1+G2} (predominantly from glucose) in the hydrolysate was much lower than Y_{G1} in the controls. Y_{X+A+M} (primarily from xylose) obtained here was also much lower than the Y_X (= 0.24) observed by Schaffner and Toledo (1991).

5.6.1 Model Simulation of Frequent but Non-Continuous Broth Removal

For any component *i*, the material balance made during the period between two

consecutive broth removals is

$$\frac{d}{dt}(VC_i) = FC_i^0 + Vr_i \qquad (6)$$

where

t is time (h), with $t = 0$ corresponding to the moment after the first removal and $t = \tau$ to the moment right before the second removal (thus, τ represents the interval between removals);

V is the medium volume (L) at the instant t;

C_i is the concentration (g/L) of component i (which is cell or reducing sugar concentration in this model simulation; cellulase activity, although possible, is not simulated here) and C_i^0 is the concentration in the fresh feed (which is 0 g/L for cell concentration and 13.5 g/L for reducing sugar concentration, respectively);

F is the medium feed rate (L/h); and

r_i is the volumetric rate of generation (g/L-h) for component i.

Due to the continuous feeding, V increases with t from V^0 (at $t = 0$) to V^τ (at $t = \tau$), according to the following equation

$$V = V^0 + Ft = V^{avg} + F(t - \tau/2). \qquad (7)$$

where $V^{avg} = (V^0 + V^\tau)/2$, representing the average medium volume during the interval. In addition, the periodical broth removal was to achieve an equivalent dilution rate, i.e.,

$$D = \frac{F}{V^{avg}}. \qquad (8)$$

By substituting Equations (3) and (4), Equation (2) can be rearranged into the following form:

$$\frac{dC_i}{dt} = r_i + \frac{D}{1 - D\left(\tau/2\right) + Dt}\left(C_i^0 - C_i\right) \quad (9)$$

Following the simple unstructured model,

for cell concentration (X) $r_i = (\mu - k_d)X$ (10)

for substrate (reducing sugar) concentration (S) $r_i = -\frac{\mu X}{Y_{X/S}^M}$ (11)

As in Equation (2), the specific cell growth rate μ (1/h) can be described by the Monod equation, i.e.,

$$\mu = \frac{\bar{\mu} S}{K + S}.$$

Substituting Equations (10) and (11) into Equation (9) gives two simultaneous ordinary differential equations (ODEs): one for *dX/dt*, the other for *dS/dt*. The two ODEs and the Monod equation were coded in Excel's Visual Basics for Applications, with the two ODEs being solved numerically using the fourth-order Runge-Kutta method (Liengme 2002). Using the kinetic parameters reported earlier in the Results section, i.e., $\bar{\mu}$ = 0.096 (1/h), K = 0.00057 g/L, $Y_{X/S}^M$ = 0.676, and k_d = 0.016 (1/h), the profiles of cell and reducing sugar concentrations could then be simulated for the systems with frequent periodical broth removal. As an example, the simulated results for the system with D = 0.05 (1/h) and τ = 0.25 h (i.e., 15 min) are shown in Figure 5.3 for two cycles of broth removal. The results expected from "true" continuous culture are also shown in Figure 5.3 for comparison. The simulation clearly demonstrated that the operation of frequent broth removal can achieve essentially the same outcomes (on average, very slight over-approximation in reducing sugar concentrations and under-approximation in

cell concentrations) as the ideal continuous culture while eliminating the practical difficulties associated with the use of simple overflow for continuous culture of mycelial fungi.

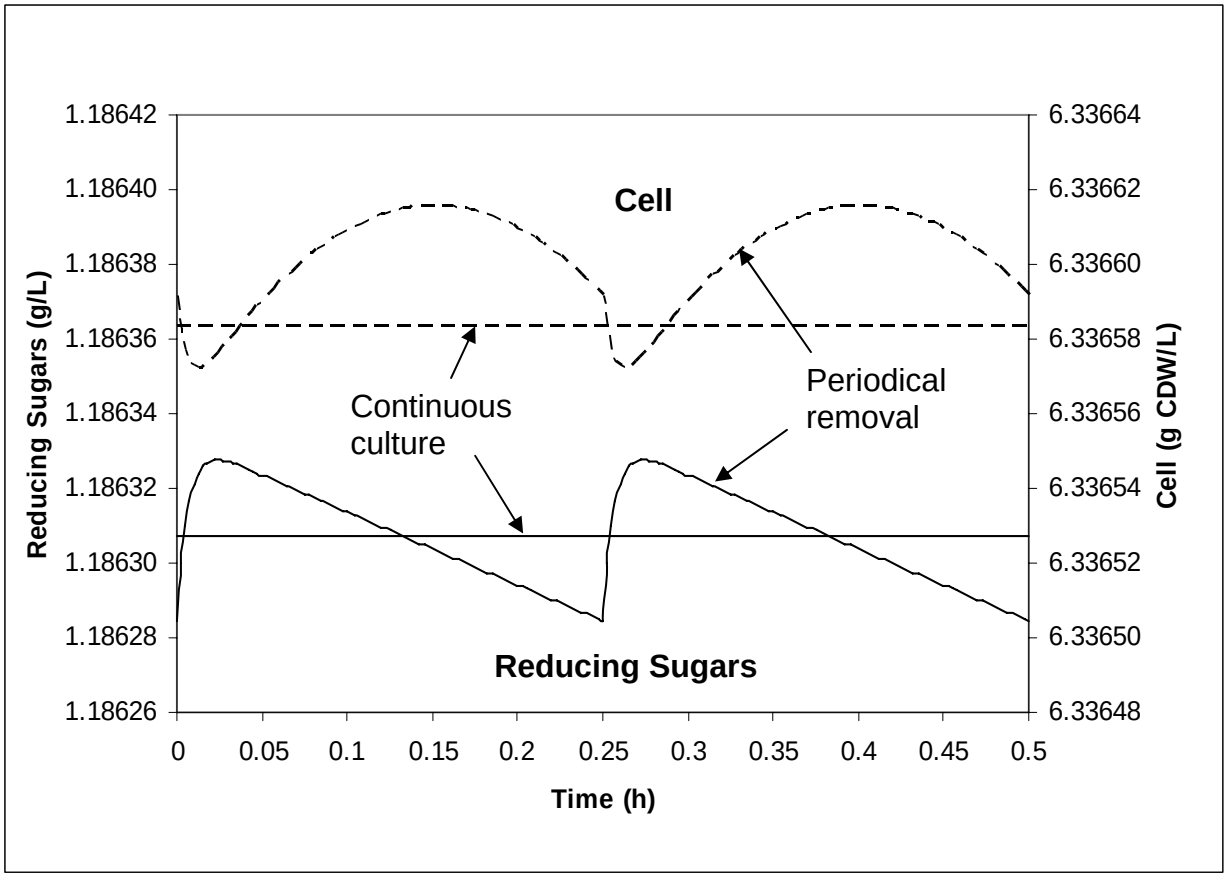

Figure 5.3 the simulated results for the system with D = 0.05 1/h and τ = 0.25 h (i.e., 15 min)

5.6.2 Inhibitory Effects and Air-Stripping Detoxification of Acid Hydrolysate

The extent of inhibition exerted by the acid hydrolysate was found rather sensitive to the hydrolysate preparation procedure. The previous batch fermentation was conducted with the hydrolysate prepared in a same batch (Lo et al. 2005). The continuous culture

study reported in the Results section was carried out with another batch of hydrolysate. Yet another batch of hydrolysate was prepared but not used in the continuous culture study because of the severe inhibition observed: the medium prepared from this hydrolysate failed to support any cell growth when used for pre-culturing inoculum, and the medium caused the cells to gradually turn to spores when added as the feed to a continuous culture originally maintained on a lactose-based medium.

5.7 Conclusions

The hardwood hydrolysate prepared with a stronger acid (during the boiling phase of hydrolysis) was found inhibitory to cells. The inhibition could be reduced by a simple approach of aerating the acidified hydrolysate, presumably via removal of volatile compounds such as acetic acid.

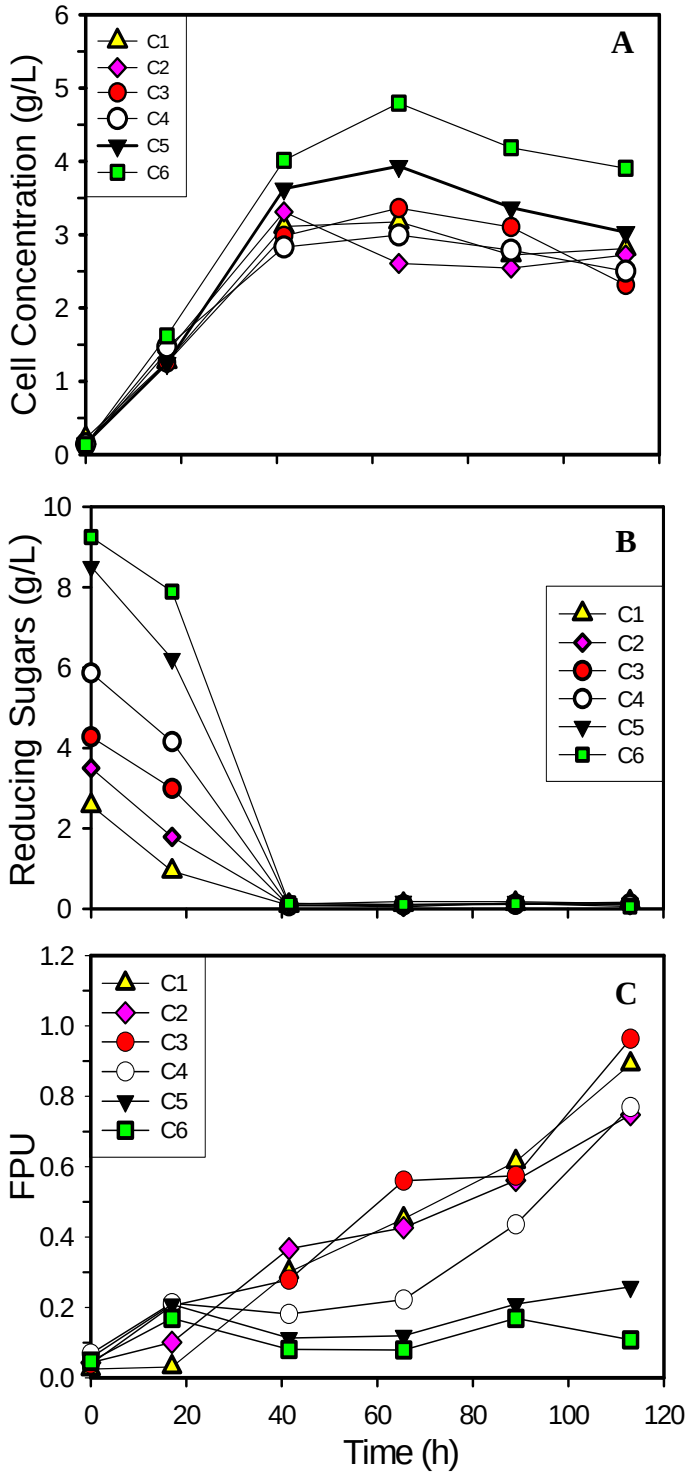

Figure 5.4 – Profiles of (a) cell growth, (b) reducing sugar concentration, and (c) cellulase production observed in the control systems. The errors were based on the standard deviation in analytical methods for each analysis.

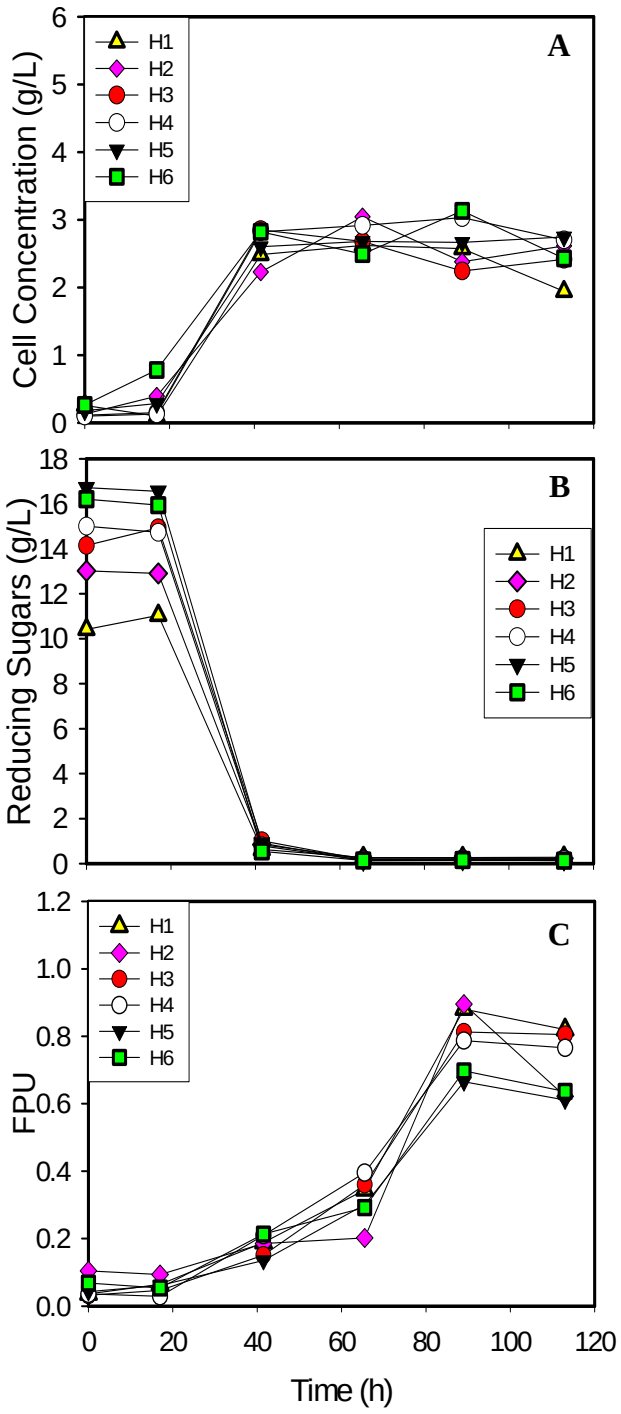

Figure 5.5 – Profiles of (a) cell growth, (b) reducing sugar concentration, and (c) cellulase production observed in the systems containing sawdust hydrolysates prepared with different lengths of boiling. The errors were based on the standard deviation in analytical methods for each analysis.

CHAPTER VI

FERMENTATION BEHAVIOR OF *TRICHODERMA REESEI* RUT C-30 FOR CELLULASE PRODUCTION USING LACTOSE AS SUBSTRATE

6.1 Abstract

Lactose is recognized as an inexpensive, soluble substrate that has reasonably good inducing capability for cellulase production by *Trichoderma reesei*. The current knowledge suggests that the fungus does not uptake lactose directly. Instead, lactose is hydrolyzed by extracellular enzymes, lactase and cellulase components, to glucose and galactose for subsequent ingestion. The fermentation behaviors of *T. reesei* grown on lactose have not been mechanistically investigated or modeled to consider this critical extracellular hydrolysis step. In this study, *T. reesei* Rut C-30 was grown in both batch and continuous culture systems using the following substrates: lactose, lactose and glycerol mixtures, glucose, galactose, as well as glucose and galactose mixtures. The concentrations of substrate and its monosaccharide constituents, and the activities of lactase and cellulase enzymes were measured along with the cell concentration.

Glucose was found, from the batch fermentation study, to strongly repress the galactose consumption. The continuous culture results were used to calculate the lactose hydrolysis rate and the specific production rates of cellulase and lactase. Both lactase and cellulase (measured as Filter Paper Unit) could hydrolyze lactose. Their individual contributions were model-estimated using the calculated lactose hydrolysis rates in the continuous culture. The specific production rates of cellulase and lactase were used to establish the effects of dilution rate and different sugar concentrations on the synthesis of these enzymes. A model was then developed to incorporate these relationships, together with the cell growth and substrate consumption kinetics, to describe the overall fermentation behaviors. All of the experimental results were used in model fitting to generate a set of best-fit model parameters. The study provided significant conceptual and quantitative insights to the lactose metabolism and cellulase production by *T. reesei.*

6.2. Introduction

Utilization of lignocellulosic biomass as renewable energy and chemical feedstock is an important component of the overall solution to the global warming caused by CO_2 emission, to the US dependence on imported oil, and to the inevitable decline in petroleum supply. Lignocellulose hydrolysis to simple sugars is a critical first step in many potential usages of biomass, including production of bioethanol and other fermentation products. Cellulase is the group of enzymes capable of hydrolyzing cellulose to glucose. Economic production of cellulase is therefore critically important to the renewable biomass utilization.

Cellulase is industrially produced by fermentation processes using the fungi *Trichoderma reesei.* Cellulase expression requires induction. While cellulose is the natural inducer, repeated addition of the solid substrate to bioreactors, while maintaining long-term sterile operation, presents serious challenges. Lactose is currently considered as the most feasible inexpensive, inducing soluble substrate for the cellulase production by *T. reesei.* It is important to improve fundamental understanding on the fermentation behaviors of *T. reesei* using lactose as the substrate.

Despite the repeated efforts, Seiboth et al. (2005) were not able to detect activity of either a lactose permease (to transport lactose into the cells) or an intracellular β-galactosidase (to hydrolyze lactose into simple sugars for catabolism) in *T. reesei.* Lactose metabolism in the fungi is therefore believed to be initiated by extracellular hydrolysis to glucose and galactose prior to ingestion (Seiboth et al. 2005). *T. reesei* is known to produce at least two groups of extracellular enzymes that are capable of lactose hydrolysis, i.e., lactase (β-D-galactosidase, EC 3.2.1.108) and cellulase (Zanoelo et al. 2004). However, the origin of lactose's ability to induce cellulase expression remains uncertain. On one hand, a study showed that galactokinase was essential for the gene transcription of endoglucanase (cbh1 and cbh2, important components of cellulase) in lactose fermentation (Seiboth et al. 2004). As galactokinase is induced by galactose, the finding implied that the inducing ability could have come from the galactose formed from lactose hydrolysis. The hypothesis however did not hold upon further investigation: Galactose did not induce cellulase production in batch culture (Seiboth et al. 2004). In continuous cultures, endoglucanase activity was detected only at a very low level and only at the lowest dilution rate studied (D = 0.015); furthermore, similar activity was found in the culture fed with a mixture of galactose and glucose as the substrate (Karaffa

et al. 2006). An alternative mechanism for lactose's inducing ability is the intracellular conversion of glucose to sophorose, which is a known potent inducer for cellulase (Vaheri et al. 1979).

Model development is a mathematical tool to describe and quantify biological systems. Although the true reactions happened *in vivo* cannot be completely revealed, the key metabolic features can be modeled to allow estimation of the empirical kinetic parameters by fitting the model with the measurable *in vitro* concentrations of important substances. In this study, *T. reesei* Rut C-30 was grown in both batch and continuous culture systems using various substrates, including lactose, lactose and glycerol mixtures, glucose, galactose, as well as glucose and galactose mixtures. Measuring the concentrations of these compounds, the activities of resultant lactase and cellulase enzymes, as well as the cell concentrations in these different culture systems enabled us to set up a relatively complete mechanistic model to describe the fermentation behaviors of *T. reesei* Rut C-30. Several previous efforts on modeling *T. reesei* Rut C-30 fermentation using lactose as substrate have been reported (Busto et al. 1996; Tholudur et al. 1999; Muthuvelayudham and Viruthagiri 2006). All were based on batch fermentation results. Rather few continuous culture studies have been conducted using lactose as the

substrate: for *T. reesei* Rut C30, the cellulase production was reported by Bailey and Tahtiharju (2003) and the lactase production by Castillo and co-workers (Castillo et al. 1984). Chaudhuri and Sahai (Chaudhuri and Sahai 1994) used a different strain, *T. reesei* C5, in his study and reported results (without lactase activities) only for a few dilution rates. Ryu et al. (Rye et al. 1979) studied a two-stage continuous culture system using yet another strain, *T. reesei* MCG77. None have investigated on the roles of lactose hydrolysis or have developed models as complete as the one reported here. The experimental results, the model developed, and the best-fit parameters obtained are reported in this work. The model successfully described the hydrolysis of lactose, the lactose-induced synthesis of lactase and cellulase enzymes, the substrate consumption and the cell growth in continuous culture. Without clear understanding, the actual inducing mechanism(s) in the lactose-supported systems has been left out in the current model.

6.3. Materials and Methods

Trichoderma reesei Rut C30 (NRRL 11460), stored in a 4 ^{o}C refrigerator on potato dextrose agar plates, was incubated for 24 hours at 28 ^{o}C before being transferred into 50

ml of potato dextrose broth in a 250-ml Erlenmeyer flask. The transferred cells were allowed to grow for 48 hours at room temperature with mild agitation and then used as the inoculum for the study conducted in a fermentor (Newbrunswick Bioflo 110). The working volume of the culture media was 1.5 L for batch fermentations and 1.0 L for continuous cultures. The medium composition was modified from that reported by Mandels (Mandels 1976): 2.0 g/L KH_2PO_4; 1.4 g/L $(NH_4)_2SO_4$; 1.0 g/L Peptone; 0.3 g/L Urea; 0.3 g/L $MgSO_4 \cdot 7H_2O$; 0.3 g/L $CaCl_2$; 0.2 ml/L Tween 80; 5.0 mg/L $FeSO_4 \cdot 7H_2O$; 2.0 mg/L $CoCl_2$; 1.6 mg/L $MnSO_4 \cdot H_2O$; 1.4 mg/L $ZnSO_4 \cdot 7H_2O$. The culture conditions were controlled at temperature 25 ± 1 °C; pH 5.0 ± 0.2, by automatic addition of 2N NaOH; dissolved oxygen (DO) about 70% of air saturation, using mixtures of pure oxygen and air adjusted by a control algorithm in Labview (Version 7.1, National Instrument, Austin, TX); and agitation 500 rpm with a 6-blade turbine. Olive oil was automatically added as the anti-foam agent. The composition of the feed medium used in the continuous culture was exactly the same as that in the batch fermentation, with the carbon source evaluated in the particular study.

Table 6.1 Batch and Continuous Culture Systems Investigated, Including the Carbon Substrates Used.

Operation Mode	Dilution Rate (h^{-1})	Carbon Substrates (g/L)
Batch	0 (2 runs for each)	Glucose: 10
		Galactose: 10
		5 Glucose + 5 Galactose
Continuous	0.04 – 0.12	Lactose: 10
	0.1	Lactose: 5, Glycerol: 5
	0.1	Lactose: 1, Glycerol: 10
	0.11	Lactose: 1, Glycerol: 9

There were two types of continuous culture systems, lactose and lactose plus glycerol as substrates, including total nine dilution rates in lactose system and three dilution rates in lactose-glycerol system, as well as six batches presented in this paper. Each data point in batch systems was obtained in a way of averaging values from three measurements that were taken at same time. For each dilution rate, three samples were taken at different time (not at same residence time) in the continuous culture systems.

Continuous culture was carried out in 1-L working volume. The feeding was started when the initial batch culture reached the early stationary phase (i.e., attained the

maximum cell concentration). After about 3 residence times, samples were taken periodically during the next 3-5 residence times and measured for the intracellular protein and substrate concentrations, to ensure the reach of steady state at the studied dilution rate. Samples taken during the steady state period were then subjected to additional analyses for the concentrations of glucose and galactose (if lactose was the substrate) and the activities of cellulase and lactase. The feed rate was then changed and the above procedures repeated for the study of culture behaviors at the new dilution rate.

Both the feeding and the culture removal from fermentor were done with pumps (Masterflex L/S, Cole-Parmer Instrument Company, Barrington, IL) computer-controlled using LabView software. The feeding was done mostly continuously using very small pump tubing. But, the pumps for feeding at very low dilution rates (< 0.065 1/h) and for culture removal at all dilution rates were activated periodically by LabView. This periodical pumping design enabled the use of higher pump rates and, for culture removal, larger pump tubing; thereby, preventing the tubing from being clogged by solids and cells and removing the spent culture more representatively. It was observed that if continuously pumped at slow rates or by the simple overflow mechanism, the broth removed might contain less mycelial cells, resulting in an artificial effect of cell retention.

The periodical culture removal was performed every 15 min at lower dilution rates and 10 min at higher dilution rates. These durations were much shorter than the residence times used in the study, i.e., 500-1,500 min. A computer model simulation confirmed that such high-frequency "fed-batch" operation is expected to give the same average culture behaviors as a true continuous culture (results not shown).

6.3.1. Sample Analyses

The lactose and galactose concentrations were analyzed using an enzymatic bioanalysis kit (Boehringer Mannheim/R-biopharm 176 303). Lactose was first hydrolyzed by β-galactosidase to galactose and glucose. The galactose along with NAD+ (Nicotinamide-adenine dinucleotide) as proton acceptor was later catalyzed by β-galactose dehydrogenase followed by the formation of D-galactonic acid and NADH. The amount of NADH was determined by light absorbance at 340 nm. The reading was converted to lactose and galactose concentration according to the equation listed in the reference. (Detailed description in Ch 3) Glucose concentration was measured by using the common PGO enzymes test kit (Sigma P7119). For the measurement of lactase enzyme activity, we followed the same procedure used by Castillo et al. (Castillo et al.

1984). The method for determining the activity of cellulase, in terms of Filter Paper Unit (FPU), has been described also in Chapter 3.

6.4. Model

The model has been developed according to the profiles observed in the batch fermentation and the continuous culture systems conducted in this study. The detailed experimental profiles are described in the Results section. It should also be noted that the results from the glycerol-containing systems were used only for fitting the enzyme (lactase and cellulase) synthesis equations and not for the cell growth or the substrate consumption equations. Glycerol is a known neutral substrate that did not induce or repress cellulase expression by *T. reesei* (Ilmen 1997). Glycerol was included here in the continuous culture study (as shown in Table 6.1) to create systems that had the same high cell growth (or dilution) rates but different sugar concentrations. The results from these systems enabled evaluation of the induction and repression effects of different sugars, i.e., lactose, glucose and galactose. (In the lactose-only continuous culture systems, the lactose, glucose and galactose concentrations were intrinsically determined by the dilution rate. The effects of growth rate and these sugar concentrations could not be

separated.) Nonetheless, since the focus of this study was on the use of lactose as cellulase-inducing substrate, the cell growth rate and yield on glycerol were not examined further.

6.4.1. Specific Cell Growth Rate

In the (glycerol-free) culture media used, the cells could grow on up to three possible carbon (and energy) substrates: peptone, glucose and galactose. Direct uptake of lactose for growth was assumed to be negligible. Glucose and galactose were either added directly (as in the batch fermentation systems) or produced from extracellular lactose hydrolysis (as in the continuous culture systems). A Monod-type dependency of the specific growth rate (μ, in 1/h) on substrate concentrations (*Pep*, *Glu* and *Gal*) was assumed:

$$\mu = \mu_{Pep} + \mu_{Glu} + \mu_{Gal} = \frac{\overline{\mu}_{Pep} Pep}{K_{Pep} + Pep} + \frac{\overline{\mu}_{Glu} Glu}{K_{Glu} + Glu\left(1 + \frac{Pep}{K_{Pep}^{i,Glu}}\right)} + \frac{\overline{\mu}_{Gal} Gal}{K_{Gal} + Gal\left(1 + \frac{Pep}{K_{Pep}^{i,Gal}} + \frac{Glu}{K_{Glu}^{i,Gal}}\right)} \quad (1)$$

where $\overline{\mu}$'s are the maximum specific growth rate (1/h) and K's are the Monod (half-saturation) constants (g/L) for the particular substrate. The first term on the right hand side describes the growth dependency on peptone concentration. As the cells strongly

preferred peptone over the sugars as substrate (observed in this and other studies (IImen et al. 1997)), the second term, for growth on glucose, included a potential inhibition by peptone, with the inhibition constant, $K_{Pep}^{i,Glu}$ (g/L). The growth on galactose was described in the third term, with both inhibition terms by peptone and glucose and the corresponding inhibition constants $K_{Pep}^{i,Gal}$ and $K_{Glu}^{i,Gal}$ (g/L). The inclusion of these inhibition terms were justified by the results from the batch fermentation systems, particularly the diauxic growth observed in the system with a mixture of glucose and galactose as the substrate (Figure 6.3). Detailed description is given in the later Results section.

6.4.2. Specific Rates of Cellulase and Lactase Syntheses

The specific synthesis rates of cellulase and lactase, SR_{FPU} and SR_{La} (both in activity Unit/g dry cells-h), were originally considered to have the following common form:

$$SR = (\alpha\mu + \beta)\frac{L}{K_L + L(1 + \frac{Glu}{K_{Glu}^i} + \frac{Gal}{K_{Gal}^i})}$$

The first part on the right hand side, in the form of $\alpha\mu + \beta$, was modeled according to the common *Leudeking-Piret* kinetics to include both growth-dependent and growth-

independent synthesis (Leudeking and Piret 1959). The second part was to account for the effects of different sugars: induction by lactose and potential end-product repression by glucose and galactose. It is known that lactose is the inducer for both cellulase and lactase (Castillo et al. 1984; Chaudhuri and Sahai 1993). The inducing effects of lactose were modeled with a Monod-type dependency, anticipating that the positive effects would reach the saturation levels at high lactose concentrations (with the half-saturation concentrations as K_L^{FPU} and K_L^{La}, both in g/L of lactose). It is also known that glucose represses the cellulase synthesis (Ilmen 1997). The repression effect was accounted for using the inhibition constant $K_{Glu}^{i,FPU}$ (g/L of glucose). It was unknown if glucose represses the synthesis of lactase or if galactose (like glucose) would repress the syntheses of either enzyme. The inhibition terms were anyway included with the corresponding inhibition constants $K_{Glu}^{i,La}$, $K_{Gal}^{i,FPU}$ and $K_{Gal}^{i,La}$ (all in g/L). The significance of any of these inhibition terms was expected to be revealed from the magnitude of the best-fit inhibition constant: the smaller the inhibition constant, the stronger the inhibition effect.

After closer examination of the enzyme activity profiles obtained in the continuous culture study, the above form was later modified to the following equations:

Cellulase: $$SR_{FPU} = \beta^{FPU} \frac{L}{K_L^{FPU} + L(1 + \frac{Glu}{K_{Glu}^{i,FPU}} + \frac{Gal}{K_{Gal}^{i,FPU}})} \quad (2)$$

Lactase: At $\mu \leq \mu_c$ (a critical specific growth rate),

$$SR_{La} = \beta^{La} \frac{L}{K_L^{La} + L(1 + \frac{Glu}{K_{Glu}^{i,La}} + \frac{Gal}{K_{Gal}^{i,La}})} \quad (3a)$$

At $\mu > \mu_c$,

$$SR_{La} = [\alpha^{La}(\mu - \mu_c) + \beta^{La}] \frac{L}{K_L^{La} + L(1 + \frac{Glu}{K_{Glu}^{i,La}} + \frac{Gal}{K_{Gal}^{i,La}})} \quad (3b)$$

With Equation (2), cellulase synthesis was reduced to be independent of growth rate. Except at very low dilution rates (< 0.5 1/h) where spores and different morphology were observed (more description in the Results section), the cellulase activity was observed to decrease with increasing D, gradually for the most part and more significantly at D > 0.11 1/h. Presumably the gradual decrease resulted from the faster removal with increasing D and the more significant decrease at high D could have been promoted by the end-product repression due to higher glucose and galactose concentrations present (Figure 6.4b).

Lactase synthesis, on the other hand, had a clearly growth-dependent component in its kinetics (Figure 6.4a). This is consistent with the primary role of lactase in hydrolyzing lactose to ingestible substrates (glucose and galactose) to support active cell growth. Lactase has been reported as a cell growth-related enzyme in lactose-based

media and high lactase activity was present at high dilution rates (Seiboth et al. 2005; Fekete et al. 2007). However, only a basal level of lactase activity was found at low D (< 0.65 1/h) before the activity started to increase rapidly with increasing D (Figure 6.4a). Modifications were therefore made to describe the specific rate of lactase synthesis by the above equations (3a) and (3b).

6.4.3. Rate of Lactose Hydrolysis

Since both lactase and cellulase (at least its β-glucosidase component (Zanoelo et al. 2004) could hydrolyze lactose, the rate of lactose hydrolysis R_L^H (g/L-h) was modeled with contributions from both enzymes:

$$R_L{}^H = k_{La}^H La \frac{L}{K_L^{H,La} + L} + k_{FPU}^H FPU \frac{L}{K_L^{H,FPU} + L} \quad (4)$$

The rate of lactose hydrolysis by each enzyme was expected to follow the Michaelis-Menten kinetics, i.e., showing the 1st order kinetics with respect to the enzyme activity (in Unit/L) and a transitional 1st-to-0th order with respect to the substrate (lactose) concentration. k_{FPU}^H and k_{La}^H are the rate constants (g/activity Unit-h), and $K_L^{H,FPU}$ and $K_L^{H,La}$ are the Michaelis-Menten constants for lactose (g/L).

6.4.4. Model for Culture Processes

The following material balance equations were used to fit the experimental results of all the batch and continuous cultures conducted in this study. For the batch culture results, the dilution rate (D, 1/h) = 0. For the continuous culture results, all the accumulation terms, on the left hand side of the equations, reduced to 0.

Cell: $$\frac{dX}{dt} = (\mu - k_d - D)X \quad (5)$$

where X is cell concentration (g/L DCW), t is culture time (h), μ (1/h) is calculated according to Equation (1) as described earlier, and k_d is the cell death constant (1/h).

Lactose: $$\frac{dL}{dt} = -R_L^H + D(L_0 - L) \quad (6)$$

where $R_L{}^H$ is the lactose hydrolysis rate given in Equation (4) and L_0 is the lactose concentration in the fresh (feed) medium. This term was used only for the systems with lactose-containing media.

Glucose: $$\frac{dGlu}{dt} = R_L^H\left(\frac{180}{342}\right) - \frac{\mu_{Glu}}{Y_{X/Glu}}X - D \cdot Glu \quad (7)$$

Galactose: $$\frac{dGal}{dt} = R_L^H\left(\frac{180}{342}\right) - \frac{\mu_{Gal}}{Y_{X/Gal}}X - D \cdot Gal \quad (8)$$

In the above equations, the first terms describe the sugar generation from lactose hydrolysis (the number in the parentheses accounts for the molecular weight ratio between the simple sugars and lactose) and the second terms describe the sugar

consumption for cell growth (μ_{Glu} and μ_{Gal} are given in Equation (1), and $Y_{X/Glu}$ and $Y_{X/Gal}$ are the cell yields from the sugars, in g CDW/g sugar). Again, the first terms were used only for systems with lactose-containing media.

Peptone: $$\frac{dPep}{dt} = -\frac{\mu_{Pep}}{Y_{X/Pep}} X - D \cdot Pep \quad (9)$$

Cellulase: $$\frac{dFPU}{dt} = SR_{FPU} X - D \cdot FPU \quad (10)$$

Lactase: $$\frac{dLa}{dt} = SR_{La} X - D \cdot La \quad (11)$$

In Equations (10) and (11), the specific enzyme production rates *SR*'s are calculated according to Equations (2) and (3).

6.4.5. Model Fitting

As the model included many simultaneous equations, the following three-step strategy was used to determine the best-fit model parameters. First, the growth-related parameters were determined by fitting the model with the results from the batch fermentations. These parameters included the maximum specific growth rates, i.e., $\overline{\mu_{Pep}}$, $\overline{\mu_{Glu}}$ and $\overline{\mu_{Gal}}$; the Monod constants for growth, i.e., K_{Pep}, K_{Glu} and K_{Gal}; the death constant k_d; the cell yields, i.e., $Y_{X/Pep}$, $Y_{X/Glu}$ and $Y_{X/Gal}$; and the growth-inhibition constants, i.e., $K_{Pep}^{i,Glu}$, $K_{Pep}^{i,Gal}$ and $K_{Glu}^{i,Gal}$. The model fitting was done with Excel and the

built-in Solver program, to minimize the sum of squares of the errors between the experimental results and the corresponding model-predicted values. The pertinent equations (1, 5, 7-9) were coded in Excel's Visual Basics for Applications, with the four simultaneous ordinary differential equations, i.e., (5) and (7)-(9), being solved numerically using the fourth-order Runge-Kutta method (Liengme 2002).

Second, the parameters involved in the enzyme synthesis and lactose hydrolysis, were approximated using the results obtained in continuous cultures, including those made with glycerol-containing media. According to Equations (6), (10) and (11), at the steady state, R_L^H, SR_{FPU} and SR_{La} can be calculated as

$$R_L^H = D(L_0 - L)$$
$$SR_{FPU} = \frac{D \cdot FPU}{X}$$
$$SR_{La} = \frac{D \cdot La}{X}$$

using the experimentally measured lactose concentration (L), enzyme activities (FPU and La), and cell concentration (X) at each dilution rate (D). Thus calculated rates were then used in fitting of Equations (2)-(4) to determine the various parameters involved in the cellulase and lactase syntheses as well as in the lactose hydrolysis (by cellulase and lactase, respectively). .

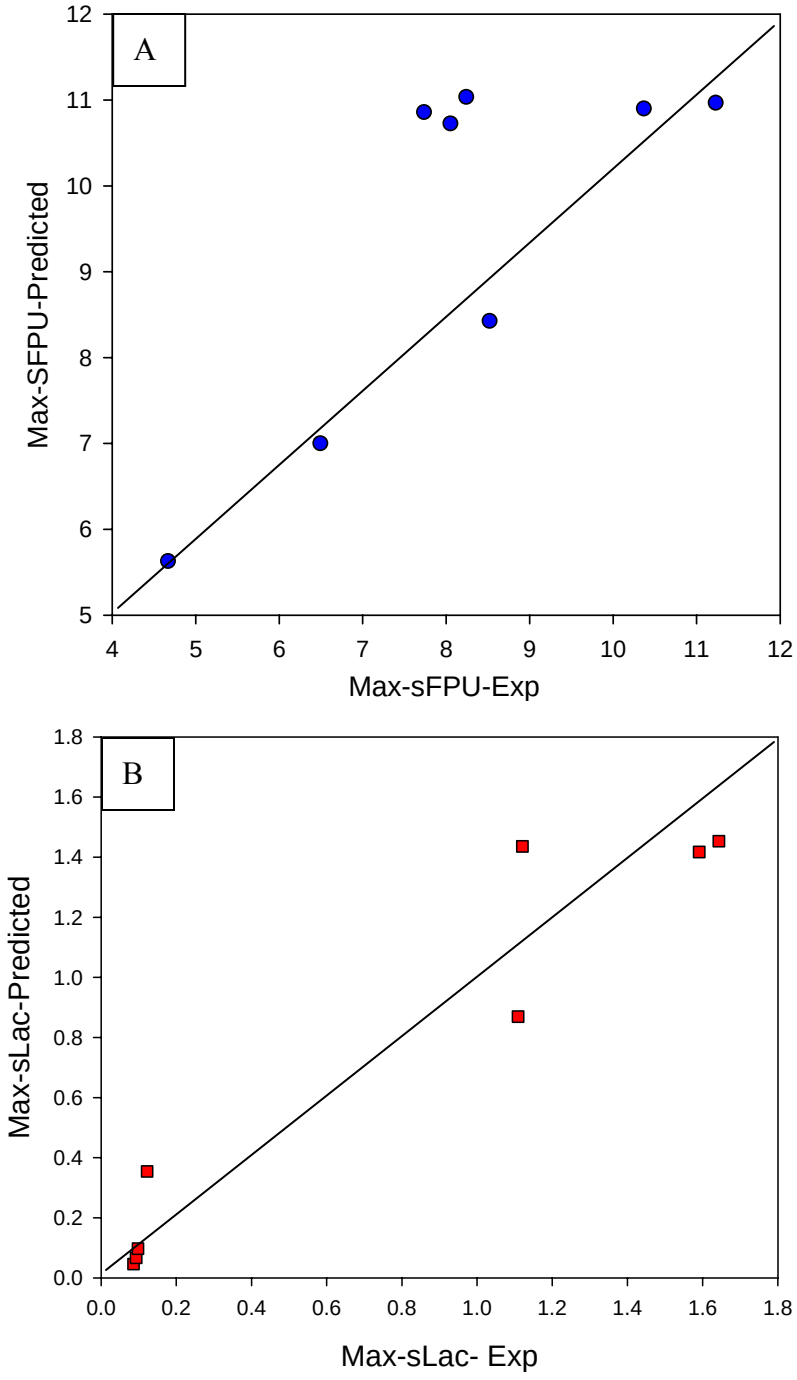

Figure 6.1 The comparison of experimental result and best-fit model result of maximum specific rates of cellulase (A) and lactase (B) synthesis.

Finally, all the parameter values estimated as described above were fine-tuned with all the batch- and continuous-culture results obtained in this study, except those with glycerol-containing media (because not enough experimental runs were done to allow good estimation of growth-related parameters on glycerol). The fine tuning was primarily to reconcile the model prediction with the continuous-culture results of cell growth and glucose and galactose concentrations that had not been used in the previous fitting.

6.5. Results

The best-fit model parameters are summarized in Table 6.4. Some parameters are described in this Results section where the culture profiles are described. More discussion is given in the Discussion section. In the following section, the experimentally observed profiles in batch- and continuous-culture studies are presented to provide the general, qualitative pictures of the culture behaviors. The model predicted profiles, using the best-fit parameters, are also shown to illustrate the quality of model description.

6.5.1. Batch Culture Profiles in Media with Glucose, Galactose or Their Mixtures as Carbon Source

6.5.1.1. Faster Growth on Glucose than on Galactose

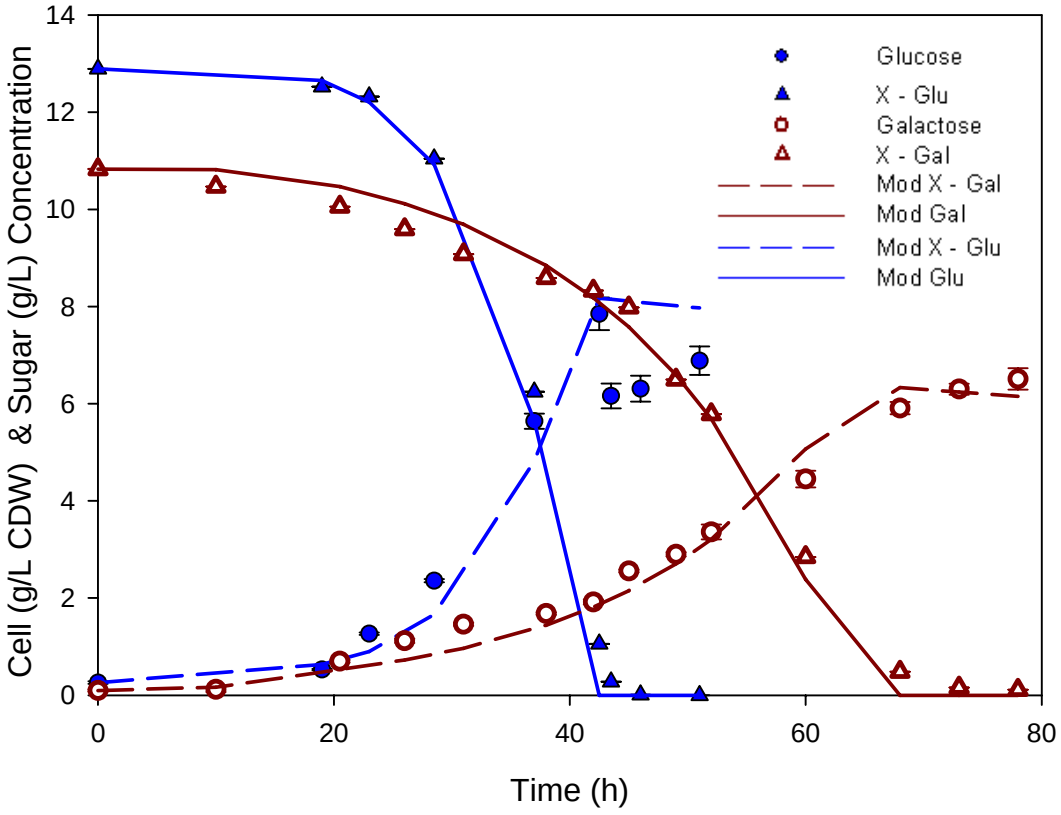

Figure 6.2 The batch culture profiles of *T. reesei* grown on glucose and galactose

The batch culture profiles of *T. reesei* grown on glucose and galactose are compared in Figure 6.2. (Repeated fermentations were made for each substrate system, although the profiles shown in Figure 6.2 were only for the representative experiments.) The data points are the experimentally measured results; the lines are the model predicted profiles (using the best-fit parameters obtained with all the experimental results in the

study). As the media were all carbon-limited, cell growth stopped at exhaustion of the sugars. Cells clearly grew faster in the glucose-based medium than in the galactose-based medium, resulting in a much larger best-fit $\overline{\mu_{Glu}}$ (0.128 1/h) than $\overline{\mu_{Gal}}$ (0.060 1/h). The cell yields (g CDW/g substrate) on the two monosaccharides were more comparable: 0.61 for glucose and 0.59 for galactose.

6.5.1.2. Diauxic Growth on Mixtures of Glucose and Galactose

To simulate the mixtures of glucose and galactose generated from the lactose hydrolysis, batch fermentations were also made in media of mixed sugars. The culture profiles in a representative experiment (out of two repetitions) are shown in Figure 6.3. A clearly diauxic growth was observed in the mixed-sugar system: cells selectively consumed glucose first and started to use galactose only after glucose was nearly depleted. This diauxic behavior can be attributed to the catabolite repression effect of glucose on the growth with galactose. The best-fit value of the empirical "inhibition" constant $K_{Glu}^{i,Gal}$ was 0.31 g/L.

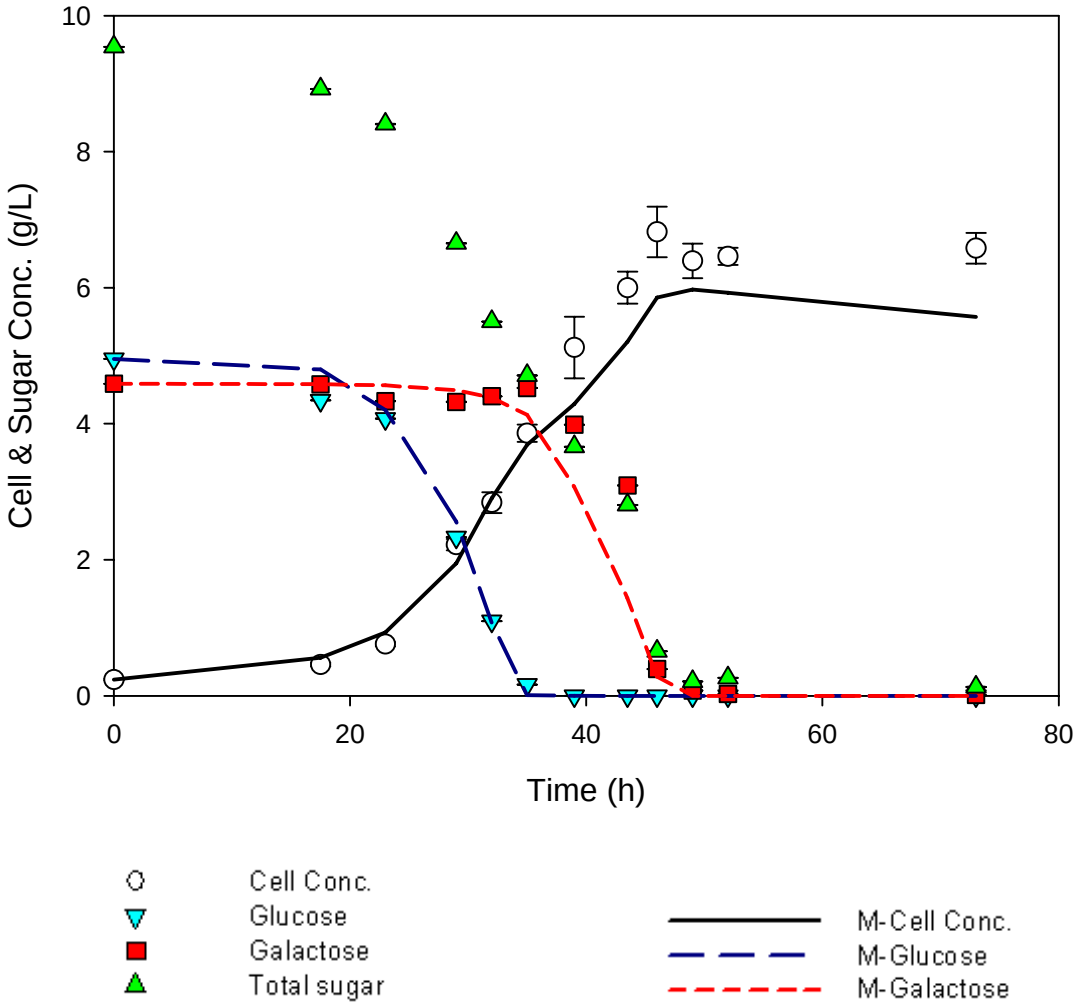

Figure 6.3. The diauxic growth of *T. reesei* in medium of mixed sugars, glucose and galactose.

6.5.1.3. Effects of Peptone on Cell Growth

Being a complex substrate, peptone concentration could not be measured. But, an increase-then-decrease profile of pH was reproducibly observed in the batch cultures. The initial increase was attributed to the characteristic release of ammonium accompanying the peptone metabolism, and the later decrease to the characteristic consumption of ammonium and production of organic acids accompanying the sugar metabolism. The pH profile also corresponded well with the disappearance of foaming tendency of the medium. The initial foaming was believed to be caused by the presence of surface-active proteins/peptides in the peptone. The foaming tendency decreased as the peptone proteins/peptides were consumed. Peptone was therefore indeed the preferred substrate that was consumed first by the cells. The best-fit cell yield on peptone was 0.46 g CDW/g peptone and the best-fit $\overline{\mu}_{Pep}$ was 0.196 1/h. The best-fit inhibition constants of peptone on glucose and galactose, i.e., $K_{Pep}^{i,Glu}$ and $K_{Pep}^{i,Gal}$, were both 0.34 g/L. As the peptone concentration in the fresh media was maintained at about 0.5 g/L, the inhibition effect of peptone on cell growth on the two sugars did not appear to be very serious.

6.5.2. Continuous Culture Profiles in Lactose-Based Medium

The steady-state properties for the continuous cultures grown in the lactose-based, carbon-limited medium, at dilution rates from 0.04 to 0.12 1/h, are summarized in Figure 6.4. Each data point was the average of at least three samples taken for analysis after the culture had reached the steady state. The model predicted results are shown as the lines.

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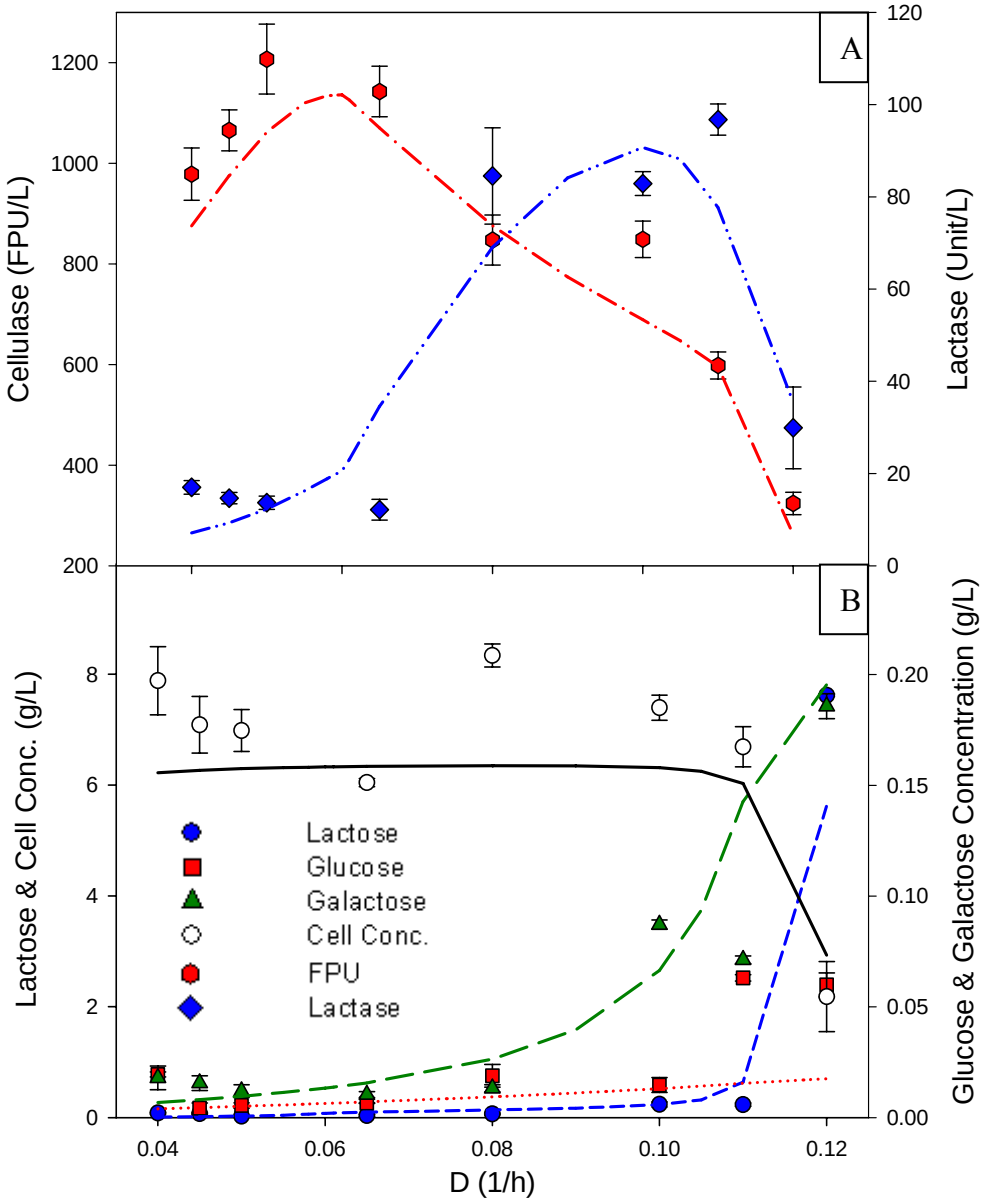


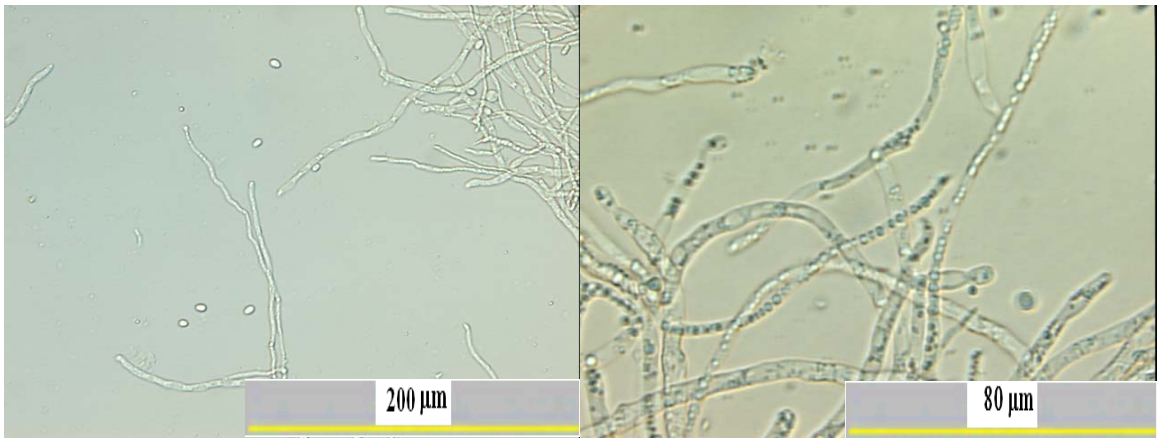
Figure 6.4. Continuous culture profiles of *T. reesei* grown in the lactose-based medium: (a) Activities of cellulase (FPU) and lactase at different dilution rates; (b) Concentrations of cell, lactose, glucose and galactose at different dilution rates.

6.5.2.1. Observations at Dilution Rates Lower than 0.05 1/h

Continuous cultures at the dilution rates of 0.03 and 0.035 1/h were also attempted. The culture, however, would start to form spores (and turn green) within 24 hours after the dilution rate was reduced from 0.04 1/h. Meaningful steady states could not be reached and no further analyses were performed on these systems, except that some samples were taken for morphology examination (describe in the next paragraph). This observation was very different from those previously reported in the literature, where the steady state culture properties were given at dilution rates as low as 0.021 1/h (Castillo et al. 1984; Pakula et al. 2005). The discrepancy might result from our use of the high-frequency fed-batch culture removal that prevented the artificial cell accumulation at the low dilution rates (potentially occurring with the simple overflow or continuous slow-rate pumping). The previous reports did not specify how the culture removal was done. At low dilution rates from 0.04 to 0.065 1/h, the specific rate of cellulase synthesis decreased as dilution rate decreased and Pakula et al.'s results showed the same trend (Pakula et al. 2005). This could result from the change of morphology during the transition of endogenous metabolism.

Cell morphology was also examined under microscope, with samples taken from different dilution rates. Some of them are shown in Figure 6.5. Many spores were found in the samples taken at D = 0.03 1/h and 0.035 1/h, the latter shown in Figure 6.5a. In addition to the presence of spores, the mycelia in these low D systems had very different diameters; some very thin mycelia were observed. The mycelia became more uniform in diameter as D increased to 0.04 1/h (Figure 6.5b) and 0.045 1/h, although spores could still be found occasionally. Spores essentially disappeared and the mycelia were smooth and uniform at D > 0.05 1/h (e.g., a sample at D = 0.065 1/h was shown in Figure 6.5c). The mycelia became thicker in diameter as D increased to 0.1 1/h (Figure 6.5d).

(A) D = 0.035 h^{-1} (B) D = 0.04 h^{-1}



(C)D = 0.65 h^{-1} (D) D = 0.1 h^{-1}

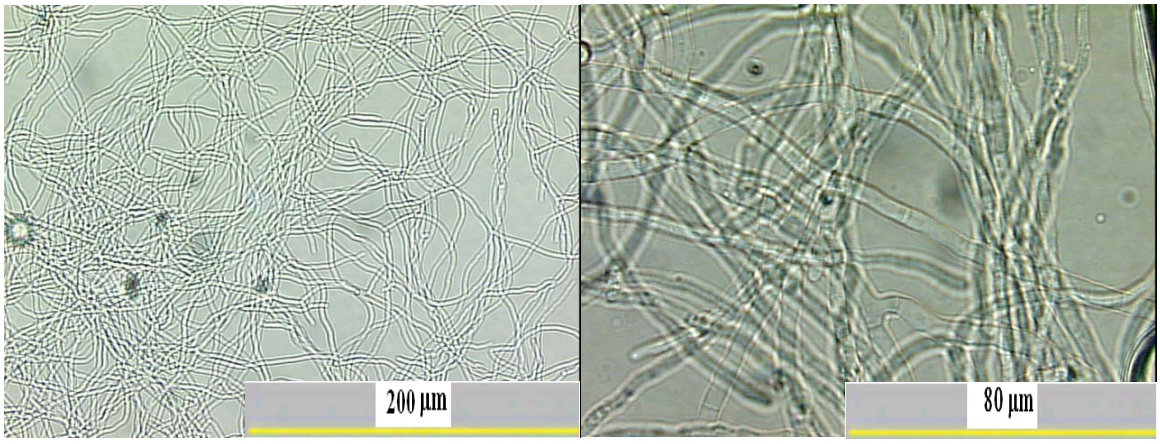


Figure 6.5. The results of cell morphology at different dilution rates, i.e., 0.035, 0.04, 0.065, and 0.1 h^{-1}. (a) and (c) were taken under 400 X magnification, and (b) and (d) under 1,000 X.

6.5.2.2. Cell and Sugars Concentrations

The continuous culture profiles for cell growth and substrate consumption are largely typical. The cell concentration remained relatively the same till the dilution rate approached the wash-out point, ~ 0.12 1/h in this study. The lactose concentration also increased sharply, from 0.3 g/L to about 7 g/L, only at the highest dilution rate. The concentration of glucose, the preferred sugar substrate, remained very low (< 0.06 g/L) throughout while the galactose concentration started to accumulate earlier (at D > 0.065 1/h). The accumulation of galactose largely reflected the slower growth on this substrate than on glucose: the substrate consumption rate = $\mu X/Y$ and, with comparable Y on the two monosaccharides, the much smaller μ_{Gal} gave much slower galactose consumption. Since glucose and galactose were produced at the same rate by lactose hydrolysis, the slower galactose consumption led to faster increase of its remaining concentration. The faster galactose accumulation did not appear to be caused strongly by the glucose repression, which effected the diauxic growth in the batch culture with the mixed-sugar medium (Figure 6.3), because the best-fit inhibition constant $K_{Glu}^{i,Gal}$ (= 0.31 g/L) was much larger than the glucose concentrations (< 0.06 g/L) remaining in the spent media. Nonetheless, the highest galactose concentration observed, about 0.2 g/L at D = 0.12 1/h,

was still much lower than the lactose concentration (7 g/L). Lactose hydrolysis was clearly the critical step that limited the faster growth of *T. reesei a* in the lactose-based medium. More discussion on this limitation is given in a later section.

It should be noted that at D = 0.04 to 0.11 1/h, the average cell concentration from the experimental data shown in Figure 6.4b was 7.2 g-CDW/L. (The standard deviation was 0.7 g/L and all of the data points were within 95% confidence interval, except those at D = 0.04 and 0.08 1/h, with 7.8 and 8.3 g-CDW/L, respectively.) After subtracting the contribution from peptone, the above average corresponded to an about 70% cell yield from lactose. This cell yield appeared to be higher than those reported in the literature, i.e., 40% (Ryu et al. 1979) to 60% (Pakula et al. 2005). Furthermore, the experimental data points were all larger than the model-predicted values, the latter being affected by the lower cell yields observed in the batch fermentations. This discrepancy was likely an artifact caused by the conversion from intracellular protein concentrations (IP) to cell dry weight concentrations (CDW). As described in Materials and Methods in Chapter 3, the correlation between IP and CDW was established from the batch fermentation results. A nonlinear correlation was revealed, as shown in Figure 4.1 Chapter 4, and attributed to the different protein contents in cells at different stages of batch growth: the protein

content was about 13.4% at the early stage (when cell concentrations were low) but only about 8.8% at the later stage (when cell concentrations were high). The higher protein content at the early stage was at least partially caused by the presence of peptone, as opposed to the absence of peptone at the later stage. In the continuous cultures, except at D = 0.12 1/h, the cell concentrations were high so that the values reported in Figure 6.4b were essentially converted from the measured IP using the protein content of about 8.8%. The continuous culture systems were however continuously fed with the fresh medium containing peptone. It is not unfeasible that the peptone availability, however brief, might cause the intracellular protein contents to be higher than the 8.8% found in the batch cultures at the later peptone-free stage. It was calculated that the discrepancy shown in Figure 6.4b, between the cell concentrations converted from the experimental IP data and those predicted by the model, could be largely reconciled if the equation used to convert IP to CDW for these continuous culture systems had assumed a protein content of about 9.9% instead of 8.8%.

6.5.2.3. Cellulase Synthesis

The experimental results for cellulase activity had a slight increase-then-decrease profile, with the maximum FPU being reached around D = 0.05-0.06 1/h (Figure 6.4a). A similar profile was reported by Pakula et al. (2005) although there the maximum FPU was attained at D = 0.031 1/h and the sharp drop was observed at D = 0.045 1/h. Wash-out was reported to occur also at a much lower dilution rate, D = 0.076 1/h. One possibility is the different culture removal methods used in the two studies, as discussed before. The other is that DO was maintained high (~ 70% air saturation) in our study using mixtures of oxygen and air as the influent gas while only air was used in the literature study. Our experience indicated that oxygen limitation would occur in our system if only air was used for aeration. In addition, the process had higher culture temperature, 30 ^{o}C, and different medium composition (Pakula et al. 2005).

The experimental results are described well by the model (Figure 6.4a). As mentioned earlier in the Model section, the model assumed the specific cellulase productivity to be growth-independent but subject to lactose induction and potential glucose and galactose repression (Equation 2). In addition, to avoid the uncertainty due to the presence of spores and different morphology at D < 0.05 1/h, Equation 2 was fit only

with the results at D ≥ 0.05 1/h. The experimental results and the best-fit curve for the specific cellulase production rates SR_{FPU} are shown in Figure 6.1a. The best-fit parameters are given in Table 6.4. The following points should be noted:

(1) The best-fit repression constant by glucose $K_{Glu}^{r,FPU}$ was 0.071 g/L while that by galactose $K_{Gal}^{r,FPU}$ was > 10 g/L. These values implied that the cellulase synthesis in the continuous culture was not repressed by galactose and only very weakly repressed by glucose (because of the low glucose concentrations remaining in the media). The fact that cellulase synthesis was not repressed by galactose was not surprising since the primary function of cellulase was to hydrolyze cellulose and galactose was not a common end product encountered in that hydrolysis. Furthermore, cellulase synthesis is known to be repressed by glucose (IImen et al. 1997) and *T. reesei* Rut C30 is a hyper-producing mutant less sensitive to glucose repression (Montenecourt and Eveleigh 1977), supporting the weak glucose repression observed in this study. The slow decrease of FPU as D increased from 0.065 to 0.11 1/h (Figure 6.4a) just reflected the faster dilution while the synthesis rate remained constant. The much lower FPU at D = 0.12 1/h was also (mainly) caused by the lower cell concentration, instead of the reduced specific production rate.

(2) While the model was only fitted with the results obtained at D ≥ 0.05 1/h, it turned out to describe the slight increase of FPU at the lower D reasonably well. This was because the lactose concentrations were expected to increase slightly with increasing D. At smaller D, the lactose concentrations were lower than the best-fit half-saturation constant for lactose induction on FPU synthesis ($K_L^{FPU} = 0.014$ g/L) so that the increasing lactose concentrations led to the increasing FPU. At larger D, the effect of higher induction by increasing lactose concentrations eventually leveled off and the decreasing trend of FPU began (due to the faster dilution as described above).

The volumetric cellulase production rates (= X × SR_{FPU}) are also shown in Figure 6.6a. The fastest production occurred at D = 0.065 - 0.11/h. In general, the downstream separation, purification (if necessary), packaging and transportation are less expensive with higher product concentrations. The optimal D for production is likely at 0.05 - 0.065 1/h where both the cellulase activity and the production rate were near the maximum levels.

6.5.2.4. Lactase Synthesis

The experimental profile for lactase activity was also shown in Figure 6.4a. Lactase activity was minimal at D ≤ 0.65 1/h, reached high levels at D = 0.8-0.11 1/h, and then dropped at D = 0.12 1/h. A similar lactase profile was observed by Fekete et al. (Fekete et al. 2007) in the continuous culture study of *T. reesei* QM 9414 in a lactose-based medium. The operation range was from 0.015 to 0.042 1/h and wash out happened at 0.05 1/h. 0.042 1/h had highest lactase activity Table 6.2 below. However, the high lactase activity was again reported at a different range of dilution rate, 0.015-0.042 1/h, from that observed in this study (0.08-0.11 1/h).

The experimental profile for lactase activities was well described by the current model as shown in Figure 6.4a. As described earlier, the specific lactase synthesis rate was modeled by Equation (3), which included a same basal rate (β^{La}) throughout the range of D and a growth-dependent component when D was higher than certain critical level, i.e., $\alpha(\mu - \mu_c)$. The experimental results and model-predicted values for the specific and volumetric lactase production rates are shown in Figure 6.6. The best-fit parameters are given in Table 6.4. While the model described the experimental results well, the mechanism responsible for the absence of growth-dependency at the low D remained

unknown. Proteolysis by proteases (which are known to be produced by *T. reesei* under low-growth conditions (Hagspiel et al. 1989; Haab et al. 1990; Dienes et al. 2007)) might play a role here. At late cultivation stages (corresponding to low dilution rates), protease has been found that it could partially modify the structure of CBH I and II, but the activities of EG I and b-glucosidase remained the same (Hagspiel et al. 1989).

The following points should also be discussed:

(1) The best-fit half-saturation induction constant of lactose on lactase synthesis K_L^{La} (= 0.080 g/L) turned out to be larger than that on cellulase synthesis (K_L^{FPU} = 0.014 g/L). This is rather surprising since lactase is supposedly the more dedicated enzyme than cellulase for lactose hydrolysis. On the other hand, this might also shed some light on the low, basal level of lactase synthesis at low D where lactose concentrations were very low. More study is warranted to investigate if β-glucosidase (or other cellulase components) had higher affinity to lactose than lactase had (at low lactose concentrations) while lactase had higher maximum lactose hydrolysis rates (at high lactose concentrations) than β-glucosidase (or other cellulase components).

(2) The best-fit repression constant of glucose on lactase synthesis $K_{Glu}^{r,La}$ was > 100 g/L, while that of galactose $K_{Gal}^{r,La}$ was 0.12 g/L. The values implied that lactase synthesis was not repressed by glucose but weakly repressed by galactose: the galactose repression was only apparent at the highest D (= 0.12 1/h) as shown in Figure 6.6 by the slight increase of SR_{La} from the value at D = 0.11 1/h to that at D = 0.12 1/h. (If the galactose repression was absent, SR_{La} would have increased linearly with D due to its growth-dependency.) It is interesting to note that both lactase and cellulase syntheses were selectively repressed (weakly) by one of the end-product monosaccharides and, more importantly, each by a different end product: cellulase by glucose and lactase by galactose.

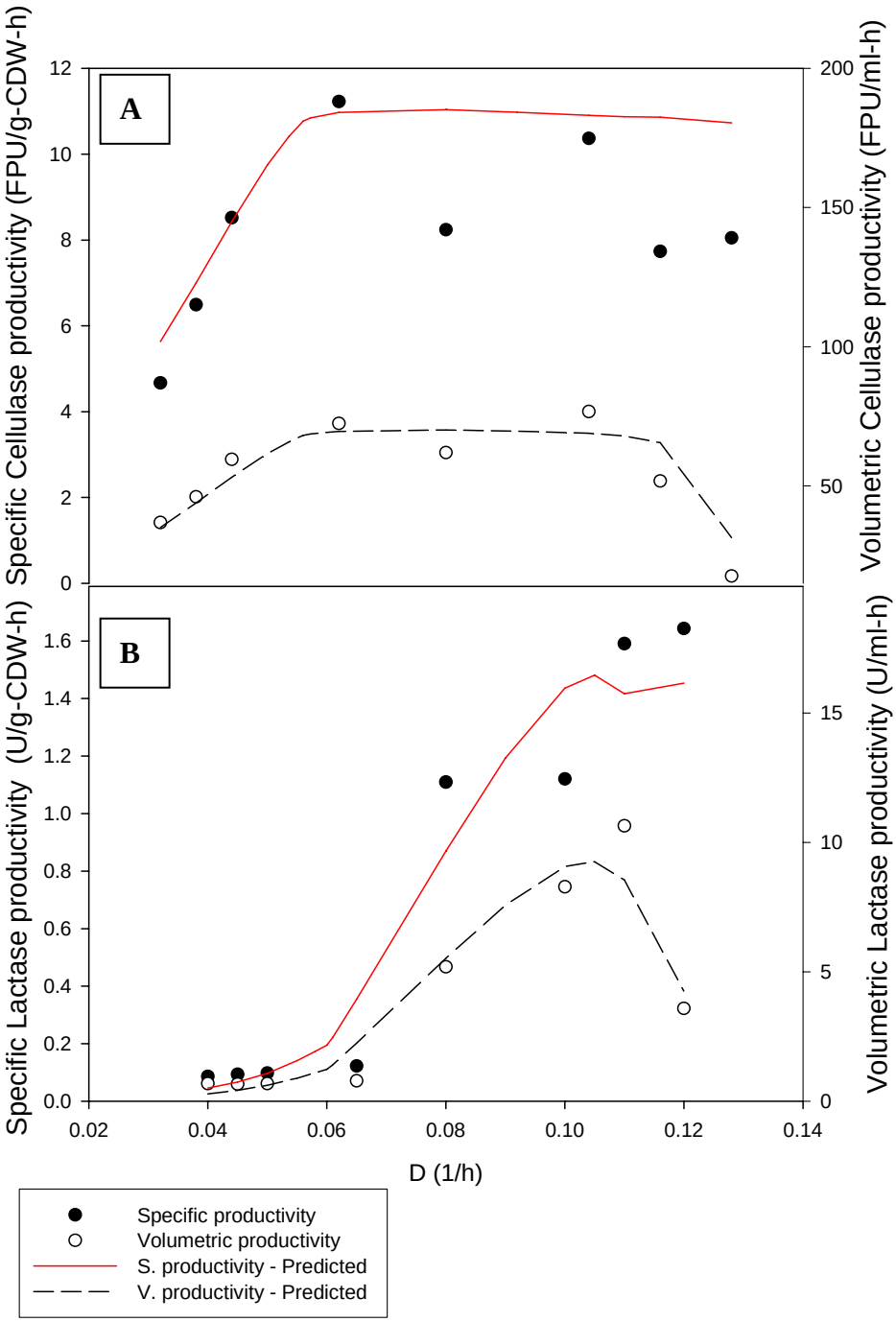


Figure 6.6. The volumetric productivity and specific productivity of cellulase A) and lactase B).

6.5.2.5. Lactose Hydrolysis

The volumetric rates of lactose hydrolysis at different D are shown in Figure 6.7, together with the model-predicted contributions to hydrolysis by cellulase and lactase. The primary contribution shifted from cellulase to lactase after D was increased beyond 0.065 1/h, as expected from the profiles of the two enzymes (Figure 6.4a). Both experimental and model-predicted results showed a linear increase of the lactose hydrolysis rate with increasing D, up to 0.11 1/h. This trend was very reasonable as the increasing lactose hydrolysis rates were necessary to support the faster cell growth rates. However, at D = 0.12 1/h the lactose hydrolysis rate dropped significantly, obviously caused by the insufficient activities of the hydrolytic enzymes (lactase and cellulase) present at this dilution rate. As shown in Figure 6.6b, the specific cellulase production rate remained constant, as expected from its growth-independent characteristics. No significant end-product repression on cellulase synthesis was apparent. On the other hand, the growth-dependent lactase synthesis needed to increase with increasing D, to provide the substrates (glucose and galactose) for faster cell growth. As shown in Figure 6.6b, the specific lactase production rate failed to increase proportionally as D increased from 0.11 1/h to 0.12 1/h. This was attributed to the galactose repression, because of the faster

increase in galactose concentration at higher D, which in turn was caused by the slower consumption of galactose by the cells. This galactose repression on lactase synthesis appeared to be the ultimate limiting factor in the lactose-based growth of *T. reesei*.

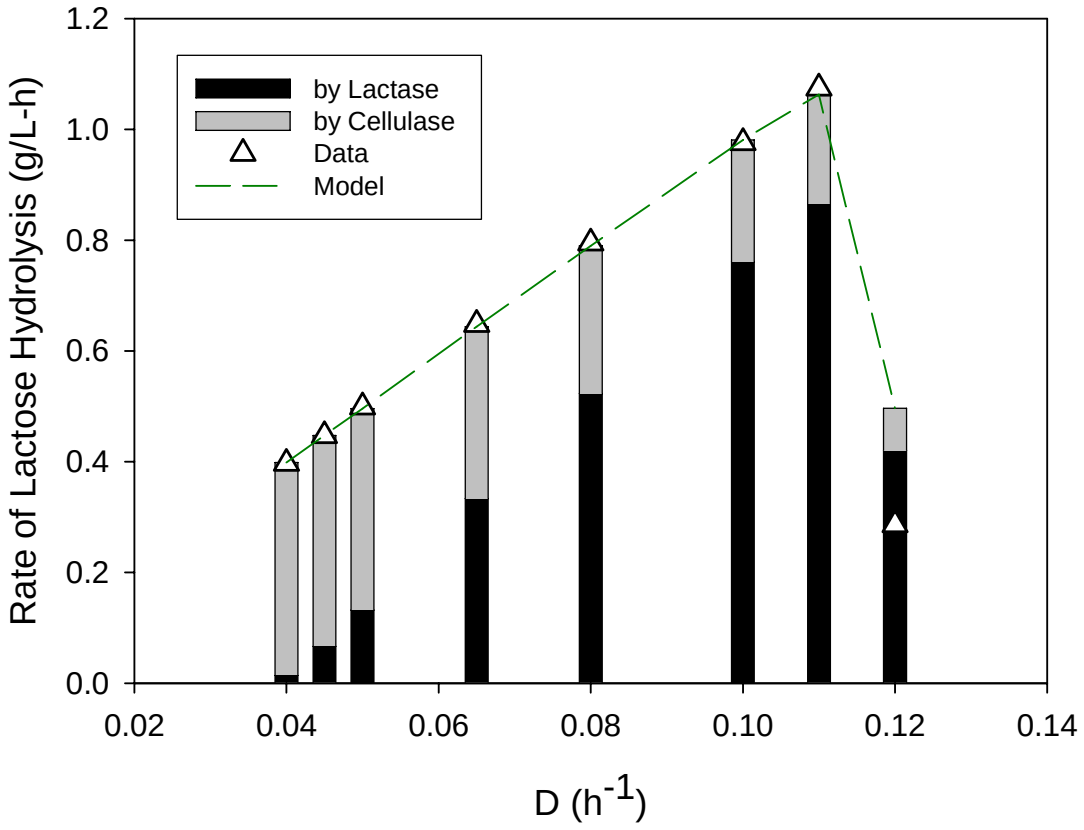


Figure 6.7. Rates of lactose hydrolysis, including individual contributions from lactase and cellulase, at different dilution rates.

6.5.2.6. Use of Mixed Glycerol and Lactose as Substrate

As described earlier, to separate the positive effect of induction by lactose and the negative effects of repression by glucose and/or galactose, which were generated from lactose hydrolysis, mixtures of glycerol and lactose were used as substrate in the feed media for some continuous culture experiments. The glycerol-containing systems were studied only at high dilution rates, D = 0.1 1/h and 0.11 1/h, because only in such systems there might be high enough sugar concentrations to cause appreciable repression effects. These systems included: at D = 0.1 1/h, mixed 5 g/L glycerol and 5 g/L lactose (i.e., 50% glycerol), mixed 10 g/L glycerol and 1 g/L lactose (i.e., 91% glycerol), pure 12.6 g/L glycerol (i.e., 100% glycerol); and at D = 0.11 1/h, mixed 9 g/L glycerol and 1 g/L lactose (i.e., 90% glycerol).

To show the effects of different % of glycerol in the feed substrate, the results of specific cellulase and lactase synthesis rates (SR_{FPU} and SR_{La}) as well as the remaining lactose concentrations in these systems are summarized in Figure 6.6. For comparison the results in the systems with pure 10 g/L lactose (i.e., 0% glycerol) at the two dilution rates are also included in Figure 6.8. As expected, the lactose concentrations decreased significantly with the increasing glycerol content in the substrate. The decrease in

specific enzyme synthesis rates was however not as dramatic, particularly at the high dilution rate D = 0.11 1/h. The observations mainly reflected the low lactose concentrations required for induction (K_L^{FPU} = 0.014 g/L and K_L^{La} = 0.080 g/L). While none of the glycerol-containing systems so far investigated gave higher cellulase or lactase synthesis rates than the rates in pure lactose systems, the findings did not preclude the possibility of better performance at higher dilution rates, e.g., D ≥ 0.12 1/h, where the end-product repression, particularly the galactose repression on lactase synthesis, was much stronger. More studies on the mixed glycerol and lactose substrate systems are warranted. It should also be pointed out that these glycerol-containing systems did serve the designed role of separating D and lactose concentrations to enable more accurate fitting of the model, particularly for the rates of cellulase and lactase syntheses and lactose hydrolysis.

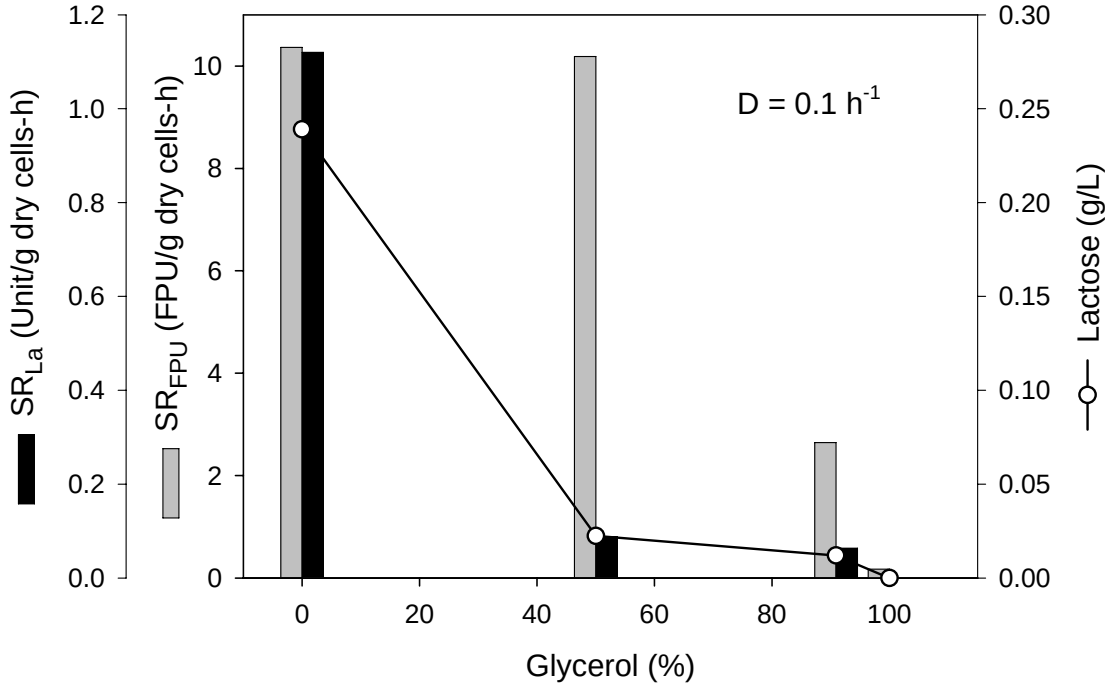


Figure 6.8. Specific synthesis rates of cellulase and lactase as well as lactose concentrations obtained in continuous culture systems (at D = 0.1 h^{-1}) with media containing both lactose and glycerol, with glycerol constituting different weight percentages of total C substrate.

6.6. Discussion

Cell Growth Parameters

$\overline{\mu_{Glu}}$, $\overline{\mu_{Gal}}$, $\overline{\mu_{Lac}}$ and K_{Glu}, K_{Gal} , K_{Pep} (Monod constant)

The maximum specific growth rates on lactose, glucose and galactose obtained in this study are compared with those reported in the literature in Table 6.2.

Table 6.2 The list of previous studies on $\overline{\mu}$, $Y_{x/s}$ at different substrates

T. reesei	Substrate	Culture conditions	$\overline{\mu}$ (1/h)	$Y_{x/s}$	reference
QM-9123	Glucose	30 °C pH 4.0	0.15	0.53	(Brown and Zainudeen 1977)
Trichoderma sp.	Glucose	30 °C pH 4.0	0.13	0.74	(Nagai et al. 1976)
MCG-77	Lactose	2-stage 32 °C	0.1	0.44	(Ryu et al. 1979)
C5	Lactose	30°C pH 5.0	0.07	0.58	(Chaudhuri and Sahai 1994)
C5	Lactose	30°C pH 5.0	0.066	0.63	(Chaudhuri and Sahai 1993)
Rut C30	Lactose	30 °C pH 4.8	0.076	0.6	(Pakula et al. 2005)
QM9414	Galactose	30 °C pH 5.0	0.075	~ 0.5	(Fekete et al. 2007)
Rut C30 (this work)	Glucose	25 °C pH 5.0	0.128	0.612	
Rut C30 (this work)	Galactose	25 °C pH 5.0	0.06	0.59	

Results obtained in this study compare reasonably well with those reported in the literature. The literature values for lactose as substrate showed a large range, i.e., from

0.066 to 0.1 1/h. The variation was partly due to the different strains employed and might also reflect the difficulties in operating the continuous culture of molds.

There were three substrates used in this study. According to our model, the overall specific growth rate was dependent of the concentrations of three limited substrates, glucose, galactose, and peptone. Therefore, each part of substrates contributed the part of its rate to the overall lactose maximum specific growth rate. On the other hand, the maximum specific growth rate for lactose-medium could be seen in continuous culture, which was close to the wash out rate D = 0.12 1/h. Based on our assumption of that lactose was completely hydrolyzed and could not be taken by cells directly, this indicated that the lactose maximum specific growth rate could possibly be lower than the rate of using pure glucose, the highest rate $\overline{\mu_{Glu}}$. This result accorded with model prediction and the experiment of Castillo et al. (1984) in lactose continuous culture operated at D = 0.11 1/h, which did not report wash out. Ryu et al. (1979) demonstrated two-stage lactose continuous culture on *T. reesei* MCG77 with 0.1 1/h, $\overline{\mu_{Lac}}$. In contrast, Pakula et al. (2005) reported an estimated lactose maximal specific growth rate was 0.076 1/h in continuous culture and 0.073 1/h in batch culture with same strain but higher culture temperature, 30 ^{o}C, and medium composition. Chaudhuri and Sahai (1994) also reported

0.07 1/h, $\overline{\mu_{Lac}}$, used *T. reesei* C5, using Monod's equation for estimation, which might not be accurate. Besides, the culture was under partial substrate limitation condition.

The value of $\overline{\mu_{Glu}}$ from model fitting was 0.128 1/h in our best-fitting result. Brown and Zainudeen (1977) reported the highest operated dilution rate was at 0.11 1/h, no wash out, by using *T. viride* culture at pH 4.0 and the value of $\overline{\mu_{Glu}}$ from their model fitting was 0.154 1/h, glucose as sole substrate. The $\overline{\mu_{Pep}}$ had highest model predicted growth rate, 0.196 1/h, which explained early cell growth on the utilization of peptone. The $\overline{\mu_{Glu}}$ was bigger than $\overline{\mu_{Gal}}$, suggesting cells grew faster in pure glucose-based medium and this coincided with the experimental results. The $\overline{\mu_{Gal}}$ was 0.06 1/h and Fekete et al. (2007) reported a continuous culture using galactose and *T. reesei* QM 9414 at dilution rate 0.075 1/h without washout.

The larger value of $\overline{\mu_{Glu}}$ than $\overline{\mu_{Gal}}$ reflected the faster growth of cells on glucose than on galactose. Besides, the path way of converting galactose to energy differed from glycolysis. The Leloir pathway took few more steps to convert galactose to Glucose-6-Phosphate before entering glycolysis (Frey 1996). Peptone had advantage on many amino acids and some trace elements to provide a good development of cells (Clausen et al. 1985; Kurbanoglu and Kurbanoglu 2002).

As expected, all of the best-fit Monod constants for substrates, K_{Pep}, K_{Gal}, and K_{Glu}, were very small, indicating the good affinity of cells to these readily consumable substrates.

$Y_{X/Pep}$, $Y_{X/Glu}$, and **$Y_{X/Gal}$**

$Y_{X/Pep}$, $Y_{X/Glu}$, and $Y_{X/Gal}$ had 0.46, 0.61, and 0.59 g-CDW/g-sub, respectively. Because the Leloir pathway requires extra enzymes to convert galactose to G-6-P, these extra enzymes cost more carbon element to synthesis when compared with glycolysis. Considering energy efficiency on substrates, galactose induced β-galactosidase when cell grew in exponential growth phase and this cost part of carbon and nitrogen to the synthesis of enzyme (Fekete et al. 2007). The Leloir pathway took few more steps to convert galactose to Glucose-6-Phosphate before entering glycolysis (Frey 1996). Glucose was definitely a good energy supplier since the energy capture efficiency was about 40% (Bailey and Ollis 1944). The low cell yield from peptone was partly because it was also a nitrogen source (not solely a carbon and energy source), and perhaps partly because not all of the ingredients in the complex substrate were consumable by the cells. $Y_{X/Glu}$ was close to around 64% conversion to per gram glucose.

k_d

T. reesei Rut C30 would turn into spores, if they were starved. The best-fit death constant was 0.003 1/h.

Hydrolysis of lactose

The rate of lactose hydrolysis was controlled by the activities of two enzymes, lactase and cellulase. The kinetic parameters κ_{FPU}^{H}, κ_{La}^{H}, $K_L^{H,FPU}$, and $K_L^{H,La}$, determined by the fitting of equation (4), were 0.0005 g/L-h, 0.012 g/L-h, 0 g/L, and 0.15 g/L, respectively. The κ_{FPU}^{H}, κ_{La}^{H} were the rate constants of lactose hydrolysis by cellulase and lactase and apparently. With a zero $K_L^{H,FPU}$, it is indicated that the rate of lactose hydrolysis by cellulase was at maximum rate all the time in the continuous culture, which could have been completely saturated by lactose. Besides, the model also suggested that the β-glucosidase had better affinity with lactose than that with lactase due to having a lower $K_L^{H,FPU}$ than $K_L^{H,La}$. Gamauf et al. (2007) reported that lactase, purified from *T. reesei* had less affinity when compared to other β-galactosides as substrates and other lactase made by other species at optimal condition, which explained the slow metabolism of lactose in *T. reesei*. From the results of model prediction shown in figure 6.6b and 7,

the profile of the rate of lactose hydrolysis was proportional to the rate of lactase synthesis, which adequately accounted for its dominant role in lactose hydrolysis.

Rates of enzyme synthesis people did find the galactose inhibition but they used pure lactase with high galactose concentration.

It has been proved that cellulase synthesis was affected by the end-production repression (IImen et al. 1997), and *T. reesei* Rut C30 was a hyper-producing cellulase mutant (Montenecourt and Eveleigh 1977). Because of the low residual glucose concentration at entire range of dilution rates in continuous culture, the specific rate of cellulase synthesis was remained quite stable within all range of tested dilution rates, within the mean of 95% of confident interval, except for the last point at 0.04 1/h dilution rate with different morphology, in figure 6.6b. This indicated the culture age (correlation with cell growth) did not affect the productivity of cellulase, and the productivity was completely controlled by the end-product repression effect. In addition, Seiboth et al. (2005) concluded that increased expression or activity of β-galactosidase somehow inhibited the transcription of cellulase, which was consistent with the observed profile of enzyme synthesis in this study.

$K_{Glu}^{i,FPU}$, $K_{Gal}^{i,FPU}$, $K_{Glu}^{i,La}$, $K_{Gal}^{i,La}$ were the competitive enzyme inhibition constants, indicating the inhibition effect on cellulase or lactase by sugars, 0.071, 10, 800481, and 0.12 g/L, respectively. A small value of $K_{Glu}^{i,FPU}$ was expected due to end-product repression. High values of $K_{Gal}^{i,FPU}$, and $K_{Glu}^{i,La}$ indicated the rate of cellulase synthesis was not inhibited by galactose and glucose did not repress the rate of lactase synthesis as well. From our experimental result (figure 6.4), glucose inhibited the consumption of galactose, and Seiboth et al. (2005) showed glucose regulated the transcription of lactase (*bga*1 gene). Nonetheless, in the continuous culture, glucose concentration was constantly low throughout all dilution rates, and this might result in a high $K_{Glu}^{i,La}$ value when the continuous culture result was used to fit the rates of enzyme synthesis. In addition, this could also indicate the higher affinity of cellulase promoter to repressor than that of lactase promoter. Likewise, the concentration of galactose was constantly close to 10 times lower than the value of $K_{Gal}^{i,La}$ (0.12 g/L) from dilution rates 0.04 to 0.11 1/h in the continuous culture. Therefore, a small $K_{Gal}^{i,La}$ value might result from the increase of galactose concentration at dilution rates from 0.11 to 0.12 1/h as lactase activity fell from 101.5 to 34.8 U/L in Figure 6.4a.

K_L^{FPU}, and K_L^{La} were small, 0.014 and 0.08 g/L, which showed good induction ability on both enzymes by lactose. Although the residual concentration of lactose was low in continuous culture, fairly good enzyme activities were still observed.

According to the best-fit results, α^{FPU}, β^{FPU}, α^{La}, and β^{La} were 0 U/g-CDW, 13.6 U/(g-CDW-h), 58.8 U/g-CDW, and 0.42 U/(g-CDW-h), respectively. With both positive α^{FPU}, and β^{FPU} for FPU, indicated cellulase enzyme was partial growth- and non-growth associated. Instead, a positive α^{E}, and negative β^{E} were given for lactase to be completely growth-associated. In addition, negative β^{E} also indicated that lactase could be degraded when cells stopped growing or had inadequate carbon source. Since the effect of specific growth rate on lactase production has been described in (α^{La} μ + β^{La}), the possibility of inhibition by high expression of cellulase or degradation by proteases should take into consideration.

Glycerol was easy digested and none-repressing carbon source for *T. reesei*. With combination of glycerol and lactose, high accumulation of lactose concentration could take place at high dilution rates when glycerol completely satisfied the needs of *T. reesei* on highest 748.4 FPU/L, did not surpass the highest cellulase, 1190 FPU/L at D = 0.05 1/h, induced by lactose as inducer and energy source in induction ability. Besides, the

accumulation of sugars did not happen, except 1 g/L of lactose plus 9 g/L of glycerol at D = 0.11 1/h with residual 0.888 g/L of glycerol. Still, this result showed comparable FPU with that of 10 g/L of lactose at same dilution rates. This implied the 5 g/L of lactose at D = 0.1 1/h and 1 g/L of lactose at D = 0.11 1/h were sufficient to induce same amount of cellulase as 10 g/L of lactose did.

6.7 Conclusion

Lactose is a good inducer and energy supplier for *T. reesei* Rut C30. The study provided significant conceptual and quantitative insights to the lactose metabolism and cellulase production by *T. reesei*. The model was developed to incorporate overall relationships in fermentation behaviors in batch and continuous cultures. The model fitting was based on the assumption that lactose was completely hydrolyzed by both cellulase and lactase for further energy usage. The results showed how the complete inhibition on uptake of galactose by glucose was for *T. reesei*, the higher enzyme activity of cellulase at lower dilution rate, and higher lactase activity at higher dilution rates. Although the mechanism of cellulase production process by using lactose as a substrate

still remained unclear, with developing this model, we brought one step closer to the fundamentally understanding the behavior of lactose induction.

Table 6.3. NOMENCLATURE

$\overline{\mu_{Pep}}$	maximum specific growth rate by peptone (1/h)
$\overline{\mu_{Glu}}$	maximum specific growth rate by glucose (1/h)
$\overline{\mu_{Gal}}$	maximum specific growth rate by galactose (1/h)
K_{pep}	Saturation constant of peptone (g/L)
K_{Glu}	Saturation constant of glucose (g/L)
K_{Gal}	Saturation constant of galactose (g/L)
κ_{FPU}^{H}	Rate constant of cellulase in hydrolysis of lactose (g/L-h)
κ_{La}^{H}	Rate constant of lactase in hydrolysis of lactose (g/L-h)
$K_{L}^{H,FPU}$	Saturation constant of cellulase in hydrolysis of lactose (g/L)
$K_{L}^{H,La}$	Saturation constant of lactase in hydrolysis of lactose (g/L)
R_{L}^{H}	Rate of lactose hydrolysis (g-lactose/g-CDW-h)
$K_{Glu}^{i,FPU}$	Inhibition constant of cellulase synthesis by glucose (g/L)
$K_{Gal}^{i,FPU}$	Inhibition constant of cellulase synthesis by galactose (g/L)
$K_{Glu}^{i,La}$	Inhibition constant of lactase synthesis by glucose (g/L)
$K_{Gal}^{i,La}$	Inhibition constant of lactase synthesis by galactose (g/L)
$K_{Pep}^{i,Glu}$	Inhibition constant of glucose by peptone (g/L)

Table 6.3. NOMENCLATURE (Continued)

$K_{Pep}^{i,Gal}$	Inhibition constant of galactose by peptone (g/L)
$K_{Glu}^{i,Gal}$	Inhibition constant of galactose by glucose (g/L)
α^{FPU}	Cell growth coefficient of growth dependence in L-P kinetic for cellulase (U/g-CDW)
β^{FPU}	Cell growth coefficient of growth dependence in L-P kinetic for cellulase (U/g-CDW-h)
α^{La}	Cell growth coefficient of growth dependence in L-P kinetic for lactase (U/g-CDW)
β^{La}	Cell growth coefficient of growth dependence in L-P kinetic for lactase (U/g-CDW-h)
K_L^{FPU}	Saturation constant for the rate of cellulase synthesis (g/L)
K_L^{La}	Saturation constant for the rate of lactase synthesis (g/L)
SR_e	Rate of lactase synthesis (Unit/g dry cells-h)
SR_{FPU}	Rate of cellulase synthesis (Unit/g dry cells-h)
SR_e^{Max}	Maximum rate of lactase synthesis (Unit/g dry cells-h)
SR_{FPU}^{Max}	Maximum rate of cellulase synthesis (Unit/g dry cells-h)
$Y_{x/Glu}$	Cell yield coefficients by glucose (g-cell/g-glucose)
$Y_{x/Ga}$	Cell yield coefficients by galactose (g-cell/g-galacose)

Table 6.3. NOMENCLATURE (Continued)

$Y_{x/Pep}$	Cell yield coefficients by peptone	(g-cell/g-peptone)
$Y_{x/Lac}$	Cell yield coefficients by peptone	(g-cell/g-lactose)
K_d	death rate constant (1/h)	

Table 6.4. Best-fit values and fitting restriction for model

Parameter	Best-fit values	Fitting restriction range
$\overline{\mu_{Pep}}$	0.196	0.01 - 1
$\overline{\mu_{Glu}}$	0.128	0.01 - 1
$\overline{\mu_{Gal}}$	0.06	0.01 - 1
K_{pep}	0.003	0.0001-1
K_{Glu}	0.02	0.0001-1
K_{Gal}	0.013	0.0001-1
κ_{FPU}^{H}	0.0005	0.0001-1
κ_{La}^{H}	0.012	0.0001-1
$K_{L}^{H,FPU}$	0	0.0001-1
$K_{L}^{H,La}$	0.15	0.0001-1
α^{FPU}.	0	0.001-100
β^{FPU}	13.6	0.001-100
α^{La}	58.8	0.001-100
β^{La}	0.42	0.001-100
$K_{Glu}^{i,FPU}$	0.071	0.0001-1

Table 6.4. Best-fit values and fitting restriction for model (Continued)

Parameter	Best-fit values	Fitting restriction range
$K_{Gal}^{i,FPU}$	10	0.0001-10^6
$K_{Glu}^{i,La}$	800481	0.0001-10^6
$K_{Gal}^{i,La}$	0.12	0.0001 - 1
$K_{Pep}^{i,Glu}$	0.34	0.0001 – 1
$K_{Pep}^{i,Gal}$	0.34	0.0001 - 1
$K_{Glu}^{i,Gal}$	0.31	0.0001 - 1
K_{L}^{FPU}	0.014	0.0001 - 1
K_{L}^{La}	0.08	0.0001 - 1
$Y_{x/Glu}$	0.61	0.01 - 1
$Y_{x/Gal}$	0.59	0.01 - 1
$Y_{x/Pep}$	0.46	0.01 - 1
K_d	0.003	0.00001 - 1

CHAPTER VII

CELLULASE PRODUTION USING MIXED CULTURE TO INCLUDE IN SITU SYNTHESIS OF SOPHOROLIPIDS AS SORUCES OF SOPHOROSE INDUCER

7.1 Abstract

Recently, we have discovered a new way to produce cellulase in mixed culture of *Trichoderma reesei* Rut C30 and *Candida bombicola*. Presumably the sophorolipids (SLs) produced by *C. bombicola* provided the necessary induction for cellulase synthesis. We postulated that the sophorolipids were hydrolyzed outside the cells to sophorose, which was a well known inducer for cellulase synthesis. Glucose was a typical carbon source, used together with a lipid source (fatty acids or glycerides) for SLs production by *C. bombicola*. Since glucose would repress cellulase synthesis by *T. reesei* Rut C30, glycerol was evaluated as the substitute because it was known not to induce or repress the cellulase synthesis and because it was expected to become very inexpensive, as the main by-product from biodiesel-manufacturing processes. Glycerol was first tested at different concentrations for its potential inhibition on the mixed culture and the results showed that

20 g/L of glycerol hindered cell growth and utilization of glycerol. Nine (9) batch culture systems with different combinations of *T. reesei*-to-*C. bombicola* ratio and glycerol-to-soybean oil ratio were also carried out for choosing the best combination for cellulase production. The chosen system was validated again and the same combination in the chosen system also was tested with the presence of excessive nitrogen source, which had lower cellulase productivity. In addition, the rate of SLs degradation was also investigated by using pure *T. reesei* culture broth with addition of SLs and glycerol. The rate of SLs degradation in culture broth with the presence of glycerol was also determined to be approximately 0.016 g-SLs/(g CDW-h). Without glycerol in culture broth, the degradation of SLs did not occur. In the cell-free systems, with or without glycerol, also did not find the SLs degradation.

7.2 Introduction

Over the past two decades, the process of cellulase production has been studied by trying different substrates such as cellulose, galactose, lactose, sophorose, carboxymethylcellulose (CMC) and various cellulosic hydrolysates (Mandels et al. 1962; Cowling and Brown 1969; Castillo et al. 1984; Karaffa et al. 2006). Although lactose is currently considered the most economically feasible inducing substrate for industrial cellulase production, sophorose is known to be the most powerful soluble inducer (Mandels et al. 1962). Sophorose is, however, very expensive ($200/10mg, Sigma). Therefore, sophorolipids, the biosurfactants consisting of sophorose and long chain fatty acids, were evaluated in this study as a candidate inducer of cellulase production processes.

Sophorolipids are a group of sophorose-containing glycolipids that can be produced in large quantities (> 120 g/L) by *Candida bombicola* fermentation using mixtures of glucose and lipid source as the substrate (Davila et al. 1992). The suitable lipid source included fatty acids (such as oleic acid, linoleic acid), soybean oil, rapeseed oil, hydrocarbons (such as hexadecane, heptadecane), and materials or wastes containing the above (Marchal et al. 1993; Hu 2000; Cavalero David and Cooper David 2003). For

example, Hu and Ju (2001) obtained highest SLs production, 0.84 g/g-hexadecane, by using hexadecane among other two substrates, glucose and soy bean oil, fermented with glucose-based medium under nitrogen-limited condition. The result showed sophorolipids contained two types of SLs, non-acetylated lactonic SLs and mono-acetylated acidic SLs, both possibly containing $(CH_2)_n$, n = 13 -15 (Hu and Ju 2001).

In this study the feasibility of producing cellulase using sophorolipids as the inducer was evaluated. Mixed resting cultures of *Trichoderma reesei* Rut C30 and *C. bombicola*, at different population ratios, were used to investigate if the sophorolipids produced in situ could indeed promote, by induction, the cellulase production. While glucose is the most commonly used sugar for sophorolipid production, glucose is known to repress cellulase synthesis at concentrations above certain threshold (e.g., the repression constant was found 0.0707 g/L of glucose in a previous continuous culture study using lactose as the inducing substrate (Lo et al. 2008). Therefore, instead of glucose, glycerol, an inert substrate that does not induce or repress cellulase synthesis, was used in this study as the primary substrate in the mixed culture systems. A small amount of lipid source (soybean oil in this study) was also provided for initial synthesis of sophorolipids.

As shown schematically in Figure 7.1, the hypothesized mechanisms involved in the mixed culture included the following: First, the yeast synthesized sophorolipids using glycerol and soybean oil as substrates. The sophorolipids were then hydrolyzed into sophorose and fatty acids. The fatty acids could be recycled for sophorolipid synthesis. More importantly, the fungus would produce cellulase using sophorose as the inducer and glycerol as the carbon and energy source.

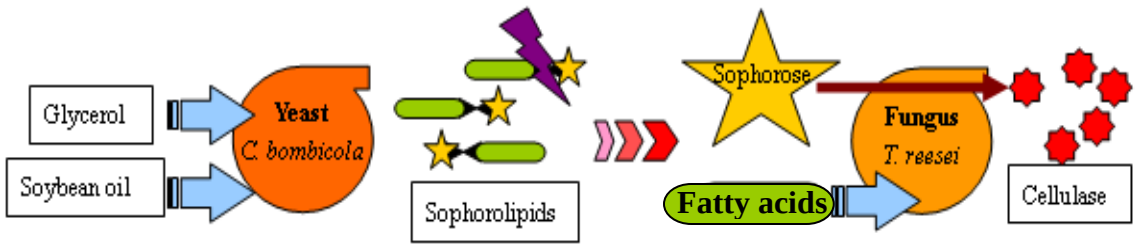


Figure 7.1 postulate of mixed culture design

This, to our best knowledge, was the first report on cellulase production using sophorolipids as the inducer. In addition to the evaluation of the feasibility of using mixed cultures of *T. reesei* Rut C30 and *C. bombicola* for cellulase production as designed, the sophorolipid degradation rate and mechanism(s), namely, cell-associated or extracellular, in *T. reesei* Rut C30 broth were also investigated.

7.3 Materials and Methods

In this section, the operation procedure of each of batch fermentation processes is described.

7.3.1 Preparation of inoculum

Basic culture transfer and culture inoculation, for *T. reesei* Rut C30, followed the procedures described in Chapter 3, preparation of inoculum section. For the yeast, we bought *C. bombicola* (ATCC 22214) from American Type Culture Collection (ATCC) and followed same procedure as *T. reesei* Rut C30 had.

Cell growth and medium preparation

50 ml of a prepared inoculum of *T. reesei* or *C. bombicola*, after 48-hour incubation, was added into a 3L flask containing 1 L of the production medium. The production medium composition for *T. reesei* was given in Chapter 3, essentially the same as that used by Mandels et al.(1962), except that glycerol was used instead of glucose. The production medium for *C. bombicola* had the following composition, Glucose 100 g/L, 2^{nd} C-source 100 g/L, Sodium citrate 5.0 g/L, Yeast extract 5.0 g/L, $(NH_4)_2SO_4$ 5.0 g/L, KH_2PO_4 1.0 g/L, $MgSO_4$ 4.0 g/L, $FeSO_4 \cdot 7H_2O$ 0.1 g/L, $CaCl_2$ 0.1 g/L, NaCl 0.1 g/L. The

composition was the same as that used by Hu (2000) but with glucose being replaced by glycerol.

The above medium for the yeast was N-limited and sophorolipids were largely produced during the prolonged stationary phase. The initial medium pH was adjusted to 6.0. Along with the cultivation, pH was allowed to decrease naturally to 3.5 and thereafter controlled at that value by automatic addition of 1 N NaOH. The overall process of sophorolipid production typically took 3 days, with 1.5-2 days for cell growth and the rest for the stationary phase.

For preparing the cells to be used in the mixed culture study, both cultures of *T. reesei* and *C. bombicola* were grown to the maximum cell concentrations, and then harvested aseptically by centrifugation. The culture condition for *T. reesei* Rut C30 in the 1 L production medium was the same as that described in Chapter 3, using glycerol as a carbon source. The culture of *C. bombicola* used same aforementioned medium with 10 g/L of glucose and no secondary substrate operated at initial pH 6.0, room temperature without pH control.

The composition of production medium for mixed culture study contained 2.0 g/L of K_2HPO_4, 0.3 g/L of $MgSO_4$, 0.4 g/L of $CaCl_2$, 0.2 mL/L of Tween 80 and same amount of

trace-elements as described in the Chapter 3. As the mixed culture study was to be done with resting cells, this medium was essentially N-free, without the common N-sources peptone, urea, and $(NH_4)_2SO_4$ in the cellulase-producing medium. The carbon source was a mixture of glycerol and secondary substrate, soybean oil (Valu Time, Skokie, IL) with the amounts being varied according to the experimental design, as described later.

7.3.2 Mixed culture study

Mixed culture process is used for evaluation on cellulase production by using yeast and fungus with glycerol as primary carbon source and soybean oil as secondary carbon source.

7.3.2.1 The study of glycerol effect

The study was done simultaneously in two flasks containing the fresh Mixed Culture Medium with 2.5 g/L of glycerol and 2.5 g/L of soybean oil as the C source. Each flask was inoculated with the same amount (2 g/L) of centrifugation-harvested yeast cells and fungal cells (T:C ratio = 1:1). After 24 hours of growth, one culture was then added with 20 g/L of glycerol and 1 g/L of soybean oil, while the other with 10 g/L of glycerol and 1

g/L of soybean oil. According to our hypothesis, only very small amounts of soybean oil would be needed if the fatty acids used to form sophorolipids were reused after the sophorolipids were hydrolyzed and sophorose consumed for cellulase induction. The study was therefore done to evaluate if such low-soybean-oil feed, with 10:1 and 20:1 glycerol-to-soybean oil ratios, would be adequate for cellulase production by the mixed culture. The study was done at room temperature, pH controlled at 5 with addition of 2 N NaOH solution, and with maximum agitation using stir bars driven by magnetic stir plates (Corning Incorporated Life Sciences, Model PC – 410).

7.3.2.2 9-system study with varying fungus-to-yeast population ratio and glycerol-to-soybean oil ratio:

After collecting resting cells from each pure yeast and fungus batch culture, the allocation of yeast and fungus cells followed the designed T/C ratio and G/O ratio table listed below. Each system was carried out with 50 ml working volume of mixed culture medium, initially adjusted to pH 5, in 500 ml flask, placed in a shaker (Model 4703, Queue Orbital shaker, Parkersburg, WV) at 250 rpm and 25 °C for 152 hours, without pH control.

T: *T. reesei Rut C30*, C: *C. bombicola*, G: glycerol, O: soybean oil

The total cell concentration was 5 g/L and the culture lasted 152 hours. For example, T:C = 2.5 g/L Yeast : 2.5 g/L fungus. For the ratio of glycerol and soybean oil, glycerol was kept at same amount in all 9 experiments for 5 g/L, and this made soybean oil concentrations in different ratio as 10 g/L in G:O = 1:2, 5 g/L in G:O = 1:1, and 2.5 g/L in G:O = 2:1.

Table 7.1 Allocation of yeast and fungus in each studied system.

T:C = 4:1 G:O= 1:2 1	T:C = 2:1 G:O= 1:2 4	T:C = 1:1 G:O= 1:2 7
T:C = 4:1 G:O= 1:1 2	T:C = 2:1 G:O= 1:1 5	T:C = 1:1 G:O= 1:1 8
T:C = 4:1 G:O= 2:1 3	T:C = 2:1 G:O= 2:1 6	T:C = 1:1 G:O= 2:1 9

7.3.2.3 3-system study comparing glucose versus glycerol as C source and N-containing versus N-free medium:

System 8 was found the best combination of mixed culture for cellulase production in the above study. Further investigation on this combination was made in a study with 3

shake flasks. Flask #1 was kept exactly the same as System 8 above. Flask #2 differed only in using glucose in place of glycerol. Flask#3 was the same as Flask #1 except for the addition of 1 g/L of $(NH_4)_2SO_4$ in the medium. The results of Flasks #1 and #2 were to be compared for the effects of the two C sources. The results of Flask #3 would shed some light on the effects of presence of N source.

7.3.3 Degradation of SLs in *T. reesei* Rut C30 culture

The initial condition of fungus culture was the same as that mentioned earlier, with the Mandel medium and 10 g/L of glycerol in shake flasks. Pure fungus culture, 2 L working volume, was ready for harvest when the cell concentration reached maximum. Culture broth was centrifuged and separated into cell cake and supernatant. For the study, eight shake flasks, 200 ml each, were used. The flasks were divided into 4 groups (two flasks identical for each group), namely, the cell-free systems with and without glycerol initially, and the cell-containing systems with and without glycerol initially. Each of the cell-free systems had 200 ml of the supernatant collected by centrifugation from the pre-grown *T. reesei* Rut C30 culture broth, and the cell-containing systems had the whole broth with 2.5 g/L (CDW) of cells. Each of the glycerol-containing systems was

supplemented with 5 g/L of glycerol initially. Fixed amounts of crude sophorolipids were added into each flask: about 3 g/L of sophorolipids in the systems with glycerol, and about 1 g/L of sophorolipids in the systems without glycerol.

7.3.4 Analytic method:

The analytic methods applied in this chapter were described in Chapter 3 including glycerol concentration, cellulase activity, cell concentration, and sophorolipids concentration. The procedure of crude sophorolipids and purification extracted from fermentation broth was also described in Chapter 3.

7.4 Results and Discussion

The preliminary study on cellulase production by mixed culture, *T. reesei* Rut C30 and *C. bombicola* was investigated.

7.4.1 The study of glycerol effect

Two different glycerol concentrations were tested for effect of glycerol on cell activity. Both were carried out for 300 hours under the same condition with cellulase

production by using mixed culture, with 20 g/L (system 1) and 10 g/L (system 2) of glycerol for each flask. The results were shown in figure 7.2. The initial condition was designed for sophorolipids production by *C. bombicola*. For the first 24 hours in both systems, sophorolipids were expected to be produced by *C. bombicola* for certain amount under N-limited medium. After 24 hours, the supplement of glycerol was designed for cell growth and maintenance. It approximately took 25 hours for cells to start consuming glycerol in system 2, which was a typical adopting time to a new environment for cells and 75 hours in system 1. Cell growth occurred in system 2 on the following 50 hours from 3.3 g/L to 5 g/L of total cells. The nitrogen source could come from the residual N sources introduced with the pregrown yeast culture, which had high amounts of nitrogen (5 g/L of yeast extract and 2 g/L of $(NH_4)_2SO_4$) in the fresh medium. On the contrary, there was no sign of cell growth on system 1 with 20 g/L of glycerol. Cellulase was produced in both systems, both around 0.25. The finding clearly indicated the inducing ability of sophorolipids for cellulase production by *T. reesei* because glycerol is an inert substrate that does not induce cellulase synthesis (as shown by the results in the continuous culture of *T. reesei* Rut C30 using glycerol as substrate, Chapter 6).

Proper medium osmotic pressure is essential to cell growth. Medium osmotic pressure increased as glycerol concentration increased. And this increased difference of osmotic pressure between outside environment and cytoplasm caused the decrease in cell viability (Beney et al. 2001). In addition, the permeability of glycerol to cell membrane was high and this permeability varied with strains dramatically (Davis et al. 2000). However, the permeability of glycerol in *T. reesei* and *C. bombicola* and the effect of glycerol by its permeability have not been reported yet.

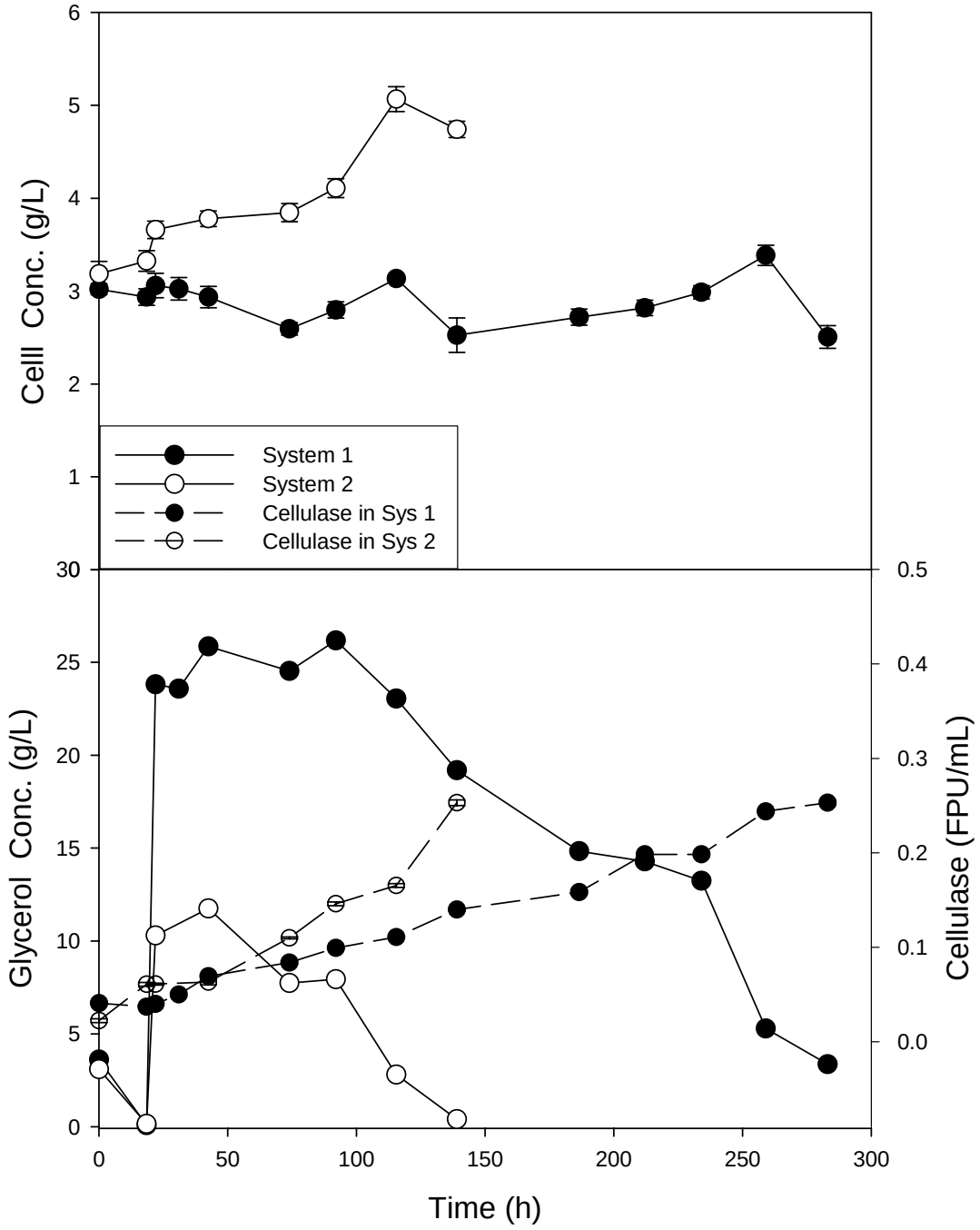


Figure 7.2 The glycerol effect on cell growth and cellulase production

7.4.2 9-system study:

The experimental design followed the description above-mentioned on the preparation of 9-system study. Only cellulase activity was monitored in this study. In terms of the effects of glycerol-to-soybean oil (G:O) ratio, the systems with higher G:O ratios (2:1) generally performed much better (except System 6, reasons unknown) than the systems with lower G:O ratio (1:2, Systems 1, 4 and 7). This finding agreed with the hypothesized mechanisms shown in Figure 7.1: Glycerol, compared to soybean oil, appeared to be a better substrate for general metabolism. It is, however, unknown to what extent the fatty acids released from sophorolipid hydrolysis were reused by the yeast for new sophorolipid synthesis. Future study is needed to evaluate the upper limit of G:O ratio before the sophorolipid synthesis is negatively affected.

As for the effects of cell concentration ratio between *T. reesei* and *C. bombicola* (T:C ratio), the systems with higher ratio of the fungus (Systems 1-3, with T:C = 4:1) did not show better cellulase production. In fact, the productivity was even poorer in Systems 1 and 2, than in Systems 7 and 8, when the G:O ratio used was lower. The observation implied that the cellulase production in these mixed culture systems was limited by the production of inducing sophorolipids. While the implication was not in disagreement

with the general hypothesis, it was rather surprising initially as sophorose was known to induce cellulase synthesis at very low concentrations, 100 μg/ml (Mandels et al. 1962; Nogawa et al. 2001). One possible explanation for the above results is that the sophorose formed from sophorolipid hydrolysis was rapidly consumed or hydrolyzed (to glucose) by the cells. The situation of sophorose depletion was likely even worse in the systems with lower G:O ratios because less glycerol was available and sophorose was a much more preferred substrate than soybean oil for cell metabolism. Which culture, the yeast or the fungus, played more important role in this sophorose consumption/hydrolysis in unknown. It should be pointed out that the yeast (alone) does not consume sophorolipids even during prolonged starvation (Hu 2000). The fungus is definitely able to effectively uptake sophorose, using it as the cellulase inducer and as the substrate. Once the sophorolipids are hydrolyzed by *T. reesei*, the yeast could be able to rapidly uptake the glucose hydrolyzed by β-glucosidase before assimilation.

Overall, the best results (0.5 FPU/ml) were found in Systems 8 and 9, with the ratios of T:C = 1:1 and G:O = 1:1 and 2:1, respectively. System 8 was chosen for further investigation in the subsequent study.

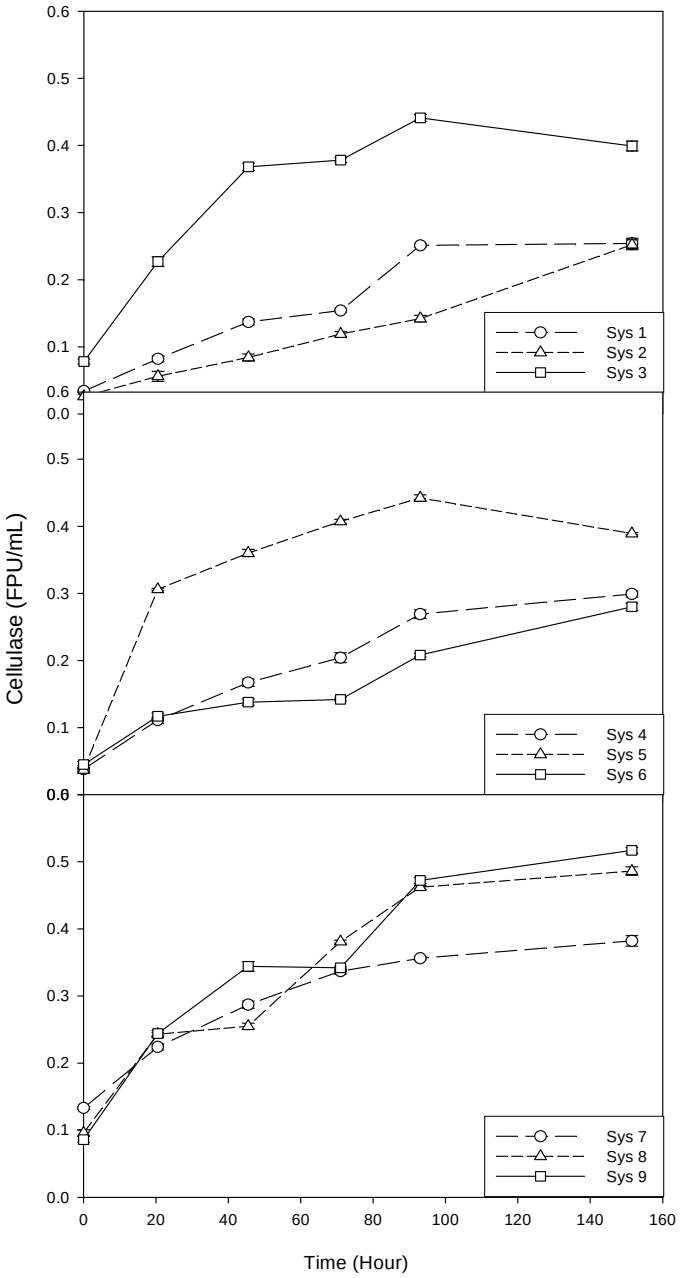


Figure 7.3 The evaluation of cellulase production on different G:O (Glycerol:Soybean oil) ratio and T:C (Fungus:Yeast) ratio in 9 systems

7.4.3 3-System study

In order to validate cellulase production in mixed culture, three more test runs were carried out. These included a system repeating the same setup as System 8, a system with addition of 1 g/L of $NH_4(SO_3)$ in the mixed culture medium, and a system with the same amount of glucose in exchange for glycerol. The results were shown in figure 7.4. The system with 1 g/L of $NH_4(SO_3)$ showed lower maximum cellulase activity, around 0.2 FPU/ml. As sophorolipids are known to be produced under N-limitation (Hu 2000), this finding again implied that sophorolipid synthesis was the limiting factor for cellulase production by the mixed culture. The other two systems had better results, around 0.4 FPU/ml. The system with glucose appeared to perform slightly worse than the system with glycerol. The finding might reflect the net effect of two counteracting mechanisms. On one hand, glucose had a (negative) repression effect on cellulase synthesis by *T. reesei*. On the other hand, *C. bombicola* produced sophorolipids at higher rates with glucose as the substrate (in addition to the lipid precursor) than with glycerol as the substrate (Ju et al. 2007). The net effect appeared to be slightly more favorable with glycerol as the substrate.

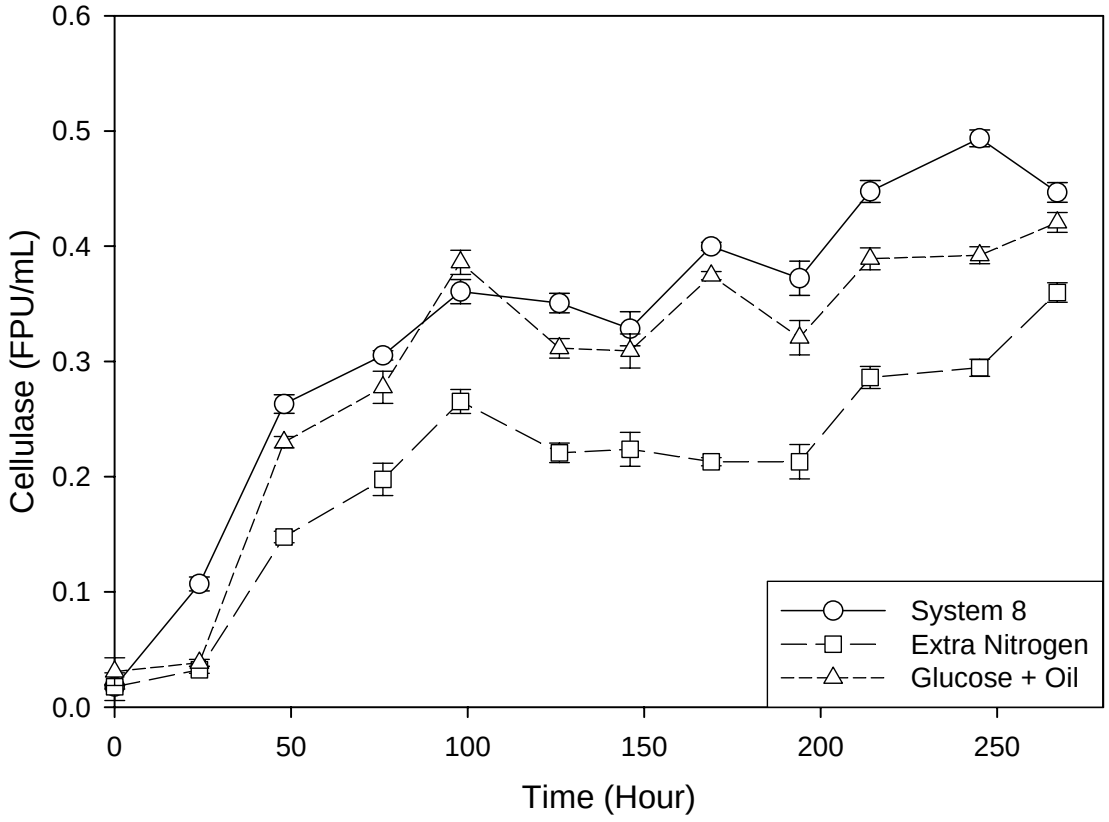


Figure 7.4 The results of validation of system 8 and evaluation of cellulase production with excessive N, or excessive C- source.

7.4.4 Degradation rate of SLs in pure fungus culture

Cellulase has been produced in mixed culture. Therefore, the assumption of this process should be verified. Four pairs of experiments in shaker were carried out for the study in degradation rate of sophorolipids in pure fungus culture. The two flasks in each

pair had identical condition and medium. The final presentation in figure 7.5 was the data from averaged numbers in each pair result.

Since previous studies showed SLs were degraded by the presence of mixed fungus and yeast. The cell-free systems could be a good control base line for this study. Assuming supplement of glycerol could encourage cell growth with the unused nitrogen source from the culture broth, therefore, the higher SLs concentration could give us clearer change of SLs concentration over time. As a result, the degradation of SLs only occurred in system required glycerol and fungus. Therefore, the results suggested *T. reesei* Rut C30 might produce some enzyme to break down the SLs when glycerol was present. The synthesis of this enzyme could require carbon source or could not be in carbon-limited condition. Besides, in the system with cells without glycerol, the SLs concentration did not change. This implied the fungus did not digest SLs directly. Also, SLs did not naturally decompose or get degraded by anything in a fungus-free broth. The degradation rate of SLs was approximately at 0.016 g-SLs/(g CDW-h).

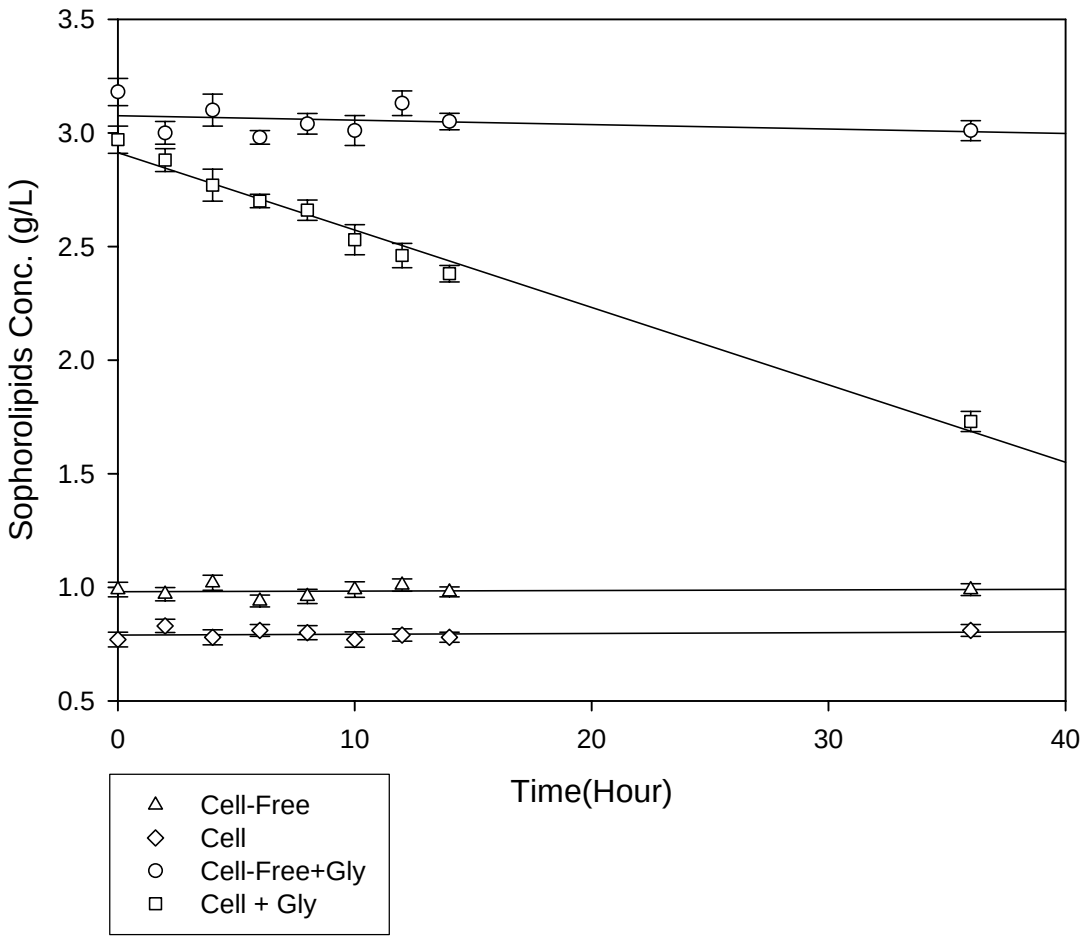


Figure 7.5. The study of rate of SLs degradation by *T. reesei* Rut C30

7.5 Conclusion

In summary, the production of cellulase by mixed culture was very clear and feasible. High glycerol concentration, 2% wt./w, could affect cell growth of mixed culture of *T. reesei* Rut C30 and *C. bombicola*. With 1% of glycerol in the medium, cells adapted themselves to the condition fairly well. The one with best performance in the study was

picked and validated. Also, the degradation of SLs was tested. The results matched with our assumption that the derived substance induced cellulase production.

Here, based on the results above, the suggestions for the next step is to operate a mixed batch/Fed-batch cultures. First, yeast was grown in N-limited medium to produce sophorolipids at stationary phase. With certain amount of remaining glycerol concentration in broth, 6N NaOH was added to kill all yeasts and then culture was hold for 3 hours. The pH in broth then would adjust to pH 5 with 6N HCl. Secondly, the broth with chemically sterilized or autoclaved yeast culture would start adding into a late exponential-growth phase *T. reesei*, cultured in C-limited medium. The addition rate of yeast broth and the amount of remaining glycerol concentration would be determined experimentally.

CHAPTER VIII

CONCLUSIONS

8.1 Conclusions

(1) Essential operational factors for cellulase production in *T. reesei* include culture condition and nutritional factors. Optimized operational factors provide a good culture condition to cells for a specific purpose, such as cellulase production. The effect of these factors on cellulase production was studied by using *Trichoderma reesei* Rut C30 in a BioFlo 110 bioreactor. The following conclusions could be drawn: First, the cellulase activity produced in the fermentation was found very low, around 0.18 FPU/ml, at a high agitation rate of 1000 rpm, but was much better, around 1.6 FPU/mL, at 500 rpm. Second, the carbon-limited medium gave best results among the media limited by different nutrients. Third, the acetic acid present in the dilute-acid pretreated hard wood hydrolysate indeed affected the survival rate of *T. reesei* when cultured in Petri dishes, containing the mixture of hydrolysate and potato dextrose agar. With low

concentrations of acetic acid, the cells had better survival rates. Fourth, a newly designed pH-based feed control strategy was our first attempt to achieve long-term high productivity of cellulase. The control strategy was based on the true cell metabolism reflected by the different slopes of pH change.

(2) Dilute acid pretreated hydrolysate was evaluated for its ability to cellulase production and cell growth. The hydrolysate generated by a dilute acid pretreatment method containing by-products, such as furfural, acetic acid, levulinic acid, and hydroxymethylfurfural (HMF). After pretreatment, the overliming process was needed for the removal of by-products. However, this process could not completely remove these by-products. By adding on dry air-blowing step after overliming, an increase of cell survival rate was observed. In addition, a series of comparisons, between hydrolysate and the combinations of cellulose plus glucose corresponding to the concentrations of oligomers and monosaccharides in tested runs, showed comparable cellulase production by using hydrolysates as an inducer and carbon source. The hydrolysate with highest oligomer concentration, selected from aforementioned batch study, was evaluated in continuous culture for a complete profile of cellulase production at different metabolic stages. The results showed stable rates of specific cellulase

production in the tested range and the total cellulase activity was comparable to that in a lactose system.

(3) A model was developed to describe the culture behaviors of *H. jecorina* Rut C30 grown on lactose. Lactose hydrolysis, to glucose and galactose, was concluded as the rate-limiting step for cell growth in the lactose-based media. Between the two monosaccharides, cells grew much faster on glucose ($\overline{\mu}_{Glu}$ = 0.128 1/h) than on galactose ($\overline{\mu}_{Gal}$ = 0.060 1/h), with comparable cell yields (0.61 and 0.59 g dry cells/g sugar, respectively). Glucose was also found to repress the consumption of galactose, resulting in clear diauxic growth in batch fermentations with media containing both glucose and galactose.

Lactose induced syntheses of both cellulase and lactase. Cellulase synthesis was essentially growth-independent and subject to weak glucose repression ($K_{Glu}^{r,FPU}$ = 0.071 g/L). Lactase synthesis was strongly growth-dependent, except at D < ~0.65 1/h where a basal level production was observed. Lactase synthesis appeared to be subject to weak galactose repression ($K_{Gal}^{r,La}$ = 0.12 g/L). The repressed lactase synthesis limited the lactose hydrolysis rate at the highest D (= 0.12 1/h) observed and, in turn, restricted the maximum cell growth rate attainable in the lactose-based medium.

(4) The preliminary study was performed to prove the postulated mechanism of cellulase induction by sophorolipids. Presumably the sophorolipids (SLs) produced by *C. bombicola* provided the necessary induction for cellulase synthesis. The process is using a mixed culture of a yeast, *C. bombicola*, and a fungus, *T. reesei* Rut C30. A series of mixed cultures having different cell concentration ratios of *C. bombicola* to *T. reesei* and different C-source ratios of glycerol to soybean oil, were evaluated for their cellulase production under nitrogen-limited condition. The one with best performance in the study was picked and validated. Also, the degradation of SLs was tested. The results matched with our assumption that the derived substance induced cellulase production. In addition, glycerol is a neutral carbon source for the cellulase production. With 1% of glycerol in the medium, cells adapted themselves to the condition fairly well. However, high glycerol concentration (2%) caused a significantly long lag phase in cell growth. Overall, the postulated mechanism was feasible and further optimization was plausible to perform better cellulase production.

8.2 Recommendations for future research

(1) The model successfully predicted the rate of lactose hydrolysis and cell concentration. However, the rates of cellulase and lactase synthesis deviated from the model prediction at low dilution rates. According to the experimental data, the explanation of these phenomena requires more information on the change of cell's morphology as dilution rates vary. Therefore, more sophisticated mechanistic model, relating the ratio of the two types of cell morphology, may improve the cell growth model prediction.

(2) Based on the results in Chapter 7, the suggestions for the next step was to operate a mixed batch/Fed-batch culture. First, yeast was grown in N-limited medium to produce sophorolipids at stationary phase. With certain amount of remaining glycerol concentration in broth, 6N NaOH was added to kill all remaining yeasts and then the culture was held for 3 hours. The pH in broth has then adjusted to pH 5 with 6N HCl. Secondly, a broth with chemically sterilized or autoclaved yeast culture would start adding into a late exponential-growth phase of *T. reesei* cultured in C-limited medium. The addition rate of yeast broth and the amount of remaining glycerol concentration would be determined experimentally.

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