UTILIZATION AND PRODUCTION OF SINGLE ACTIVITY ENZYMES FROM *ASPERGILLUS NIDULANS*

By

MICHAEL MUELLER

Diplom Ingenieur (FH) in Biotechnology
Fachhochschule Bingen
Bingen, Germany
2007

Master of Science in Biosystems Engineering
Oklahoma State University
Stillwater, OK
2009

Submitted to the Faculty of the
Graduate College of the
Oklahoma State University
in partial fulfillment of
the requirements for
the Degree of
DOCTOR OF PHILOSOPHY
July, 2012

UTILIZATION AND PRODUCTION OF SINGLE

ACTIVITY ENZYMES FROM

ASPERGILLUS NIDULANS

Dissertation Approved:

Dr. Mark R. Wilkins

Dissertation Adviser

Dr. Hasan Atiyeh

Dr. Nurhan Dunford

Dr. Yu Mao

Dr. Andrew J. Mort

Outside Committee Member

Dr. Sheryl A. Tucker

Dean of the Graduate College

TABLE OF CONTENTS

Chapter Page

Chapter Page

LIST OF TABLES

Table Page

Table Page

LIST OF FIGURES

Figure Page

Figure Page

Figure Page

Figure Page

CHAPTER I

INTRODUCTION

The Energy Independence and Security Act of 2007 is the driving force for biomass utilization as feedstocks for biofuels. It says that by 2022, 36 billion gallons of bioethanol must be used per year in the US, of which are 16 billion gallons of ethanol produced from cellulose while ethanol from starch is limited to 15 billion gallons (EPA 2011). Since the conversion of lignocellulosic materials to fermentable sugars is still facing challenges for commercialization, universities and research institutes are focusing on this technology. Several funding sources are available for performing research in biofuels. The US Department of Energy (DOE), the US Department of Agriculture (USDA) and the Sun Grant Initiative, a national network of land-grant universities and federally funded laboratories, and others provide funding for selected proposals.

Liquid high-density fuels are still the major source of energy for transportation. Especially in aviation, liquid fuels cannot be replaced by electric batteries or heavy fuel cells since the energy density of liquid fuels cannot be achieved (Hemighaus et al. 2006). However, it is common knowledge that petroleum as the source for liquid fuels will be depleted eventually. Therefore, an early investment in fuels derived from biomass is necessary. In case oil is depleted, not only transportation must gain its energy from biofuel,

but also the chemical, pharmaceutical and bioproduct industries will be dependent on biomass as their sole feedstock. Currently, the biofuels industry utilizes easily fermentable sugars for ethanol, or plant oils for biodiesel production. However, as it becomes clear that many industries are dependent on biomass, which is also limited on an area basis, a complete utilization of the organic matter is required to achieve optimum efficiency.

In order to achieve a complete utilization of the organic matter of a plant, one must understand the composition of a plant. As crude oil is composed of different chemicals, which are separated in the refinery into asphalt, liquefied petroleum, jet fuel, diesel fuel, heating oil, gasoline and other valuable products, products from a plant can also be separated into several chemicals in the biorefinery. Using corn as an example, its kernel can be separated into starch and oil. The starch can serve as food or be further processed to bioethanol. The oil can be used for cooking or biodiesel production. The rest of the plant, also known as stover, can be separated into lignin, cellulose and hemicellulose. Lignin can be burned for power production or used as substrate for the biopolymer industry. The cellulose and hemicellulose can be used as feedstock for bioethanol production. Figure 1.1 shows a schematic of how biomass could be processed in a biorefinery to produce different chemicals with corn as an example. The same principle can be used for other common biomass feedstocks, such as woody biomass, herbaceous biomass and agricultural residues.

Where theory seems straight forward, the praxis faces major difficulties in providing technology that can perform these steps economically. Where the left-hand side of Figure 1.1 is already done and the base for major bioethanol production in the United States, the right-hand side deals with high energy and water usage, low enzymatic activities and overall low

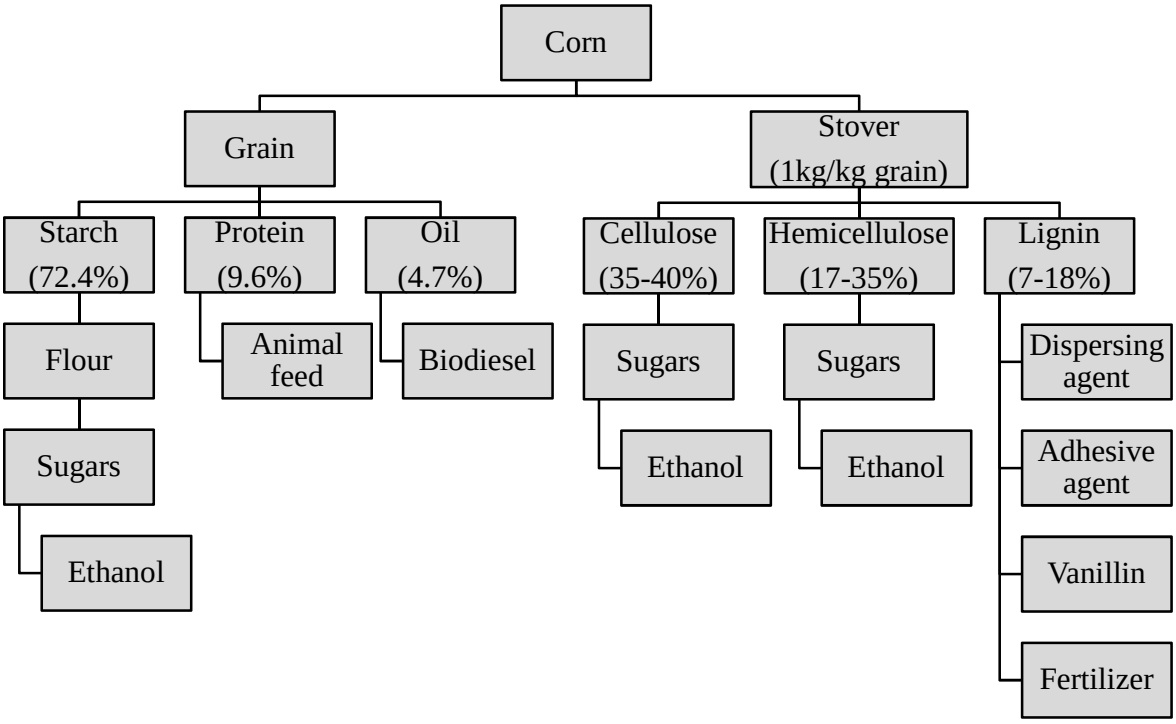


Figure 1.1: Schematic of biorefinery with corn as example (Cheng 2010).

efficiencies. The major difficulty is the breakdown of the complete lignocellulosic structure to fermentable sugars, while minimizing formation of inhibitors and loss of sugars. Evolution made lignocellulose, and especially lignin, highly recalcitrant. Lignocellulose gives mechanical strength to plants and is resistant to microbial breakdown. Therefore, finding enzymes that break down cellulose, hemicellulose and lignin is rather difficult. Fortunately, a breakdown is not impossible, which can be observed when plants rot in the field. However, some of these processes are time intensive and of little interest for industrial purposes. The best role models for biomass breakdown in nature are white-rot and brown-rot fungi. White-rot fungi excrete laccases and other enzymes to break down and utilize lignin, hemicellulose and cellulose, leaving a white material (Fritsche 2002). Brown-rot fungi are specialized in the breakdown of cellulose by excreting cellulases and leave the brown lignin (Fritsche 2002). Current technologies apply, compared to nature, harsh physical or chemical processes to achieve rapid lignin breakdown or dissolution. Subsequently, cellulolytic and hemicellulolytic enzymes are applied to hydrolyze the cellulose and hemicellulose to sugars. Due to the harshness of the pretreatment, losses of sugars and byproducts and/or inhibitors are created. It has long been recognized that a mix of enzymes containing multiple activities towards cellulose, hemicellulose and lignin can improve the overall conversion efficiency. Moreover, where in the past the focus for lignocellulosic bioethanol was based on cellulose, current research tries to utilize hemicellulose as feedstock too. As mentioned above, we can face biomass shortages, so it is of highest importance to use as much of the organic matter for product formation as possible.

A reason for the high cost of lignocellulosic biofuels production is the production of hydrolytic enzymes with high and tailored activities. An ideal enzyme has high activity over

a large spectrum of pH changes, has optimum activity at ambient temperatures, is not affected by substrate or product inhibition, and has a long stability over time, while being cheap to produce. Current enzyme production uses ether submerged fermentation (SmF) or solid state fermentation (SSF). SSF has already been proven to achieve higher enzyme productivities by reduced protease production (Viniegra-González et al. 2003). However, SSF requires a leaching step to remove the enzyme from the solid matrix, which will dilute the enzyme concentration. Furthermore, it is very difficult to apply continuous operation on a SSF system. A trickle bed reactor can be the solution to the problem. It allows the culture to grow on a solid surface by optimum oxygen and nutrient supply. When a recycle is implemented, the substrate utilization and product formation can be improved. However, a major drawback can be the uncontrolled growth of mycelia, which can lead to clogging of pipes and apparatuses. Limiting the growth of the organism by continuous product formation can be a potential solution. A trickle bed reactor can only achieve its full potential when the growth of the organism can be limited while the enzyme production is unaffected. Then the system could be viewed as a stationary catalyst. When the recycle flow rate is chosen appropriately high, the assumption could be made that the dissolved solids concentration is uniform throughout the reactor, which allows the usage of continuously stirred tank reactor (CSTR) models.

Currently, literature tries to achieve zero-growth rate systems in order to maintain a constant cell mass. Zero-growth rate systems can be achieved by reducing the energy and carbon supply to a level equal to the maintenance energy required by the organism to survive, but without growth. It is not known if such a system can be kept active over a prolonged period of time. A solution could be the usage of mutant strains that are unable to utilize a

certain essential coenzyme. These strains need this particular coenzyme in the medium to grow. By removing the coenzyme from the medium after the organism is grown, the growth could be limited while the enzyme production can continue. Such a system has not been proposed in the literature. Different mutants of *Aspergillus nidulans* can be found with a multitude of different markers. Among others, these markers can be co-enzymes and need to be fed to the organism to allow growth.

The first part of the present study investigates the synergistic effects of enzymatic hydrolysis of grain sorghum stover by using multiple enzymes. Conversion of cellulose to glucose is compared between untreated and pretreated grain sorghum stover.

The second part of this study investigates the feasibility of limiting the growth by coenzyme limitation for continuous enzyme production. This system is then applied to a laboratory scale trickle bed reactor to investigate the feasibility of the reactor system over a period of time.

1.1 References

Cheng JJ. 2010. Biological process for ethanol production. In: Cheng JJ, editor. Biomass to renewable energy processes. New York: CRC Press. p 229-234.

EPA. 2011. Renewable Fuel Standard (RFS) Program. US Department of Energy.

Fritsche W. 2002. Mikrobiologie. Fischer G, editor. Berlin: Spektrum Akademischer Verlag Heidelberg.

Hemighaus G, Boval T, Bosley C, Organ R, Lind J, Brouette R, Thompson T, Lynch J, Jones J. 2006. Alternative jet fuels. Chevron Corporation.

Viniegra-González G, Favela-Torres E, Aguilar CN, Rómero-Gomez SJ, Díaz-Godínez G, Augur C. 2003. Advantages of fungal enzyme production in solid state over liquid fermentation systems. Biochemical Engineering Journal 13(2-3):157-167.

CHAPTER II

OBJECTIVES

1. Determine the synergistic effects of enzyme mixtures for maximum enzymatic hydrolysis of grain sorghum stover untreated and pretreated with liquid hot water pretreatment.

2. Determine the feasibility of continuous client enzyme production with an *Aspergillus nidulans* mutant containing a pyridoxine marker by limiting the supply of pyridoxine.

3. Determine the feasibility of using the limited growth system with pyridoxine limitation in a trickle bed reactor for prolonged client enzyme production.

.

CHAPTER III

REVIEW OF LITERATURE

3.1 Lignocellulosic substrates

Plant material that is composed mainly of cellulose, hemicellulose and lignin is considered lignocellulosic material. Lignocellulosic material was traditionally a by-product of the agricultural and forest industries. Three types of lignocellulosic material can be identified: agricultural residues, herbaceous biomass and woody biomass (Lin and Tanaka 2006). Typical agricultural lignocellulosic residues are corn stover, wheat straw, and rice straw. According to the Billion-Ton-Study update by Perlack (2011), the USA produces 111 million metric tons of agricultural residues in the baseline scenario in 2012. Table 3.1 shows typical values for cellulose, hemicellulose and lignin contents of agricultural residues. Using average values, a mass of 44.4 million tons of cellulose could be produced. Assuming complete cellulose conversion to bioethanol, 25 million tons (32 billion liters) of ethanol could be produced annually. With 24 MJ/L for ethanol and 34.2 MJ/L for gasoline, the bioethanol could replace 22 billion liters (5 billion gallons) of gasoline.

Herbaceous biomass is grown as feedstock for the biofuels industry. Several herbaceous feedstocks show promising fiber compositions to qualify for the biofuels

industry. Among others, switchgrass, miscanthus, and Coastal Bermuda grass are the main species. Common compositions can be seen in Table 3.1. Optimum feedstock characteristics for biofuel production are high cellulose and hemicellulose concentration and low lignin, moisture and ash content. The composition varies greatly with the state of plant maturity. Therefore, harvest should take place before lignification is initiated, since lignification reduces biodegradability (Jung and Vogel 1992). Woody biomass can be divided into two categories: softwood and hardwood. Softwoods are gymnosperms and include all evergreens (Cheng 2010). Examples of softwoods are pine and spruce. Hardwoods have broad leaves and are angiosperms. Examples of hardwoods are poplar, willow and oak (Fengel and Grosser 1975). The difference in cellulose, hemicellulose and lignin content between softwoods and hardwoods is shown in Table 3.1. It is not the author's intention to rank the different feedstocks as good and bad. It is the author's opinion that all resources need to be considered and evaluated in their usage as feedstock, since different areas with different climates promote the growth of different feedstocks in a sustainable manner. Furthermore, a mix of all substrates will be needed to overcome the future demand for the biofuels, bioproduct and chemical industry. However, each substrate with its unique characteristics requires an individually optimized conversion process.

3.1.1 Cellulose

Cellulose is the main constituent of plants and serves to give the plant structure and mechanical strength (as opposed to starch, which serves as an energy source) (O'Sullivan 1997). Cellulose is a linear polymer composed of glucose molecules with β-1,4-glycosidic bonds. Six polymorphs of cellulose have been identified (I, II, III, IV, V,

Table 3.1: Typical composition (% dry basis) of lignocellulosic biomass (Cheng 2010).

Biomass		Cellulose	Hemicellulose	Lignin
Agricultural waste	Corn stover	35 – 40	17 – 35	7 – 18
	Wheat straw	33 – 50	24 – 36	9 – 17
	Rice straw	36 – 47	19 – 25	10 – 24
Herbaceous biomass	Switchgrass	28 – 37	24 – 27	15 – 18
	Miscanthus	38 – 43	24 – 27	22 – 25
	Coastal Bermuda grass	26	31	20
Woody biomass	Hardwoods	40 – 50	15 – 20	20 – 25
	Softwoods	40 – 50	11 – 20	27 – 30

and VI) (O'Sullivan 1997). Cellulose I is the form found in nature. By application of heat, mercerization, or liquid ammonia the cellulose forms II to IV are interconverted (O'Sullivan 1997). Since cellulose I is the natural occurring form of cellulose, it is of main interest and described in more detail. Multiple chains of β-1,4- glycosidic bound glucose molecules together form a cellulose microfibril (Cheng 2010). Multiple microfibrils form a cellulose fibril. Due to the β-1,4-glycosidic bond, the OH group from C6 on one chain forms a hydrogen bond with the OH group from C3 on a neighboring chain. These intermolecular bonds together with intramolecular bonds are responsible for the rigid structure of cellulose and its crystallinity (Cheng 2010). Figure 3.1 shows the H-bonds between two strands of cellulose chains.

3.1.2 Hemicellulose

Hemicellulose is a heterogeneous branched polymer with a backbone composed of xylose, mannose or glucose. Other main branched constituents are glucose, arabinose, mannose, galactose, phenolic groups, acetyl groups and sugar acids (Cheng 2010; Saha 2003). Its composition varies greatly with plant type. Where softwoods are mainly composed of glucomannans, hardwoods are mainly composed of xylans (Girio et al. 2010). Six groups of major hemicelluloses can be identified: glucuronoxylans (GX), galactoglucomannans (GGM), arabinoglucuronoxylans (AGX), xyloglucans (XG), arabinoxylans (AX) and complex heteroxylans (CHX). Table 3.2 shows an overview of the hemicelluloses, their predominant source, backbone structure and side chains.

Hardwood hemicelluloses are mainly composed of GX. The content can range between 15% and 30% of the dry mass (Alén 2000). GX consists of a linear backbone of

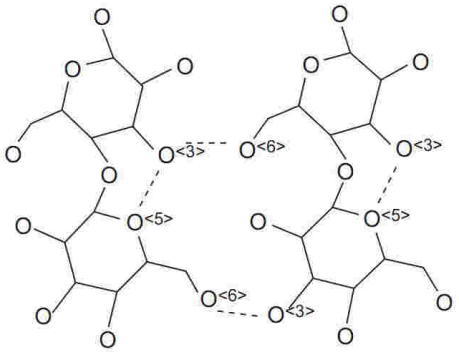


Figure 3.1: H-bonding of two cellulose I strands. Adapted from O'Sullivan (1997).

Table 3.2: Main types of polysaccharides present in hemicelluloses (information based mainly on (Alén 2000; Carpita and Gibeaut 1993; De Vries and Visser 2001; Ebringerova et al. 2005; Pereira et al. 2003).

Polysaccharide type	Abb[1]	Biological origin	%db[2]	Backbone	Side chains	Linkages	Dp[3]
Arabinogalactan	AG	Softwoods	1-3	β-D-Gα*l*p	β-D-Gal*p*, α-L-Ara*f*, β-L-Ara*p*	β-(1→6) α-(1→3) β-(1→3)	100 – 600
Xyloglucan	XG	Hardwoods, grasses	2-25	β-D-Glc*p*, β -D-Xyl*p*	β-D-Xyl*p*, β-D-Gal*p*, α-L-Ara*f*, α-L-Fuc*p*, Acetyl	β-(1→4) α-(1→3) β-(1→2) α-(1→2) α-(1→2)	
Galactoglucomannan	GGM	Softwoods	10-25	β-D-Mαn*p*, β-D-Glc*p*	β-D-Gal*p* Acetyl	α-(1→6)	40 – 100
Glucomannan	GM	Softwoods, hardwood	2-5	β-D-Mαn*p*, β-D- Glc*p*, β-D-Xyl*p*			40 – 70
Glucuronoxylan	GX	Hardwoods	15-30	β-D-Xyl*p*	4-O-Me-α-D-Glc*p*A, Acetyl	α-(1→2)	100 – 200
Arabinoglucuronoxylan	AGX	Grasses, cereals, softwoods	5-10	β-D-Xyl*p*	4-O-Me- α -D-Glc*p*Aβ -L-Ara*f*	α-(1→2) α-(1→3)	50 – 185
Arabinoxylans	AX	Cereals	0.15-30	β-D-Xyl*p*	α-L-Ara*f*Feruloy	α-(1→2) α-(1→3)	
Glucuronoarabinoxylans	GAX	Grasses, cereals	15-30	β-D-Xyl*p*	α-L-Ara*f*, 4-O-Me- α -D-Glc*p*A, Acetyl	α-(1→2) α-(1→3)	
Homoxylans	HX	Algae		β-D-Xyl*p*			

[1] Abbreviation, [2] % of dry mass, [3] Degree of polymerization

β-(1,4) glycosidic linked β-D-xylopyranosyl units. One in ten xylose molecules has an α-1,2-linked uronic acid group and some are acetylated at C2 and C3. GX may contain small amounts of L-rhamnose and galacturonic acid (Girio et al. 2010).

GGM is the main hemicellulose in softwoods with 10% to 25% of the dry mass (Pereira et al. 2003). The linear backbone is composed of β-(1,4)-glycosidic linked b-D-glucopyranosyl and b-D-mannopyranosyl units (Girio et al. 2010). The units are partially acetylated at C2 or C3. It was observed that GGM appear to be water soluble when the galactose content is increased (Timell 1965).

The major component of non-wood hemicellulose is AGX. AGX are also a minor component in softwoods (Girio et al. 2010). The backbone is composed of xylopyranose units with 4-O-methyl-α-D-glucopiranosyl uronic acid and α-L-arabinofuranosyl units (Timell 1965). Compared to hardwoods, AGX may be less acetylated.

Hardwoods are predominantly composed of XG. XG also can be found in small amounts in grasses. The backbone of XGs are composed of β-1,4-linked glucose residues and 75% of these residues are substituted with xylose at O-6. XGs form hydrogen bonds with cellulose microfibrils and support the structural integrity of the cellulose network (Carpita and Gibeaut 1993; De Vries and Visser 2001; Mishra and Malhotra 2009). The cellulose microfibrils are coated with XG, which limits their aggregation. Tethers bind the microfibrils and regulate the mechanical properties of the cell wall (Johansson et al. 2004).

The major hemicellulose structures in cereal grain cell walls are arabinoxylans. While similar to hardwoods, AXs contain a higher amount of arabinose (Girio et al. 2010). The linear backbone of AX is composed of β-1,4-xylose units and is substituted

with α-L-arabinofuranosyl units at 2-O and/or 3-O positions. The extractability of xylan is restricted by physical and/or covalent interactions with other cell wall components (Girio et al. 2010).

Cereals, seed, gum exudates and mucilages contain complex heteroxylans where the xylose backbone has single uronic acid and arabinosyl residues as well as various mono- and oligoglycosyl side chains (Girio et al. 2010).

3.1.3 Lignin

Lignin is, after cellulose, the second most abundant organic polymer in lignocellulosic biomass. As a compound of the cell wall of higher plants, lignin confers mechanical strength and plays a vital role in vascular transport (Martone et al. 2009). Lignin also protects cellulose and hemicellulose from microbial breakdown, making wood persistent and a useful building material (Vanholme et al. 2010). It is a three-dimensional amorphous heteropolymer (Uzal et al. 2009), and is composed of hydroxycinnamyl alcohols (or monolignols), coniferyl alcohol and sinapyl alcohol. A minor compound is p-coumaryl alcohol (Boerjan et al. 2003). From the monolignols result guaiacyl, syringyl and p-hydroxyphenyl units, which are incorporated into the lignin polymer. In general, softwoods (gymnosperms) and hardwoods (angiosperms) have different major lignin monolignol compounds. Softwoods are mainly composed of guaiacyl units (with minor p-hydroxyphenyl units) and hardwoods are mainly composed of guaiacyl and syringyl units (Vanholme et al. 2010). Figure 3.2 shows the lignin structure of poplar as predicted from NMR analysis.

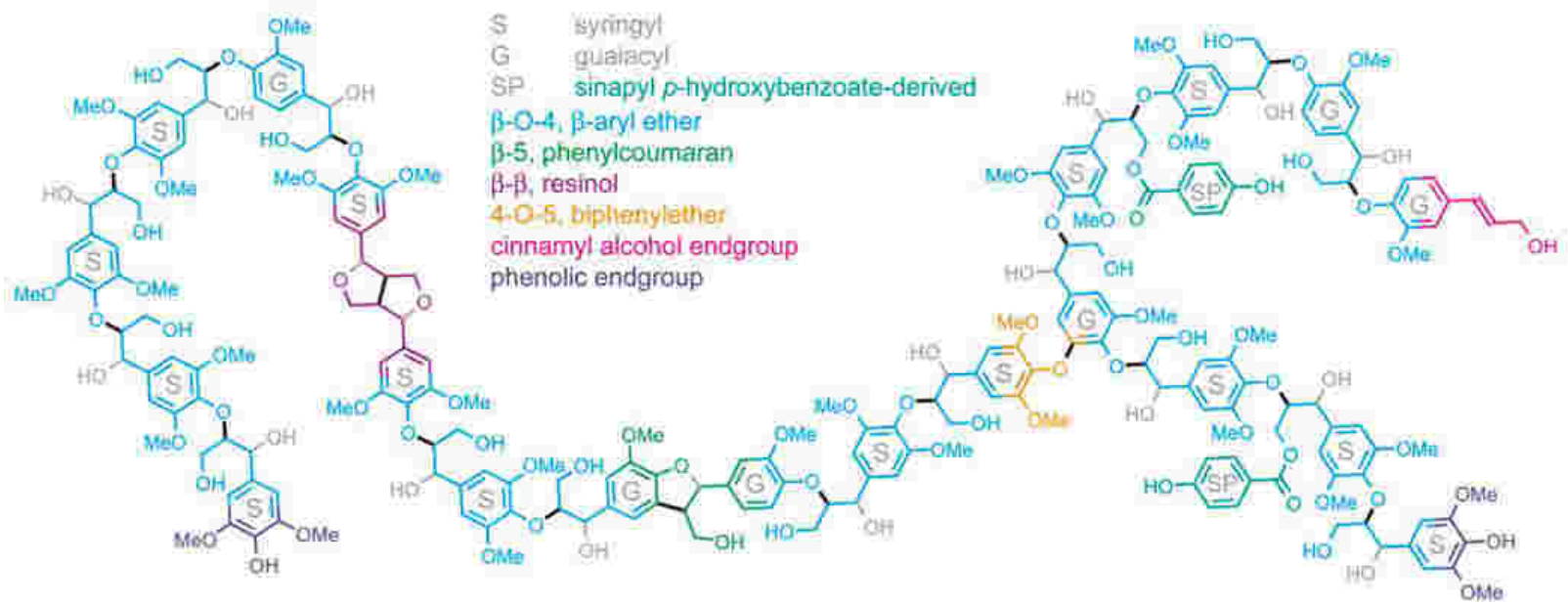


Figure 3.2: NMR predicted lignin structure of poplar (Stewart et al. 2009).

3.1.4 Grain sorghum stover

Grain sorghum is a common cereal crop in Nigeria, the United States, India, Mexico and other countries. It is the third most used grain for food in the United States, and used second, after corn, as a feedstock for commercial bioethanol production (Anonymous 2010). Kim and Dale (2004) reported that North America has a potential of producing 1.89 gigaliter (GL) bioethanol from sorghum straw. However, to improve the conversion efficiency of grain sorghum to ethanol, it is necessary to not only utilize the grain, but also the plant residues, also known as stover. The stover is mainly composed of structural carbohydrates (cellulose, hemicellulose, and lignin). In a previous study, the cellulose and hemicellulose content was found to be 35% (dry basis (db)) and 28% (db), respectively and the lignin content was 5.6% (db) (Salem et al. 1994). Powell et al. (1991) compared the chemical composition of conventional grain sorghum, intermediate and forage sorghum stover. The cellulose content in stover varied between 31.3% (db) for grain sorghum and 25.3% (db) for intermediate sorghum stover. Hemicellulose was highest in grain sorghum stover with 28.2% (db) and lowest in intermediate sorghum stover with 21.7% (db).

3.2 Pretreatment of lignocellulose

The ultimate compound derived from biomass for many applications in biofuel, bio-product and chemical industries is sugar. In its monomeric form, it can be used for many applications and converted to other compounds. Whereas the formation of sugar from starch or sugar crops is cheap and can be achieved in high yields, the sugar formation from lignocellulose is still costly. However, in order to relieve the pressure of

starch and sugar crops' demand for non-food applications, lignocellulose-to-sugar technology is being researched to find pretreatment and enzymatic hydrolysis techniques than can reduce costs and increase yields.

The difficulty in converting lignocellulose to sugar comes from the structure in which the polymers are arranged within the fiber matrix. A typical plant fiber is composed of three major compounds: cellulose, hemicellulose, and lignin. Cellulose, present as a linear polymer, is surrounded by the hemicellulose, which is present as a branched polymer. Both, cellulose and hemicellulose are surrounded by lignin. Lignin is nature's answer to rapid microbial breakdown. Lignin is highly recalcitrant and protects cellulose and hemicellulose from enzymatic breakdown. These characteristics allow us to use wood as a building material. However, it is this recalcitrance that needs to be overcome in order to convert the cellulose and hemicellulose to useful sugars. A dead tree in the forest is broken down by microorganisms. White-rot fungi degrade lignin and brown-rot fungi hydrolyze cellulose. Unfortunately, these processes have slow reaction rates and are not of high interest for industry.

Several techniques are available to pretreat lignocellulose. The purpose of a pretreatment technique is to (Alvira et al. 2010):

- break and remove lignin
- de-crystallize cellulose
- increase porosity
- improve accessibility of enzymes
- enhance biodigestability

Figure 3.3 shows a schematic of the arrangement of lignin, hemicellulose and cellulose to each other, as well as the effect of pretreatment on a fiber matrix. An optimized pretreatment technique must improve the formation of sugars, avoid the degradation of sugars or loss of carbohydrates, avoid the formation of inhibitors, not require a size reduction of biomass, be effective at low moisture content, be cost-effective, allow lignin recovery and have a minimum power and heat requirement (Alvira et al. 2010). No pretreatment technique has been developed yet that fulfills all requirements. Detailed descriptions of available pretreatment techniques are available elsewhere (Alvira et al. 2010; Bjerre et al. 1996; Chang et al. 2001; Duff and Murray 1996; Girio et al. 2010; Kumar et al. 2009; Millet et al. 1976; Monties 1991; Mosier et al. 2005a; Playne 1984; Sun and Cheng 2002; Zheng et al. 1998).

Liquid hot water pretreatment was found to be a cost effective method to pretreat agricultural residues and grasses and is described below (Faga et al. 2010; Mosier et al. 2005b; Mosier et al. 2005a; Suryawati et al. 2008b).

3.2.1 Liquid hot water pretreatment

The main objective of liquid hot water pretreatment (LHWP) is to dissolve hemicellulose with a reduced inhibitor formation (Alvira et al. 2010). During LHWP, the material is treated with water at high temperatures and pressures. The temperatures can vary between 160ºC and 240ºC (Alvira et al. 2010). The water is maintained in liquid form in a completely enclosed reactor. LHWP does not require any chemicals, besides water, for the reaction. The pH of the material after the process is closer to neutral compared to other common pretreatment techniques, such as dilute acid or alkaline

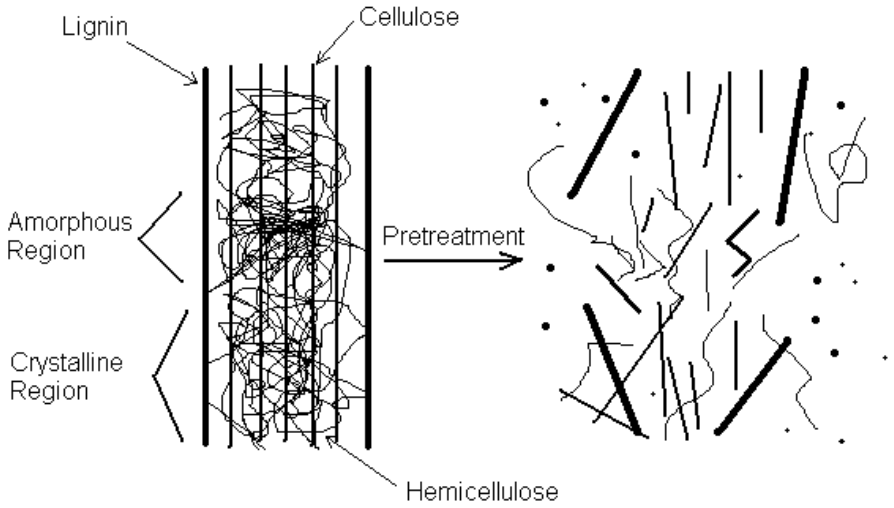


Figure 3.3: Effect of pretreatment on biomass scheme. Lignin gets disrupted and hemicellulose dissolved. Adapted from Hsu et al. (1980) and Mosier et al. (2005a).

pretreatment, which simplifies the neutralization process required for downstream enzymatic hydrolysis and fermentation. The lack of chemicals also reduces the cost of the process. Since no corrosive chemicals are used, cheaper reactor equipment can be chosen (Mosier et al. 2005b). For batch treatment, a steel reactor is filled with an appropriate amount of biomass and water (Suryawati et al. 2008). Solids concentrations can range between 2 and 100 (w/w) liquid-to-solid ratio (Girio et al. 2010).

The reactor is equipped with a stirring device to achieve uniform heat and mass distribution. Temperature and pressure control are used to monitor the process. The reactor is heated, which consequently leads to an increase in pressure. When the desired temperature is reached, it is held constant for a certain time. The holding time can vary between 5 and 15 minutes (Suryawati et al. 2008). After the pretreatment, temperature and pressure are reduced to normal. The pH of pure water drops to 5 when heated to 200°C (Weil et al. 1998).

With a high dielectric constant, ionic substances are dissociated. Forty to sixty percent of the biomass is dissolved (Mosier et al. 2005a). For corn stover, the optimal conditions found by Mosier et al. (2005b) were 190°C for 15 min. Ninety percent of the cellulose was converted to glucose after cellulase treatment in that study. Suryawati et al. (2008b) conducted a simultaneous saccharification and fermentation (SimSF) of Kanlow switchgrass pretreated with LHWP. The researchers set the conditions to 200°C for 10 min with a 10% dry biomass loading. Under these conditions 43.9% of the dry solids were solubilized. The glucan content in the solids was increased from 36.6% to 56.6%. The conversion efficiency from cellulose to ethanol was 80%. Hemicellulose can be dissolved completely during LHWP; however, the dissolved pentoses can be further

degraded to furfural (Mosier et al. 2005a). The holding time and temperature need to be chosen carefully, since the degree of lignin removal, cellulose de-crystallization and hemicellulose dissolution are positively correlated to inhibitor formation. With constant holding time, a relatively high temperature can lead to good lignin removal and de-crystallization, but also high inhibitor formation. With constant temperature, an increase of the holding time leads to higher lignin removal and de-crystallization, but also higher inhibitor formation (Suryawati et al. 2008).

3.3 Enzymatic hydrolysis of biomass

After physico-chemical pretreatments, enzymatic hydrolysis is necessary for sugar formation. In general, yeast does not express extracellular enzymes for the degradation of polysaccharides. For biological ethanol production, cellulose and hemicellulose need to be hydrolyzed to simple sugars. Several manufacturers provide enzyme cocktails for the degradation of cellulose or hemicellulose. However, enzyme production and enzymatic hydrolysis are still expensive and need to be optimized. The complete degradation of cellulose to glucose involves three enzyme activities: endoglucanase, exoglucanase, and beta-glucosidase. Endoglucanases hydrolyse cellulose to oligosaccharides. Exoglucanase converts the oligosaccharides to cellobiose, which is hydrolyzed to glucose by beta-glucosidase (Cheng 2010) (Figure 3.4).

Hemicellulose degradation needs a variety of enzymes, since hemicellulose is composed of a variety of sugars. The main constituent of many hemicelluloses are xylans with a homopolymeric xylose backbone with random side chains composed of arabinose,

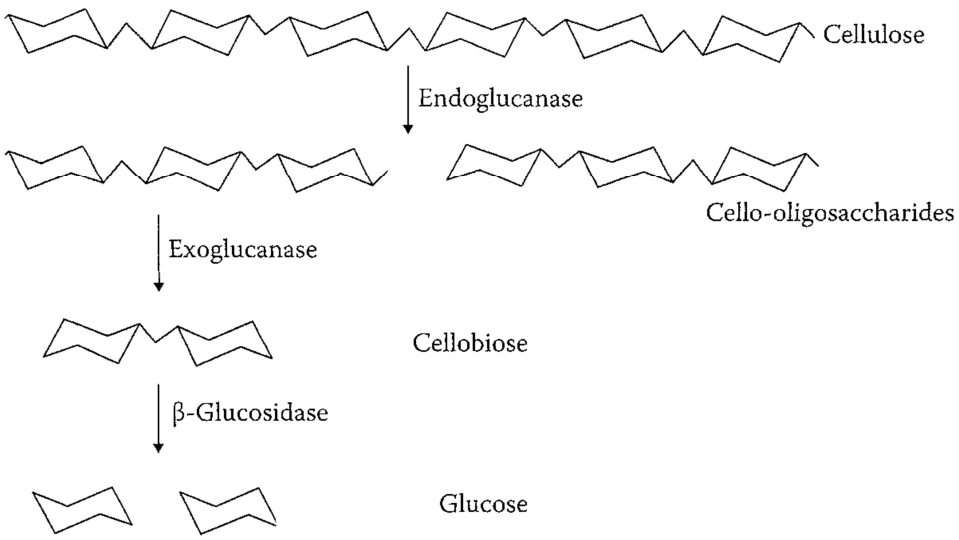


Figure 3.4: Enzymatic processes to convert cellulose into glucose (adapted from Cheng 2010).

glucoronic acid, ferulic acid and acetic acid. Xylanase is an endo-enzyme for xylans. Beta-xylosidase degrades xylo-ologosaccharides to xylose. Pectin, a family of heteropolymeric and homopolymeric polysaccharides dominated by galacturonic acid units and homogalacturonan, is another major component of hemicellulose and involves pectinase for degradation (Cheng 2010). The bonds between hemicellulose and lignin can be cleaved by ferulic acid esterase.

While the degradation of cellulose is widely understood and enzymes with high activities can be obtained, the degradation of hemicellulose is more complex. Each substrate has a unique hemicellulose composition. Furthermore, the presence of hemicellulose and lignin can inhibit cellulose degradation simply by the fact that the cellulose is shielded by lignin and hemicellulose from the enzymes. An ideal enzyme cocktail hydrolyzes all cell wall components for optimum sugar release.

3.3.1 Limiting factors involved in decreasing enzymatic hydrolysis of biomass

One major factor that decreases the enzymatic hydrolysis of biomass as time progresses is the formation of inhibitors. For cellulose hydrolysis a main inhibitor is cellobiose, the product of endo- and exo-glucanases, which competes for the active site of exo-glucanases (Cheng, 2010). This competitive product inhibition can be reduced by using beta-glucosidase, which will hydrolyze cellobiose to glucose (Cheng, 2010). The increasing concentration of glucose over time also leads to product inhibition of beta-glucosidase, which can be avoided by using simultaneous saccharification and fermentation (SimSF) (Sun and Cheng, 2002).

A second important factor leading to a decreased enzymatic hydrolysis rate is the biomass composition and structure. As discussed in more detail in the next paragraph, synergistic effects were observed when using enzyme mixtures (Banerjee et al. 2010; Beukes et al. 2008; Beukes and Pletschke 2010; Gottschalk et al. 2010). This occurs since cell wall components hinder the access of single enzymes to the specific substrate. The first hindrance is given by lignin, which can be removed with physico-chemical methods. The second hindrance is hemicellulose, which is a network surrounding the cellulose fibers. Therefore, as time progresses, less specific substrate can be reached by the corresponding enzymes, which reduces the hydrolysis rate.

3.3.2 Optimization of enzymatic hydrolysis with enzyme mixtures

Optimization of enzymatic hydrolysis can be achieved by expressing enzymes with higher activities, increasing the protein excretion of host organisms, or using multiple enzymes to achieve synergistic effects. Beukes et al. (2008) conducted a test on sugarcane bagasse to see the synergistic affects between xylanase A, endo-glucanase E. and mannanase A and compared the results to specific substrate mixtures composed of birchwood xylan, carboxymethylcellulose and locust bean gum. The researchers performed a factorial design with varying enzyme concentrations but with a total enzyme volume of 40 µl in each test. The degree of synergism was calculated by dividing the resulting activity of the combination of two enzymes by the sum of the individual activity of each enzyme. The substrate, sugarcane bagasse, was treated with the enzyme mixtures for 30 min at 50°C and pH 5.5. Using two enzyme combinations on a birchwood xylan/carboxymethylcellulose mix, the highest activity with 1078.11 U/mg was observed

with a xylanase/endoglucanase molar ratio of 25/75. This test also showed the highest degree of synergism of 3.59. The same enzyme mix, xylanase/endoglucanase, in a different molar ratio (75/25) achieved an activity on sugarcane bagasse of 9.50×10^{-2} U/mg and the highest degree of synergism (4.65). With all three enzymes together, the best result on a synthetic substrate mix (birchwood xylan, carboxymethylcellulose, locust bean gum) was found with a molar ratio of 25% xylanase, 25% endoglucanase and 50% mannanase. The degree of synergism was 4.46 (activity values were not given). On sugarcane bagasse, the same molar ratio achieved an activity of 2.97×10^{-1} U/mg with a degree of synergism of 2.73. Using all three enzymes increased the activity about 3 times compared to the xylanase/endoglucanase mix. However, the degree of synergism was less with all three enzymes together than with only two enzymes. It could be concluded that a mixture of enzymes is more effective for biomass breakdown than the usage of single enzymes. The sugarcane bagasse used in the experiment was not further pretreated, and it can be expected that an appropriate pretreatment would lead to even higher activities and degrees of synergism. This can also be seen in the great differences in activities achieved on specific substrates compared to sugarcane bagasse. It is believed that lignin directly affects enzymes' ability to reach cellulose and reduces hydrolysis.

A follow up work was published (Beukes and Pletschke 2010) and incorporated a pretreatment on sugarcane bagasse before the enzymatic hydrolysis. The treatment used was lime pretreatment with the following conditions: 0.4 g lime/g dry bagasse, 70°C, for 36 h at 100 rpm. The researchers used arabinofuranosidase, xylananse and mannanse in various combinations. As expected, the enzymatic hydrolysis showed with all enzyme combinations higher activities with pretreated bagasse than with non-pretreated bagasse.

The highest activity of about 600 U sugar produced was achieved with a combination of all three enzymes with a molar ratio of 37.5% arabinofuranosidase, 25% mannanase and 37.5% xylananse on pretreated sugarcane bagasse, while the same enzyme mix achieved only 60 U on un-treated sugarcane bagasse. However, the degree of synergism was about 2.2 lower as with a test on non-pretreated bagasse with a molar ratio of 12.5% arabinofuranosidase and 87.5% xylanase. It could be concluded that a mix of enzymes leads to synergism and that incorporated pretreatment leads to better enzymatic hydrolysis.

Another test with a synthetic enzyme mixture was performed on ammonia fiber explosion (AFEX) pretreated corn stover (Banerjee et al. 2010). The researchers investigated combinations of commercially available enzymes with combinations of different enzymes with known single activities. The first part included the following commercially available enzymes: Spezyme CP, Novozyme 188, Accellerase 1000 and Multifect-xylanase. The enzyme loading was based on total protein concentration. The total protein loadings were 7.5, 15 and 30 mg/g glucan. Spezyme CP was tested with Novozyme 188 and Accellerase 1000 was tested with Multifect-xylanase. The highest glucan conversion of 64.3% was achieved with 50 mass% Accellerase 1000 and 50 mass% Multifect-xylanase, and a total protein concentration of 30 mg/g glucan. Spezyme CP and Novozyme 188 achieved only 57.4% with the same protein concentrations. These enzyme combinations also showed the highest xylan conversion of 44.7% and 38.6% with Accellerase/Multifect and Spezyme/Novozyme, respectively.

The enzymes with single activities were cellobiohydrolase1 (CBH1), cellobiohydrolase2 (CBH2), endoglucanase1 (EG1), exoglucanase3 (EX3), beta-

glucosidase (BG), and beta-xylanase (BX) (Banerjee et al. 2010). In terms of glucose release, the best combination in their study was with all six enzymes with the following proportions: 46% CBH1, and all other enzymes with 11% each. The glucan conversion was 39.3%. Xylan conversion under these conditions was 22.8%; however, the best xylan conversion of 28.0% was achieved with following proportions: 5% CBH1, 28% EG1, 5% BG, 52% EX3, 5% BX and 5% CBH2. This set was not as efficient as the commercial enzyme solutions, indicating that some enzyme activities were missing.

Gottschalk et al. (2010) used mixtures of different hydrolytic enzymes expressed by *Trichoderma reesei* and *Aspergillus awamori* to hydrolyze steam-treated sugarcane bagasse. The enzymes produced by *T. reesei* were carboxymethyl cellulase, beta-glucosidase and xylanase. *A. awamori* expressed the same enzymes with the addition of ferulic acid esterase. The highest glucose yield of 81.1% was achieved after 72 h with 50% enzyme solution from *T. reesei* and 50% enzyme solution from *A. awamori*. The 50/50 combination had the following activities in IU/g: 111, carboxymethyl cellulase; 205, beta-glucosidase; 489, xylanase; and 0.7, ferulic acid esterase. With this combination a xylose yield of 91.0% was achieved.

It can be concluded that a combination of enzyme activities can lead to an improved breakdown of lignocellulosic biomass, where EG, EX, BG, BX, ferulic acid esterase, arabinofuranosidase and mannanase are the key enzymes.

3.4 Filamentous fungi

Filamentous fungi are eukaryotes belonging to the deuteromycetes (Madigan and Martinko 2006). *Penicillium*, *Aspergillus* and *Candida* are typical examples of this group

(Madigan and Martinko 2006). Filamentous fungi grow by extension of cells at the end of an existing cell. The cells divide and create branches. A single branch is called a hyphae. These hyphae grow as a network that is called the mycelia. Filamentous fungi can also create spores, which are resistant against dryness, heat, freezing and certain chemicals (Madigan and Martinko 2006). The life cycle of filamentous fungi starts at an existing mycelium. Hyphae grow upwards (into the air) and at the ends conidiospores grow. At the end of the conidiospores, the conidias (spores) are formed (conidiation), which get transported by air or by being carried. When the conditions are beneficial for growth, the spores will sprout and become hyphae (Madigan and Matinko 2006). The growth of filamentous fungi on solid surfaces is different than in liquid medium. Unlike bacteria or yeast, filamentous fungi are adapted to growth on solid surfaces (Prosser and Tough 1991). The extension of hyphae facilitates the exploration of the environment for new nutrients (Prosser and Tough 1991). Young mycelia grow in the form of a circle, with an initial exponential phase followed by a deceleration phase leading to a linear increase of the colony radius (Prosser and Tough 1991). Given enough medium surface, the linear growth phase can continue indefinitely. The growth of mycelia can be expressed by the following equation:

$K = \mu \cdot w$ (Prosser and Tough 1991)

K is the radial growth rate, μ is the specific growth rate and w is the width of the peripheral growth zone and may be determined experimentally.

The branching density depends on nutrient concentration around and underneath the colony. Reduced nutrient concentration leads to a reduced branching density, which allows the mycelia to grow faster in length, finding other areas with higher nutrient concentration. With high nutrient concentration, the branching density increases in order to utilize more of the nutrients (Prosser and Tough 1991). Colony differentiation appears when nutrients in the center of the colony are depleted. Then the colony has an outer ring of vegetative hyphae and an inner center of sporulating cells.

The growth of filamentous fungi in liquid medium is in some aspects similar to the growth characteristics of unicellular organisms. While turbidity can be used to measure cell mass of unicellular organisms, often it cannot be used for filamentous fungi, since growth leads to the formation of pellets (Prosser and Tough 1991). Furthermore, since mycelia adhere to surfaces, growth in continuous stirred tank reactors can lead to fouling of pipelines or accumulation of the organisms on the agitator or tank walls, leading to a non-uniform distribution within the tank (Prosser and Tough 1991). The pellet formation can lead to mass transfer issues in fermentation processes, where nutrients and oxygen need to diffuse to the middle of the pellet and products need to diffuse back into the medium (Huang and Bungay 1973). Two growth forms need to be distinguished characterizing filamentous fungi growth in liquid medium. The first form of growth that will be discussed is the dispersed mycelia, which is equivalent to a homogeneous mixed culture of unicellular organisms (Prosser and Tough 1991). In a batch culture, the growth is characterized by a lag phase, followed by exponential growth. Linear growth can be a result of limitations in a continuously supplied nutrient, e.g. oxygen. After the exponential growth phase, a deceleration phase is initiated. The

deceleration phase is due to substrate limitation, accumulation of inhibitory compounds, changes in pH and/or formation of secondary metabolites (Prosser and Tough 1991). Oxygen limitation can be a result of a change in rheological properties. Mycelial cultures lead to non-Newtonian fluid properties, reflected as shear thinning or pseudoplastic behavior where the apparent viscosity decreases with increasing agitation (Allen and Robinson 1990; Blakebrough et al. 1978; Pedersen et al. 1993). Nutrient and heat transport are also affected by the rheological properties (Prosser and Tough 1991). An increase in viscosity also leads to reduced mixing efficiency and an increase in process cost (Prosser and Tough 1991).

The second growth form is the formation of pellets. It is important to know that the form of filamentous fungi in liquid medium shows a continuous range from dispersed mycelia to pellets (Prosser and Tough 1991). In contrast to dispersed mycelia, a pellet solution behaves like a Newtonian fluid (Kim et al. 1983), which is beneficial for mixing purposes. Furthermore, no fouling inside the reactor or piping takes place (Prosser and Tough 1991). A pellet stained with cresyl-violet shows four distinguished layers (Whittler et al. 1986). The outermost layer is heavily stained and is composed of growing hyphae. The next layer is less heavily stained and shows signs of autolysis. When a hollow pellet is present, a third layer can be seen showing an irregular wall structure. The fourth layer is a hollow center. The hollow center is believed to be a result of limited nutrient and oxygen transfer causing autolysis (Prosser and Tough 1991). The formation of pellets in liquid medium depends on a variety of factors. The most important factor is agitation (Metz and Kossen 1977). Agitation causes the removal of hyphae from the pellet surface and direct rupture of pellets (Taguchi et al. 1968). In general increasing

agitation rate decreases the pellet size (Prosser and Tough 1991). The growth medium composition also affects pellet formation as well as extracellular polysaccharides, inoculum concentration, specific growth rate, aeration, surfactants, pH and suspended solids.

3.5 Enzyme production

The production of enzymes in large scale is currently performed in solid state fermentations (SSF) or submerged cultures (SmF). One of the largest cellulolytic enzyme producers, Novozymes located in Denmark, uses submerged culture techniques that are a well-kept secret (Novozyme 2010a). Bacteria and fungi are used in fed-batch or continuous systems.

The University of Sao Paulo conducts intense research in xylanase production (Betini et al. 2009; de Carvalho Peixoto-Nogueira et al. 2009). Their main focus is in optimizing the enzyme expression of xylanases for the paper bleaching industry. The researchers found *Aspergillus niveus* and *Aspergillus fumigates* as xylanase producers with high enzyme activities. *A. fumigates* was found to produce the highest activity among the organisms tested with a culture medium by Vogel (1964) showing an specific activity of 109 U/mg protein (total activity = 348 U) (de Carvalho Peixoto-Nogueira et al. 2009). They also compared static to agitated enzyme production and found the activity with the static culture to be 2.3-fold higher than in the agitated culture (de Carvalho Peixoto-Nogueira et al. 2009). Further improvement was achieved by using SSF on inexpensive carbon sources (wheat bran, corncob, rice straw, triturated rice straw, oatmeal, sugarcane bagasse, cassava flour, *Eucalyptus grandis* sawdust), and carbon source mixtures (corncob + wheat bran, rice straw + wheat bran, triturated rice straw +

wheat bran) (Betini et al. 2009). The best activity was achieved with *A. niveus* on wheat bran (2 g solid substrate with 4 ml distilled water at 70 – 80% relative humidity at 30°C for 96 h) with a value of 928 total U. The researchers reported that the specific activity is higher in all carbon source mixtures than in wheat bran alone. Unfortunately, the values for specific activity (U/mg protein) are not given.

Xylanase and cellulase production using mixed SmF was performed by Garcia-Kirchner et al. (2002). The researchers used 4 g of sugarcane bagasse with 180 ml of medium with spores from *Penicillium* sp. CH-TE-001 and/or *Apergillus terrus* CH-TE-013. The flasks were incubated at 29°C on a rotary shaker at 180 rpm. They found a higher cellulase and xylanase production with the mixed culture compared to the single cultures. The enzyme mix, which was not further characterized, showed the highest activity on carboxymethyl cellulose (CMC) and birchwood xylan after 5 days with a value of 1.4 U/ml and 5 U/ml, respectively. No values of protein concentration were given.

A detailed description of SSF is given by Mitchell et al. (2000). This paragraph will concentrate on the general principles. SSF has been used over centuries for food production, namely the Koji technology, where rice is fermented to produce rice wine, also called “sake”. In SSF, microorganisms grow on solid substrate particles with the absence of visible liquid water (Mitchell et al. 2000). The typical water content ranges between 12 and 80 wt% (Cannel and Moo-Young 1980). Wheat bran, sugarcane bagasse and soy bran are, among others, typical substrates for enzyme production (Mitchell et al. 2000). The substrate is characterized as a low cost waste material from agriculture. The general process involves the pretreatment of the substrate including grinding and optional

physico-chemical treatments, followed by inoculation usually with a filamentous fungi and active or passive aeration. The reactor may or may not be agitated and humidity may be controlled. After completion the products need to be leached out (Mitchell et al. 2000).

Typical reactor types for SSF are tray, drum and packed bed bioreactors. Tray reactors are simple to use without active agitation or aeration. The trays' filling height is limited, since overfilling can lead to anaerobic processes and/or overheating (Tunga et al. 1999) with temperature and humidity as the only control parameters (Durand et al. 1997). Due to the large area required, large scale usage of tray reactors is limited. Drum reactors incorporate mixing and can lead to increased heat and mass transfer. The growth can be improved, but sheer forces can limit the final product formation (Nigam et al. 2004). Therefore, the handling on large scale can be difficult. Packed bed reactors are columns, where the substrate is kept in place by a perforated platform. Forced aeration is applied at the bottom of the column (Mitchell and Lonsane 1992). Product retrieval, non-uniform growth and limited heat and mass transfer are common problems of packed bed reactors (Lonsane et al. 1985; Mitchell and Lonsane 1992).

Enzyme productivity is often higher in SSF than compared to submerged fermentation (SmF) (Viniegra-González and Favela-Torres 2004). Viniegra-González et al. (2003) reviewed enzyme production using SSF and SmF in terms of physiological differences that explain the higher enzyme titers in SSF. In an experiment conducted by Romero-Gómez et al. (2000), five times higher invertase concentrations were produced with SSF compared to SmF using *Aspergillus niger* due to higher biomass production. Another experiment conducted by Díaz-Gódínez et al. (2001) for the production of pectinase showed again higher titers using SSF than SmF due to higher biomass

production. They also found that the production with SSF leads to an apparent resistance to catabolite repression, which was indicated by high pectinase titers with SSF using high initial substrate (in this case sucrose) concentrations. In SmF high sucrose concentrations led to an inhibition of product formation. It was also observed that pectinase production with SmF had higher breakdown of pectinase by contaminant protease. An experiment with restricted growth by introduction of a steric hindrance was performed by Aguilar et al. (2001). For the production of tannase, *A. niger* grew on finely ground polyurethane foam (PUF), which had a higher density (113 g/L) than normal sized PUF (15 g/L) with the purpose of restricting mycelia growth. Again, higher titers of tannase were produced with SSF than with SmF, with values of 14,000 U/L and 2,800 U/L, respectively. The biomass levels were found to be 4.5 g/L and 11.5 g/L for SSF and SmF, respectively. Even with limited growth by physical hindrance, SSF performed better than SmF, which was also due to a reduced protease activity in SSF. Protease activity was found to be eight times higher in SmF. Table 3.3 summarizes the advantages and disadvantages of SSF and SmF.

Biofilm reactors have been investigated for many applications with many different reactor types (Cheng et al. 2010). Types of biofilm reactors are: stirred-tank, trickling filter, rotating-disk, membrane biofilm, fluidized-bed and airlift. The choice of reactor depends on the final product, process complexity and costs. Table 3.4 shows the advantages and disadvantages of each reactor type.

Current production processes are too expensive to utilize enzymes on a large scale for low cost products like bioethanol. Novozymes claims on their homepage that they

Table 3.3: Advantages and disadvantages of SmF and SSF.

Culture mode	Advantages	Cause	Disadvantage	Cause
SmF	Continuous production		High apparent viscosity	Due to dispersed mycelia
	Very well understood with well established models		Reduced nutrient transfer	Due to high apparent viscosity
			Reduced oxygen	Due to high apparent viscosity
			Cell degradation	Due to reduced mass transfer
			Reduced productivity	Due to clump or pellet formation and elevated protease formation
			Shear stress of cells	Mixing
SSF	Higher biomass formation	Intact macroscopic mycelia	No continuous fermentation	
	Higher productivity	Intact macroscopic mycelia and higher biomass	Leaching step required	
	Reduced protease formation		Mixing is not possible or reduced	
			Heat transfer	Due to limited mixing

Table 3.4: Comparison of different types of biofilm reactors. Adapted from Cheng et al. (2010).

Reactor type	Advantages	Disadvantages
Stirred-tank	High cell concentration and productivity; long-term production	Shear stress of cells; more mixing needed
Trickling filter	High cell concentration and productivity; low power requirement	Cell fouling; mixing problem; difficult to recover products
Rotating-disk	High cell concentration; good for aerobic strains	Semi-continuous production; high risk of contaminations
Membrane biofilm	High cell concentration and productivity; easy for product separation	Cell fouling, constraints in scale-up applications
Fluidized-bed reactors	Uniform particle mixing and temperature gradient; long-term production	High energy requirement; long biofilm establishment time; shear stress
Airlift	High cell concentration and productivity; relatively low shear stress	High energy requirement; long biofilm establishment time

have reduced their enzyme usage costs to $0.50 per gallon ethanol produced (Novozyme 2010b). To enhance productivity, a trickling-filter is proposed in this dissertation. The macrostructure of the fungus is maintained by keeping the mass transfer at an optimum. The support is inert, since the substrates for enzyme expression and growth are in a dissolved form.

3.5.1 Trickle bed reactor

Trickle bed reactors (TBR) support the growth of microorganisms on a solid surface with continuous or a periodic supply of medium. The medium that trickles down can also be recycled to the top of the column. TBRs have historically been used in the waste water treatment industry. The medium trickles down the solid support without completely submerging the organisms. The distribution of the medium on top of the column must be uniform to avoid channeling throughout the column. Channeling can lead to reduced retention time of the medium, which subsequently leads to a non-uniform growth of the microorganism and an inefficient use of the surface.

In comparison to SmF and SSF, TBR has the advantage to be a continuous process with a mycelium growth that supports high productivity. TBR does not require a leaching step as is needed for SSF. TBR also allows the fungus to grow as a mycelium, which was found to have a higher productivity compared to pellet growth in SmF (Viniegra-González et al. 2003).

Not much research has been done to investigate enzyme production with TBR. Lenz and Hölker (2004) used a lab scale TBR to produce laccase with *Pleurotus ostreatus* (a white-rot fungi). Sugar cane bagasse was used as the solid support. The

researchers achieved 30 times higher enzyme production with the TBR compared to SmF.

The use of a TBR also allows a semi-continuous process, where enzyme is periodically removed and replaced with fresh medium (Couto and Toca-Herrera 2007). This technology should be investigated further and also tested on a larger scale. This technology helps to overcome some of the difficulties that occur with SmF (e.g. high water usage) and SSF (e.g. upscaling), while showing the advantages of both in terms of easy and standardized process control while the organism grows on a solid support with optimized oxygen and mass transport.

3.5.2 Modeling of enzyme production from microorganism growing on surfaces of packing bed reactors

In order to predict enzyme production in a reactor, an appropriate model has to be established. At steady state the substrate consumption in a packing bed or plug flow reactor can be modeled with the following equation (Shuler and Kargi 2002):

$$S_0 - S = \eta \frac{q_P \cdot \overline{X}}{Y_{P/S}} \frac{L \cdot a \cdot A}{F} H \qquad \text{(Eq. 3.1)}$$

Where S_0 = initial (entering) substrate concentration [g/L], S = exiting substrate concentration [g/L], η = effectiveness factor (ratio of the rate of substrate consumption in the presence of diffusion limitation to the rate of substrate consumption in the absence of diffusion limitation), q_p = specific rate of product formation [g product/g cell · h], $\overline{X}$ = average dry cell concentration in g per L bed, A = cross sectional area of column [cm^2], F

= feed flow rate [L/min], $Y_{P/S}$ = product from substrate yield, H = column height [cm], L = biofilm thickness or the characteristic length of the support particle ($L=V_P/A_P$), where V_P and A_P are the volume and surface area of the support particle and a = biofilm or support particle surface area per unit reactor volume (cm^2/cm^3).

The exiting substrate concentration and product concentration are constant. For this model growth is assumed to be negligible. The effectiveness factor is a constant, which incorporates diffusion limitation. In case of no diffusion limitation, $\eta = 1$. With diffusion limitation, η depends on the shape of the support or particles and needs to be calculated with the Thiele modulus. Several equations are available to estimate η.

From the substrate utilization the exiting product concentration can be obtained:

$$P = Y_{P/S}(S_0 - S) \qquad \text{(Eq. 3.2)}$$

In the start up phase, medium is continuously pumped in a recycle without the addition of fresh medium. The system is considered a batch system.

For growth associated products the productivity is:

$$q_P = Y_{P/X} \cdot \mu_g \qquad \text{(Eq. 3.3)}$$

Where $Y_{P/X}$ = product yield from biomass and μ_g = specific growth rate [h^{-1}].

Assuming a different scenario in which fresh medium continuously flows in during the growth phase, then the specific biomass growth rate in a plug flow reactor can be modeled using the following equation (assuming the decay rate is zero) (Tchobanoglous et al. 2003):

$$\mu_g = \frac{Y_{X/S} r_m (S_0 - S)}{(S_0 - S) + k_S \ln\left(\frac{S_0}{S}\right)} \qquad \text{(Eq. 3.4)}$$

The decay rate is the rate in which the cells die and is also called death rate (μ_d). In case a recycle is added, the equation is altered to:

$$\mu_g = \frac{Y_{X/S} r_m (S_0 - S)}{(S_0 - S) + (1+\alpha) k_S \ln\left(\frac{S_i}{S}\right)} \qquad \text{(Eq. 3.5)}$$

$$S_i = \frac{S_0 + \alpha S}{1+\alpha}$$

Where $Y_{X/S}$ = biomass from substrate yield, r_m = maximum specific substrate utilization [g substrate/g cell · h], S_0 = influent concentration [g/L], S = effluent concentration [g/L], S_i = influent concentration [g/L] to reactor after dilution with recycle flow, α = recycle ratio and k_s = half saturation constant [g/L].

If SSF is assumed, Luedeking and Piret (1959) modeled the product formation of fungi with the following relationship:

$$\frac{dP}{dt} = Y_{P/X}\frac{dX}{dt} + kX \qquad \text{(Eq. 3.6)}$$

Where P = product concentration [g/L], $Y_{P/X}$ = product yield (g product/g biomass), k = secondary coefficient of product formation or destruction [h^{-1}] and X = biomass [g/L]. Viniegra-González et al. (2003) related the cell mass formation as a function of biomass.

$$X(t) = \frac{X_M}{1-((X_M - X_0)/X_0)e^{-\mu_M t}} \qquad \text{(Eq. 3.7)}$$

Where μ_M maximum specific growth rate [h^{-1}], X_0 = initial biomass concentration [g/L] and X_M = equilibrium level of X [g/L] for which dX/dt = 0.

For substrate formation:

$$S(t) = S_0 - \frac{X - X_0}{Y_{X/S}} - \frac{mX_M}{\mu_M}\ln\left[\frac{X_M - X_0}{X_M - X}\right] \qquad \text{(Eq. 3.8)}$$

Where m = maintenance coefficient (g S/ g X · h)

For product formation:

$$P(t) = P_0 + Y_{P/X}(X - X_0) + \frac{kX_M}{\mu_M}\ln\left[\frac{X_M - X_0}{X_M - X}\right] \qquad \text{(Eq. 3.9)}$$

3.6 Product formation under zero growth

Most biotechnological processes are used for the production of metabolites (e.g. organic acids, alcohols, extracellular proteins, etc.). The growth of the organism and, therefore, the accumulation of biomass is often not the priority. For these processes the biomass that is produced can be considered a byproduct. The formation of biomass uses carbon and energy compounds that could potentially be used for product formation. In many studies it was proposed to limit carbon and energy supply in chemostats to a level where the supplied energy is equal to the maintenance energy of the cell (Boender et al. 2009; Schrickx et al. 1993; Schrickx et al. 1995). Then, the organism will approach a zero growth rate when the energy consumed is near the maintenance ratio (Pirt 1965). It was shown that submerged filamentous fungi cultures conidiate when nitrogen and carbon are limited (Broderick and Greenshields 1981; Galbraith and Smith 1969; Ng et al. 1972). Previous studies used retentostat cultures to analyze product formation under carbon and energy limited conditions. To achieve a retentostat, a chemostat is equipped with a filter with a pore size smaller than the size of the organism (0.2 μm), which traps the cells in the reactor. After the desired cell mass is reached, the continuously supplied medium has limited carbon and energy content to reduce the metabolic rate. Jørgensen et al. (2010) reported a growth rate of *A. niger* approaching zero after 6 days when grown in a retentostat under carbon and energy limited conditions. However, the growth yield was constant, leading to an increase in biomass over time. An interesting observation from the study was how the percent of respired carbon changed over time (Jørgensen et al. 2010). With decreasing specific growth rate, the percent carbon respired increased. After two days conidiation was observed. This led to an increase of melanin that rapidly increased

after four days. Stress on the cells, initiated by carbon and energy limitation, induces the transcription of *brlA* (Skromne et al. 1995). The transcription factor *brlA* is a positive regulator for conidiation (Adams and Timberlake 1990). From the results presented in previous studies, (Jørgensen et al. 2010; Schrickx et al. 1993; Schrickx et al. 1995; Verseveld et al. 1991), it is not conclusive if a continuous product formation with zero growth rate over a prolonged period of time using carbon and energy limitation is feasible. The results show that over the test period the biomass concentration increases until the end of the experiment. Additionally, no literature of zero growth rate experiments was found with filamentous fungi grown on surfaces. Furthermore, no literature was found initiating a zero growth rate by limiting other essential nutrients in the medium that do not supply the organism with carbon or energy. Examples could be the limitation of vitamins or coenzymes necessary for certain cell functions.

3.6.1 Coenzyme limitation for zero growth rate on *A. nidulans* mutants

As mentioned above, no literature was found testing coenzyme limitation on product formation using filamentous fungi. Coenzymes serve, as the name indicates, as additional functional groups for enzymes in metabolism. Coenzymes link to an enzyme covalently and activate its active catalytic site. Examples of coenzymes are pyridoxine, biotin, thiamine and riboflavin.

A. nidulans mutants can be purchased from the Fungal Genetics Stock Center (FGSC) with various markers. Markers are compounds that are essential for cell growth, which these mutants cannot synthesize for themselves, since they lack a particular gene for synthesis. Several mutants require coenzymes in the medium for growth. A complete

list of available *A. nidulans* mutants with different markers is available online (FGSC 2011).

In theory, when the fungus grows on normal medium and is then exposed to medium missing the marker, it will stop growing but continue to produce the client enzymes of interest. Therefore, a real zero growth rate would be achieved for continuous enzyme production. The following sections will describe potential coenzyme limitations.

3.6.1.1 Pyridoxine limitation

Pyridoxine is a vitamin and precursor for pyridoxal-5'-phosphate (PLP). PLP is involved in many reactions in the amino acid metabolism. One reaction is transamination, where an a-amino acid is converted to an a-keto acid (Voet and Voet 2004). PLP acts as a coenzyme together with the enzyme in the form of a Schiff base. It is also involved throughout amino acid biosynthesis in the conversion of aspartate to lysine, threonine to methionine and a-ketoisocaproate to leucine (Voet and Voet 2004). PLP is a trace element and only needed in very low concentrations. One *A. nidulans* strain, FGSC# 773, is a mutant unable to synthesize its own pyridoxine. Hence, when pyridoxine is removed from the medium, growth is limited. *A. nidulans* expresses XynB as a client protein induced by a maltose promoter (Segato et al. 2011). It became of interest to investigate the performance of a culture previously grown on medium with pyridoxine, placed on medium without pyridoxine. In theory, the limitation of pyridoxine would reduce cell growth to a minimum, allowing the use of the remaining pyridoxine to maintain essential cell functions including protein secretion. However, to prolong cell function and protein production, pyridoxine would need to be replenished frequently.

3.6.1.2 Biotin limitation

Biotin is a cofactor involved in carboxylation reactions. It binds covalently to enzymes by amide linkages (Voet and Voet 2004). Some of the enzymes that use biotin as a prosthetic group are pyruvate carboxylase, acetyl-CoA carboxylase and propionyl-CoA carboxylase. Pyruvate carboxylase is a CO_2 carrier that forms a carboxyl substituent and catalyzes the reaction from pyruvate and HCO_3^- to oxaloacetate, which is then used for gluconeogenesis. Acetyl-CoA carboxylase catalyzes the first step in fatty acid biosynthesis; whereas, propionyl-CoA carboxylase is involved in the oxidation of odd-chain fatty acids (Voet and Voet 2004).

Assuming biotin would be removed from the medium after an *A. nidulans* mutant with a biotin marker is grown, the organism would not be able to perform fatty acid synthesis and carboxylation of pyruvate. Fatty acids are used for cell wall formation. The lack of building a cell wall is assumed to inhibit growth. However, it would be interesting to analyze how the organism would react when biotin is missing in the medium and if it continues the production of client proteins.

3.6.1.3 Limitation of thiamine

Besides biotin or pyridoxine, *A. nidulans* mutants with a thiamine marker are available. In order to achieve zero growth by limiting thiamine, it is important to understand the function of thiamine in the metabolism. Thiamine, a precursor for thiamine-pyrophosphate (TPP), is essential for alcoholic fermentation. More specifically, it is used as a coenzyme for pyruvate decarboxylase catalyzing the reaction from pyruvate to acetaldehyde (Voet and Voet 2004). Since alcoholic fermentation occurs predominantly in anaerobic environments, it can be assumed that the limitation of

thiamine would not have the desired effect of limiting growth of an *A. nidulans* culture growing under aerobic conditions.

3.7 References

Adams TH, Timberlake WE. 1990. Developmental repression of growth and gene expression in *Aspergillus*. Proceedings of the National Academy of Sciences 87(14):5405.

Aguilar CN, Augur C, Favela-Torres E, Viniegra-González G. 2001. Production of tannase by Aspergillus niger Aa-20 in submerged and solid state fermentations: influence of glucose and tannic acid. Journal of Industrial Microbiology and Biotechnology 26(5):296-302.

Alén R. 2000. Structure and chemical composition of wood. In: Stenius P, editor. Forest Products Chemistry. Helsinki: Fapet Oy. p 12-57.

Allen DG, Robinson CW. 1990. Measurement of rheological properties of filamentous fermentation broths. Chemical engineering science 45(1):37-48.

Alvira P, Tomás-Pejó E, Ballesteros M, Negro MJ. 2010. Pretreatment technologies for an efficient bioethanol production process based on enzymatic hydrolysis: A review. Bioresource technology 101(13):4851-4861.

Annonymous. 2010. Sorghum. U.S. Grains Council.

Banerjee G, Car S, Scott-Craig JS, Borrusch MS, Aslam N, Walton JD. 2010. Synthetic enzyme mixtures for biomass deconstruction: production and optimization of a core set. Biotechnology and Bioengineering 106:707-720.

Betini JHA, Michelin M, Peixoto-Nogueira SC, Jorge JA, Terenzi HF, Polizeli M. 2009. Xylanases from Aspergillus niger, Aspergillus niveus and Aspergillus ochraceus produced under solid-state fermentation and their application in cellulose pulp bleaching. Bioprocess and biosystems engineering 32(6):819-824.

Beukes N, Chan H, Doi RH, Pletschke BI. 2008. Synergistic association between Clostridium cellulovorans enzymes XynA, ManA and EngE against sugarcane bagasse. Enzyme and Microbial Technology 42:492-498.

Beukes N, Pletschke BI. 2010. Effect of lime pre-treatment on the synergistic hydrolysis of sugarcane bagasse by hemicelluloses. Bioresource technology 101:4472-4478.

Bjerre AB, Olesen AB, Fernqvist T. 1996. Pretreatment of wheat straw using combined wet oxidation and alkaline hydrolysis resulting in convertible cellulose and hemicellulose Biotechnology and Bioengineering 49:568-577.

Blakebrough N, McManamey WJ, Tart KR. 1978. Rheological measurements on *Aspergillus niger* fermentation systems. Journal of Applied Chemistry & Biotechnology 26:453-461.

Boender LGM, De Hulster EAF, Van Maris AJA, Daran-Lapujade PAS, Pronk JT. 2009. Quantitative physiology of Saccharomyces cerevisiae at near-zero specific growth rates. Applied and environmental microbiology 75(17):5607.

Boerjan W, Ralph J, Baucher M. 2003. Lignin biosynthesis. Annual review of plant biology 54:519-46.

Broderick A, Greenshields R. 1981. Sporulation of *Aspergillus niger* and *Aspergillus ochraceus* in continuous submerged liquid culture. Journal of general microbiology 126(1):193.

Cannel E, Moo-Young M. 1980. Solid state fermentation systems. Process Biochemistry 15:2-7.

Carpita NC, Gibeaut DM. 1993. Structural models of primary cell walls in flowering plants: consistency of molecular structure with the physical properties of the walls during growth. The Plant Journal 3(1):1-30.

Chang VS, Nagwani M, Kim C-H, Holtzapple MT. 2001. Oxidative lime pretreatment of high-lignin biomass. Applied Biochemistry and Biotechnology 94:1-28.

Cheng JJ. 2010. Biological process for ethanol production. In: Cheng JJ, editor. Biomass to renewable energy processes. New York: CRC Press. p 229-234.

Cheng KC, Demirci A, Catchmark JM. 2010. Advances in biofilm reactors for production of value-added products. Applied microbiology and biotechnology 87(2):445-456.

Couto SR, Toca-Herrera JL. 2007. Laccase production at reactor scale by filamentous fungi. Biotechnology advances 25(6):558-569.

de Carvalho Peixoto-Nogueira S, Michelin M, Betini JHA, Jorge JA, Terenzi HF, de Lourdes Teixeira de Moraes Polizeli M. 2009. Production of xylanase by Aspergilli using alternative carbon sources: application of the crude extract on cellulose pulp biobleaching. Journal of Industrial Microbiology and Biotechnology 36(1):149-155.

De Vries RP, Visser J. 2001. *Aspergillus* enzymes involved in degradation of plant cell wall polysaccharides. Microbiology and Molecular Biology Reviews 65(4):497-522.

Diaz-Godinez G, Soriano-Santos J, Augur C, Viniegra-Gonzalez G. 2001. Exopectinases produced by Aspergillus niger in solid-state and submerged fermentation: a comparative study. Journal of Industrial Microbiology and Biotechnology 26(5):271-275.

Duff SJB, Murray WD. 1996. Bioconversion of forest products industry waste cellulosics to fuel ethanol: a review. Bioresource Technology 55:1-33.

Durand A, Almanza S, Renaud R, Maratray J. 1997. Solid state fermentations: an attractive alternative to submerged-liquid fermentations. Agro Food Industry Hi-Tech 8:39-42.

Ebringerova A, Hromadkova Z, Heinze T. 2005. Hemicellulose. Polysaccharides I:1-67.

Faga BA, Wilkins MR, Banat IM. 2010. Ethanol production through simultaneous saccharification and fermentation of switchgrass using Saccharomyces cerevisiae D5A and thermotolerant Kluyveromyces marxianus IMB strains. Bioresource technology 101(7):2273-2279.

Fengel D, Grosser D. 1975. Chemische Zusammensetzung von Nadel-und Laubhölzern. European Journal of Wood and Wood Products 33(1):32-34.

FGSC. 2011. Aspergillus Resources. Kansas City: Fungal Genetics Stock Center.

Galbraith JC, Smith J. 1969. Sporulation of *Aspergillus niger* in submerged liquid culture. Journal of general microbiology 59(1):31.

Garcia-Kirchner O, Muñoz-Aguilar M, Pérez-Villalva R, Huitron-Vargas C. 2002. Mixed submerged fermentation with two filamentous fungi for cellulolytic and xylanolytic enzyme production. Applied biochemistry and biotechnology 98(1):1105-1114.

Girio F, Fonseca C, Carvalheiro F, Duarte L, Marques S, Bogel-Lukasik R. 2010. Hemicelluloses for fuel ethanol: A review. Bioresource technology 101(13):4775-4800.

Gottschalk LMF, Oliveira RA, Bon EPdS. 2010. Cellulases, xylanases, β-glucosidase and ferulic acid esterase produced by *Trichoderma* and *Aspergillus* act synergistically in the hydrolysis of sugarcane bagasse. Biochemical Engineering Journal 51:72-78.

Huang MY, Bungay HR. 1973. Microbe measurements of oxygen concentration in mycelial pellets. Biotechnology and Bioengineering 15:1193.

Johansson P, Brumer III H, Baumann MJ, Kallas ÅM, Henriksson H, Denman SE, Teeri TT, Jones TA. 2004. Crystal structures of a poplar xyloglucan endotransglycosylase reveal details of transglycosylation acceptor binding. The Plant Cell Online 16(4):874-886.

Jørgensen TR, Nitsche BM, Lamers GE, Arentshorst M, van den Hondel CA, Ram AF. 2010. Transcriptomic insights into the physiology of *Aspergillus niger* approaching a specific growth rate of zero. Applied and environmental microbiology 76(16):5344-5355.

Jung HJG, Vogel KP. 1992. Lignification of switchgrass (Panicum virgatum) and big bluestem (Andropogon gerardii) plant parts during maturation and its effect on fibre degradability. Journal of the Science of Food and Agriculture 59(2):169-176.

Kim J, Lebeault J, Reuss M. 1983. Comparative study on rheological properties of mycelial broth in filamentous and pelleted forms. Applied microbiology and biotechnology 18(1):11-16.

Kim S, Dale BE. 2004. Global potential bioethanol production from wasted crops and crop residues. Biomass and Bioenergy 26:361-375.

Kumar P, Barrett DM, Delwiche MJ, Stroeve P. 2009. Methods for pretreatment of lignocellulosic biomass for efficient hydrolysis and biofuel production. Industrial & Engineering Chemistry Research 48(8):3713-3729.

Lenz J, Hölker U. 2004. Trickle-film processing: an alternative for producing fungal enzymes. BIOforum Europe 6.

Lin Y, Tanaka S. 2006. Ethanol fermentation from biomass resources: current state and prospects. Appl Microbiol Biotechnol 69:627-642.

Lonsane BK, Ghidyal NPG, Budiatman S, Ramakrishna SV. 1985. Engineering aspects of solid-state fermentation. Enzyme and Microbial Technology 7:258-265.

Luedeking R, Piret EL. 1959. A kinetic study of the lactic acid fermentation. Batch process at controlled pH. Journal of Biochemical and Microbiological Technology and Engineering 1(4):393-412.

Madigan MT, Martinko JM. 2006. Brock Mikrobiologie. München: Pearson Studium.

Martone PT, Estevez JM, Lu F, Ruel K, Denny MW, Somerville C, Ralph J. 2009. Discovery of lignin in seaweed reveals convergent evolution of cell-wall architecture. Current Biology 19(2):169-175.

Metz B, Kossen NWF. 1977. The growth of moulds in form of pellets - a literature review. Biotechnology and Bioengineering 19:781-799.

Milbrandt A, Laboratory NRE. 2005. A geographic perspective on the current biomass resource availability in the United States: United States. Dept. of Energy.

Millet MA, Baker AJ, Scatter LD. 1976. Physical and chemical pretreatment for enhancing cellulose saccharification. Biotech. Bioeng. Symp. 6:125-153.

Mishra A, Malhotra AV. 2009. Tamarind xyloglucan: a polysaccharide with versatile application potential. J. Mater. Chem. 19(45):8528-8536.

Mitchell D, Berovic M, Krieger N. 2000. Biochemical engineering aspects of solid state bioprocessing. New Products and New Areas of Bioprocess Engineering:61-138.

Mitchell DA, Lonsane BK. 1992. Definition, characteristics and potential. In: Doelle HW, Mitchell DA, Rolz CE, editors. Solid substrate cultivation. London: Elsevier Science Publishers.

Monties B. 1991. Plant cell walls as fibrous lignocellulosic composites: relations with lignin structure and function. Animal Feed Science and Technology 32(1-3):159-175.

Mosier N, Hendrickson R, Ho N, Sedlak M, Ladisch MR. 2005b. Optimization of pH controlled liquid hot water pretreatment of corn stover. Bioresource Technology 96:1986-1993.

Mosier N, Wyman C, Dale B, Elander R, Lee YY, Holtzapple M, Ladisch M. 2005a. Features of promising technologies for pretreatment of lignocellulosic biomass. Bioresource Technology 96:673-686.

Ng A, Smith J, McIntosh A. 1972. Conidiation of *Aspergillus niger* in continuous culture. Archives of Microbiology 88(2):119-126.

Nigam P, Robinson T, Singh D. 2004. Solid-state fermentation: an overview. In: Arora DK, editor. Handbook of fungal biotechnology. New York: Marcel Dekker, Inc.

Novozyme. 2010a. Fermentation. Novozyme. http://www.novozymes.com/en/innovation/our-technology/production-processes/fermentation/Pages/default.aspx

Novozyme. 2010b. Novozymes Cellic® CTec2. The first commercially viable enzyme for cellulosic ethanol production. http://www.bioenergy.novozymes.com/cellulosic-ethanol/cellic-product-information/

O'Sullivan AC. 1997. Cellulose: the structure slowly unravels. Cellulose 4(3):173-207.

Pedersen AG, Bundgaard-Nielsen M, Nielsen J, Villadsen J, Hassager O. 1993. Rheological characterization of medium containing *Penicillium chrysogenum*. Biotechnology and bioengineering 41(1):162-164.

Pereira H, Graça J, Rodrigues JC. 2003. Wood chemistry in relation to quality. In: Barnett JR, Jeronimidis G, editors. Wood quality and its biological basis. Oxford: Blackwell Publishing.

Perlack RD, Stokes BJ. 2011. U.S. Billion-Ton Update: Biomass Supply for a Bioenergy and Bioproducts Industry. U.S. Department of Energy. ORNL/TM-2011/224. Oak Ridge National Laboratory, Oak Ridge, TN. 227p.

Pirt S. 1965. The maintenance energy of bacteria in growing cultures. Proceedings of the Royal Society of London. Series B, Biological Sciences:224-231.

Playne MJ. 1984. Increased digestibility of bagasse by pretreatment with alkalis and steam explosion. Biotechnology and Bioengineering 26:426-433.

Powell J, Hons F, McBee G. 1991. Nutrient and carbohydrate partitioning in sorghum stover. Agronomy journal 83(6):933-937.

Prosser IJ, Tough AJ. 1991. Growth mechanisms and growth kinetics of filamentous microorganisms. Biotechnology 10(4):253-274.

Romero-Gomez S, Augur C, Viniegra-González G. 2000. Invertase production by Aspergillus niger in submerged and solid-state fermentation. Biotechnology Letters 22(15):1255-1258.

Saha BC. 2003. Hemicellulose bioconversion. Journal of industrial microbiology & biotechnology 30(5):279-291.

Salem HB, Nefzaoui A, Rokbani N. 1994. Upgrading of sorghum stover with anhydrous ammonia or urea treatments. Animal feed science and technology 48(1-2):15-26.

Schrickx JM, Krave AS, Verdoes JC, van den Hondel CA, Stouthamer AH, van Verseveld HW. 1993. Growth and product formation in chemostat and recycling cultures by Aspergillus niger N402 and a glucoamylase overproducing transformant, provided with multiple copies of the glaA gene. Journal of general microbiology 139(11):2801.

Schrickx JM, Raedts MJ, Stouthamer AH, Verseveld HWv. 1995. Organic acid production by *Aspergillus niger* in recycling culture analyzed by capillary electrophoresis. Analytical Biochemistry 231:175-181.

Segato F, Damásio ARL, Gonçalves TA, Lucas RC, Squina FM, Decker S, Prade RA. 2011. Secretion of client proteins in Aspergillus. Submitted.

Shuler ML, Kargi F. 2002. Bioprocess Engineering Basic Concepts. Upper Saddle River: Prentice Hall PTR.

Skromne I, Sánchez O, Aguirre J. 1995. Starvation stress modulates the expression of the *Aspergillus nidulans* brlA regulatory gene. Microbiology 141(1):21.

Sun Y, Cheng J. 2002. Hydrolysis of lignocellulosic materials for ethanol production: a review* 1. Bioresource Technology 83(1):1-11.

Suryawati L, Wilkins MR, Bellmer DD, Huhnke RL, Maness NO, Banat IM. 2008. Simultaneous Saccharification and Fermentation of Kanlow Switchgrass Pretreated by Hydrothermolysis using *Kluyveromyces marxianus* IMB4. Biotechnology and Bioengineering 101:894-902.

Taguchi H, Yoshida T, Tomita Y, Teramoto S. 1968. The effects of agitation shock on mycelia on agitation of mycelial suspensions. Journal of Fermentation Technology 46:814.

Tchobanoglous G, Burton FL, Stensel HD. 2003. Wastewater engineering - Treatment and Reuse. New York: McGraw-Hill.

Timell TE. 1965. Wood hemicelluloses. Advances in Carbohydrate Chemistry 20:409-483.

Tunga R, Banerjee R, Bhattacharyya BC. 1999. Studies of some physical parameters for large scale protease production by SSF. Bioprocess Engineering 21:107-112.

Uzal EN, Gómez Ros LV, Pomar F, Bernal MA, Paradela A, Albar JP, Ros Barceló A. 2009. The presence of sinapyl lignin in Ginkgo biloba cell cultures changes our views of the evolution of lignin biosynthesis. Physiologia Plantarum 135(2):196-213.

Vanholme R, Demedts B, Morreel K, Ralph J, Boerjan W. 2010. Lignin biosynthesis and structure. Plant physiology 153(3):895-905.

Verseveld HW, Metwally M, Sayed M, Osman M, Schrickx JM, Stouthamer AH. 1991. Determination of the maximum product yield from glucoamylase-producing Aspergillus niger grown in the recycling fermentor. Antonie van Leeuwenhoek 60(3):313-323.

Viniegra-González G, Favela-Torres E. 2004. Basic principles for the production of fungal enzymes by solid-state fermentation. In: Arora DK, editor. Handbook of fungal biotechnology. New York: Marcel Dekker, Inc.

Viniegra-González G, Favela-Torres E, Aguilar CN, Rómero-Gomez SJ, Díaz-Godínez G, Augur C. 2003. Advantages of fungal enzyme production in solid state over liquid fermentation systems. Biochemical Engineering Journal 13(2-3):157-167.

Voet D, Voet JG. 2004. Biochemistry. Hoboken: John Wiley & Sons, Inc.

Vogel HF. 1964. Distribution of lysine pathways among fungi: evolutionary implications. American Naturalist 98:435-446.

Weil JR, Brewer M, Hendrickson R, Sarikaya A, Ladisch CM. 1998. Continuous pH monitoring during pretreatment of yellow poplar wood sawdust by pressure cooking in water. Applied Biochemistry and Biotechnology 70:99-111.

Whittler R, Baumgartl H, Lubbers DW, Schugerl K. 1986. Investigation of oxygen transfer into *Penecillium chrysogenum* pellets by microbe measurement. Biotechnology and Bioengineering 28:1024.

Zheng YZ, Lin HM, Tsao GT. 1998. Pretreatment for cellulose hydrolysis by carbon dioxide explosion. Biotechnol Prog 14:890-896.

CHAPTER IV

OPTIMIZATION OF ENZYMATIC HYDROLYSIS OF GRAIN SORGHUM STOVER

4.1 Abstract

Agricultural residues have been identified as an abundant and renewable resource for the biofuels, bioproducts and chemical industries. Grain sorghum stover (GSS) is a potential waste material in the US. The complex cell wall structure of agricultural residues is composed of lignocellulosic fibers. The conversion of lignocellulosic material to fermentable sugars has proven to be an economic challenge. Physico-chemical pretreatment followed by enzymatic hydrolysis is required to produce the desired monomers. In order to achieve optimum utilization of organic matter, multiple enzyme activities are necessary to hydrolyze cellulose and hemicellulose. Cellulose requires endo- and exoglucanase as well as beta-glucosidase for complete breakdown. For hemicellulose xylanase, ferulic acid esterase and mannanse, among others, have been shown to improve enzymatic hydrolysis. The present study investigated the enzymatic hydrolysis of untreated and liquid hot water pretreated GSS by using Cellic CTec2 as the cellulolytic enzyme combined with xylanase B (XynB), ferulic acid esterase (FAE) and mannanase (Man) for hemicellulose breakdown. The pretreatment improved the glucose conversion from 25.4% with a CTec2/XynB combination to 76.6% with CTec2 as the only enzyme. The addition of XynB, FAE and Man did not improve the glucose conversion of pretreated GSS. Xylose conversion showed the same pattern for the different enzyme mixtures. On non-pretreated GSS, xylose conversion was highest with CTec2/XynB with 17.7%; while, on pretreated GSS, xylose conversion was 68.1% with CTec2 alone. Synergistic effects on glucose conversion were observed with a XynB/FAE combination, with a degree of synergy (DS) of 1.83 and 16.2 on pretreated and non-pretreated GSS, respectively. Milder pretreatment conditions should be investigated, since milder conditions lead to higher hemicellulose content. With higher hemicellulose content, XynB and FAE could affect the glucose conversion by reducing heat and/or time requirement for the pretreatment.

.

4.2 Introduction

The utilization of biomass, such as wood residues, grasses, and agricultural residues, for the production of value added products from sugar fermentation (e.g. bioethanol) is only possible when the carbohydrate structure can be hydrolyzed to sugars in high yields in an efficient and cost effective way. After physico-chemical pretreatments, enzymatic hydrolysis is necessary for sugar formation. Enzyme production and enzymatic hydrolysis are expensive and need to be optimized.

Whereas cellulose degradation is widely understood, hemicellulose degradation is still under intense investigation. Hemicellulose degradation needs a variety of enzymes since hemicellulose is composed of a variety of sugars. The main constituent of many hemicelluloses are xylans with a homopolymeric xylose backbone with random side chains composed of arabinose, glucuronic acid, ferulic acid and acetic acid (Cheng 2010). Xylanase is an endo-enzyme for xylans. Beta-xylosidase degrades xylo-oligosaccharides to xylose. Pectin, a family of heteropolymeric and homopolymeric polysaccharides dominated by galacturonic acid units and homogalacturonan, is another major component of hemicellulose and involves pectinase for degradation (Cheng 2010). The bonds between hemicellulose and lignin can be cleaved by ferulic acid esterase (Cheng 2010).

Optimization of enzymatic hydrolysis can be achieved by expressing enzymes with higher activities, increasing the protein excretion of host organisms, or using multiple enzymes to achieve synergistic affects. Beukes et al. (2008) showed that a mixture of enzymes is more effective for biomass breakdown than the use of single enzymes. Xylanase A, endo-glucanase E and mannanase A with a molar ratio of 25%, 25%, and 50%, respectively, achieved a degree of synergism of 4.46. The sugarcane

bagasse used in the experiment was not further pretreated, and it can be expected that an appropriate pretreatment would lead to even higher activities and degrees of synergism. Since lignin affects the enzymes' ability to reach cellulose, a pretreatment that removes lignin would lead to improved hydrolysis.

A follow up work to Beukes et al. (2008) was published (Beukes and Pletschke 2010) and incorporated a lime pretreatment (0.4 g lime/g dry bagasse, 70°C, for 36 h at 100 rpm) on sugarcane bagasse before enzymatic hydrolysis. The highest activity of about 600 μmol/min sugar produced was achieved with a combination of three enzymes with the molar ratio of 37.5% arabinofuranosidase, 25% mannanase and 37.5% xylananse; whereas, the same enzyme combination achieved only 60 U on non-pretreated bagasse, showing that pretreatment improves enzymatic hydrolysis. The degree of synergy was also 2.2 times higher on pretreated bagasse compared to non-pretreated bagasse with the same enzyme mixture.

Another test with a synthetic enzyme mixture was performed on ammonia fiber explosion (AFEX) pretreated corn stover (Banerjee et al. 2010). The researchers investigated Spezyme CP, Novozyme 188, Accellerase 1000, and Multifect-xylanase in various combinations, as well as various enzymes with single activities. The total protein concentrations used were 7.5, 15, and 30 mg/g total stover glucan. The highest glucan conversion of 64.3% was achieved with 50 mass% Accellerase 1000 and 50 mass% Multifect-xylanase, and a total protein concentration of 30 mg/g glucan. These conditions also showed the highest xylan conversion of 44.7%. The enzymes that were tested with single activity were: cellobiohydrolase1 (CBH1), cellobiohydrolase2 (CBH2), endoglucanase1 (EG1), exoglucanase3 (EX3), beta-glucosidase (BG) and beta-xylanase

(BX). The highest glucan conversion of 39.3 % was achieved with 46% CBH1 and all other enzymes with 11% each. Xylan conversion under these conditions was 22.8%; however, the best xylan conversion was achieved with the following proportions: 5% CBH1, 28% EG1, 5% BG, 52% EX3, 5% BX and 5% CBH2. This set of single activity enzymes was not as efficient as the commercial enzyme solutions, indicating that some enzyme activities are missing in the set.

Gottschalk et al. (2010) used mixes of different enzymes from *Trichoderma reesei* and *Aspergillus awomori* on steam-treated sugarcane bagasse. The enzymes produced by *T. reesei* and were carboxymethyl cellulase, beta-glucosidase and xylanase. *A. awamori* expressed the same enzymes with the addition of ferulic acid esterase. The highest glucose yield of 81.1 % was achieved after 72 h with 50% enzyme solution from *T. reesei* and 50% enzyme solution from *A. awamori*. The 50/50 combination had the following activities in IU/g: 111, carboxymethyl cellulase; 205, beta-glucosidase; 489, xylanase; and 0.7, ferulic acid esterase. With this combination a xylose yield of 91.0 % was achieved.

The present study focuses on the enzymatic hydrolysis of raw and liquid hot water pretreated grain sorghum stover (GSS) with Xylanase B (XynB), beta-mannanase (Man), ferulic acid esterase (FAE) and Novozymes' Cellic CTec2 (Ctec2). The effect of pretreatment on raw GSS for enzymatic hydrolysis was evaluated, as well as the effect of different enzyme combinations for glucose and xylose conversion. The degree of synergy (DS) was calculated for enzyme mixtures.

4.3 Materials and Methods

4.3.1 Compositional analysis

Compositional analysis of GSS was performed before and after liquid hot water pretreatment according to the procedures provided by the National Renewable Energy Laboratory (NREL). The first step involved a successive water and ethanol extraction to remove extractable compounds. A Dionex Accelerated Solvent Extractor model 200 was used as described by NREL/TP-510-42619 (Sluiter et al. 2008e). For both solvents, a pressure of 10.34 MPa at 100°C and 3 cycles for 7 min each were used. The samples were air dried until all visible liquid was evaporated. To achieve a higher degree of drying, the samples were dried in a vacuum oven at 40°C for 24 h. The mass of extract was determined. Additionally, an HPLC analysis of the water-extract was performed to determine sugar composition.

The structural carbohydrates and lignin of the extracted GSS were analyzed according to NREL/TP-510-42618 (Sluiter et al. 2008d). The biomass with previously removed extractable compounds was subject to a two-step acid hydrolysis in 72% sulfuric acid at 30°C for 60 min and subsequently with 4% sulfuric acid at 121°C for 20 min in pressurized tubes in an autoclave. The liquid fraction was subject to HPLC analysis to determine structural carbohydrates. Acid soluble lignin was measured with UV/VIS spectroscopy at 320 nm. The remaining solids were burned in a muffle furnace to determine the acid insoluble lignin.

Furthermore, the moisture and ash content were determined with NREL/TP-510-42621 (Sluiter et al. 2008a) and NREL/TP-510-42622 (Sluiter et al. 2008b), respectively.

After the pretreatment, structural carbohydrates and lignin composition of the pretreated GSS solids were determined as described above. Additionally, the liquid fraction, called the prehydrolyzate, was analyzed for sugars and degradation products via HPLC. All samples for HPLC sugar analysis were neutralized with sodium carbonate and filtered through 0.2 μm filters immediately after acid hydrolysis.

4.3.2 Pretreatment

The GSS was pretreated using a 1-L Parr pressure reactor (Parr Series 4520, Parr Instrument Company, Moline, IL). With a solid loading of 10% (based on dry weight) and a total mass of 600 g, the reactor was heated while the biomass was constantly stirred at 500 rpm. After achieving the reaction temperature of 190°C or 200°C, the temperature was held for 10 min. To terminate the reaction, the reactor was placed into an ice bath for swift cooling. The valves were kept closed at all times.

At a temperature of about 40°C, the reactor was opened and the slurry was removed and vacuum filtered through Whatman #5 filter paper. Samples for compositional analysis were removed from the liquid and solid fraction. The solids were washed using 2 L of 60°C warm DI-water to remove all soluble solids, including soluble carbohydrates and inhibitor compounds.

4.3.3 HPLC analysis

Concentrations of cellobiose, glucose, xylose, galactose, arabinose and mannose were analyzed on an HPX-87P column (Bio-Rad, Sunnyvale, Ca.). The eluent was HPLC grade DI-water with a flow of 0.6 ml/min at 85°C and compounds were detected by a

refractive index detector (1100 Series Agilent, Santa Clara, CA, USA) (Sluiter et al. 2008c). For the degradation products furfural and 5-hydroxymethylfurfural, an HPX-87H column (Bio-Rad, Sunnyvale, Ca.) was used with 0.01 N H_2SO_4 as solvent with a flow rate of 0.6 ml/min at 60°C (Sluiter et al. 2008c). Refractive index detection was also used to detect the degradation compounds.

4.3.4 Enzyme production for factorial design

A stainless steel tray was used for culturing. The tray was filled with 500 ml medium that contained a minimal medium with 50.0 ml/L 20x Clutterbuck salts (120 g/L $NaNO_3$, 10.4 g/L KCl, 10.4 g/L $MgSO_4$, 30.4 g/L KH_2PO_4), 1.0 ml/L 1000x trace elements (22 g/L $ZnSO_4 \cdot 7H_20$, 11 g/L H_3BO_3, 5.0 g/L $MnCl_2 \cdot 7H_2O$, 5.0 g/L $FeSO_4 \cdot 7H_2O$, 1.6 g/L $CoCl_2 \cdot 5H_2O$, 1.6 g/L $CuSO_4 \cdot 5H_2O$, 1.1 g/L $Na_2MoO_4 \cdot 4H_2O$, 50 g/L Na_2-EDTA), 10.0 g/L glucose monohydrate, 50.0 g/L maltose monohydrate and 1 ml/L of a 1 g/L pyridoxine solution. The pH of the medium was adjusted to 6.5 using 10 N NaOH. Genetically modified strains of *Aspergillus nidulans* were used for XynB, Man and FAE production. The modification procedure is explained elsewhere (Segato et al. 2011). Spores of *A. nidulans* cultured on three agar plates containing minimal medium were manually scratched off the surface and added to the medium in a tray under sterile conditions. The final spore concentration in each tray was 10^7 spores/ml. Spore count was performed with a Reichert Bright-Line Hemacytometer (Hausser Scientific, Horsham, PA). The trays were incubated at 50°C for 48 h. Since the fungus grew on top of the liquid, a separation of liquid from fungus was achieved by filtration through a filter paper vacuum filtration unit. After filtration the enzyme solution was concentrated using an

Amicon ultrafiltration unit (Millipore, Billerica, MA, USA) with a nominal molecular weight limit of 10,000 daltons using a pressure of 276 kPa and polyethersulfone membrane (Millipore, Billerica, MA, USA) with a diameter of 76 mm. The final volume of enzyme solution was 30 to 40 ml. The concentrated enzyme solution was washed to remove residual sugar by diluting with 250 ml ammonium acetate buffer (25 mM, pH 6) and re-concentrated using the ultrafiltration unit.

4.3.5 Determination of protein concentration

The protein concentration was determined using a Bradford assay. Forty µl of Bradford commassie solution was pipetted into a well of a 96-well plate. The amount of enzyme solution depended on the protein concentration received after filtration. A typical amount was 5 µl. DI water was added to achieve a total volume of 200 µl. The blank well contained only 40 µl Bradford solution and 160 µl DI water. The absorbance was measured using a UV-Vis 96-well plate reader (Tecan Infinite M200, Männedorf, Switzerland) at 595 nm.

4.3.6 Determination of enzyme activity

All enzyme solutions were analyzed for their specific activity on specified substrates. Table 4.1 summarizes the activities and lists the enzymes with the substrate. Sigmacell cellulose and xylan from beechwood were obtained from Sigma-Aldrich (St. Louis, MO, USA). Insoluble wheat arabinoxylan and locust bean gum were obtained

Table 4.1: Overview of enzyme activity with specific substrates.

Enzyme	Substrate	Total protein concentration [g/l]	Activity [U/mg]	Volumetric Activity [U/ml]
CTec2 (10% dilution)	Sigmacell Cellulose, Typ 20	8.31	0.45	3.74
XynB	Xylan from beechwood >90% xylose residues	1.09	83.82	91.36
Man	Locust (Carob) bean gum	1.123	0.81	0.91
FAE	Wheat arabinoxylan insoluble (gel) form	1.11	0.00079	0.00088

from Megazyme (Wicklow, Ireland) and Spectrum (Gardena, CA, USA), respectively. All substrates were dissolved/suspended in 25 mM ammonium acetate buffer pH 6.0 to a final concentration of 1.0 %. The locust bean gum solution was homogenized and boiled. The mixture was allowed to cool over night with constant stirring. To remove insoluble material, the locust bean gum solution was centrifuged at 6,000 rpm for 10 min (Stalbrand et al. 1993). For XynB, Man, and CTec2, 49 µl of substrate solution and 1 µl of enzyme solution were added to 1.5 ml centrifuge tubes. The tubes were inoculated in a water bath at 50°C for various times (depended on enzyme, varied between 7 and 180 min). After inoculation, the tubes were removed from the water bath and 50 µl of dinitrosalicylic acid (DNS) reagent were added immediately to terminate the enzymatic hydrolysis. The tubes were placed in a second water bath at a temperature of 100°C for 5 min to achieve color formation (Miller 1959). After the reaction time, 100 µl of the liquid was transferred in a 96-well plate and analyzed at 575 nm for reducing sugar concentration. The enzyme blank contained 49 µl of 25 mM ammonium acetate buffer pH 6.0 and 1 µl of enzyme solution. The substrate blank contained 49 µl of substrate solution and 1 µl of 25 mM ammonium acetate buffer pH 6.0. FAE activity was determined with 190 µl of wheat arbinoxylans (insoluble) suspension and 10 µl of FAE solution added into 1.5 ml centrifuge tubes. The enzyme and substrate blanks contained buffer instead of substrate or enzyme, respectively. The solutions were incubated at 50 ºC for 2 h. After hydrolysis, the tubes were centrifuged for 5 min at 15,000 rpm. An aliquot of 100 µl was transferred into a 96-well UV-plate. The absorbance of ferulic acid was measured at 310 nm (Faulds and Williamson 1994). With the following equation, the specific activity was calculated based on the spectrophotometer values.

$$U = \left(\left((A - 0.047) / F \right) \times \left(\frac{V_{assay}}{t} \right) \right) / p_{enzyme} \quad \text{(Eq. 1)}$$

With U = specific activity [µmol/mg*min], A = absorbance, F = calibration factor, V_{assay} = assay volume (200 µl), t = incubation time [min], and p_{enzyme} = mass protein in enzyme used [mg].

4.3.7 Enzymatic hydrolysis of grain sorghum stover

For the enzymatic saccharification of GSS, the NREL procedure NREL/TP-510-42629 (Selig et al. 2008a) serves as a basis for the procedure, but was modified for a mixture of the provided enzymes.

A sample amount of GSS with an equivalent of 0.1 g glucan (based on dry basis) was placed into a 20 ml glass scintillation vial. The pH was kept constant at 6.0 by adding 5 ml of a 25 mM ammonium acetate buffer. To avoid bacterial growth, 100 µl of a 2% sodium azide solution were added. DI water was added prior to the enzyme solution. The amount of DI water depended on the volume of the enzyme solution that was added. The total volume was 10 ml. The volume of the enzyme solution depended on the protein content. When more than one enzyme was added, the volume was divided by the number of enzymes to achieve equal protein mass proportions. Table 4.2 shows the dosage for each test in terms of mg protein of enzyme per g glucan in each test tube. A 0 h sample was taken and served as a blank for the substrate and enzymes. The reaction conditions were 50°C with a reaction time of 168 h. Samples were taken at 0, 48, 120, and 168 h. The samples were analyzed via HPLC as described above.

Table 4.2 shows the enzyme combinations and corresponding protein concentrations. In order to obtain the degree of synergy (DS), each enzyme was tested alone on unpretreated and pretreated GSS in different concentrations. The dosage was 3.75, 2.5, and 1.88 mg total protein/g glucan. Equations 2 to 4 were used to calculate DS from the glucan conversion (GC). A value >1 means a synergistic effect is present.

$$DS_2 = \frac{GC_{enzyme1+2}}{\sum_{1\_3.75}^{2\_3.75} GC} \qquad \text{(Eq. 2)}$$

With DS_2 = degree of synergy for two-enzyme mix and $GC_{enzyme1+2}$ = glucose conversion with two-enzyme mix. The denominator is the summation of glucose conversions achieved with the respective two enzymes with a dosage of 3.75 mg total protein/g glucan.

$$DS_3 = \frac{GC_{enzyme1+2+3}}{\sum_{1\_2.5}^{3\_2.5} GC} \qquad \text{(Eq. 3)}$$

With DS_3 = degree of synergy for three-enzyme mix and $GC_{enzyme1+2+3}$ = glucose conversion with three-enzyme mix. The denominator is the summation of glucose conversions achieved with the respective three enzymes with a dosage of 2.5 mg total protein/g glucan.

$$DS_4 = \frac{GC_{enzyme1+2+3+4}}{\sum_{1\_1.88}^{4\_1.88} GC} \qquad \text{(Eq. 4)}$$

Table 4.2: Protein concentration levels in factorial design in mg protein per g glucan and in parenthesis U/g glucan.

Test #	XynB	FAE	CTec2	Man
1	-	-	-	-
2	7.5 (629)	-	-	-
3	-	7.5 (0.006)	-	-
4	-	-	7.5 (3.38)	-
5	-	-	-	7.5 (6.08)
6	-	3.75 (0.003)	3.75 (1.69)	-
7	-	-	3.75 (1.69)	3.75 (3.04)
8	3.75 (315)	3.75 (0.003)	-	-
9	3.75 (315)	-	3.75 (1.69)	-
10	3.75 (315)	-	-	3.75 (3.04)
11	-	3.75 (0.003)	-	3.75 (3.04)
12	-	2.5 (0.002)	2.5 (1.13)	2.5 (2.03)
13	2.5 (210)	2.5 (0.002)	-	2.5 (2.03)
14	2.5 (210)	2.5 (0.002)	2.5 (1.13)	-
15	2.5 (210)	-	2.5 (1.13)	2.5 (2.03)
16	1.88 (157)	1.88 (0.0015)	1.88 (0.85)	1.88 (1.52)

With DS_4 = degree of synergy for four-enzyme mix and $GC_{enzyme1+2+3+4}$ = glucose conversion with four-enzyme mix. The denominator is the summation of glucose conversions achieved with the respective four enzymes with a dosage of 1.88 mg total protein/g glucan.

4.4 Results and Discussion

4.4.1 Chemical composition of grain sorghum stover

GSS was pretreated at 190°C and 200°C using liquid hot water pretreatment. The chemical composition of raw and liquid hot water pretreated GSS is listed Table 4.3. The pretreatment caused the solubilization of hemicellulose, and a reduction of xylan from 16.5% in raw GSS to 3.6% in 200°C pretreated GSS and to 8.7% in 190°C pretreated GSS. With an increase of pretreatment temperature, and therefore an increase in severity of the pretreatment, more hemicellulose (represented by xylan) was solubilized. The extractives were removed as well, leading to an increase of glucan and acid insoluble lignin in pretreated GSS. The magnitude of glucan and acid insoluble lignin increase is dependent on the pretreatment temperature. The glucan content increased from 27.6% to 47.7% at 190°C and to 57.1% at 200°C. The acid insoluble lignin content increased from 17.5% to 31.0% at 190°C and to 35.7% at 200°C. Similar increases in glucan and xylan solubilization was achieved by Faga et al. (2010) and Suryawati et al (2008a). Both studies used the same pretreatment conditions (10 min, 200ºC) on switchgrass. The reduction in xylan was caused by degradation of xylose to furfural,

Table 4.3: Chemical composition of raw (dried) and liquid hot water pretreated grain sorghum stover. Pretreatment conditions: 10% solids and 190ºC and 200 ºC, 10 min.

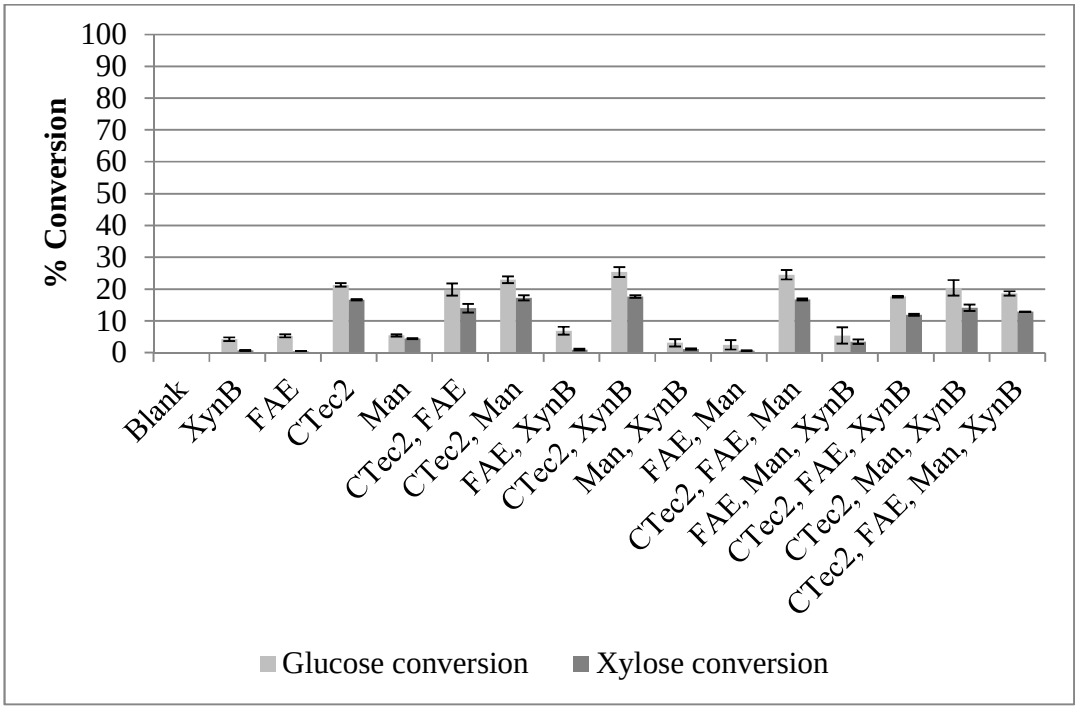
Compound	Before pretreatment	After pretreatment	
		190°C	**200°C**
Glucan	27.6%	47.7%	57.1%
Xylan	16.5%	8.72%	3.6%
Extractables	25.4%	-	-
Lignin (AIL)	17.5%	31.0%	35.7%
Lignin (ASL)	9.5%	0.49%	3.8%

5-hyrdoxymethylfurfural and acetic acid at high temperatures, which has been shown in the literature (Palmquist and Hahn-Hägerdal 2000). The substrate pretreated at 190ºC was chosen for the enzymatic hydrolysis since the hemicellulose content is higher compared to the substrate pretreated at 200ºC.

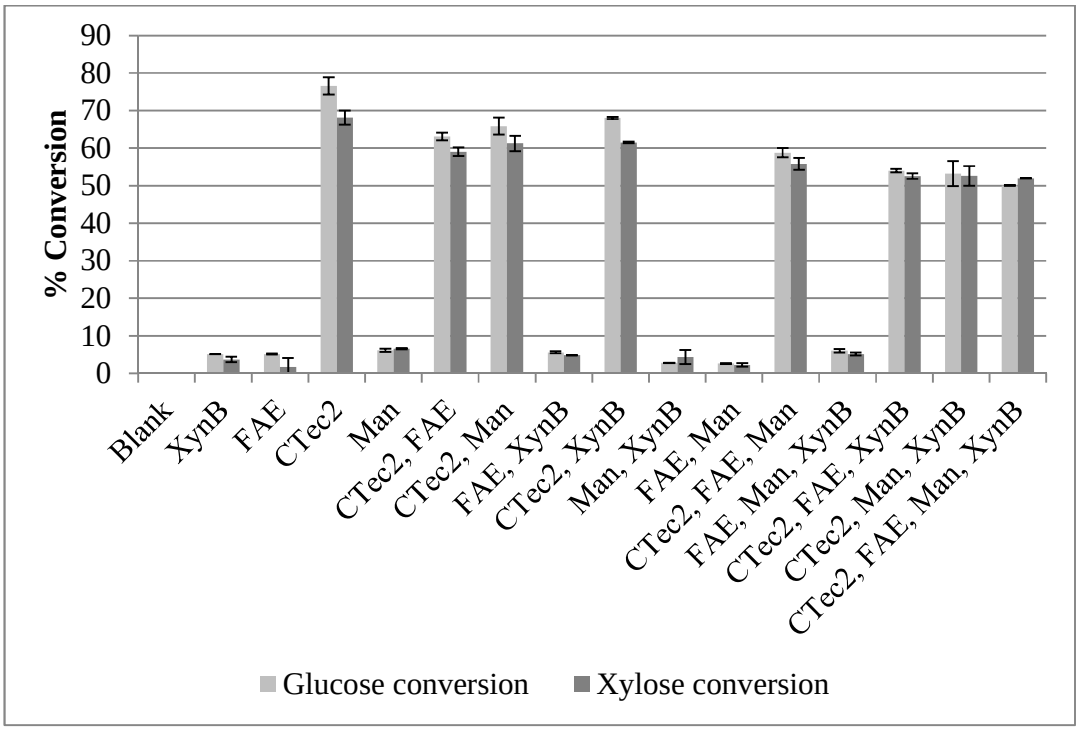
4.4.2 Enzymatic hydrolysis of grain sorghum stover

The objective was to maximize glucose conversion of GSS using liquid hot water pretreatment and a mixture of cellulolytic and hemicellulolytic enzymes. The first observation was that the pretreatment has a positive effect on digestibility, which was also demonstrated in other studies (Alvira et al. 2010; Beukes and Pletschke 2010; Bjerre et al. 1996; Chang et al. 2001; Kumar et al. 2009; Millet et al. 1976). The maximum glucose conversion without pretreatment was 25%; whereas, the maximum glucose conversion with pretreatment was 77% (Figure 4.1 A and B). The lignin and hemicellulose structure interfered with the enzymatic hydrolysis of glucose. Pretreatment leads to a partial dissolution of hemicellulose and an opening of the lignin structure (Alvira et al. 2010). Consequently, the cellulose is more exposed for enzymatic hydrolysis compared to non-pretreated GSS, even though the AIL content is higher in pretreated GSS compared to non-pretreated GSS.

The additional hemicellulolytic enzymes, XynB, FAE, and Man, had little effect on the enzymatic hydrolysis of pretreated GSS. The pretreated GSS was best hydrolyzed with CTec2 alone with a dosage of 7.5 mg/g glucan. The glucose conversion was 77% and the xylose conversion was 68%. It is clear that the commercially available enzyme CTec2 has good hemicellulolytic activity besides cellulolytic activity. The addition of



A



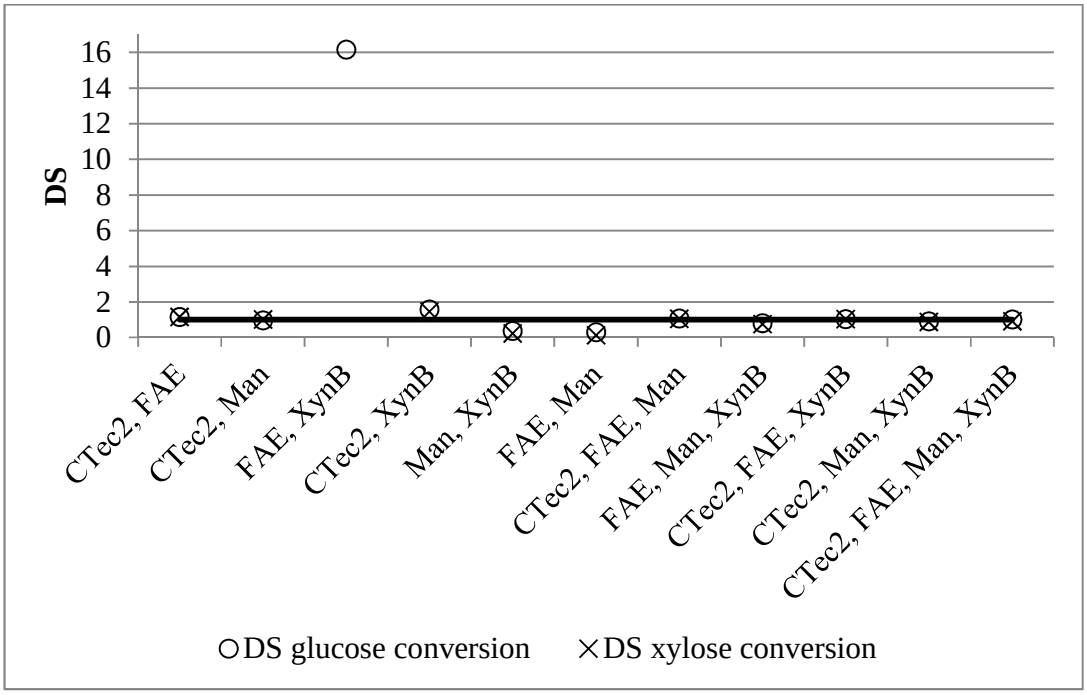
B

Figure 4.1: Comparison of glucose and xylose conversion of raw (A) and pretreated (B) grain sorghum stover using different enzyme combinations. Each test has a total protein dosage of 7.5 mg/g glucan. Each test was performed in duplicates. Error bars represent standard deviation.

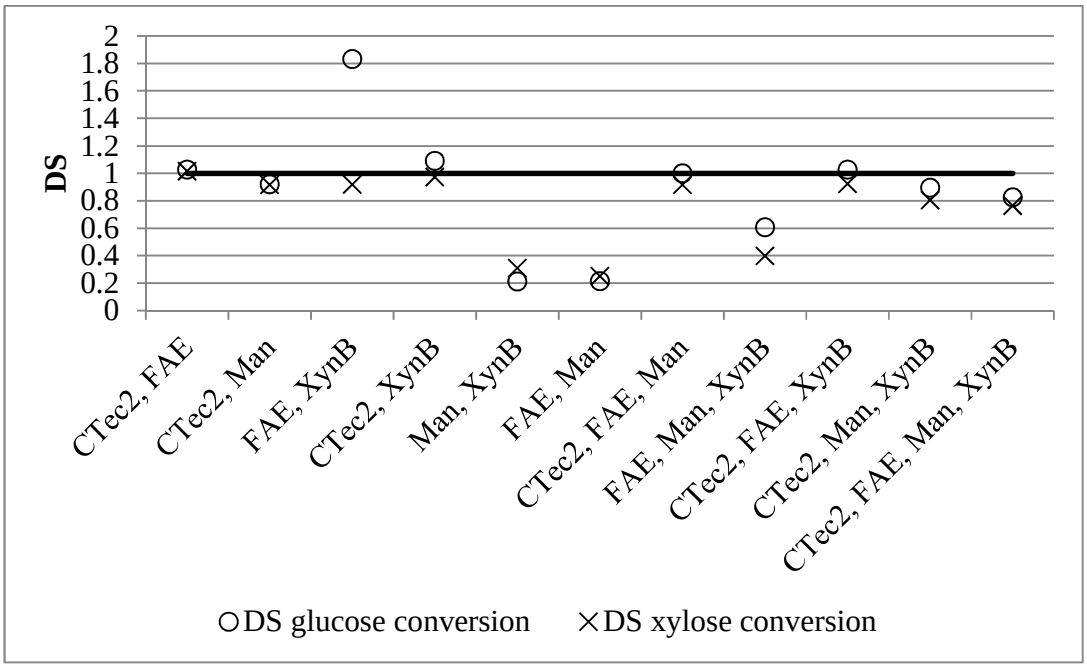
XynB did not improve xylose conversion; however, a synergistic effect was observed when half of the CTec2 dosage was replaced with XynB. The resulting glucose conversion was 68%. A combination of all four enzymes led only to a glucose conversion of 50% and a xylose conversion of 52%.

On non-pretreated GSS, XynB had a positive effect together with CTec2 on glucose and xylose conversion. The mixture of XynB and CTec2 performed better than CTec2 alone with a glucose and xylose conversion of 25% and 18%, respectively, while CTec2 alone only achieved 21% and 17% for glucose and xylose conversion, respectively. The DS was 1.5 for both glucose and xylose conversion (Figure 4.2 A and B). The highest synergistic effect was achieved with XynB and FAE. For glucose conversion on pretreated GSS, the DS was 16. For non-pretreated GSS, the DS was also highest with XynB and FAE with a value of 1.8. However, a combination of CTec2, XynB, and FAE did not improve the glucose or xylose conversion on both substrates compared to CTec2 alone.

Tabka et al. (2006) investigated the enzymatic hydrolysis of wheat straw, pretreated with diluted sulfuric acid pretreatment followed by steam explosion, with a mixture of cellulase, xylanase, and ferulic acid esterase. Their best glucose conversion of 51% was achieved with a combination of cellulose, xylanase and ferulic acid esterase. It is important to note that the researchers used a higher cellulase and ferulic acid esterase dosage than this study (CEL: 10 IU/g of cellulase; XYL: 3 IU/g of xylanase, FAE A: 10 IU/g of Ferulic acid esterase). Furthermore, Tween20 was added, which is known to improve enzyme activity over time by preventing unproductive binding of enzymes to



A



B

Figure 4.2: Comparison of degree of synergy (DS) during enzymatic hydrolysis of raw (A) and pretreated (B) grain sorghum stover for glucose and xylose conversion with different enzyme combinations. Each test has a total enzyme dosage of 7.5 mg/g glucan.

lignin. A synergistic effect between xylanase, ferulic acid esterase and acetyl xylan esterase with cellobiohydrolase was reported by Selig et al. (2008b). However, their maximum cellobiose release from liquid hot water pretreated corn stover was only 25% of the theoretical maximum (with 72.7% glucan in the substrate and a cellobiose release of 180 mg/g substrate). Glucose was only present in small amounts (10 mg/g substate).

CTec2 is a very potent enzyme for the hydrolysis of cellulose and hemicellulose specifically designed for lignocellulosic material. A better understanding of the synergistic effects of XynB, FAE, and Man used in this project could be obtained by using pure cellulolytic enzyme activities with only exo- and endo glucanase, as well as ß-glucosidase. Furthermore, more tests with higher enzyme dosages could lead to higher glucose conversion. Also, the temperature stability of the enzymes could be improved using surfactants, such as Tween20 or Tween80. Additionally, a reduction in pretreatment severity with increasing enzyme dosage could lead to a more cost effective process.

4.5 Conclusion

Pretreating GSS leads to improved conversion of cellulose to glucose, which has been demonstrated repeatedly in other studies on lignocellulosic substrates. The hemicellulolytic enzymes did not improve the enzymatic hydrolysis of pretreated GSS when combined with the commercially available enzyme Cellic CTec2. XynB did improve glucose conversion with CTec2 on non-pretreated GSS.

Additional research with different pretreatment conditions could show improved effects of XynB and FAE in combination with CTec2. At milder pretreatment conditions,

the hemicellulose fraction would be higher in the biomass where XynB and FAE could have a positive effect and DS, leading to high glucose conversion yields. Milder pretreatment conditions require less energy and/or time and contribute to a more economical process.

4.6 Future work

Additional research should be performed with higher enzyme dosages and with the addition of surfactants, which have been shown to stabilize enzymes over time. Tween 20 and Tween 80 have been shown to improve enzymatic hydrolysis of corn stover (Kaar and Holtzapple 1998). Tween prevents thermal deactivation of the enzyme, allowing a prolonged hydrolysis and higher reaction temperatures. Increased enzyme activity and stability was also observed with addition of polyethylene glycol (Ouyang et al. 2010). After 48 h more than 90% of the initial enzyme activity remained in the solution. Sophorolipid was found to increase sugar conversion from cellulose by disrupting the cellulose structure, making it more accessible for enzymes, and by reducing the adsorption of enzyme onto the cellulose (Helle et al. 1993). Furthermore, lower temperatures could be tested on the enzymatic hydrolysis. The optimum temperature of most of the enzymes is 50°C, but the stability over time is reduced at 50°C.

4.7 References

Alvira P, Tomás-Pejó E, Ballesteros M, Negro MJ. 2010. Pretreatment technologies for an efficient bioethanol production process based on enzymatic hydrolysis: A review. Bioresource technology 101(13):4851-4861.

Banerjee G, Car S, Scott-Craig JS, Borrusch MS, Aslam N, Walton JD. 2010. Synthetic enzyme mixtures for biomass deconstruction: production and optimization of a core set. Biotechnology and Bioengineering 106:707-720.

Beukes N, Chan H, Doi RH, Pletschke BI. 2008. Synergistic association between Clostridium cellulovorans enzymes XynA, ManA and EngE against sugarcane bagasse. Enzyme and Microbial Technology 42:492-498.

Beukes N, Pletschke BI. 2010. Effect of lime pre-treatment on the synergistic hydrolysis of sugarcane bagasse by hemicelluloses. Bioresource technology 101:4472-4478.

Bjerre AB, Olesen AB, Fernqvist T. 1996. Pretreatment of wheat straw using combined wet oxidation and alkaline hydrolysis resulting in convertible cellulose and hemicellulose Biotechnology and Bioengineering 49:568-577.

Chang VS, Nagwani M, Kim C-H, Holtzapple MT. 2001. Oxidative lime pretreatment of high-lignin biomass. Applied Biochemistry and Biotechnology 94:1-28.

Faga BA, Wilkins MR, Banat IM. 2010. Ethanol production through simultaneous saccharification and fermentation of switchgrass using Saccharomyces cerevisiae D5A and thermotolerant Kluyveromyces marxianus IMB strains. Bioresource technology 101(7):2273-2279.

Faulds CB, Williamson G. 1994. Purification and characterization of a ferulic acid esterase (FAE-III) from *Aspergillus niger*: specificity for the phenolic moiety and binding to microcrystalline cellulose. Microbiology 140(4):779-787.

Gottschalk LMF, Oliveira RA, Bon EPdS. 2010. Cellulases, xylanases, β-glucosidase and ferulic acid esterase produced by *Trichoderma* and *Aspergillus* act synergistically in the hydrolysis of sugarcane bagasse. Biochemical Engineering Journal 51:72-78.

Helle SS, Duff SJB, Cooper DG. 1993. Effect of surfactants on cellulose hydrolysis. Biotechnology and bioengineering 42(5):611-617.

Kaar WE, Holtzapple MT. 1998. Benefits from Tween during enzymic hydrolysis of corn stover. Biotechnology and bioengineering 59(4):419-427.

Kumar P, Barrett DM, Delwiche MJ, Stroeve P. 2009. Methods for pretreatment of lignocellulosic biomass for efficient hydrolysis and biofuel production. Industrial & Engineering Chemistry Research 48(8):3713-3729.

Miller GL. 1959. Use of dinitrosalicylic acid reagent for determination of reducing sugar. Analytical chemistry 31(3):426-428.

Millet MA, Baker AJ, Scatter LD. 1976. Physical and chemical pretreatment for enhancing cellulose saccharification. Biotech. Bioeng. Symp. 6:125-153.

Ouyang J, Dong Z, Song X, Lee X, Chen M, Yong Q. 2010. Improved enzymatic hydrolysis of microcrystalline cellulose (Avicel PH101) by polyethylene glycol addition. Bioresource technology 101(17):6685-6691.

Palmquist E, Hahn-Hägerdal B. 2000. Fermentation of lignocellulosic hydrolysates. I: inhibition and detoxification. Bioresource Technology 74:17-24.

Segato F, Damásio ARL, Gonçalves TA, Lucas RC, Squina FM, Decker S, Prade RA. 2011. Secretion of client proteins in Aspergillus. Submitted.

Selig M, Weiss N, Ji Y. 2008a. Enzymatic saccharification of lignocellulosic biomass. National Renewable Energy Laboratory NREL/TP-510-42629.

Selig MJ, Knoshaug EP, Adney WS, Himmel ME, Decker SR. 2008b. Synergistic enhancement of cellobiohydrolase performance on pretreated corn stover by addition of xylanase and esterase activities. Bioresource technology 99(11):4997-5005.

Sluiter A, Hames B, Hyman D, Payne C, Ruiz R, Scarlata C, Sluiter J, Templeton D, Wolfe J. 2008a. Determination of total solids in biomass and total dossolved solids in liquid process samples. National Renewable Energy Laboratory(NREL/TP-510-42621).

Sluiter A, Hames B, Ruiz R, Scarlata C, Sluiter J, Templeton D. 2008b. Determination of ash in biomass. National Renewable Energy Laboratory(NREL/TP-510-42622).

Sluiter A, Hames B, Ruiz R, Scarlata C, Sluiter J, Templeton D. 2008c. Determination of sugars, byproducts, and degradation products in liquid fraction process samples. National Renewable Energy Laboratory(NREL/TP-510-42623).

Sluiter A, Hames B, Ruiz R, Scarlata C, Sluiter J, Templeton D, Crocker D. 2008d. Determination of structural carbohydrates and lignin in biomass. National Renewable Energy Laboratory(NREL/TP-510-42618).

Sluiter A, Ruiz R, Scarlata C, Sluiter J, Templeton D. 2008e. Determination of extractives in biomass. National Renewable Energy Laboratory NREL/TP-510-42619.

Stalbrand H, Siika-aho M, Tenkanen M, Viikari L. 1993. Purification and characterization of two [beta]-mannanases from Trichoderma reesei. Journal of biotechnology 29(3):229-242.

Suryawati L, Wilkins MR, Bellmer DD, Huhnke RL, Maness NO, Banat IM. 2008. Simultaneous saccharification and fermentation of Kanlow switchgrass pretreated by hydrothermolysis using Kluyveromyces marxianus IMB4. Biotechnology and bioengineering 101(5):894-902.

Tabka M, Herpoël-Gimbert I, Monod F, Asther M, Sigoillot J. 2006. Enzymatic saccharification of wheat straw for bioethanol production by a combined cellulase xylanase and feruloyl esterase treatment. Enzyme and microbial technology 39(4):897-902.

CHAPTER V

CONTINUOUS ENZYME PRODUCTION UNDER COENZYME LIMITATION

5.1 Abstract

The growth of the organisms in fermentation processes is usually not of primary interest. Growth utilizes nutrients and energy that could possibly be used for product formation. The current solution for limiting or inhibiting growth of microorganisms is the reduction of carbon and energy supply to a level equal to the maintenance energy of the organism. However, control of the exact supply of carbon and energy is difficult, and often the carbon source is used for both growth and product formation. A different approach to limiting growth can be the limitation of co-enzymes. The present study investigated the limitation of pyridoxine on an *Aspergillus nidulans* culture unable to synthesize its own pyridoxine that produces xylanase B (XynB) as client enzyme. It was observed that the growth was limited when pyridoxine was absent, while the enzyme production was unaffected. The enzyme production was similar to a culture that grew on medium with pyridoxine and achieved 1026 U after 480 h of continuous fermentation. Furthermore, the present study investigated the growth rate of *A. nidulans* and determined the productivity of XynB production under pyridoxine limitation. A maximum growth rate of 0.311 h^{-1} and a XynB productivity of 21.14 U/g·h was observed.

5.2 Introduction

Industrial scale enzyme production with filamentous fungi uses mainly two fermentation processes: solid state (SSF) or submerged fermentation (SmF). No other process has proven to be applicable due to the difficulty of culturing filamentous fungi. Filamentous fungi grow as a mycelium, which can lead to clogging of pipelines due to their affinity to surfaces.

Most biotechnological processes are used for the production of metabolites (e.g. organic acids, alcohols, extracellular proteins, etc.). The growth of the organism and, therefore, the accumulation of biomass, is often not the priority. For these processes, the cell mass that is produced can be considered a byproduct. The formation of cell mass uses carbon and energy compounds that could potentially be used for product formation. In many studies it was proposed to limit carbon and energy supply in chemostats to a level where the supplied energy is equal to the maintenance energy of the organism (Boender et al. 2009; Schrickx et al. 1993; Schrickx et al. 1995). Then, the organism will approach a zero growth rate when the energy consumed is near the maintenance ratio (Pirt 1965). It was shown that submerged filamentous fungi cultures conidiate when nitrogen and carbon are limited (Broderick and Greenshields 1981; Galbraith and Smith 1969; Ng et al. 1972). Previous studies used retentostat cultures to analyze product formation under carbon and energy limited conditions. To achieve a retentostat, a chemostat is equipped with a filter with a pore size smaller than the size of the organism (0.2 μm), which traps the cells in the reactor. After the desired cell mass is reached, the continuously supplied medium has limited carbon and energy content to reduce the metabolic rate. Jørgensen et al. (2010) reported a growth rate of *A. niger* approaching zero after 6 days when grown in

a retentostat under carbon and energy limited conditions. However, the growth yield was constant, leading to an increase in cell mass over time. An interesting observation from the study was how the percent of respired carbon changed over time (Jørgensen et al. 2010). With decreasing specific growth rate, the percent carbon respired increased. After two days conidiation was observed, leading to an increase of melanin, which rapidly increased after four days. Stress on the cells, initiated by carbon and energy limitation, induces the transcription of *brlA* (Skromne et al. 1995). The transcription factor *brlA* is a positive regulator for conidiation (Adams and Timberlake 1990). From the results presented in previous studies, (Jørgensen et al. 2010; Schrickx et al. 1993; Schrickx et al. 1995; Verseveld et al. 1991), it is not conclusive if continuous product formation with zero growth rate over a prolonged period of time using carbon and energy limitation is feasible. The results show that over the test period the cell mass concentration increases until the end of the experiment. Additionally, no literature on zero growth rate experiments was found with filamentous fungi grown on surfaces. Furthermore, no literature was found initiating a zero growth rate by limiting other essential nutrients in the medium that do not supply the organism with carbon or energy. Examples could be the limitation of vitamins or coenzymes necessary for certain cell functions.

Reducing the growth rate can maximize product formation, since the substrate is used for product formation and not for microbial growth. A potential compound present in the medium necessary for fungal growth is pyridoxine. Pyridoxine is a vitamin and precursor for pyridoxal-5'-phosphate (PLP), which is involved in many reactions in amino acid metabolism. One reaction is transamination, where an α-amino acid is converted to an α-keto acid (Voet and Voet 2004). PLP acts as a coenzyme together with

the enzyme in the form of a Schiff base. It is also involved throughout amino acid biosynthesis in the conversion of aspartate to lysine, threonine to methionine and α-ketoisocaproate to leucine. PLP is a trace element and only needed in very low concentrations. It is proposed that a limitation of pyridoxine on an *A. nidulans* culture with a pyridoxine marker in the fermentation medium would lead to a shift of substrates to protein formation. The cell matrix has enough PLP stored to perform basic reactions and protein formation without growth. No literature has been found where the limitation of pyridoxine was tested on filamentous fungi.

5.3 Materials and Methods

5.3.1 Strain and spore formation

Aspergillus nidulans with the number 733 in the Fungal Genetic Stock Culture (FGSC) was used to conduct the experiment. The strain has a pyridoxine marker. The modification of *A. nidulans* to express Xylanase B as a client protein is described elsewhere (Segato et al. 2011). Spores were kept in a 20% glycerol, 10% lactose solution as a stock culture at -80ºC. Spores were made by using a solid medium containing 9.0 g/L glucose, 50 ml/L 20X Clutterbuck salt solution (120 g/L $NaNO_3$, 10.4 g/L KCl, 10.4 g/L $MgSO_4$, 30.4 g/L KH_2PO_4), 1 ml/L 1000X trace element solution (22 g/L $ZnSO_4 \cdot 7H_20$, 11 g/L H_3BO_3, 5.0 g/L $MnCl_2 \cdot 7H_2O$, 5.0 g/L $FeSO_4 \cdot 7H_2O$, 1.6 g/L $CoCl_2 \cdot 5H_2O$, 1.6 g/L $CuSO_4 \cdot 5H_2O$, 1.1 g/L $Na_2MoO_4 \cdot 4H_2O$, 50 g/L Na_2-EDTA), 1 ml of a 1 g/L pyridoxine solution, and 15% agar. The pH was adjusted to 6.5 using 10 N NaOH. Twenty µl of stock spore solution were distributed on the agar surface. The plates were kept for 48 h at 37ºC. After incubation the plates were stored at 4ºC until usage.

5.3.2 Fermentation medium

The fermentation medium contained 47.6 g/L maltose and 9.0 g/L glucose as carbon sources. Maltose is necessary to activate the promoter for Xylanase B expression. The medium also contained 50 ml/L 20x Clutterbuck salt solution, 1 ml/L 1000X trace element solution, and 1 ml of a 1 g/L pyridoxine solution. For the maximum growth rate experiment, maltose and glucose concentrations were varied, but were kept at the same ratio (4.7:1) according to best protein formation with 47 g/L maltose and 10 g/L glucose. The pH was adjusted to 6.5 using 10 M NaOH. The medium was sterilized by autoclaving at 121ºC for 20 min.

5.3.3 Determination of maximum growth rate of *A. nidulans*

Before the maximum growth rate (μ_{max}) can be obtained, multiple experiments are necessary to investigate the specific growth rate (μ_{net}) at different initial substrate (S) concentrations. The Monod model was used as the underlying base for μ_{max} and μ_{net} determination. For μ_{net} it is required to measure cell mass concentration over time. Since the organism is a filamentous fungus, representative sampling is not possible. To overcome this problem, multiple parallel tests were started with different inoculation times. The tests were carried out in Petri dishes. Each dish was filled with 20 ml of medium with identical substrate concentration. All parallel tests were inoculated with the same amount of spore solution (20 µl) with the same spore concentration. Two dishes were inoculated for each time interval of 12, 24, 36 and 48 h. After these time intervals, the complete cell mycelium was harvested, washed with deionized water and dried at 50°C for one day. The protein concentration in the broth was analyzed by the Bradford

assay (Bradford 1976). An aliquot of broth was used for sugar analysis with HPLC. The logarithm of dry cell mass was plotted vs. time. The slope of the linear regression in the linear region of the plot represented μ_{net}. This process was repeated for different initial maltose and glucose concentrations. The obtained μ_{net} was plotted in a reciprocal form vs. 1/S. For this plot S represents the glucose concentration plus the glucose equivalent of maltose. A linear regression led to μ_{max} and K_S, which were 0.311 h^{-1} and 1.34 g/L, respectively.

5.3.4 Evaluation of XynB production with *A. nidulans* under pyridoxine limitation

Two tests with different transplanting frequencies, 24 h and 48 h, were chosen. To grow a mycelium, a Petri dish was filled with 20 ml of medium and inoculated with 20 µl of spore solution. The culture was allowed to grow at 37°C until a mycelium covered the surface of the medium. After inoculation part of the mycelium was manually transferred into a Petri dish previously filled with medium lacking pyridoxine. This culture was allowed to grow either 24 h or 48 h and then again transferred into medium without pyridoxine. This was repeated until protein production decreased. Then, the mycelium previously grown on pyridoxine-free medium was transferred into medium with pyridoxine and after either 24 h or 48 h it was again transferred into medium without pyridoxine. The cultures were transferred nine and five times for the 48 h and 24 h culture, respectively. After each transfer interval, the protein concentration was measured by the Bradford assay, the activity was determined with DNS reagent, the wet mass of transferred mycelium was obtained, the pH was measured and the sugar and organic acid concentration were measured with HPLC. Additionally to both tests, a control mycelium,

which was always placed into medium with pyridoxine, was evaluated. Both test and control were evaluated in duplicate.

5.3.5 Determination of *A. nidulans* XynB productivity under pyridoxine limitation

In order to obtain the enzyme productivity under pyridoxine limitation, a mycelium had to be grown on medium with pyridoxine and then transferred into medium without pyridoxine. A Petri dish was filled with 20 ml medium with pyridoxine and was inoculated with 20 µl of spore solution. The culture was grown for 48 h at 37°C. After 48 h the culture was transferred into medium without pyridoxine and after 48 more h it was again transferred into medium without pyridoxine. After this second transfer, the mycelium growth was examined based on visual comparison before and after incubation. After 24 h the medium was analyzed for sugar concentration with HPLC, protein concentration with Bradford assay and xylanase activity with DNS reagent. The mycelium was washed with DI water and dried for one day at 50°C to obtain the dry cell mass. The productivity was determined by calculating the total activity (U) from the volumetric activity (U/ml) and dividing it by the total dry cell mass (g) and 24 h. The enzyme activity was measured to determine the existence of XynB. The test was performed in triplicate and compared to a control culture that grew on medium with pyridoxine.

5.3.6 Determination of protein concentration

The protein concentration was determined using a Bradford assay. Forty µl of Bradford coommassie solution was pipetted into a well of a 96-well plate. In order to stay within the absorbance calibration curve limit of 2.5 at 575 nm, the amount of enzyme

solution varied depending on the protein concentration received from the test. DI water was added to achieve a total volume of 200 µl. The blank well contained only 40 µl Bradford solution and 160 µl DI water. The absorption was measured using a UV-Vis 96-well plate reader (Tecan Infinite M200, Männedorf, Switzerland) at 595 nm.

5.3.7 Determination of XynB activity

XynB was analyzed for its specific activity on xylan from beechwood (Sigma Aldrich). Forty-nine µl of 0.5% substrate solution prepared with 25 mM ammonium acetate buffer at pH 6 and 1 µl of enzyme solution were added into a well of a 96-well plate. The plate was incubated in a water bath at 50°C for 15 min. After incubation, the 96-well plate was removed from the water bath and 50 µl of dinitrosalicylic acid (DNS) reagent was added immediately to terminate the enzymatic hydrolysis. The 96-well plate was placed in a second water bath at a temperature of 100°C for 5 min to achieve the color formation. After the reaction time, 100 µl of the liquid was transferred in a 96-well reading plate and analyzed at 575 nm for reducing sugar concentration. With the following equation the specific activity was calculated based on the spectrophotometer values.

$$U = \left(\left((A - 0.047) / F \right) \times \left(\frac{V_{assay}}{t} \right) \right) / p_{enzyme} \qquad \text{(Eq. 5.1)}$$

With U = specific activity [µmol of reducing sugar/mg protein · min], A = absorbance at 575 nm, F = calibration factor, V_{assay} = assay volume (200 µl), t = incubation time [min] and p_{enzyme} = mass protein in enzyme used [mg].

The final results are given in XynB activity units per volume, U_V.

$$U_V = U \cdot C_P \qquad \text{(Eq. 5.2)}$$

With C_P = protein concentration in mg/ml.

5.3.8 Sugar and organic acid analysis

Concentrations of glucose and maltose were analyzed on an HPX-87P column (Bio-Rad, Sunnyvale, CA, USA). The eluent was HPLC grade DI-water with a flow rate of 0.6 ml/min at 85°C and a refractive index detector (1100 Series Agilent, Santa Clara, CA, USA) (Sluiter et al. 2008c). Organic acids were analyzed on a HPX-87H column (Bio-Rad, Sunnyvale, CA, USA). The eluent was 0.01 N sulfuric acid with a flow rate of 0.6 ml/min at 60ºC. A refractive index detector (1100 Series Agilent, Santa Clara, CA, USA) was used for detection (Sluiter et al. 2008c).

5.4 Results and Discussion

5.4.1 Growth rate of *A. nidulans*

Most of the literature that deals with analyzing growth kinetics of filamentous fungi either use submerged cultures or cultures grown on solid medium or surfaces. When grown as a surface culture, the parameters for growth rate are based on circumferential growth, rather than on a mass per volume unit. The present study uses a surface grown culture, but applies the same methodology as for submerged cultures. Table 5.1 shows the total cell mass, protein and substrate concentrations after 48 h, as well as the specific growth rates for the corresponding initial substrate concentrations and yields. Using parallel tests to imitate sampling at different time points worked well for

Table 5.1: Total cell mass, protein concentration, substrate concentration (glucose + glucose equivalent from maltose), specific growth rate and yields for *Aspergillus nidulans* at different initial substrate concentrations, with standard deviation of two replicates.

Initial substrate concentration [g/l]	Total cell mass after 48 h [mg]	Protein concentration after 48 h [g/L]	Substrate concentration after 48 h [g/L]	μ_{net} [h^{-1}]	$Y_{X/S}$ [g cells/g substrate]	$Y_{P/X}$ [g protein/g cells]	$Y_{P/S}$ [g protein/g substrate]
59.3	119 ± 11.3	0.019 ± 3.54E-5	52.2 ± 0.77	0.061	0.834 ± 0.02	0.0048 ± 9.1E-5	0.0027 ± 0.0003
29.7	93 ± 9.90	0.009 ± 0.002	20.0 ± 3.43	0.063	0.484 ± 0.10	0.0025 ± 7.1E-5	0.0010 ± 0.0008
11.9	79 ± 2.83	0.018 ± 0.002	1.73 ± 0.31	0.095	0.390 ± 0.002	0.0054 ± 0.0003	0.0017 ± 9.4E-5
5.93	57 ± 2.12	0.011 ± 0.0005	0.20 ± 0.02	0.194	0.493 ± 0.017	0.0058 ± 0.0007	0.0020 ± 0.0001
3.00	28 ± 2.12	0.010 ± 0.001	0	0.214	0.464 ± 0.012	0.0187 ± 0.0053	0.0034 ± 0.0003
1.19	14 ± 0.71	0.004 ± 0.0007	0	0.146	0.611 ± 0.030	0.0025 ± 0.0007	0.0017 ± 0.0007

this system and the growth curves showed the familiar pattern observed for many microorganisms. The total cell mass after 48 h increased with increasing initial substrate concentration. However, it was found that μ_{net} does not increase with substrate concentration. In fact, a typical substrate inhibition pattern in the double reciprocal plot of μ_{net} versus S was found (Figure A1). Hence, the fungus grows at a slower rate at higher substrate concentrations.

The highest μ_{net} with a value of 0.214 h^{-1} was found with initial substrate concentrations of 2.4 g/L maltose and 0.45 g/L glucose. At higher substrate concentrations, the fungus indeed grows more slowly, but continuously for at least 48 h. At lower substrate concentrations, the fungus grows fast to exploit all nutrients, but stops growing when the nutrients are depleted. The maximum specific growth rate is 0.311 h^{-1}, and K_S was 1.33 g/L.

It was found that the mycelia of filamentous fungi develop differently for different substrate concentrations in the environment. At high substrate concentration, the hyphae form a dense network with more branches per area. The purpose is to utilize more efficiently the nutrients in that area. When the substrate concentration is low, the hyphae branch less per area and grow longer in order to reach a location with higher substrate concentration (Prosser and Tough 1991). This behavior was also observed in the present study. The cultures with low initial substrate concentration were more like a gel throughout the liquid; whereas, the cultures with high initial substrate concentration developed a mycelium layer on top of the surface of the liquid at the end of the test.

For industrial applications a growth rate based on mass mycelium per time is more useful than a growth rate based on circumferential growth. In technical applications

it is often not practical to measure circumferential growth. Cultures that grow in nutrient rich medium also become thicker (more mass per area), which would not be included in measuring circumferential growth.

5.4.2 XynB production with inhibited growth

It was observed that the fungus produces the desired enzyme, XynB, even when growth is suppressed. Figure 5.1 shows the total accumulative XynB activity over time for the experiment with a transplanting frequency of 48 h. The average XynB activity was 10.0 ± 5.13 U/ml for the culture grown without pyridoxine. The control showed an average XynB activity of 9.78 ± 3.0 U/ml. It was observed that the protein concentration drops to 0.08 g/L after 192 h when grown on medium without pyridoxine. The 240 h and 288 h samples showed a yellow colored broth that smelled similar to urine. In these samples the pH dropped from 8.5 to 4.5. After 288 h the culture was transferred into medium with pyridoxine to recover. At 336 h, no sign of smell was observed and the broth color was again the normal amber color. However, even though the protein concentration was low during 240 h and 288 h, the activity was still comparable to the previous levels. This indicates a higher proportion of xylanase B in the total protein mix. No difference in XynB production was observed between the test without pyridoxine and the control.

With a transplanting frequency of 24 h, the average XynB activity was 9.63 ± 5.64 U/ml and 9.88 ± 2.06 U/ml for the culture grown without and with pyridoxine, respectively. Figure 5.1 shows the cumulative XynB activity over time. The

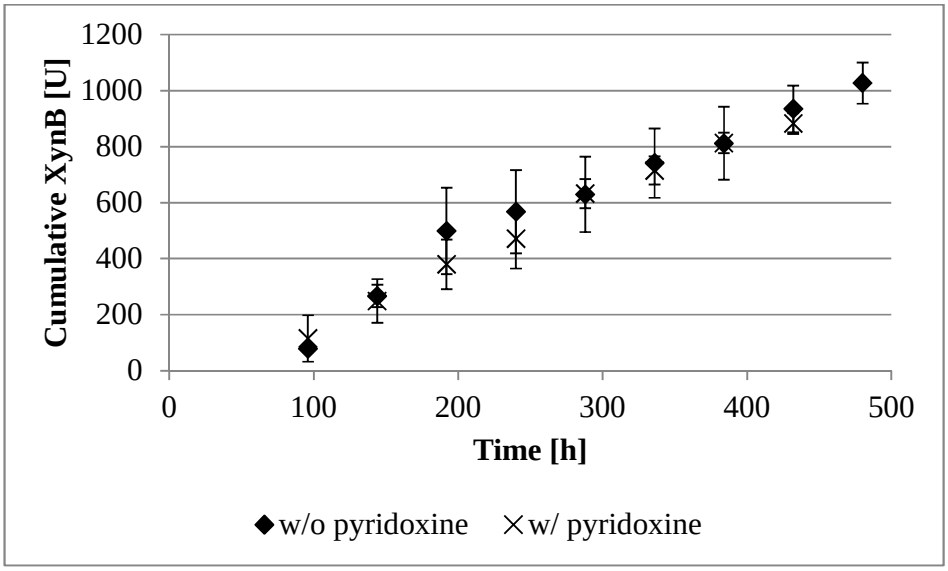


Figure 5.1: XynB production over time with limited pyridoxine and 48 h transplanting frequency. The culture was initially (0-48 h) grown on medium with pyridoxine and again at 288 h. Error bars represent standard deviation of two replicates.

phenomenon of decreased protein formation with simultaneous increase in activity, as was found for both transplanting frequencies, shows that XynB formation is independent of mycelium growth. Furthermore, it can be seen in Figure 5.2, that when the culture was transplanted with a 24 h frequency, the accumulative XynB production reaches the same levels as the culture with 48 h transplanting frequency, but at 192 h as opposed to 480 h. Hence, it is not necessary to use a 48 h frequency, since the same XynB activity is already reached after 24 h. XynB production was higher for the test without pyridoxine compared to the control when a transplanting frequency of 24 h was used.

Substrate utilization is shown in Figure 5.3. At all times maltose was completely utilized for 48 h transplanting frequency. Glucose was always utilized except at 240 h and 288 h when grown on medium without pyridoxine. At 288 h the glucose concentration increased to 12.99 g/L, indicating that the metabolism still utilizes all maltose and converts some of it to glucose, which is then not used further. With a 24 h transplanting frequency, the average residual maltose and glucose concentration was 11.39 g/L and 4.98 g/L, respectively, when grown on medium without pyridoxine (Figure 5.4). The maltose and glucose utilization was higher for the culture grown on pyridoxine than for the culture grown on medium without pyridoxine. The control showed average maltose and glucose concentrations of 5.30 g/L and 2.03 g/L, respectively. Compared to the culture grown without pyridoxine, the control showed new mycelium growth, which requires substrate and explains the faster nutrient utilization. After 192 h pyridoxine needs to be replenished for further enzyme production. The moment of pyridoxine replenishment can be monitored by measuring pH. It is recommended to replenish

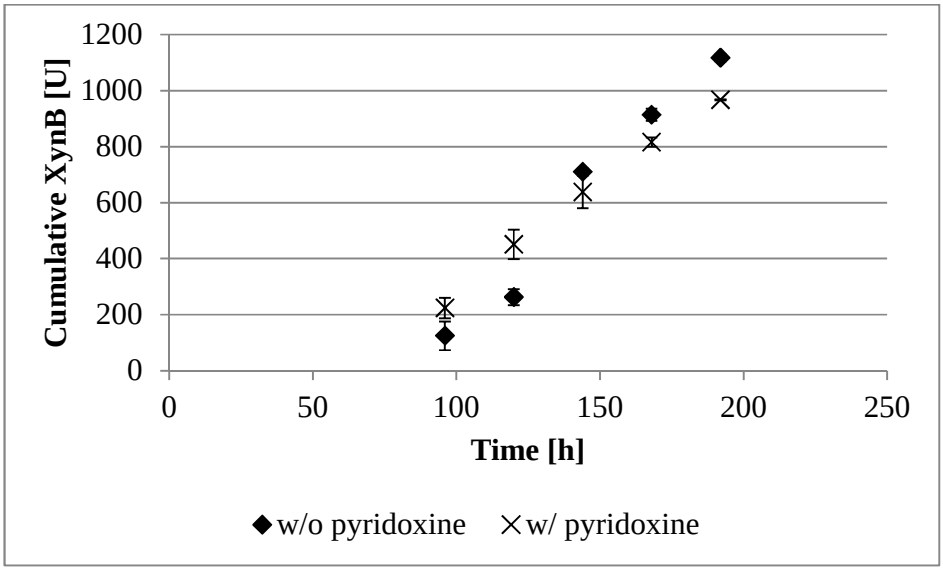
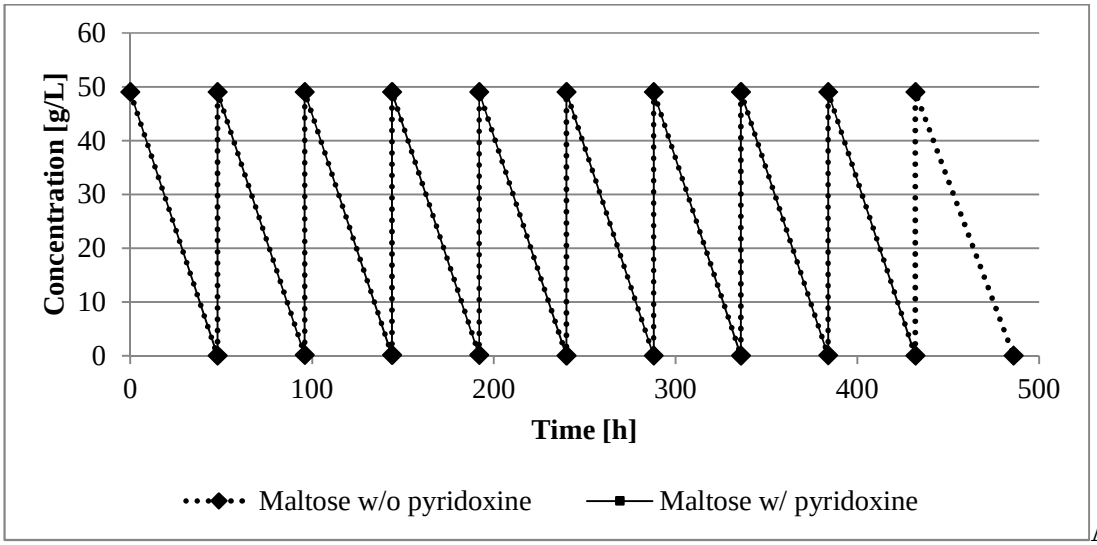
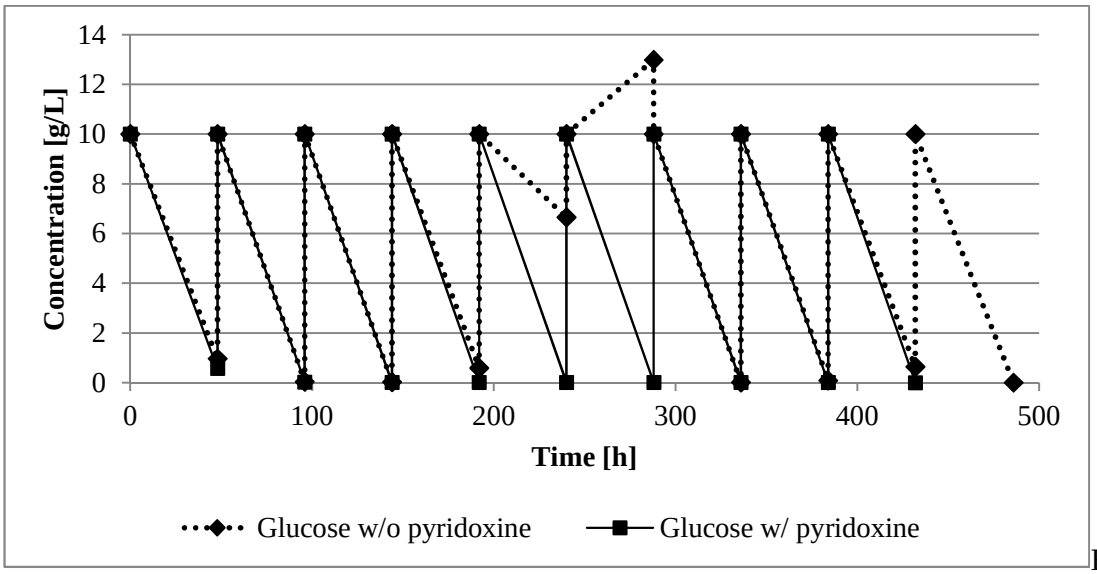


Figure 5.2: XynB production over time with limited pyridoxine and 24 h transplanting frequency. The culture was initially (0-72 h) grown on medium with pyridoxine and again at 144 h. Error bars represent standard deviation of two replicates.

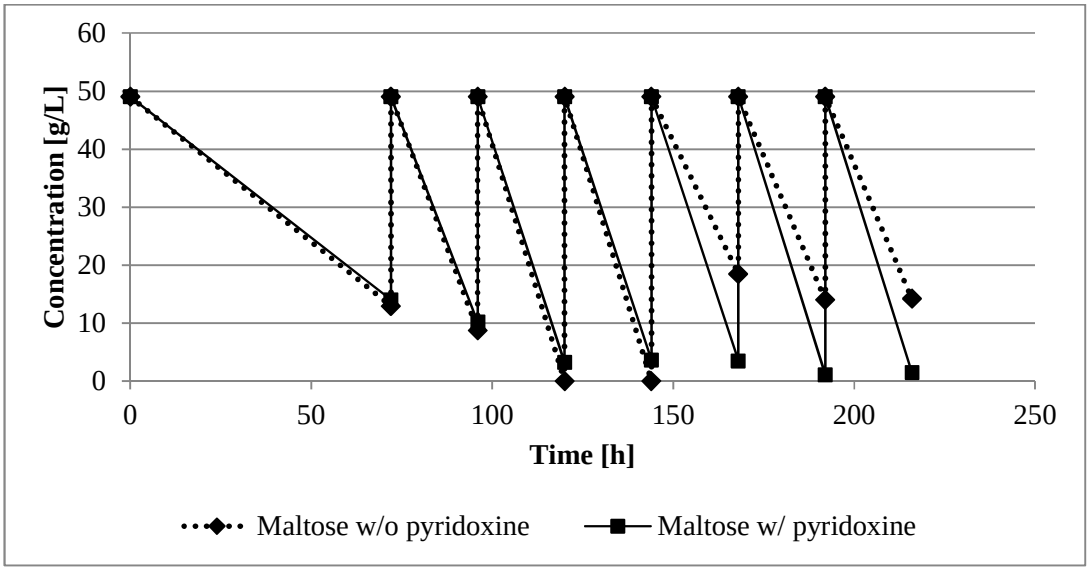


A

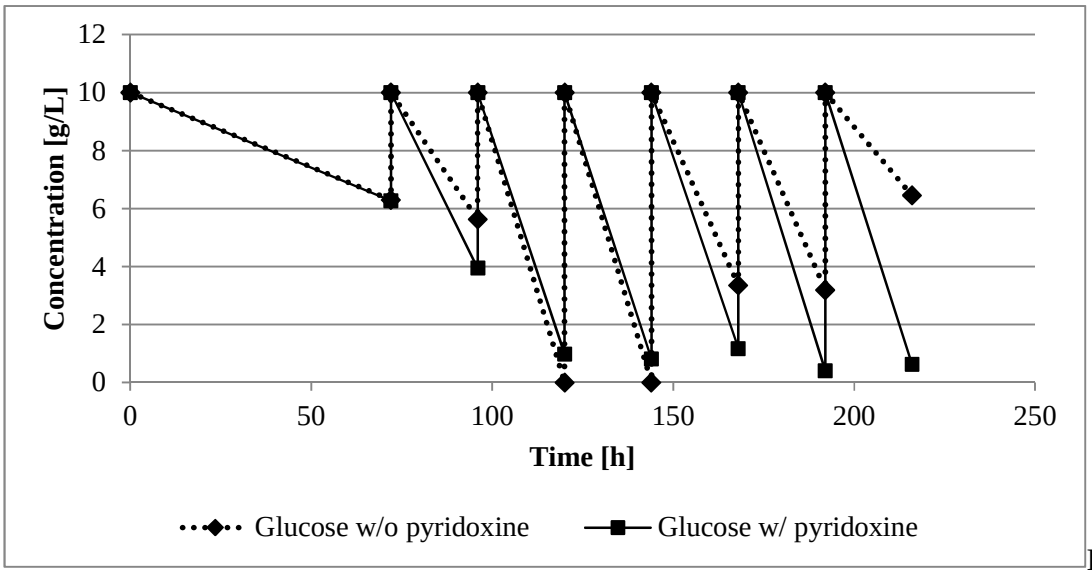


B

Figure 5.3: Maltose (A) and glucose (B) utilization during enzyme production with limited pyridoxine with 48 h transplanting frequency.



A



B

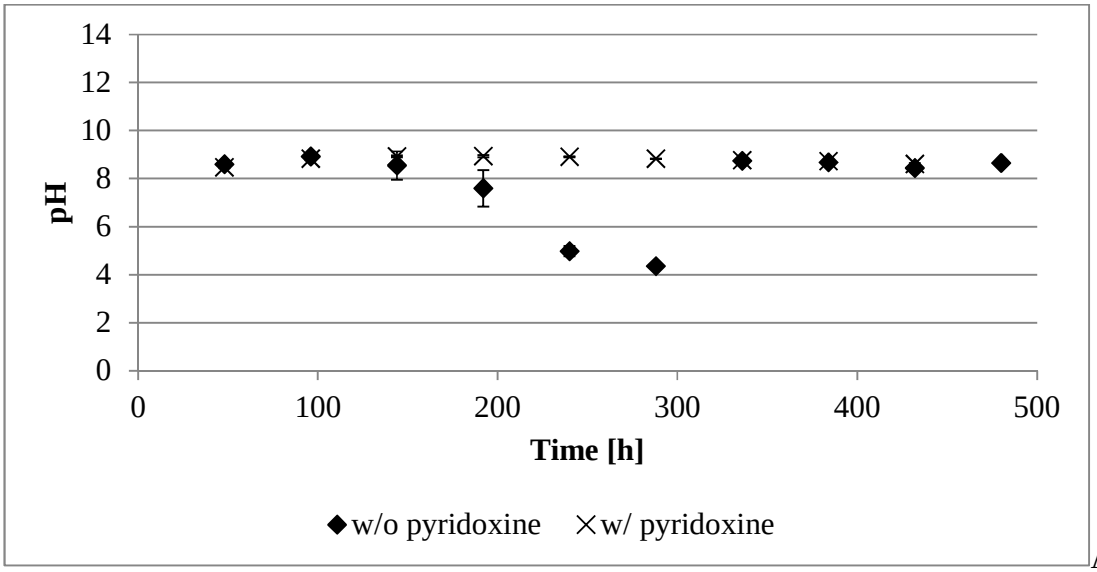
Figure 5.4: Maltose (A) and glucose (B) utilization during enzyme production with limited pyridoxine with 24 h transplanting frequency.

pyridoxine before the pH drops below 6 in order to avoid a decrease in protein production (Figure 5.5). The positive correlation of pH to protein production is a useful tool for reactor control and monitoring. Whereas online monitoring of pH is ready available, online measurement of protein concentration is more difficult.

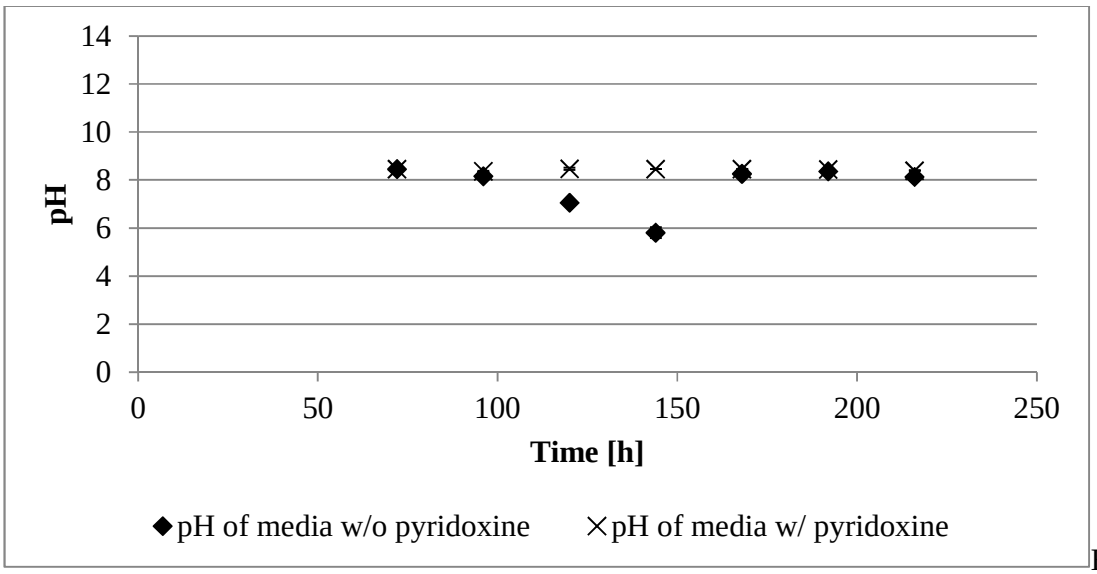
No measurement of cell mass was performed due to the impossibility of taking homogenous samples. Pictures were taken to document the constant surface area of the fungus throughout the experiment (Figures A2A and A2B). It can be seen that no visual growth of surface area (enlargement of mycelia) took place when the fungus was kept on medium without pyridoxine. The fungus's color became darker while using medium without pyridoxine.

The cause for the pH decrease over time under pyridoxine limitation appears to be linked to succinic acid production. An increase of succinic acid from 0.06 g/L to 0.26 g/L could be observed when the pH dropped between 240 h and 288 h and decreased back to 0.08 g/L at 336 h (data not shown). It is expected that pyridoxine limitation causes an increase in organic acids and/or amino acids due to the involvement of pyridoxine in amino acid metabolism. Other organic acids and amino acids either were not observed to increase or not detected with HPLC.

Besides of the changes that occur in the metabolism based on pyridoxine limitation, fungal autolysis can also be the responsible for pH decrease. Pyridoxine indirectly leads to starvation by disturbing the amino acid metabolism, which has been shown to initiate autolysis (White et al. 2002). Autolysis is a process in which the fungus enzymatically breaks down old fungal mass, in order to utilize the nutrients for fungal



A



B

Figure 5.5: pH development over time for 48 h (A) and 24 h (B) transplanting frequency. Error bars represent standard deviation of two replicates.

hyphae tip growth. This breakdown of fungal mass could release compounds responsible for pH decrease.

5.4.3 Productivity of *A. nidulans* with inhibited growth

Table 5.2 shows all factors obtained from this experiment. The goal was to determine the protein productivity of an *A. nidulans* culture when initially grown on medium with pyridoxine and then transferred onto medium without pyridoxine. The results presented are obtained after transplanting the culture a second time. After the second transplantation, no visual growth was observed with the culture without pyridoxine. The productivity was obtained and had a value of 21.14 U/g dry cell mass*h for the culture without pyridoxine and 8.09 U/g dry cell mass*h for the culture with pyridoxine. The productivity without pyridoxine was 2.6 times higher compared to the productivity with pyridoxine over the same time period, which is also represented in the yields. The protein to dry cell mass yields ($Y_{P/X}$) of the culture without pyridoxine and the culture with pyridoxine were 507.42 U/g and 194.21 U/g, respectively. The protein to substrate yield ($Y_{P/S}$) was higher for the culture with pyridoxine, 21.84 U/g, than the culture without pyridoxine, 13.95 U/g.

5.5 Conclusion

It was successfully demonstrated that a fungus mutant requiring pyridoxine for growth can maintain enzyme production even when growth is limited by limiting pyridoxine. However, the continuous enzyme production cannot go on forever. Pyridoxine needs to be replenished frequently. Since pH correlates with pyridoxine

Table 5.2: Factors obtained of productivity experiment. Total substrate = $C_{glucose}$ + $C_{maltose}$/0.947. $Y_{P/X}$ = protein to dry cell mass yield, $Y_{P/S}$ = protein to substrate yield.

Factors	**w/o pyridoxine**	**w/ pyridoxine**
Initial maltose [g/L]	47.62	47.62
Initial glucose [g/L]	10.00	10.00
Initial total substrate [g/L]	60.28	60.28
Final maltose after 24 h [g/L]	7.68 ± 1.30	6.02 ± 0.993
Final glucose after 24 h [g/L]	8.56 ± 1.90	4.92 ± 0.397
Final total substrate after 24 h[g/L]	16.67	11.28 ± 1.40
XynB concentration after 24 h [U/ml]	17.32 ± 3.15	11.27 ± 4.38
Dry cell mass [g]	0.68 ± 0.13	0.78 ± 0.08
$Y_{P/X}$ [U/g]	507.42 ± 37.54	194.21 ± 52.81
$Y_{P/S}$ [U/g]	21.84 ± 8.57	13.95 ± 5.64
Productivity [U/g · h]	21.14 ± 1.56	8.09 ± 2.20

depletion, monitoring the pH allows identifying the correct moment when pyridoxine needs to be added.

Pyridoxine limitation causes stress on the cell, which induces conidiation and melanin formation. The increased stress on the cells due to pyridoxine limitation was observed to have no effect on XynB formation. With a decrease in protein formation, the activity increases, which keeps the volumetric activity relatively constant.

5.6 Future work

The pyridoxine limitation system has shown to be a successful method of limiting growth while the enzyme production can continue. As mentioned earlier, pyridoxine is essential for the amino acid metabolism. Potential future work could be the investigation of a biotin limitation system. As pyridoxine is a marker for this *A. nidulans* mutant, other coenzymes can act as a marker. Biotin is a promising coenzyme, whose limitation could improve the client protein formation. Biotin is involved in carboxylation reactions. Pyruvate carboxylase, acetyl-CoA carboxylase, and propionyl-CoA use biotin as a CO_2 carrier (Voet and Voet 2004). Pyruvate carboxylase initiates the first step in gluconeogenesis by converting pyruvate to oxalacetate. Acetyl-CoA carboxylase is involved in fatty acid synthesis and propionyl-CoA is involved in odd-chain fatty acid oxidation (Voet and Voet 2004). Fatty acids are required for cell membrane synthesis. It is expected that the limitation of biotin for an *A. nidulans* mutant with a biotin marker would limit growth; whereas, the enzyme production would be unaffected. A petri dish experiment as was used for this experiment could be used to show within a short period of time the feasibility of the biotin limitation process. Important factors to investigate are the continuous client enzyme production and the limitation of growth over time. It is

assumed that using this system would lead to higher client protein productivity compared to the system with a pyridoxine marker because the amino acid metabolism is not affected by the limitation of biotin.

During transplanting the medium becomes darker in color over time. It is assumed that the darker coloration is due to an elevated melanin formation. An increase in melanin formation was reported when filamentous fungi initiate conidiation (Jørgensen et al. 2010). Conidiation is usually a response to energy or carbon starvation to conserve the cell until it is transported to a nutritional environment. Stress induces the transcription of *brlA* (Skromne et al. 1995) and the transcription factor *brlA* is a positive regulator for conidiation (Adams and Timberlake 1990). At the moment when all pyridoxine was depleted, the protein production and the PH were at a minimum and the medium became a light yellow. This shows that no melanin, and therefore, no conidiation took place. However, when the same culture was placed again on medium with pyridoxine, the medium became darker again and melanin was formed.

5.7 References

Adams TH, Timberlake WE. 1990. Developmental repression of growth and gene expression in *Aspergillus*. Proceedings of the National Academy of Sciences 87(14):5405.

Boender LGM, De Hulster EAF, Van Maris AJA, Daran-Lapujade PAS, Pronk JT. 2009. Quantitative physiology of Saccharomyces cerevisiae at near-zero specific growth rates. Applied and environmental microbiology 75(17):5607.

Bradford MM. 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Analytical Biochemistry 72(1-2):248-254.

Broderick A, Greenshields R. 1981. Sporulation of *Aspergillus niger* and *Aspergillus ochraceus* in continuous submerged liquid culture. Journal of general microbiology 126(1):193.

Galbraith JC, Smith J. 1969. Sporulation of *Aspergillus niger* in submerged liquid culture. Journal of general microbiology 59(1):31.

Jørgensen TR, Nitsche BM, Lamers GE, Arentshorst M, van den Hondel CA, Ram AF. 2010. Transcriptomic insights into the physiology of *Aspergillus niger* approaching a specific growth rate of zero. Applied and environmental microbiology 76(16):5344-5355.

Ng A, Smith J, McIntosh A. 1972. Conidiation of *Aspergillus niger* in continuous culture. Archives of Microbiology 88(2):119-126.

Pirt S. 1965. The maintenance energy of bacteria in growing cultures. Proceedings of the Royal Society of London. Series B, Biological Sciences:224-231.

Prosser IJ, Tough AJ. 1991. Growth mechanisms and growth kinetics of filamentous microorganisms. Biotechnology 10(4):253-274.

Schrickx JM, Krave AS, Verdoes JC, van den Hondel CA, Stouthamer AH, van Verseveld HW. 1993. Growth and product formation in chemostat and recycling cultures by Aspergillus niger N402 and a glucoamylase overproducing transformant, provided with multiple copies of the glaA gene. Journal of general microbiology 139(11):2801.

Schrickx JM, Raedts MJ, Stouthamer AH, Verseveld HWv. 1995. Organic acid production by *Aspergillus niger* in recycling culture analyzed by capillary electrophoresis. Analytical Biochemistry 231:175-181.

Segato F, Damásio ARL, Gonçalves TA, Lucas RC, Squina FM, Decker S, Prade RA. 2011. Secretion of client proteins in Aspergillus. Submitted.

Skromne I, Sánchez O, Aguirre J. 1995. Starvation stress modulates the expression of the *Aspergillus nidulans* brlA regulatory gene. Microbiology 141(1):21.

Sluiter A, Hames B, Ruiz R, Scarlata C, Sluiter J, Templeton D. 2008. Determination of sugars, byproducts, and degradation products in liquid fraction process samples. National Renewable Energy Laboratory(NREL/TP-510-42623).

Verseveld HW, Metwally M, Sayed M, Osman M, Schrickx JM, Stouthamer AH. 1991. Determination of the maximum product yield from glucoamylase-producing Aspergillus niger grown in the recycling fermentor. Antonie van Leeuwenhoek 60(3):313-323.

Voet D, Voet JG. 2004. Biochemistry. Hoboken: John Wiley & Sons, Inc.

White S, McIntyre M, Berry DR, McNeil B. 2002. The autolysis of industrial filamentous fungi. Critical reviews in biotechnology 22(1):1-14.

CHAPTER VI

CONTINUOUS XYLANASE PRODUCTION WITH *ASPERGILLUS NIDULANS* UNDER PYRIDOXINE LIMITATION USING A TRICKLE BED REACTOR

6.1 Abstract

In the present study, a new reactor system was designed and tested using *Aspergillus nidulans* with a pyridoxine marker and xylanase B (XynB) as a client enzyme. The new reactor system is a trickle bed reactor (TBR) with recycle, which utilizes the advantages of solid state fermentation (SSF) and submerged fermentation (SmF). The fungus is allowed to grow on a solid inert support, while the medium containing carbon and energy source trickles down. The uncontrolled growth of mycelia in the tubing, eventually leading to clogging, is reduced by utilizing a growth limitation by pyridoxine limitation. The fungus is unable to synthesize its own pyridoxine and is unable to grow when no pyridoxine is present in the medium, while the enzyme production is unaffected. The current study demonstrated a successful continuous operation over 18 days with high XynB titers. The reactor achieved a XynB output of 41 U/ml with an influent and effluent flow rate of 0.5 ml/min and a recycle flow rate of 56 ml/min. Production yields were 1.4 times higher compared to a static tray culture and between 1.1 and 67 times higher compared to SSF enzyme production stated in the literature.

.

6.2 Introduction

The production of enzymes in large scale is currently performed using solid state (SSF) or submerged fermentations (SmF). One of the largest cellulolytic enzyme producers, Novozymes located in Denmark, uses submerged culture techniques that are a well-kept secret (Novozyme 2010a). Bacteria and fungi are used in fed-batch or continuous systems.

The University of Sao Paulo has conducted intense research in xylanase production (Betini et al. 2009; de Carvalho Peixoto-Nogueira et al. 2009). Their main focus is in optimizing the enzyme expression of xylanases for the paper bleaching industry. The researchers found *Aspergillus niveus* and *Aspergillus fumigates* as xylanase producers with high enzyme activities. *A. fumigates* was found to produce the highest xylanase activity with a culture medium by Vogel (1964) showing an activity of 109 U/mg protein (348 total U). De Carvalho Peixoto-Nogueira et al. (2009) also compared static to agitated enzyme production and found the activity with the static culture to be 2.3-fold higher than in the agitated culture. Further improvement was achieved by using SSF on inexpensive carbon sources (wheat bran, corncob, rice straw, triturated rice straw, oatmeal, sugarcane bagasse, cassava flour, *Eucalyptus grandis* sawdust) (Betini et al. 2009). The best activity was achieved with *A. niveus* on wheat bran (2 g solid substrate with 4 ml distilled water at 70 to 80% relative humidity at 30°C for 96 h) with a value of 928 total U.

Xylanase and cellulase production using mixed submerged fermentation was performed by Garcia-Kirchner et al. (2002). The researchers used 4 g of sugarcane bagasse with 180 ml of medium with spores from *Penicillium* sp. CH-TE-001 and/or

Apergillus terrus CH-TE-013. The flasks were incubated at 29°C on a rotary shaker at 180 rpm. They found a higher cellulase and xylanase production with the mixed culture compared to the single cultures. The enzyme mix, which was not further characterized, showed the highest activity on carboxymethyl cellulose (CMC) and birchwood xylan after 5 days with a value of 1.4 U/ml and 5 U/ml, respectively. No values of protein concentration were given.

A detailed description of SSF is given by Mitchell et al. (2000). This paragraph will concentrate on the general principles. SSF has been used over centuries for food production, namely the Koji technology, where rice is fermented to produce rice wine also called “sake”. In SSF, microorganisms grow on solid substrate particles with the absence of visible liquid water (Mitchell et al. 2000). The typical water content ranges between 12 and 80 wt% (Cannel and Moo-Young 1980). Wheat bran, sugarcane bagasse and soy bran are, among others, typical substrates for enzyme production (Mitchell et al. 2000). The substrate is characterized as a low cost waste material from agriculture. The general process involves the pretreatment of the substrate, including grinding and optional physico-chemical treatments, followed by inoculation usually with a filamentous fungi and active or passive aeration. The reactor may or may not be agitated, and humidity may be controlled. After completion the products need to be leached out (Mitchell et al. 2000).

Typical reactor types for SSF are tray, drum and packed bed bioreactors. Tray reactors are simple to use without active agitation or aeration. The trays filling height is limited, since overfilling can lead to anaerobic processes and/or overheating (Tunga et al. 1999) with temperature and humidity as the only control parameters (Durand et al. 1997).

Due to the large area required, large scale usage of tray reactors is limited. Drum reactors incorporate mixing and can lead to increased heat and mass transfer. The growth can be improved, but sheer forces can limit the final product formation (Nigam et al. 2004). Therefore, the handling on large scale can be difficult. Packed bed reactors are columns where the substrate is kept in place by a perforated platform. Forced aeration is applied at the bottom of the column (Mitchell and Lonsane 1992). Product retrieval, non-uniform growth and limited heat and mass transfer are common problems of packed bed reactors (Lonsane et al. 1985; Mitchell and Lonsane 1992).

The enzyme productivity is often higher in SSF than in SmF (Viniegra-González and Favela-Torres 2004). Viniegra-González et al. (2003) reviewed enzyme production using SSF and SmF in terms of physiological differences that explain the higher enzyme titers in SSF. In an experiment conducted by Romero-Gómez et al. (2000), five times higher invertase concentrations were produced with SSF using *Aspergillus niger* due to higher biomass production than with SmF. Another experiment conducted by Díaz-Gódínez et al. (2001) for the production of pectinase showed again higher titers using SSF due to higher biomass production than with SmF. They also found that production with SSF leads to an apparent resistance to catabolite repression, which was indicated by high pectinase titers with SSF using high initial substrate (in this case sucrose) concentrations. In SmF high sucrose concentrations led to an inhibition of product formation. It was also observed that pectinase production with SmF had higher breakdown of pectinase by contaminant protease. An experiment with restricted growth by introduction of a steric hindrance was performed by Aguilar et al. (2001). For the production of tannase, *A. niger* grew on finely ground polyurethane foam (PUF), which

had a higher density (113 g/L) than normal sized PUF (15 g/L) with the purpose of restricting mycelia growth. Again, SSF produced higher titers of tannase than SmF, with values of 14,000 U/L and 2,800 U/L, respectively. The cell mass levels were found to be 4.5 g/L and 11.5 g/L for SSF and SmF, respectively. Even with limited growth by physical hindrance, SSF performed better than SmF, which was also due to a reduced protease activity in SSF. Protease activity was found to be eight times higher in SmF.

Biofilm reactors have been investigated for many applications, with many different reactor types (Cheng et al. 2010). Types of biofilm reactors are: stirred-tank reactor, trickling filter reactor, rotating-disk reactor, membrane biofilm reactor, fluidized-bed reactor and airlift reactor. The choice of reactor depends on the final product, process complexity and costs.

The enzymes used for the project described in this article are usually produced in lab scale static tray fermentation cultures. The current production processes are too expensive to utilize the enzymes on a large scale for low cost products like bioethanol. To enhance enzyme productivity, a trickle bed reactor (TBR) was designed. The macrostructure of the fungus is maintained by keeping the mass transfer at an optimum. The support is inert and the substrates for enzyme expression and growth are in a dissolved form. Furthermore, the system allows for maintaining a large cell mass with a large surface area. The reactor system is modeled for continuous enzyme production. No previous literature was found on the use of a TBR for the production of enzymes by filamentous fungi. The XynB production with the reactor was compared to a shaking flask and static tray fermentation which were used to simulate SmF and SSF.

Furthermore, the XynB production in the reactor was tested with and without growth limitation induced by pyridoxine limitation.

6.3 Materials and Methods

6.3.1 Culture medium

The medium was composed of 50.0 ml/L 20x Clutterbuck salts (120 g/L $NaNO_3$, 10.4 g/L KCl, 10.4 g/L $MgSO_4$, 30.4 g/L KH_2PO_4), 1.0 ml/L 1000x trace elements (22 g/L $ZnSO_4 \cdot 7H_20$, 11 g/L H_3BO_3, 5.0 g/L $MnCl_2 \cdot 7H_2O$, 5.0 g/L $FeSO_4 \cdot 7H_2O$, 1.6 g/L $CoCl_2 \cdot 5H_2O$, 1.6 g/L $CuSO_4 \cdot 5H_2O$, 1.1 g/L $Na_2MoO_4 \cdot 4H_2O$, 50 g/L Na_2-EDTA), 10.0 g/L glucose monohydrate, 50.0 g/L maltose monohydrate and 1 ml/L of a 1 g/L pyridoxine solution. For the pyridoxine limitation test, the medium did not contain pyridoxine. The pH was adjusted to 6.5 using 10 N NaOH. The medium was sterilized by autoclaving at 121°C for 20 min.

6.3.2 Reactor Design

Figure 6.1 shows the schematic of the TBR design. The reactor is composed of a glass column with an inside diameter of 10.5 cm. The height is 60 cm. The packing were lava rocks purchased from a local hardware store, originally used for outside grills. The diameter of the rocks varied between 3 and 4 cm. The inlets for fresh and recycled medium were placed on top of the reactor. On the bottom a stainless funnel led the medium into a 250 ml baffled flask. The baffled flask was placed on a magnetic stirrer. The whole reactor was able to fit into an autoclave for sterilization. A heating tape was wrapped around the column to keep a constant temperature of 37ºC. Three peristaltic pumps (MasterFlex 7523-20, Barnant Co., Barrington, IL, USA) were

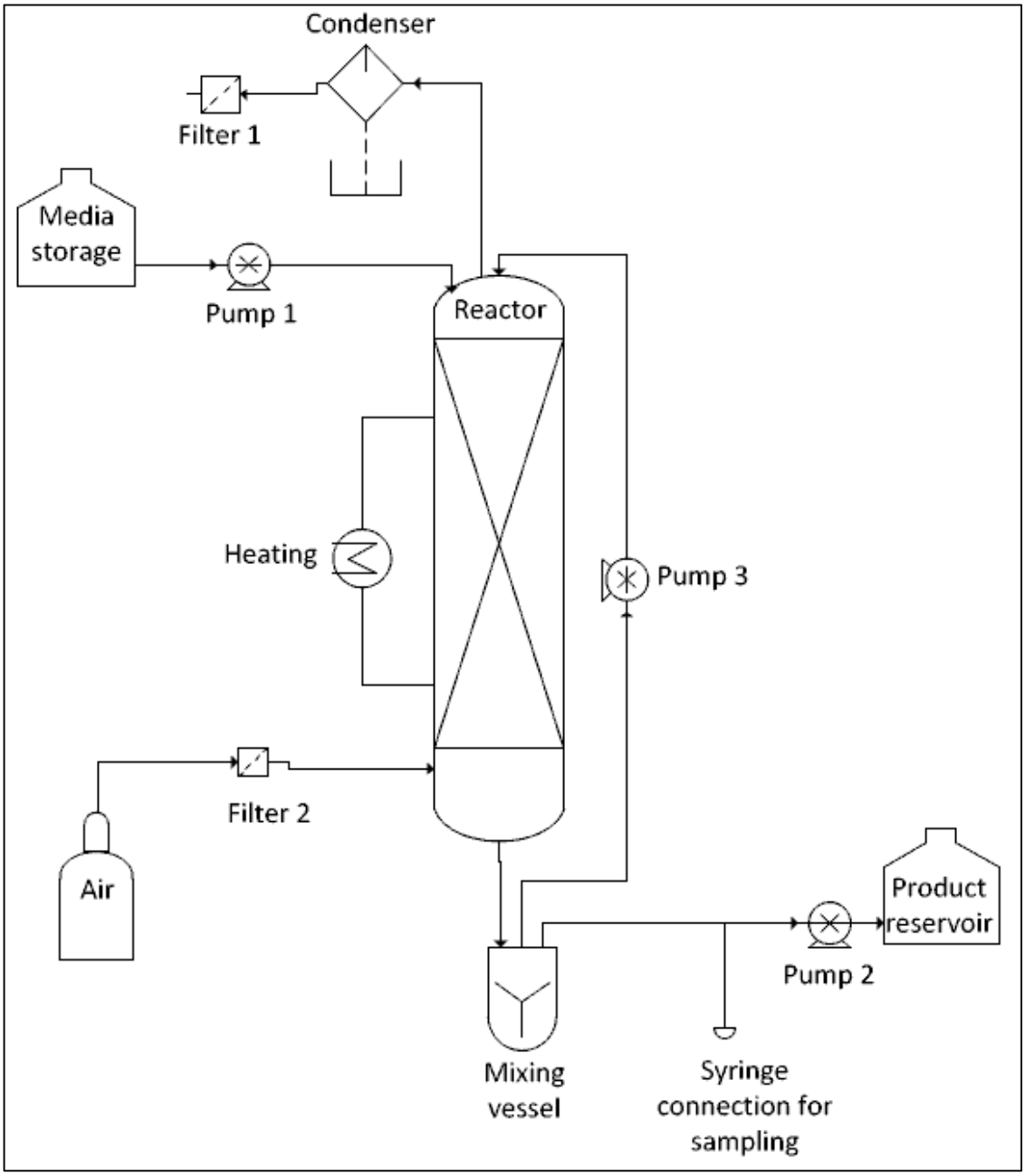


Figure 6.1: TBR set up.

used for medium flow. The influent and effluent flow rates varied from 0.5 to 1.0 ml/min. One pump was used to pump in fresh medium into the TBR. The second pump took medium out from the baffled flask at the same rate as the first pump. The third pump was used to pump medium from the baffled flask back into the reactor. The recycling of medium at various rates (224, 112, and 56 m/min) allowed higher substrate utilization, mixing and enhanced enzyme productivity. The tubing that connected the baffled flask with the product reservoir had an attachment that allowed sampling. Air was pumped into the reactor at 1.15 L/min (standard temperature and pressure) at the bottom through a 0.2 μm sterile filter and exited the column at the top.

6.3.3 Reactor model

Several models are available to design reactors based on unicellular organisms. However, for filamentous fungi applied in a TBR, no model has been established. Several parameters are needed to be determined experimentally. The specific and maximum growth rates as well as productivity of *A.nidulans* were 0.061 h^{-1} (for 59.3 g/l total substrate concentration), 0.311 h^{-1}, and 21.14 U/g · h, respectively (see Chapter V). The following assumptions were made in the development of the model:

- The fungus will have the same yields ($Y_{X/S}$, $Y_{P/S}$, and $Y_{P/X}$) in the reactor as observed with Petri dish experiments for the same initial substrate concentration.
- The specific growth rate found in the Petri dishes is equal to the specific growth rate in the reactor.
- The total available medium inside the reactor includes medium to saturate the lava rocks.
- Maltose and glucose are utilized at equal rates.

- High recycle flow rate allowed for the assumption that the reactor behaved like a continuously stirred tank reactor (CSTR).

Since it was not possible to measure the exact cell mass inside the reactor, a theoretical cell mass based on $Y_{X/S}$ was calculated. Assuming complete substrate utilization during the growth phase, the cell total mass (X) could be calculated:

$$X = Y_{X/S} \cdot S_T$$

with $Y_{X/S}$ = cell mass to substrate yield and S_T = Total substrate mass [g] in the reactor.

The substrate uptake rate R [g/h] could now be calculated using the specific substrate uptake rate and the total cell mass:

$$R = qs \cdot X$$

with qs = specific substrate uptake rate in g substrate/g cells*h.

Using the active volume, a volumetric substrate uptake rate R_V [g/L*h] can be calculated:

$$R_V = R / V_a$$

with V_a = active volume [L].

The flow rate Q was calculated using the volumetric substrate uptake rate and the substrate concentration in the inlet:

$$Q = R_V / S$$

with S = substrate concentration [g/L]

This flow rate was determined based on the assumption that all substrates are consumed according to the rates and yields determined in experiments done in Petri dishes.

6.3.4 Reactor process

The primary target of this investigation was to achieve a continuous system for enzyme production with μ_{net} equal to zero. The reactor process was divided into two

steps. The first step was the startup and growth of cell mass. The second step initiated the growth limitation with continuous product formation. This step required a change to medium without pyridoxine.

In the first step, 600 ml of medium were pumped into the reactor and recycled until all packing material was saturated with liquid. Twenty-five ml of a concentrated spore solution (8×10^7 spores/ml) containing 20.0 mg spores of *A. nidulans* (dry weight) was added with a syringe on top of the reactor. The remaining free medium, about 60 ml, were then continuously recycled at 224 ml/min without the addition or extraction of medium, until all substrates were depleted and the fungus culture was grown.

In the second step, the free medium was extracted from the reactor, and replaced with medium that had no pyridoxine. A constant flow of pyridoxine-free fresh medium was pumped into the reactor. At the same rate, medium was extracted from the reactor and collected. The medium in the mixing vessel, which had the same composition as the effluent, was frequently monitored for maltose, glucose and protein concentration analysis. The pH was also monitored frequently. When the pH started to decrease to 6.5, indicating that pyridoxine was exhausted, a 0.5 ml aliquot of a 1 g/L pyridoxine solution was added into the reactor. During step 2, the medium was recycled at various high flow rates (224, 112, 56 ml/min). This allowed the assumption that at any moment and location, the substrate concentration throughout the reactor was equal.

6.3.5 Analysis of flow rate through reactor

The glass column was filled with a known mass of air dried lava rocks. The height of the packing material was 50 cm with a column diameter of 10.5 cm. Before the flow rate could be measured, the tubing of the system was filled with DI water. A graduated

cylinder was filled with 1.0 L of DI water. At various flow rates the remaining liquid in the graduated cylinder was recorded after the system reached steady state. At zero flow rate the mass of water attached to the lava rocks (holding capacity) could be obtained at steady state. The holding capacity was also measured in a different test, where rocks were first submerged in water and then weighed after all the surface water was drained.

6.3.6 Enzyme production in static tray

A stainless steel tray was used for culturing. The tray was filled with 500 ml medium, containing a minimal medium with 50.0 ml/L 20X Clutterbuck salts (120 g/L $NaNO_3$, 10.4 g/L KCl, 10.4 g/L $MgSO_4$, 30.4 g/L KH_2PO_4), 1.0 ml/L 1000X trace elements (22 g/L $ZnSO_4 \cdot 7H_20$, 11 g/L H_3BO_3, 5.0 g/L $MnCl_2 \cdot 7H_2O$, 5.0 g/L $FeSO_4 \cdot 7H_2O$, 1.6 g/L $CoCl_2 \cdot 5H_2O$, 1.6 g/L $CuSO_4 \cdot 5H_2O$, 1.1 g/L $Na_2MoO_4 \cdot 4H_2O$, 50 g/L Na_2-EDTA), 10.0 g/L glucose, 50.0 g/L maltose and 1 ml/L of 1 g/L pyridoxine solution with an adjusted pH of 6.5. Spores of *A. nidulans* with a pyridoxine marker cultured on three agar plates containing minimal medium were manually scratched off the surface and added to the medium in the tray under sterile conditions. The trays were incubated at 37°C for 48 h. Since the fungus grows on top of the liquid, a separation of liquid from fungus was achieved by filtration through a filter paper vacuum filtration unit. After filtration the enzyme concentration was determined.

6.3.7 Comparison of tray and shaking flask enzyme production

The purpose of this experiment was to determine if the protein production was higher using a tray in comparison to using a flask. Shaking was shown in the literature to alter the growth form of filamentous fungi to pellets, while a surface grown fungi

develops a mycelium with hyphae directed to the source of nutrients. The same medium volume of 180 ml was added to a tray and a 500 ml Erlenmeyer flask. Both cultures were inoculated with spores to a final concentration of 4 x 10^5 spores/ml and grown at 50°C. The flask culture was agitated at 225 rpm. Samples were taken at 0, 24, 48, 72 and 96 h and analyzed for protein, glucose and maltose concentration.

In order to compare the enzyme production between the tray, flask and reactor operation, the total mass protein over a fixed period of time was calculated and standardized with dry cell mass. For the reactor the total dry cell mass was determined after the run was completed. The protein formation over a steady state period of time with a known flow rate was used to determine total protein produced within that time period. The following equation was used for the reactor:

$$P_{\mathrm{Re}\,actor} = \frac{\overline{p}_{T_2-T_1} \cdot Q \cdot (t_2 - t_1)}{X} \qquad \text{(Eq. 6.1)}$$

With $P_{Reactor}$ = total protein mass per dry cell mass [g/g], $p_{t2\text{-}t1}$ = average protein concentration [g/L] during the time period between t_1 and t_2 [h], Q = flow rate of continuous operation [L/h] and X = dry cell mass [g].

Equation 6.2 is used to calculate the total protein per cell mass from both, tray and flask cultures. These systems are batch fermentations, which simplifies the equation.

$$P = \frac{\overline{p} \cdot V}{X} \qquad \text{(Eq. 6.2)}$$

With P = total protein per dry cell mass (P_F and P_T for flask and tray, respectively) [g/g], p = protein concentration [g/L], V = liquid volume [L] and X = dry cell mass.

The dry cell mass is obtained by filtering the medium and cells through a filter paper at the end of the experiment and drying overnight at 105ºC.

6.3.8 Determination of protein concentration

The protein concentration was determined using a Bradford assay. Forty µl of Bradford commassie solution were pipetted into a well of a 96-well plate. The amount of enzyme solution depended on the protein concentration received. A typical amount is 40 µl. DI water was added to achieve a total volume of 200 µl. The blank well contained only 40 µl Bradford solution and 160 µl DI water. The absorbance was measured using a UV-Vis 96-well plate reader (Tecan Infinite M200, Männedorf, Switzerland) at 595 nm.

6.3.9 Determination of XynB activity

Xylanase B activity was measured on xylan from beechwood (Fisher Scientific, Pittsburgh, PA). Forty-eight µl of 1.0% substrate solution and 2 µl of enzyme solution were added into a 96-well plate. The plate was inoculated in a water bath at 50°C for 7 min. After inoculation, the 96-well plate was removed from the water bath and 50 µl of dinitrosalicylic acid (DNS) reagent were added immediately to terminate enzymatic hydrolysis. The 96-well plate was placed in a second water bath at a temperature of 100°C for 5 min to achieve the color formation. After the reaction time, 100 µl of the liquid was transferred in a 96-well reading plate and analyzed at 575 nm for reducing sugar concentration. With the following equation, the specific activity was calculated based on the spectrophotometer values.

$$U = \left(\left((A - 0.047) / F \right) \times \left(\frac{V_{assay}}{t} \right) \right) / p_{enzyme} \qquad \text{(Eq. 6.3)}$$

With U = specific activity [µmol of reducing sugar/mg protein · min], A = absorbance at 575 nm, F = calibration factor, V_{assay} = assay volume (200 µL), t = incubation time [min] and p_{enzyme} = mass protein in enzyme used [mg].

The final results are given in XynB activity units per volume, U_V.

$$U_V = U \cdot C_P \qquad \text{(Eq. 6.4)}$$

With C_P = protein concentration in mg/ml.

6.3.10 Protein analysis with SDS-PAGE

Additional characterization of the proteins was done by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). A 12% separation gel was made by mixing 1.7 ml H_2O, 1.25 ml 1.5 M Tris, 50 µl 10% SDS, 2.0 ml 30% acryl, 25 µl 10% ammonium per sulfite (APS) and 25 µl tetramethylethylendiamin (TEMED). The stacking gel was composed of 2.5 ml H_2O, 0.38 ml 0.5 M Tris, 0.03 ml 10% SDS, 0.5 ml 30% acryl, 0.03 ml APS and 3.0 µL TEMED. Samples were concentrated using ultrafiltration (Stirred Ultrafiltration Cell Model 8400, Millipore, Billerica, MA, USA). An aliquot equivalent to 10 µg protein was mixed with 15 µl of 2x Laemmli buffer (4% SDS, 20% glycerol, 10% 2-mercaptoethanol, 0.004% bromphenol blue, 0.125 M Tris HCl) and boiled for 5 min for optimal band resolution. The solution was then injected into the gel. After completion of the SDS-PAGE run, the gels were stained with Bio-Safe™ Coomassie Stain (Bio-Rad, Hercules, CA, USA) and de-stained with water.

6.3.11 Sugar and organic acid analysis

Concentrations of glucose, maltose and urea were analyzed on an HPX-87P column (Bio-Rad, Sunnyvale, Ca.). The eluent was HPLC grade DI-water with a flow of 0.6 ml/min at 85°C and a refractive index detector (1100 Series Agilent, Santa Clara, CA, USA) (Sluiter et al. 2008c). Organic acids were analyzed on a HPX-87H column (Bio-Rad, Sunnyvale, Ca.). The eluent was 0.1 N sulfuric acid with a flow rate of 0.6 ml/min at 60ºC. A refractive detector (1100 Series Agilent, Santa Clara, CA, USA) was used for detection (Sluiter et al. 2008c).

6.3.12 Calculation of yields

In order to compare the different XynB productions methods used in this study, a common base needs to be established. Cell mass and liquid volume may differ, which can lead to false conclusions if just XynB concentration is compared. Since typical enzyme activity is given in U (µmol/min), all yields are based on the volumetric XynB activity, U_V, in U/L. Substrate and cell mass concentrations are given in g/L. The product from substrate yields, $Y_{P/S}$, is calculated for the tray and flask experiment as:

$$Y_{P/S} = \frac{U/V}{(S_0 - S_{96})} \qquad [\text{U/g}]$$

With U = XynB activity in U, V = volume of fermentation broth after 96 h in L, S_0 = initial substrate concentration in g/L and S_{96} = substrate concentration at 96 h in g/L. Similarly can the product from substrate yield, $Y_{P/X}$, be calculated:

$$Y_{P/X} = \frac{U/V}{X_{96}} \qquad [\text{U/g}]$$

With X_{96} = cell mass concentration after 96 h in g/L. The initial cell mass can be neglected.

The cell mass from substrate yield ($Y_{X/S}$) was calculated with following equation:

$$Y_{X/S} = \frac{X_{96}}{(S_0 - S_{96})} \qquad [g/g]$$

6.4 Results and Discussion

6.4.1 Comparison of enzyme production in static tray and shaking flask

The purpose of this study was to show that a culture grown as a static tray culture (simulating a SSF) would produce higher titers of the client enzyme compared to a shaking flask by using the same liquid volume and spore inoculation. As it can be seen in Figure 6.2, the XynB concentration over time was about two times higher with the shaking flask culture than with the static tray after 96 h. The SDS-PAGE analysis revealed that the tray shows larger bands of XynB compared to the flask, indicating a higher purity of XynB in the total protein mix (Figure 6.3). This observation confirms the results found in previous studies comparing SSF with SmF (Viniegra-González et al. 2003), where SSF fermentations led to higher enzyme purity. However, a comparison of the yields shows that the shaking flask produces XynB more efficiently than the static tray. The product to substrate yield ($Y_{P/S}$) was 780.72 U/g for flask fermentation and 543.16 U/g for tray fermentation after 96 h. The flask culture also resulted in a greater product from cell mass yield, $Y_{P/X}$ = 1849.77 U/g, compared to the tray experiment with $Y_{P/X}$ = 480.05 U/g. Due to the high cell mass of 50.0 g/L produced in the static tray, the cell mass from substrate yield ($Y_{X/S}$) is higher, 1.13 g/g, compared to the flask, 0.42 g/g.

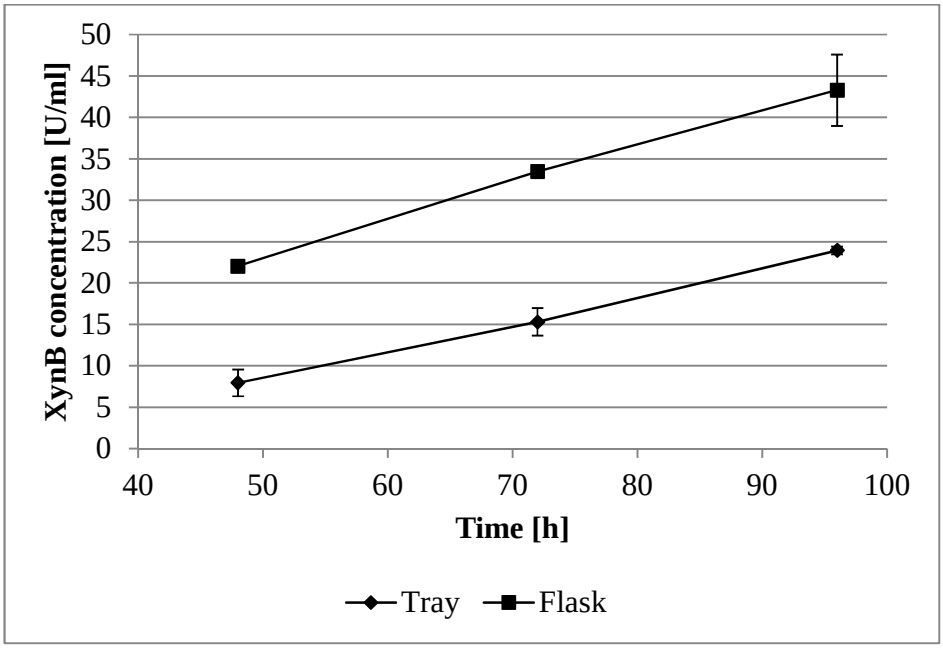


Figure 6.2: Effect of fermentation mode on the XynB production over time at 37ºC. Tray and shaked flask had the same volume of medium and inoculum concentration. Error bars represent standard deviation of two replicates.

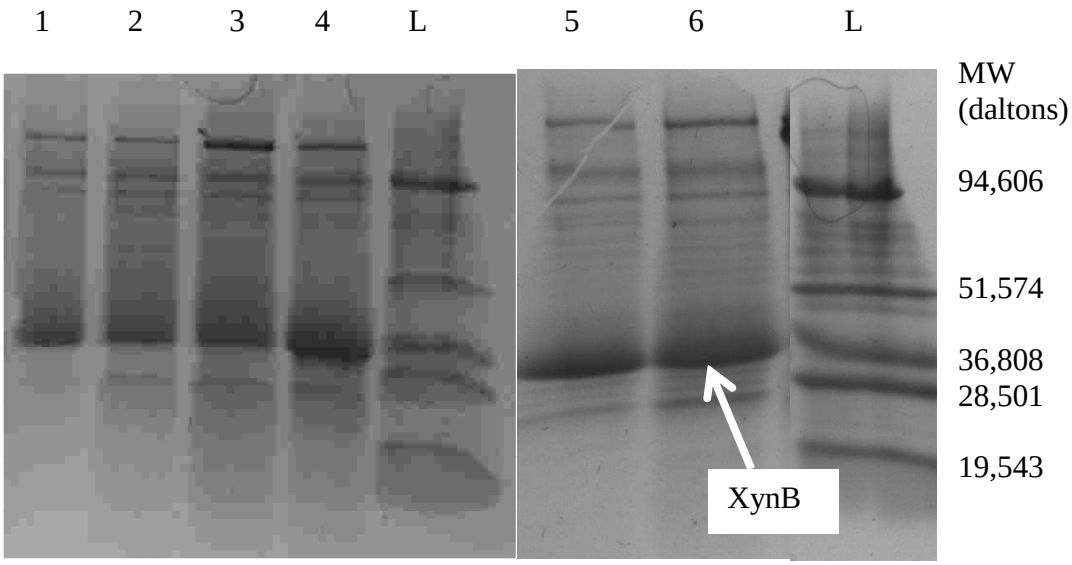
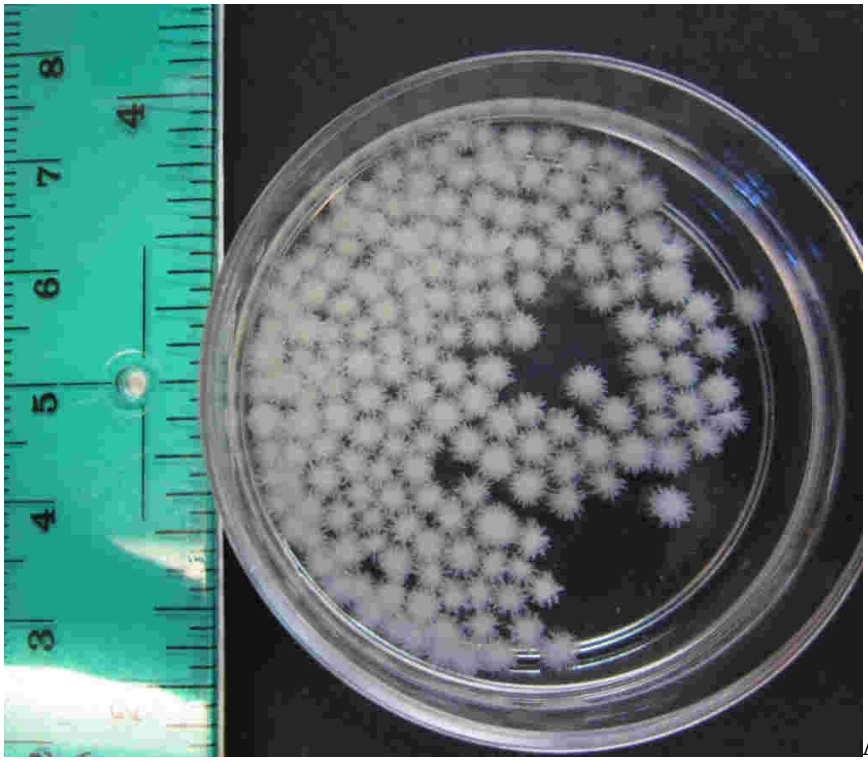


Figure 6.3: SDS-PAGE analysis of proteins produced by flask and tray cultures by *A. nidulans*. 1 = Flask 48 h, 2 = Flask 72 h, 3 = Flask 96 h, L = Ladder, 4 = Tray 48 h, 5 = Tray 72 h, 6 = Tray 96 h.

The fungal morphology was very different in both cultures. While the static tray showed a dense mycelium on top of the surface, the shaking culture produced pellets (Figure 6.4A) with a diameter of about 2-3 mm. It has been shown that the pellet size depends on the inoculum concentration. With increasing inoculum spore concentration, the pellet size decreases (Žnidaršič et al. 2000). Other factors affecting the pellet formation are shear stress (Fujita et al. 1994) and pH (Žnidaršič et al. 2000). The pellet size was found to affect the protein formation, where a decrease in pellet size causes an increased product formation (El-Enshasy et al. 1999). However, in order to extract the desired protein from the fermentation broth after fermentation, the fungal mass has to be separated, which is more difficult with the pellet culture than with the static tray culture. The density of the pellets is similar to the density of the fermentation broth (Figure 6.4B). Settling or centrifugation are less effective in separating the pellets while filtering requires a high energy demand due to the viscosity of the broth. This was also observed when the samples were filtered through 0.2 μm syringe filters for sample analysis. A relatively large amount of force was required to press the liquid through the filter. In this process the pellets needed to be disrupted to extract the liquid with the syringe. It is believed that downstream processing of a fungal pellet solution is more energy and process intensive compared to the fermentation broth from a static culture.

6.4.2 Flow rate through reactor

The mass of rocks used for this experiment was 2,501.6 g. The packing was 50 cm high with a diameter of 10.5 cm. The rocks were air dried prior to the experiment and


A

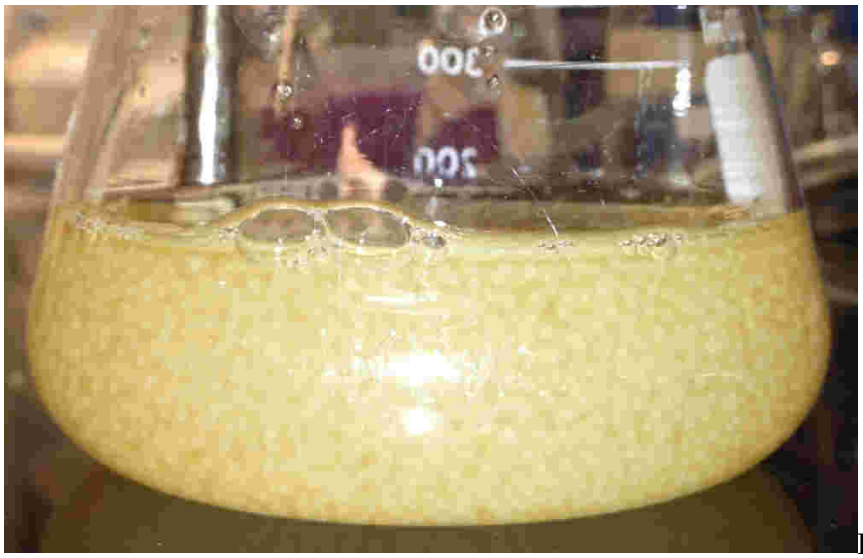

B

Figure 6.4: (A) *A. nidulans* pellets grown in a shaking flask culture at 37ºC for 96 h rotated at 225 rpm. (B) Shaking flask fermentation broth after 96 h.

the tubing was filled with water. Figure 6.5 shows the residence time over flow rate. A logarithmic relationship can be seen. Between each change of flow rate, the system was allowed to reach steady state for 30 min. It can also be seen that when no flow was applied, 560 ml of water stayed in the column. This amount of water is adsorbed to the rock surface and in capillaries within the rocks. In a separate experiment the water holding capacity was determined on a mass of water per mass of lava rocks basis. By submerging a known mass of rocks in water and weighing the rocks after draining the surface water a factor of 0.214 g water/g rocks was calculated. When this factor is applied to the mass of rocks used in this experiment the amount of water adsorbed to the rocks would be 535 g. The volume per mass ratio of the rocks was determined by a volume replacement experiment. The factor is 0.579 ml/g. Using this factor the static liquid holdup in the rocks was 0.370 ml water/ml rocks.

6.4.3 Enzyme production in reactor

6.4.3.1 Enzyme production without pyridoxine limitation

The reactor design was tested with and without pyridoxine limitation. After 42 h of inoculation, a uniform fungus formation was observed, which was when semi continuous operation was initiated. The medium was recycled with flow rates equal or higher than 51 ml/min to achieve a uniform substrate concentration throughout the column. Every 6 h a volume of 240 ml was taken out from the mixing vessel into the storage tank and replaced with fresh medium. Figure 6.6A shows the protein concentration in the reactor, as well as in the storage tank.

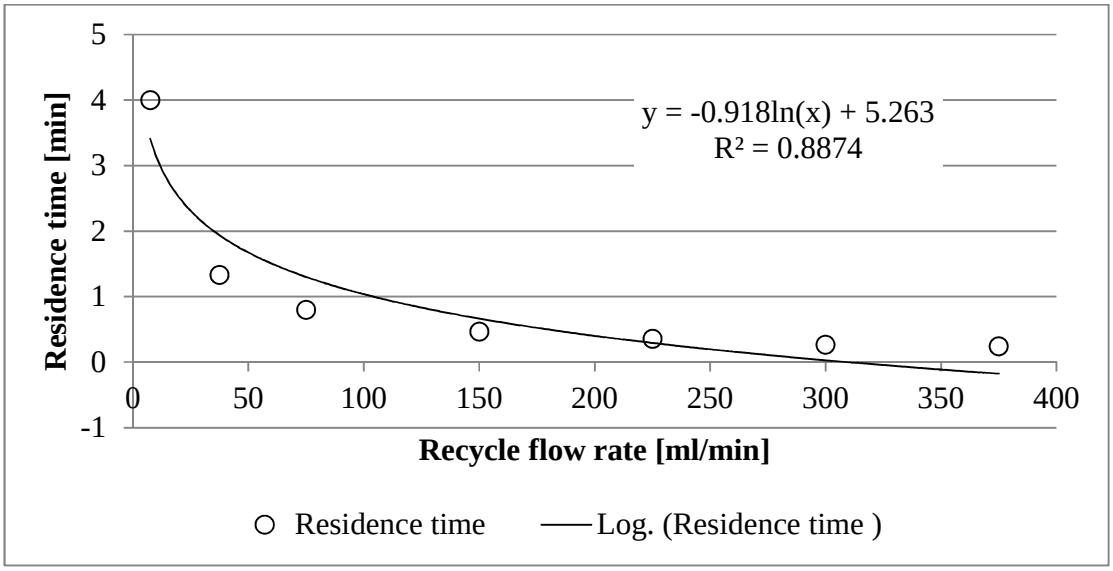
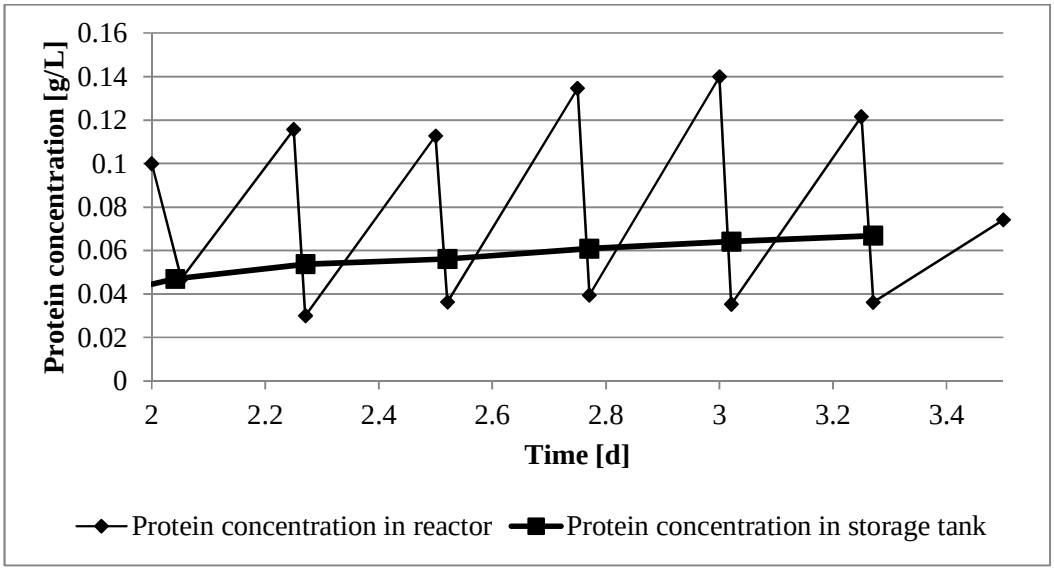
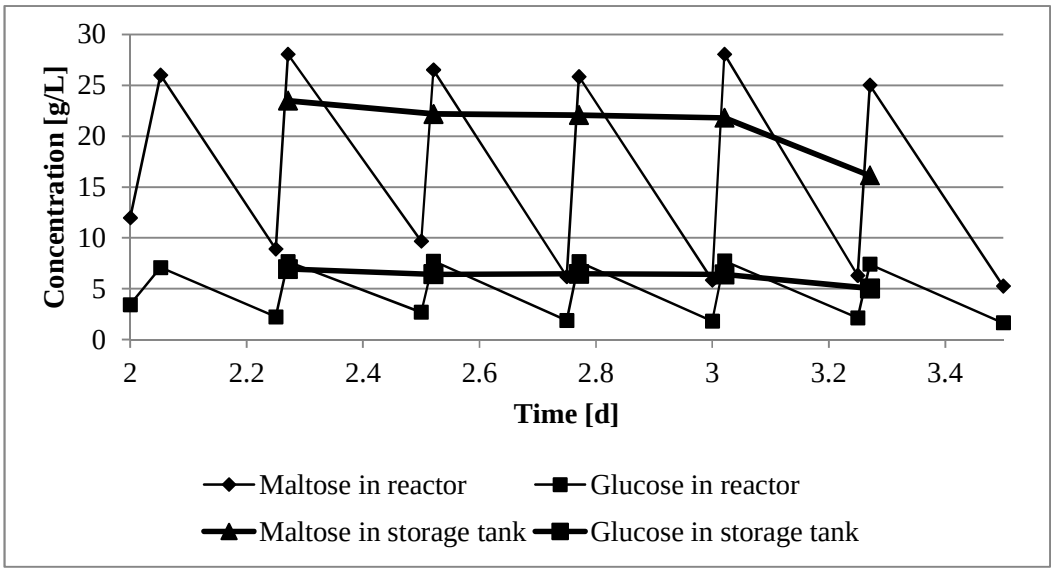


Figure 6.5: Comparison of residence time with different recycle flow rates through lava rock packing of trickle bed reactor. Test was performed with DI water at room temperature.



A



B

Figure 6.6: Protein formation (A) and substrate utilization (B) in trickle bed reactor and storage container without pyridoxine limitation using semi-continuous operation. Every 6 h 240 ml of fermentation broth were pumped out and replaced with fresh medium.

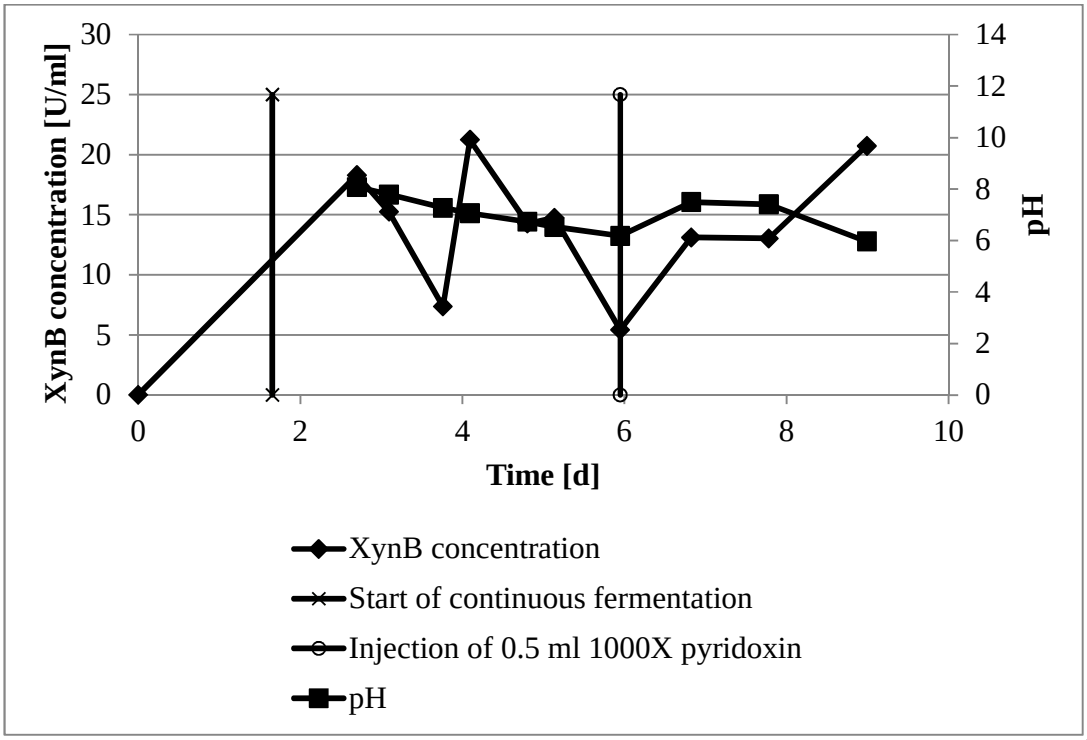
Also shown in Figure 6.6 (B) is the glucose and maltose concentration. Between day 2 and 3.3, a constant enzyme production was achieved. However, at day 3.5, the tubing in- and outlets were clogged and eventually the process was terminated. The zigzag pattern in protein concentration was due to the semi continuous operation. Right before the feeding, the concentration of protein was highest in the reactor. After feeding the reactor with fresh medium, the protein concentration was low due to a dilution with fresh medium. The protein concentration in the storage tank slightly increased from 0.047 g/L at day 2 to 0.067 g/L after 3.3 days. During the 6-h-periods, 80% of glucose and 77% of maltose were utilized. However, as can be seen in Figure 6.6B, the maltose and glucose concentration in the storage tank was higher. Including the storage container, a maltose utilization of 53% and a glucose utilization of 35% was achieved. The activity was not measured during the reactor run. Using an average activity per protein concentration of 575 U/g protein, obtained from later experiments, an estimated average XynB production of 31 U/ml was produced and present in the storage tank. While this test demonstrated a possible continuous enzyme production with a trickle bed reactor, the high risk of clogging needed to be addressed in order for the reactor to be operational over a prolonged time.

6.4.3.2 Enzyme production with pyridoxine limitation

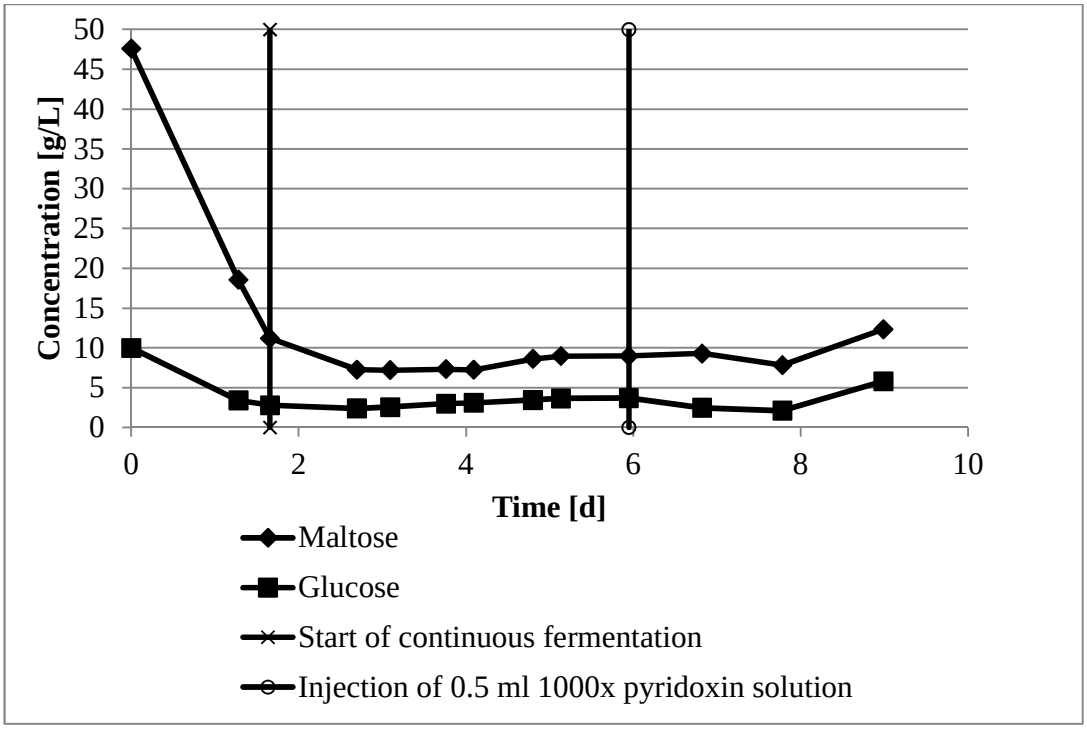
A continuous enzyme production with limited risk of clogging was achieved by reducing growth with pyridoxine limitation. In a petri dish experiment, the feasibility to produce protein continuously without fungal growth was demonstrated (Chapter V). This concept was applied to the reactor design.

In the first experiment, the reactor was filled with 2,502 g of lava rocks. The water holding capacity of the lava rocks was previously determined. A volume of 550 ml of medium was required to saturate 2,502 g of rocks. After autoclaving the reactor was filled with 700 ml of medium with pyridoxine, which was recycled at a flow rate of 224 mL/min until all rock surfaces were wetted and a steady level of 160 ml of medium in the mixing vessel was achieved. A fungal spores suspension (8×10^7 spores/ml) containing 20.0 mg spores (dry weight) was injected and allowed to grow for 1.7 days. After 1.7 days the medium inside the reactor was allowed to trickle down into the mixing vessel and then was pumped out. The same amount of fresh medium without pyridoxine (150 ml) was added and recycled at a flow rate of 224 ml/min until the desired level of medium (160 ml) in the mixing vessel was reached. Then the system was switched to continuous operation with an in- and effluent outflow rate of 1.0 ml/min ($D = 0.0014\ h^{-1}$). After 2.1 days of inoculation, a XynB activity of 14.4 U/ml was reached, which was held for 4 days (Figure 6.7A). As expected the pH dropped from 8.08 at 2.7 d to 6.18 at 6.0 d. The same behavior was observed during the petri dish experiment in Chapter V and is an indicator when pyridoxine needs to be replenished. At 6.0 d, 0.5 ml of 1000x pyridoxine solution was added. As can be seen in Figure 6.7A, the XynB activation increased again to 13.1 U/ml and the pH increased to 7.49.

The substrate concentration can be seen in Figure 6.7B. After continuous production was initiated, the concentration of maltose was 7.3 g/L, which is equivalent to a substrate utilization rate of 85%. The concentration of glucose was 3.0 g/L, which is equivalent to a substrate utilization rate of 75%.



A



B

Figure 6.7: XynB Production and pH development (A) and substrate utilization (B) with 1.0 ml/min influent and effluent flow rate. Recycle flow rate was 224 ml/min.

Due to contamination, the continuous product formation stopped after 7.8 days. However, the process was allowed to continue while an attempt to remove the contamination was tried by the addition of ampicillin. During this period it was observed that the protein concentration dropped to a minimum of 0.017 g/L after 9 days. The activity measured was 1194.79 U/mg. This would result in a volumetric activity of 20.3 U/ml. Even with reduced protein production, the XynB metabolism was unaffected.

In a second experiment, the fermentation conditions were modified to achieve an improved XynB production. The lava rocks from the previous experiment were replaced with a fresh set with a total mass of 2,353 g. The reactor was filled with 740 ml pyridoxine containing medium and recycled to achieve a uniform wetting of the lava rocks and a steady liquid level in the mixing vessel (160 ml). The same amount of spores was added as in the previous experiment (8×10^7 spores/ml). After 1.9 days continuous operation was started with an influent and effluent flow rate of 0.5 ml/min ($D = 0.0007\ h^{-1}$). Table 6.1 shows an overview of the most important results. A continuous XynB production was achieved for 7 days with a concentration of 17.81 U/ml (Figure 6.8). After day 6.9 0.5 ml of 1000x pyridoxine solution were added since a decrease in XynB and pH was observed. At day 9.3 the recycle flow rate was reduced by 50% to 112 ml/min. The change of flow rate resulted in an increase of XynB production to an average of 28.09 U/ml and increased the productivity from 3.6 U/g cells*h to 5.7 U/g cells*h. A decrease of recycle flow rate of 50% doubles the residence time. The medium is longer in contact with the mycelium, which explains the increase in XynB concentration. Further improvement of XynB production was achieved when the temperature was increased from 32ºC to 37ºC at day 13.3. The XynB concentration increased to 33.36 U/ml and

Table 6.1: Comparison of product yields at different time intervals with 0.5 ml/min flow rate.

Time [d]	Average product formation [U/ml]	Average sugar concentration [g/L]	Dry cell mass [g]	$Y_{P/S}$ [U/g]	$Y_{P/X}$ [U/g]
2 – 9	17.81	21.62	n.a.	494.78	-
9 – 13	28.09	16.23	n.a.	678.56	-
13 – 17	33.36	7.48	n.a.	665.25	-
18 - 19	40.26	4.34	148.8	755.58	626.08

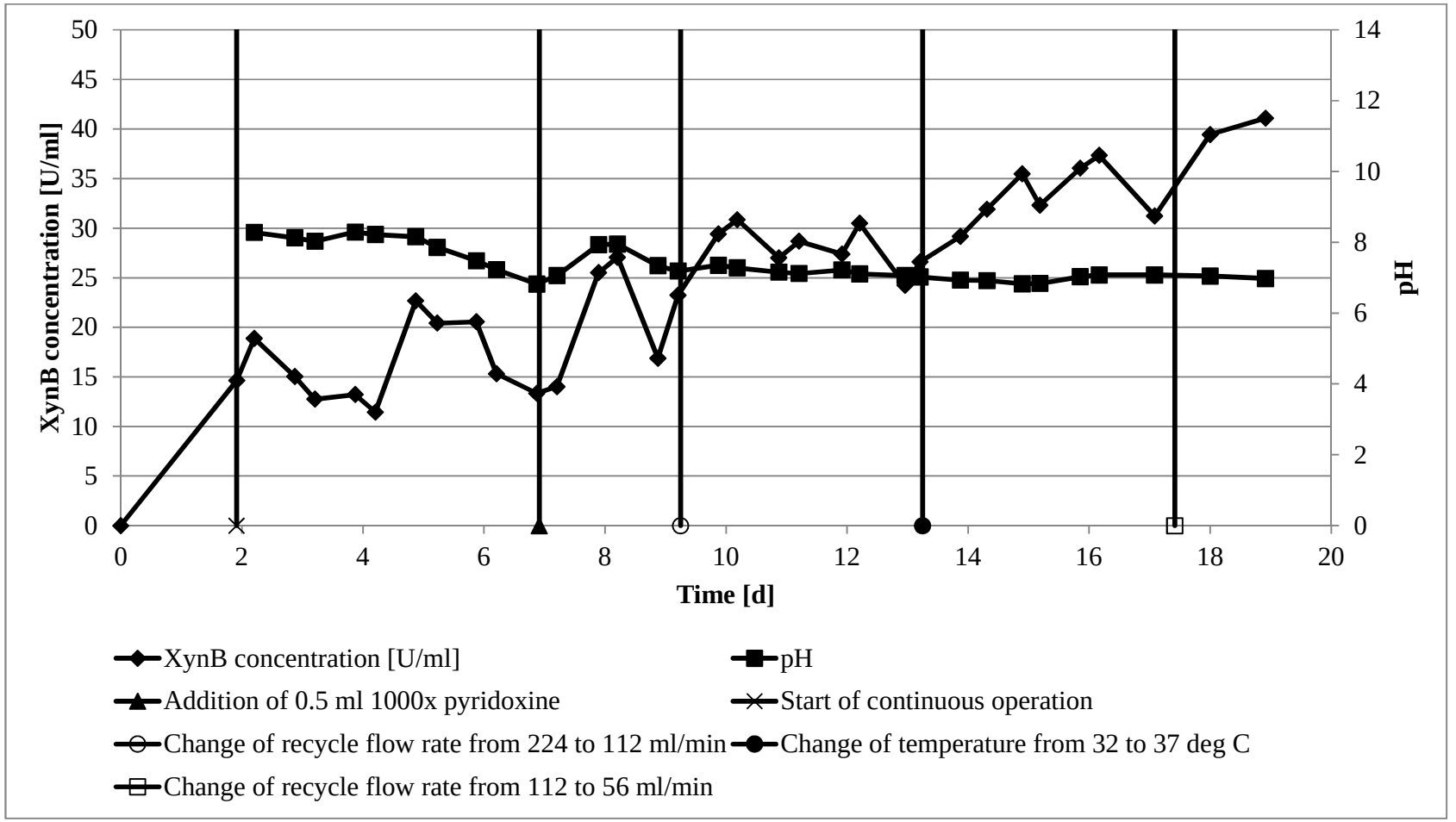
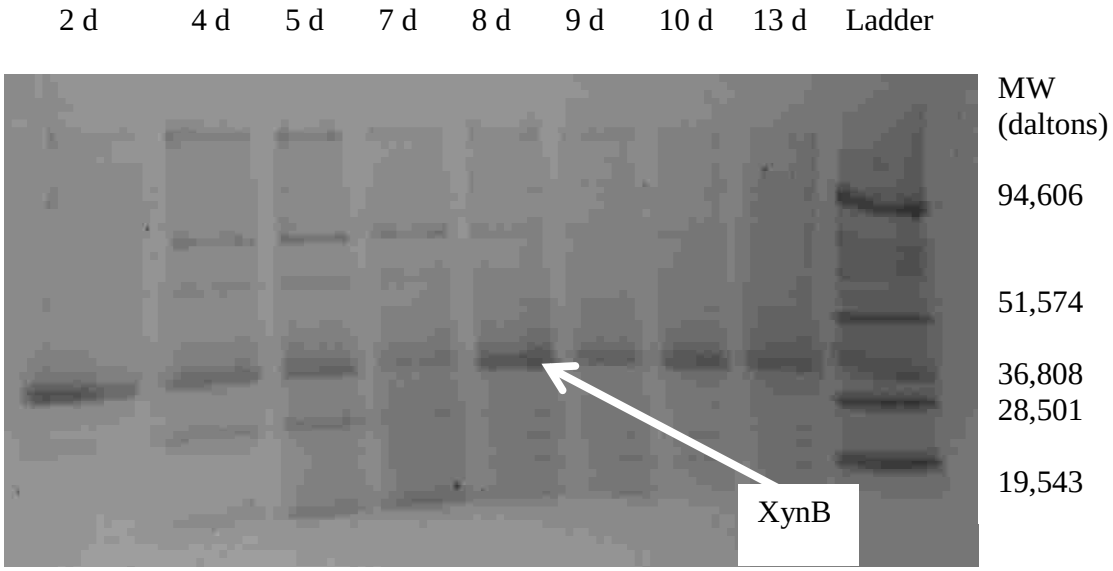


Figure 6.8: XynB production and pH development in trickle bed reactor. Continuous flow in and out was set at 0.5 ml/min. Initial recycle flow rate was 224 ml/min.

productivity increased to 6.7 U/g cells*h. The improvement by adjusting the temperature to 37ºC was expected since 37ºC is the optimum growth temperature for *A. nidulans*. At day 17.4 the flow rate was again reduced by 50% to 56 ml/min, which increased of XynB production to 40.26 U/ml and a productivity of 8.1 U/g cells*h. Further confirmation of XynB production was given by SDS-PAGE analysis. Figure 6.9 shows the XynB bands for selected time points. The XynB bands are the most predominant bands in the pictures and indicate a high purity of XynB.

The reactor produced high titers of XynB. A comparison of XynB with SSF xylanase production in the literature is not directly possible since the present study is a continuous process; whereas; SSF is performed in batch mode. Typical xylanase production results from SSF are given in U/g of solid substrate, while this study uses U/ml. Azin et al. (2007) reported a xylanase production of 600 U/g substrate using *Trichoderma longibrachiatum* on a mixture of wheat bran and wheat straw. Converting the xylanase concentration to U/ml by incorporating the substrate mass and volume liquid used for enzyme extraction results in a volumetric activity of 35.3 U/ml. Similarly, the xylanase production achieved by Gessesse and Mamo (1999) was reported to be 600 U/g using an alkaliphilic *Bacillus* sp. on wheat bran. Conversion to volumetric activity gives 0.6 U/ml. Park et al. (2002) reported an about 10-fold higher xylanase production of 5,000 U/g substrate compared to other literature findings. The researchers used *A. niger* on rice straw. When substrate mass and extraction liquid volume are incorporated to the result, the volumetric activity was 125 U/ml. The difference with other findings may be explained by the usage of a different organism.

The current reactor design also produced higher XynB concentrations compared



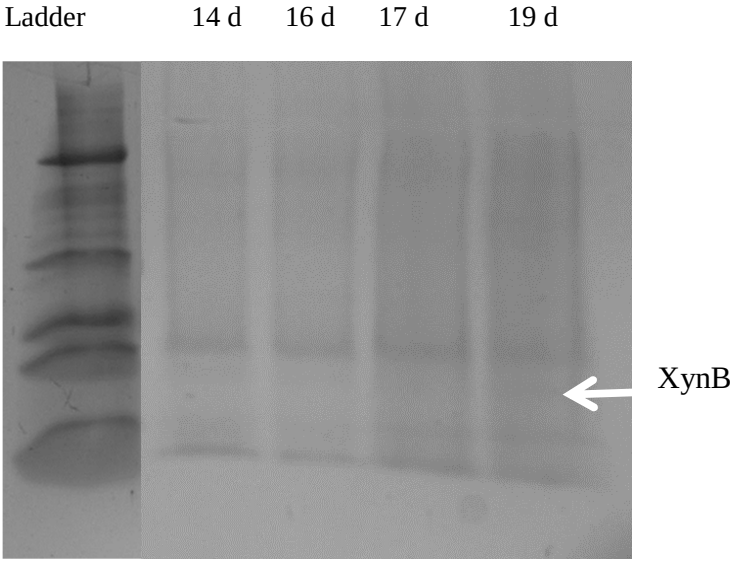


Figure 6.9: SDS-PAGE analysis of reactor XynB production at different selected time points.

to the static tray culture and reached the level of the shaking flask culture. Product from cell mass and substrate yield were both higher for the reactor compared to the static tray. The reactor achieved an $Y_{P/X}$ of 626.1 U/g and an $Y_{P/S}$ of 755.7 U/g (Table 6.1). By recycling the medium and increasing the surface for mycelium growth, the reactor likely allows improved mass transfer over the static tray.

The substrate consumption throughout the reactor run increased with changes in operation conditions. The highest maltose and glucose utilization was achieved at day 18 with the lowest recycle flow rate (56 ml/min) and 37°C (Figure 6.10). During the highest product formation, 92% and 78% of maltose and glucose were consumed, respectively. With a lower recycle flow rate, the residence time of flowing liquid through the packing increased, which allowed for improved diffusion of substrate into the fungus matrix. However, the mass transfer in the reactor can be further improved. Channeling occurred due to insufficient medium distribution on top of the column. The channeling led to areas in the bed where no medium or only limit amounts of medium came into contact with the mycelium. These issues could be addressed by changing the diameter to height ratio of column and with an improved medium inlet on top of the column, such as a spray nozzle.

6.4.4 Prediction of XynB production and substrate utilization

The XynB production and substrate utilization were modeled using CSTR model equations. CSTR modeling compared to packed-bed reactor modeling leads to a more simplified prediction of product formation. In this experiment the recycle flow rate was high enough, with a residence time of 22 s, to assume a constant concentration of substrates, product and cell mass throughout the reactor column and the mixing vessel. It

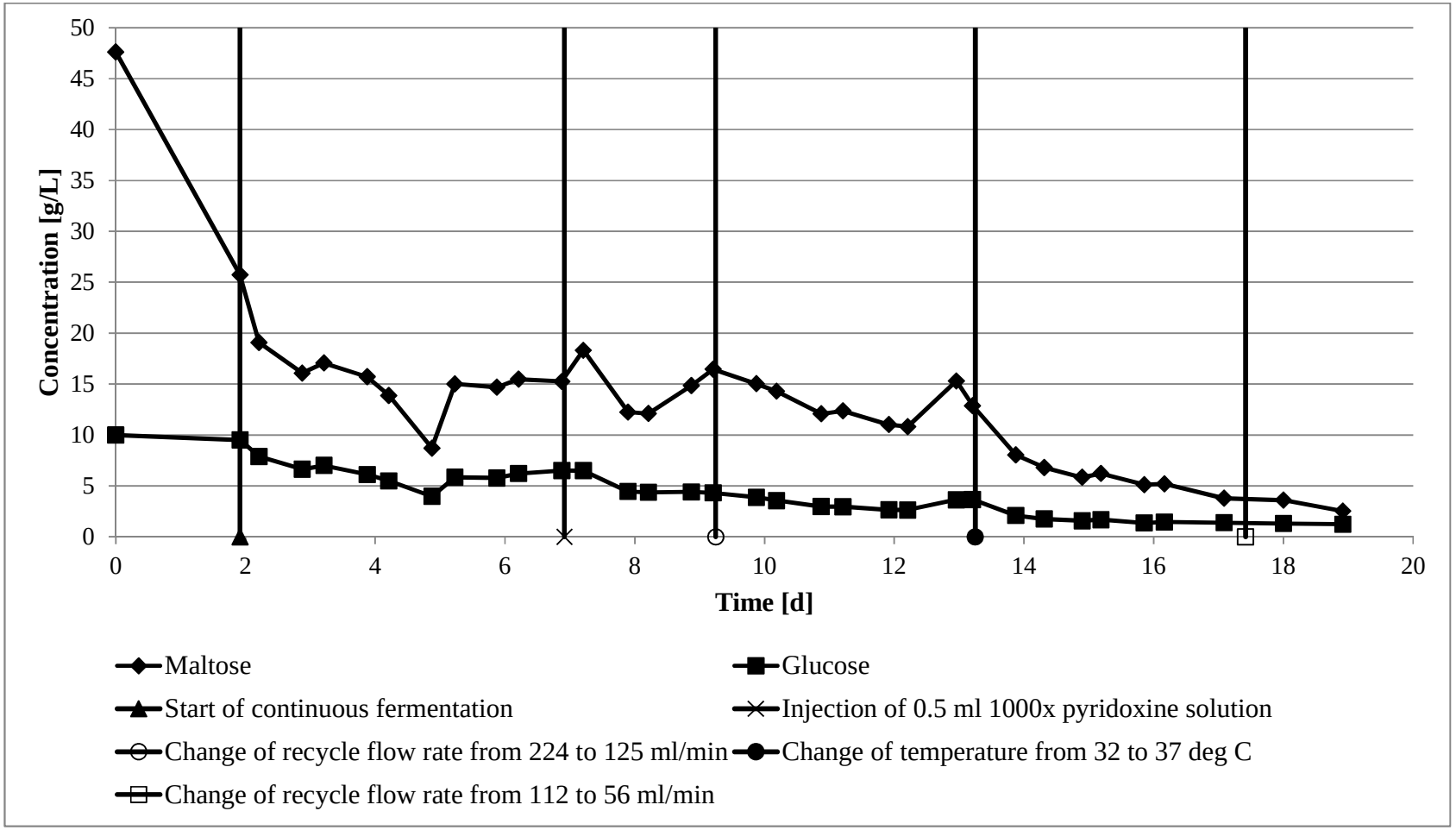
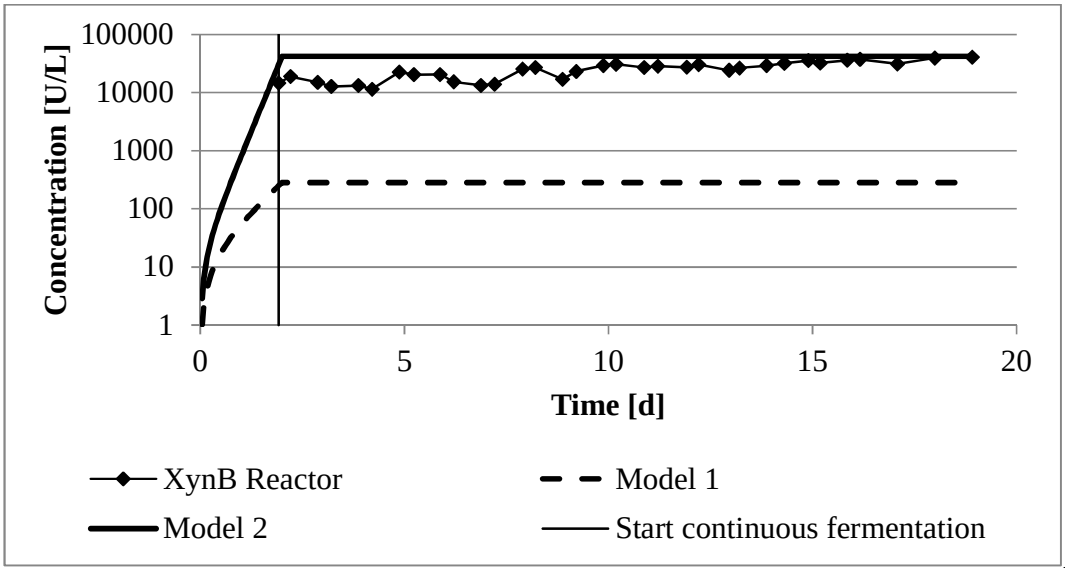


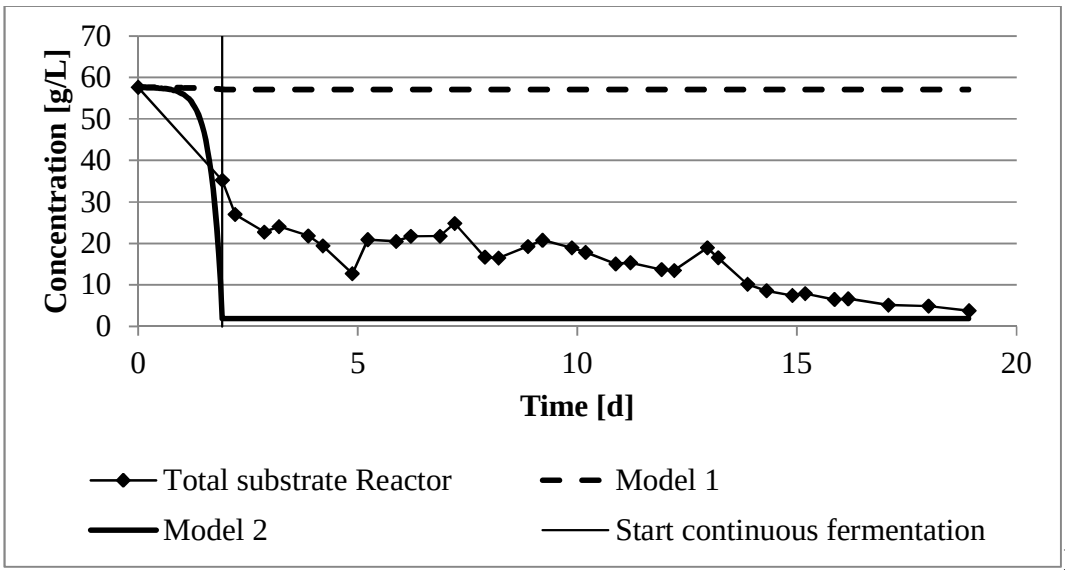
Figure 6.10: Maltose and glucose consumption in trickle bed reactor. Continuous flow in and out was set at 0.5 ml/min. The initial recycle flow rate was 224 ml/min.

was not possible to measure cell mass concentration during the experiment. With a known initial cell mass concentration, the cell mass could be predicted over time. Using this predicted cell mass, the XynB formation and the substrate utilization could be predicted. The required parameters were, S_0, which was maltose (47.6 g/L), glucose (10 g/L), or the summation of both (57.6 g/L), X_0 = 0.033 g/L, μ_{net} = 0.061 h^{-1}, $Y_{P/X}$ = 480 U/g, $Y_{X/S}$, which was different for maltose (1.2), glucose (19.2), and both together (1.13). Model 1 is based on summation of both sugars as substrate concentration. Figure 6.11A shows the actual XynB production in the reactor and the predicted XynB production (Model 1). It also shows a different prediction, Model 2, which was calculated with an adjusted net grow rate. The adjusted growth rate was back calculated using the actual XynB production at day 18 in the reactor. With the known product concentration at day 18 and $Y_{P/X,}$ the theoretical cell mass concentration can be determined. With the theoretical cell mass concentration and the known initial cell mass, the μ_{net} necessary to achieve the theoretical cell mass at 48 h can be calculated (Figure A4). As can be seen in Figure 6.11B, which shows the total substrate utilization, Model 2 is more suitable. Graphs predicting cell growth, maltose and glucose utilization can be found in the Appendix. The adjusted net growth rate was 0.164 h^{-1}. The previous μ_{net} was measured in a Petri dish experiment at equal conditions. The reactor improves the growth rate of the fungus by 270%. The reactor leads to a higher mass transfer by continuously pumping the medium in the recycle and by actively supplying the culture with air.

An alternative model (Model 3) could be established using the XynB productivity determined in Chapter V. The productivity for a culture grown on medium without pyridoxine was 21.14 U/g dry cell mass*h. The predicted dry cell mass was



A



B

Figure 6.11: Comparison of actual XynB production in reactor (A) and substrate utilization (B) to predictions using CSTR modeling. With S_0 = 57.6 g/L, $Y_{P/X}$ = 480 U/g, $Y_{X/S}$ = 1.13, μ_{net} = 0.061 h^{-1} (Model 1), and μ_{net} = 0.164 h^{-1} (Model 2). μ_{net} for Model 2 was back calculated from actual product formation in reactor. The cell concentration is assumed constant after initiating continuous fermentation.

calculated in the same manner as in Model 2. The corresponding XynB prediction was calculated using the productivity and multiplying it with the predicted cell mass and time. The resulting predicted XynB content is given as the accumulative XynB content in U. The comparison of the predicted and actual accumulative XynB content can be seen in Figure 6.12. The predicted XynB formation over time follows a lower slope than the actual XynB production in the reactor. After completion of 18 days continuous fermentation, the total cell mass inside the reactor was measured. While Model 2 predicted a cell mass 61 g, the total cell mass after 18 days was 149 g. It is unknown what percentage of the total cell mass was viable cell mass. Furthermore, it was observed that the cell mass in the reactor increased over time. Figure 6.13 shows the reactor column at different time points. At day 7 a small amount of pyridoxine solution was added in order to keep XynB production running. It is possible that some of the pyridoxine recycled through the reactor over a prolonged time, allowing the fungus to grow slowly but steadily. No more pyridoxine injection was necessary after day 7 for the rest of the test period. The pH was relatively constant over 11 days, signaling that the fungus was able to utilize pyridoxine. A more likely explanation can be found in a process called autolysis. It was shown that filamentous fungi under starvation initiate a process where in order to allow mycelium tip growth, old mycelia are broken down enzymatically to recycle cell components (White et al. 2002). This recycle of old mycelia mass could be a potential source for recycled pyridoxine and would explain why the fungal mass, viable plus dead, increases over time. A slow increase of cell mass over time would also explain the higher accumulative XynB content compared to the model prediction.

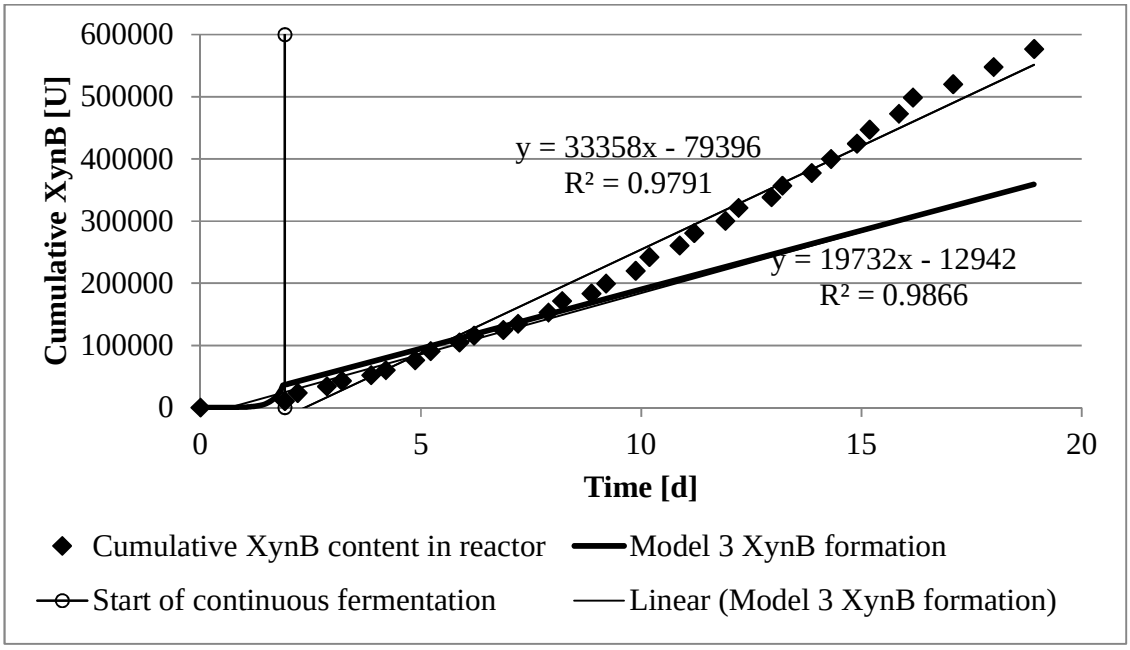


Figure 6.12: Comparison of actual cumulative XynB content in reactor to model prediction using an experimentally determined productivity of 21.14 U/g dry cell mass*h and a cell mass prediction using CSTR modeling.

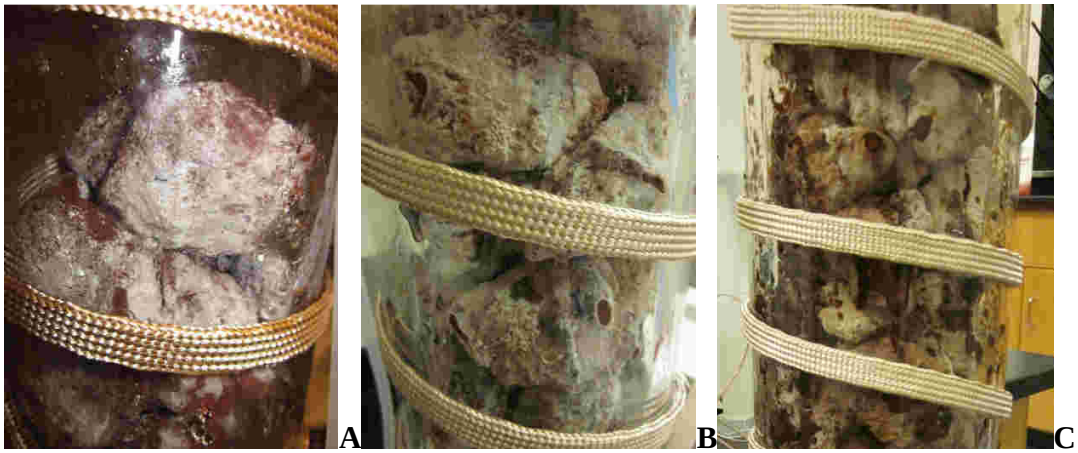


Figure 6.13: Fungal growth in trickle bed reactor on lava rock surface. Growth after 1 day (A), 2 days (B), and 9 days (C).

It is proposed that an *A. nidulans* mutant with a different marker, which is not essential for amino acid metabolism, would produce higher amounts of protein compared to the pyridoxine system. A potential marker could be biotin. Biotin is used for carboxylation reactions and its absence would not interfere with amino acid metabolism, but limit growth. Biotin is a coenzyme for pyruvate, acetyl-CoA, and propionyl-CoA carboxylase (Voet and Voet 2004). When acetyl-CoA carboxylase is inactive due to biotin depletion, no fatty acid synthesis would take place. It is assumed that growth would be limited, since membrane wall components (fatty acids) are missing.

6.5 Conclusion

It was demonstrated that a trickle bed reactor system is applicable in producing enzymes by *A. nidulans*. Fungal mycelia favor surfaces for growth and can clog pipes, tubing tube connectors, reactor inlets and outlets. A solution to these problems is limiting the growth of the organism by changing the conditions. This was successfully demonstrated by limiting the pyridoxine supply to an *A. nidulans* mutant unable to synthesize its own pyridoxine over a period of 19 days. Small amounts of pyridoxine have to be added to the system to elongate enzyme productivity.

6.6 Future work

Since the prototype of the TBR was designed and constructed from scrap parts, multiple modifications should be incorporated to achieve higher production efficiency. The main drawback of the current design was the creation of channeling. The diameter of the column was 10 cm with a column height of 50 cm. This narrow design, with a

diameter to height ratio (DH) of 0.2, leads to channeling where the trickling fluid flows along the glass wall without touching the rock. Only a small amount flows down the rocks and has contact with the fungus. Typical dimensions for trickle bed reactors have a DH between 0.5 and 1.0 (Winterbottom and King 1999).

A second improvement would be the medium distribution on top of the column. Currently, four static inlets distribute the medium. Since the location of the inlets does not change over time, the impact point of medium on the rocks is constant, also leading to channeling. An improvement could be a moving arm with multiple outlets that rotates at a constant speed as it is used in trickle bed fermenters in the waste water industry. Another option could be an agitator placed underneath one central inlet tube. The agitator would rotate and spray the medium over the column. However, in all cases large open tubes should be used to reduce the risk of clogging.

Further improvement could be achieved by using a steal basket for the solid support. This would simplify the loading and unloading of the solid support. After completion of a fermentation, the used support could be removed like a cartridge and replaced by a new one.

The mixing vessel could be replaced by a larger container with means for separating mycelia cell mass that gets washed out from the column. The product stream would contain no visual cell mass and could be processed directly for protein purification and concentration.

Furthermore, a pH probe for online pH monitoring and a CO_2 sensor for indirect cell mass measurement would simplify process control. Monitoring the pH online would also reduce the sampling volume. The current system does not allow cell mass

measurement. Only after the process is terminated can the total cell mass can be determined by measuring the mass gravimetrically.

Besides improving the reactor hardware, different solid support matrices could be tested. The lava rocks have been proven to be a successful inert support for the filamentous fungus to grow. However, lava rocks have a higher density compared to other materials that could be used and contribute greatly to the total weight of the reactor system. Especially in up-scaling, the weight of the rocks could lead to increased costs of reactor equipment to accommodate the mechanical strength of the equipment and the handling of the rocks. Furthermore, the reuse of the lava rocks requires an extensive cleaning process. It was observed that the fungus grows very persistently on the rock surface. The chemical industry uses plastic composites or metals as materials for solid support matrices, which have a high specific surface area by keeping the weight at a minimum.

The improvements mentioned above deal with the hardware and dimensions of the reactor. Further improvements involve the *Aspergillus* strain itself and its ability to produce enzyme while its growth is limited by coenzyme limitation. Future work should concentrate on the usage of an *A. nidulans* strain with biotin as a marker, leaving the amino acid metabolism undisturbed. It is hypothesized that with a biotin marker system more client enzyme could be improved than with the pyridoxine system.

6.7 References

Aguilar CN, Augur C, Favela-Torres E, Viniegra-González G. 2001. Production of tannase by Aspergillus niger Aa-20 in submerged and solid state fermentations: influence of glucose and tannic acid. Journal of Industrial Microbiology and Biotechnology 26(5):296-302.

Azin M, Moravej R, Zareh D. 2007. Production of xylanase by *Trichoderma longibrachiatum* on a mixture of wheat bran and wheat straw: Optimization of culture condition by Taguchi method. Enzyme and microbial technology 40(4):801-805.

Betini JHA, Michelin M, Peixoto-Nogueira SC, Jorge JA, Terenzi HF, Polizeli M. 2009. Xylanases from Aspergillus niger, Aspergillus niveus and Aspergillus ochraceus produced under solid-state fermentation and their application in cellulose pulp bleaching. Bioprocess and biosystems engineering 32(6):819-824.

Cannel E, Moo-Young M. 1980. Solid state fermentation systems. Process Biochemistry 15:2-7.

Cheng KC, Demirci A, Catchmark JM. 2010. Advances in biofilm reactors for production of value-added products. Applied microbiology and biotechnology 87(2):445-456.

de Carvalho Peixoto-Nogueira S, Michelin M, Betini JHA, Jorge JA, Terenzi HF, de Lourdes Teixeira de Moraes Polizeli M. 2009. Production of xylanase by Aspergilli using alternative carbon sources: application of the crude extract on cellulose pulp biobleaching. Journal of Industrial Microbiology and Biotechnology 36(1):149-155.

Diaz-Godinez G, Soriano-Santos J, Augur C, Viniegra-Gonzalez G. 2001. Exopectinases produced by Aspergillus niger in solid-state and submerged fermentation: a comparative study. Journal of Industrial Microbiology and Biotechnology 26(5):271-275.

Durand A, Almanza S, Renaud R, Maratray J. 1997. Solid state fermentations: an attractive alternative to submerged-liquid fermentations. Agro Food Industry Hi-Tech 8:39-42.

El-Enshasy H, Hellmuth K, Rinas U. 1999. Fungal morphology in submerged cultures and its relation to glucose oxidase excretion by recombinant *Aspergillus niger*. Applied biochemistry and biotechnology 81(1):1-11.

Fujita M, Iwahori K, Tatsuta S, Yamakawa K. 1994. Analysis of pellet formation of *Aspergillus niger* based on shear stress. Journal of fermentation and bioengineering 78(5):368-373.

Garcia-Kirchner O, Muñoz-Aguilar M, Pérez-Villalva R, Huitron-Vargas C. 2002. Mixed submerged fermentation with two filamentous fungi for cellulolytic and xylanolytic enzyme production. Applied biochemistry and biotechnology 98(1):1105-1114.

Gessesse A, Mamo G. 1999. High-level xylanase production by an alkaliphilic *Bacillus sp.* by using solid-state fermentation. Enzyme and microbial technology 25(1-2):68-72.

Lonsane BK, Ghidyal NPG, Budiatman S, Ramakrishna SV. 1985. Engineering aspects of solid-state fermentation. Enzyme and Microbial Technology 7:258-265.

Mitchell D, Berovic M, Krieger N. 2000. Biochemical engineering aspects of solid state bioprocessing. New Products and New Areas of Bioprocess Engineering:61-138.

Mitchell DA, Lonsane BK. 1992. Definition, characteristics and potential. In: Doelle HW, Mitchell DA, Rolz CE, editors. Solid substrate cultivation. London: Elsevier Science Publishers.

Nigam P, Robinson T, Singh D. 2004. Solid-state fermentation: an overview. In: Arora DK, editor. Handbook of fungal biotechnology. New York: Marcel Dekker, Inc.

Novozyme. 2010. Fermentation. Novozyme.

Park Y, Kang S, Lee J, Hong S, Kim S. 2002. Xylanase production in solid state fermentation by Aspergillus niger mutant using statistical experimental designs. Applied microbiology and biotechnology 58(6):761-766.

Romero-Gomez S, Augur C, Viniegra-González G. 2000. Invertase production by Aspergillus niger in submerged and solid-state fermentation. Biotechnology Letters 22(15):1255-1258.

Sluiter A, Hames B, Ruiz R, Scarlata C, Sluiter J, Templeton D. 2008. Determination of sugars, byproducts, and degradation products in liquid fraction process samples. National Renewable Energy Laboratory(NREL/TP-510-42623).

Tunga R, Banerjee R, Bhattacharyya BC. 1999. Studies of some physical parameters for large scale protease production by SSF. Bioprocess Engineering 21:107-112.

Viniegra-González G, Favela-Torres E. 2004. Basic principles for the production of fungal enzymes by solid-state fermentation. In: Arora DK, editor. Handbook of fungal biotechnology. New York: Marcel Dekker, Inc.

Viniegra-González G, Favela-Torres E, Aguilar CN, Rómero-Gomez SJ, Díaz-Godínez G, Augur C. 2003. Advantages of fungal enzyme production in solid state over liquid fermentation systems. Biochemical Engineering Journal 13(2-3):157-167.

Voet D, Voet JG. 2004. Biochemistry. Hoboken: John Wiley & Sons, Inc.

Vogel HF. 1964. Distribution of lysine pathways among fungi: evolutionary implications. American Naturalist 98:435-446.

White S, McIntyre M, Berry DR, McNeil B. 2002. The autolysis of industrial filamentous fungi. Critical reviews in biotechnology 22(1):1-14.

Winterbottom JM, King M. 1999. Reactor design for chemical engineers: CRC.

Žnidaršič P, Komel R, Pavko A. 2000. Influence of some environmental factors on *Rhizopus nigricans* submerged growth in the form of pellets. World Journal of Microbiology and Biotechnology 16(7):589-593.

APPPENDICES

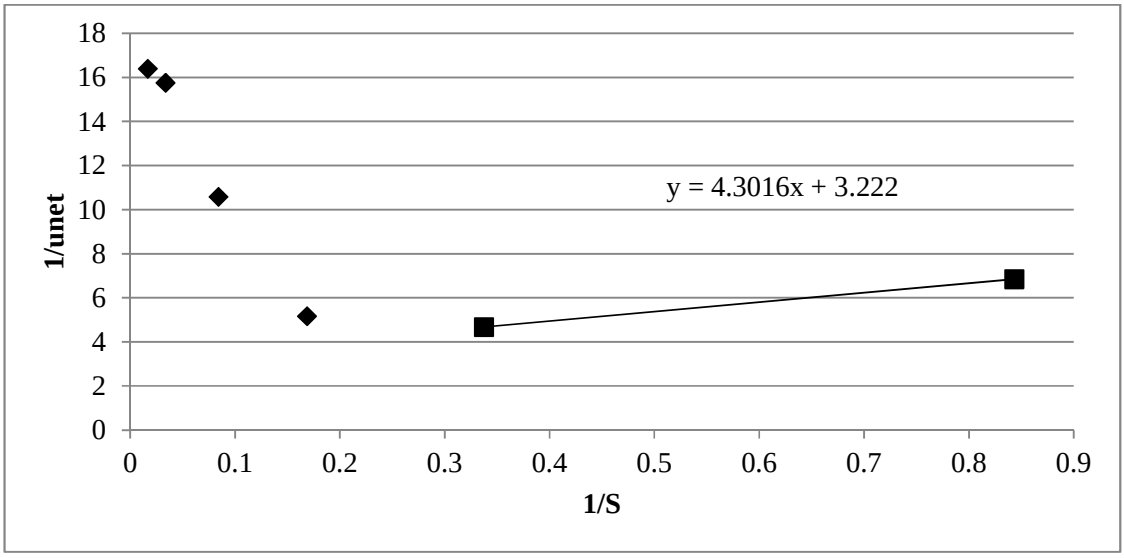


Figure A1: Double reciprocal plot of µnet and initial substrate concentration S with *A. nidulans* grown in Petri dishes.

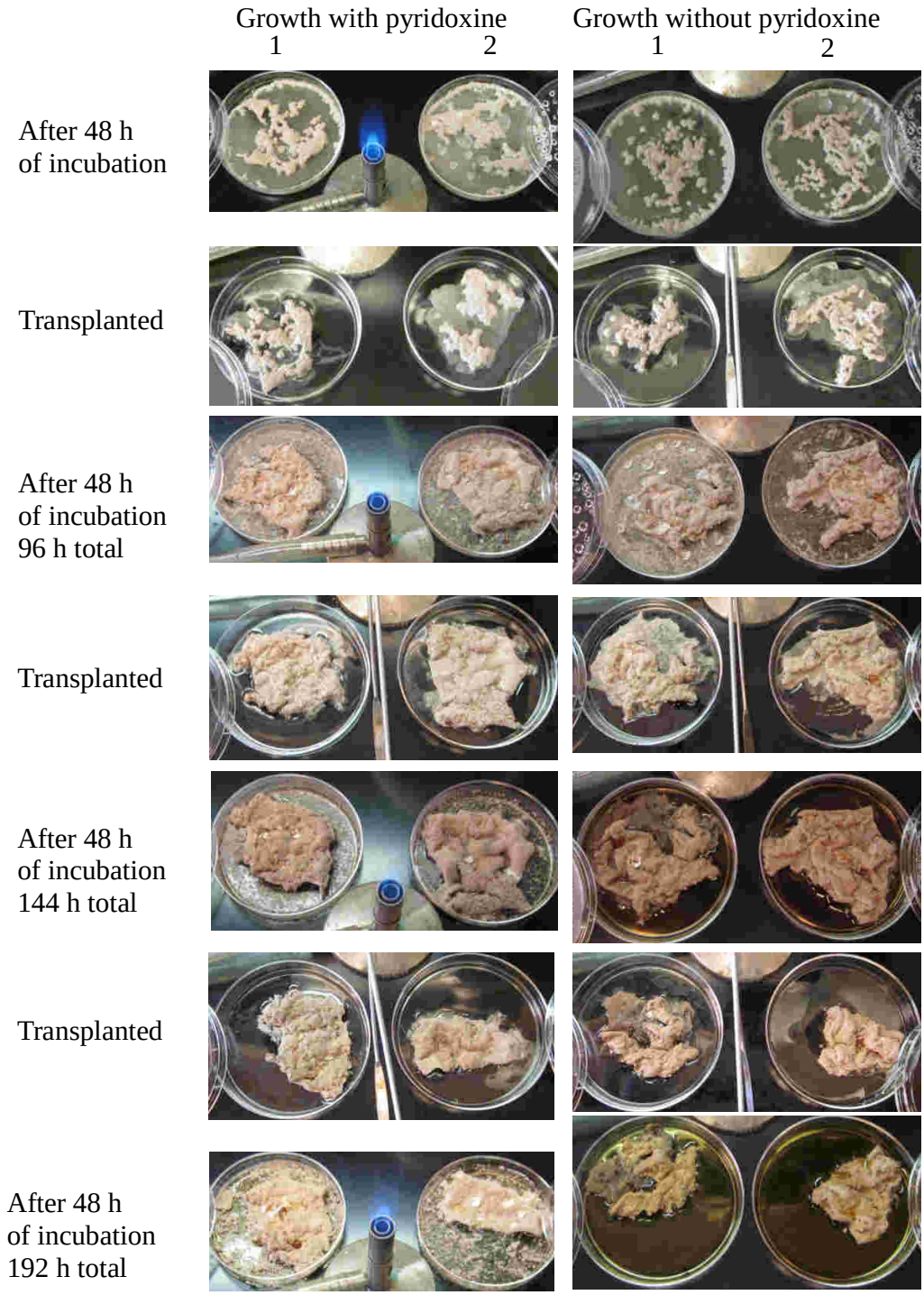


Figure A2A: Comparison of an *A. nidulans* culture grown on medium with and without pyridoxine. The visual observation shows that the culture without pyridoxine shows no mycelia formation.

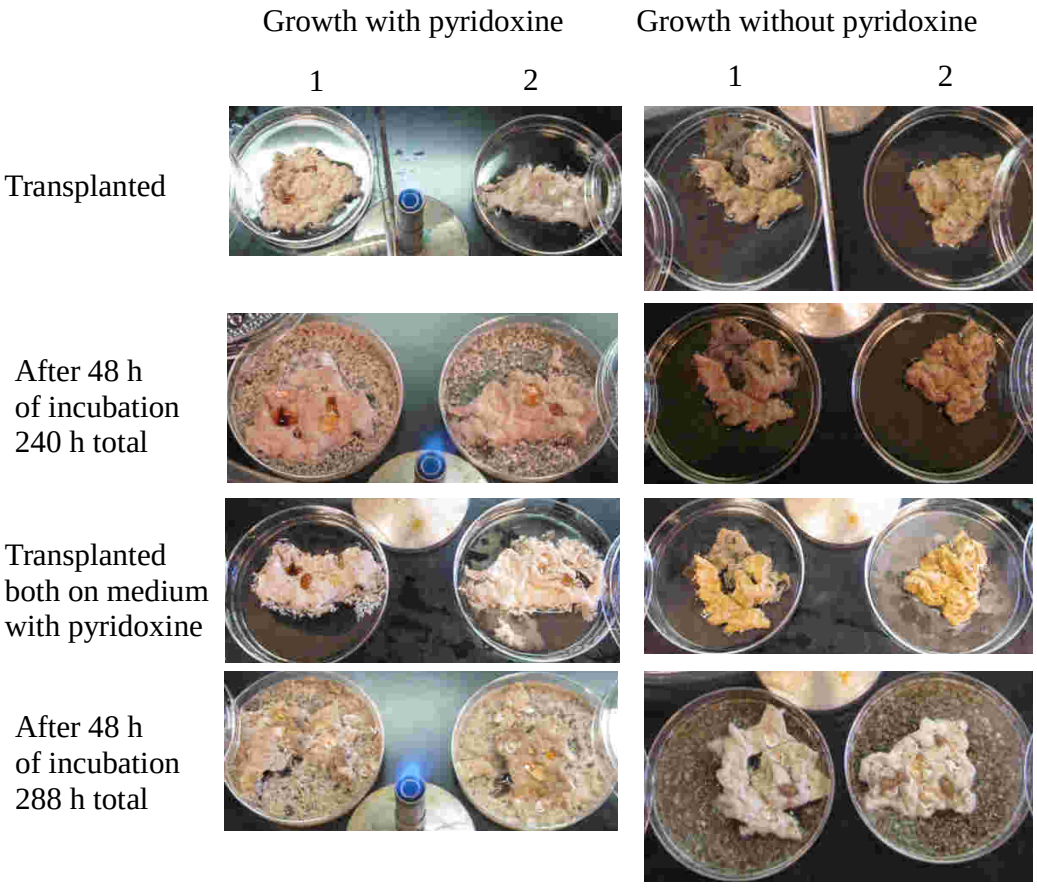
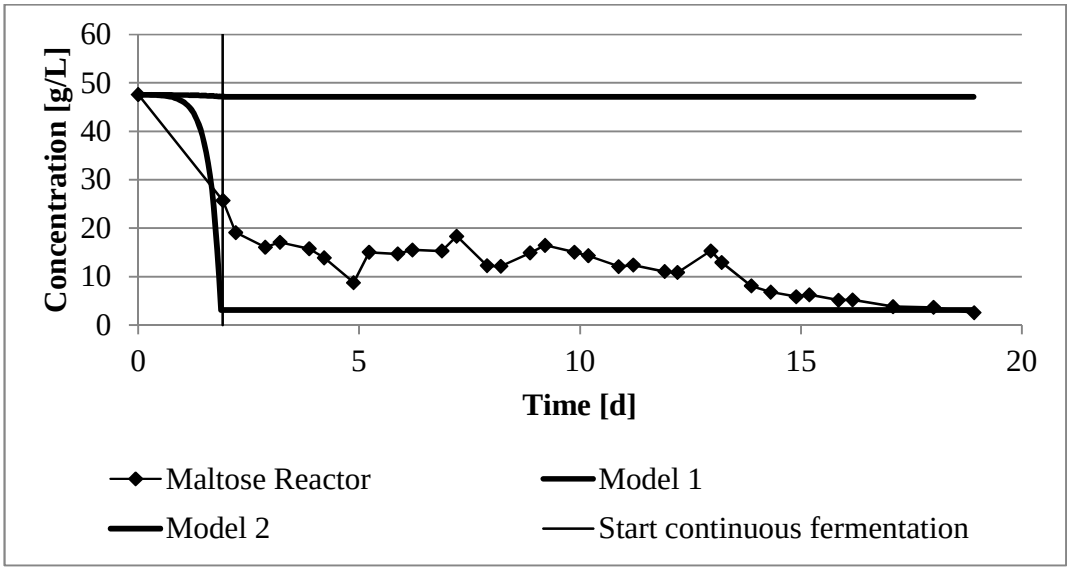
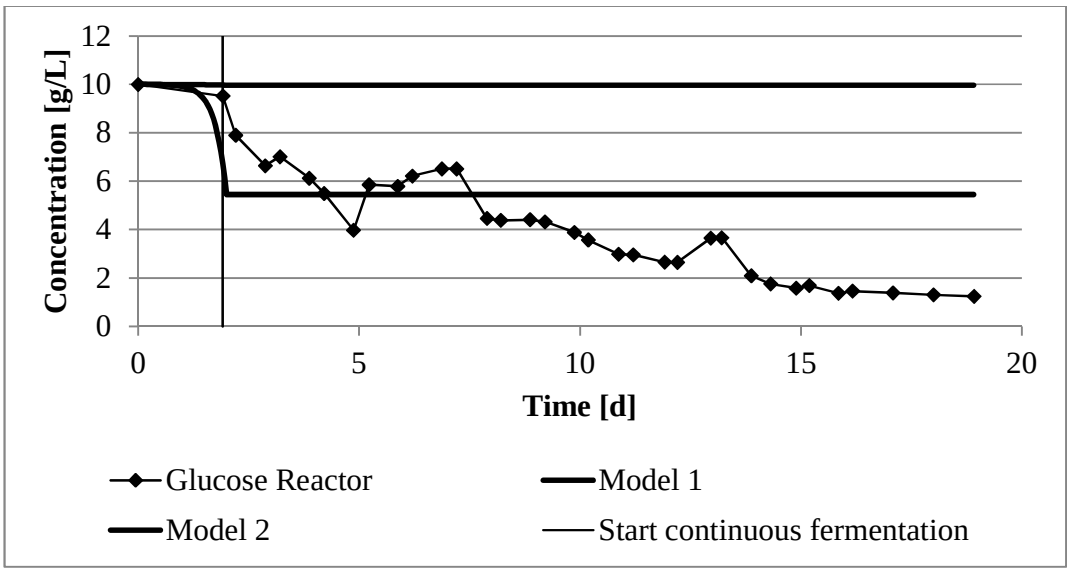


Figure A2B: Continuation of figure A2A. Comparison of an *A. nidulans* culture grown on medium with and without pyridoxine. The visual observation shows that the culture without pyridoxine shows no mycelia formation.



A



B

Figure A3: Comparison of actual maltose (A) and glucose (B) utilization to predictions using CSTR modeling. With $S_{0\_Maltose}$ = 47.6 g/L, $S_{0\_Glucose}$ = 10 g/L, $Y_{P/X}$ = 480 U/g, $Y_{X/S\_Maltose}$ = 1.2, $Y_{X/S\_Glucose}$ = 19.2, μ_{net} = 0.061 h^{-1} (Model 1), and μ_{net} = 0.164 h^{-1} (Model 2). μ_{net} for Model 2 was back-calculated from actual product formation in reactor. The cell concentration is assumed constant after initiating continuous fermentation.

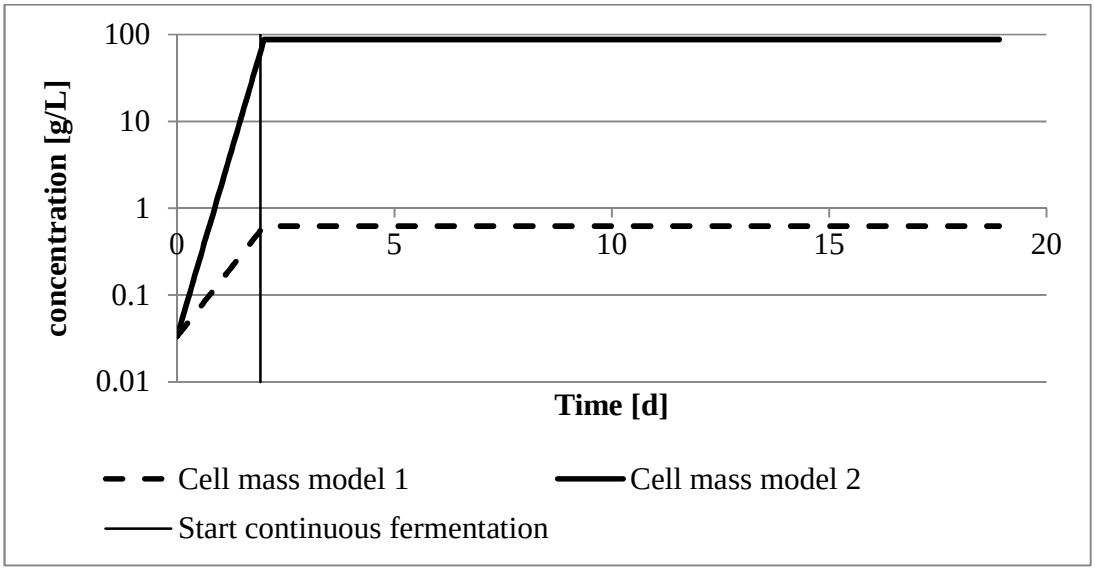


Figure A4: Model prediction of cell mass in reactor. Model 1 with μ_{net} = 0.061h^{-1} and Model 2 with μ_{net} = 0.164 h^{-1}.

VITA

Michael Mueller

Candidate for the Degree of

Doctor of Philosophy

Thesis: UTILIZATION AND PRODUCTION OF SINGLE ACTIVITY ENZYMES FROM *ASPERGILLUS NIDULANS*

Major Field: Biosystems Engineering

Biographical:

Personal Data: Born in Dernbach, Germany, on January 30, 1983
The son of Friedhelm and Margot Müller

Education:
Completed the requirements for the Doctor of Philosophy/Education in your major at Oklahoma State University, Stillwater, Oklahoma in July, 2012.

Completed the requirements for the Master of Science in Biosystems Engineering at Oklahoma State University, Stillwater, OK in May, 2009.

Completed the requirements for Diplom Ingenieur (FH) in Biotechnology at Fachhochschule Bingen, Bingen, Germany in July, 2007.

Experience:
January 2012 to May 2012:
Teaching Assistant, Oklahoma State University, Stillwater, OK, 74074

May 2009 to May 2012:
Research Associate, Oklahoma State University, Stillwater, OK, 74074

August 2007 to May 2009:
Research Assistant, Oklahoma State University, Stillwater, OK, 74074

Professional Memberships:
- American Society of Agricultural and Biological Engineers (ASABE)
- The Association of German Engineers (VDI)

Name: Michael Mueller Date of Degree: July, 2012

Institution: Oklahoma State University Location: Stillwater, Oklahoma

Title of Study: UTILIZATION AND PRODUCTION OF SINGLE ACTIVITY ENZYMES FROM *ASPERGILLUS NIDULANS*

Pages in Study: 155 Candidate for the Degree of Doctor of Philosophy

Major Field: Biosystems Engineering

Scope and Method of Study: The first objective was to determine the synergistic effects of enzyme mixtures for maximum enzymatic hydrolysis of grain sorghum stover untreated and pretreated with liquid hot water pretreatment. The second objective was to determine the feasibility of continuous client enzyme production with an *Aspergillus nidulans* mutant containing a pyridoxine marker by limiting the supply of pyridoxine. The third objective was to determine the feasibility of using the limited growth system with pyridoxine limitation in a trickle bed reactor for prolonged client enzyme production.

Findings and Conclusions: The usage of accessory enzymes together with the commercial available cellulolytic enzyme Cellic CTec2 from Novozymes did not improve the enzymatic hydrolysis of grain sorghum stover. The best glucose and xylose conversion observed was 77% and 68% respectively, achieved on pretreated grain sorghum stover with CTec2.

Pyridoxine limitation was successfully shown to have no effect on XynB production compared to a culture grown with pyridoxine. Additionally, it was observed that the culture did not grow when pyridoxine was absent.

A successful demonstration of continuous XynB production with the trickle bed reactor was shown over a period of 18 days. The reactor achieved a XynB output of 41 U/ml with an influent and effluent flow rate of 0.5 ml/min and a recycle flow rate of 56 ml/min. Production yields are higher compared to a static tray culture and SSF enzyme production stated in the literature.

ADVISER'S APPROVAL: Dr. Mark R. Wilkins