PRODUCTION OF VALUE-ADDED PRODUCTS

FROM MEAT PROCESSING CELLULOSIC WASTE

By

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To my family

1 INTRODUCTION

1.1 Statement of the Problem

Used casing waste discharged from frankfurter/sausage production constitutes a major organic waste stream produced by the meat processing industry. A typical small frankfurter plant can generate as much as 1,000 tons of spent casings annually (Sanders et al. 2000). The long, tubular-shaped casing, composed primarily of regenerated cellulose, is used to form and contain the uncooked frankfurter/sausage emulsion while it is being processed. After cooking, the spent casing is removed and transported to a dumpster for disposal. Due to the large volume of solid waste being produced daily, this industry has high costs for hauling and landfill disposal. This creates considerable economic incentive for the industry to develop and implement a process that effectively converts the cellulosic spent casing waste into useful byproducts.

It has long been known that cellulase enzymes can hydrolyze cellulosic waste materials and convert them into useful soluble byproducts. Even though this is an effective means of breaking down the spent casings to sugars, it requires the use of expensive commercial cellulase enzymes. For this to become a practical alternative, it is crucial to increase the effectiveness of the process to reduce the cost of commercial enzymes used.

The high cost of the commercial enzymes and the increased demand in many applications has caused researchers to search for more economical alternatives. This includes production of the cellulase enzymes from cellulosic materials found in

municipal solid waste (newspaper) and agricultural residue (Shin et al. 2000). These materials are composed of lignocellulosic compounds and must be pretreated to release cellulose, the primary inducer of the cellulase enzyme production. The chemical and mechanical pretreatments needed with these solid substrates are a significant, but fixed, cost. The economic viability of this process depends mainly on the cost and purity of the cellulolytic material (Abraham and Kurup, 1997).

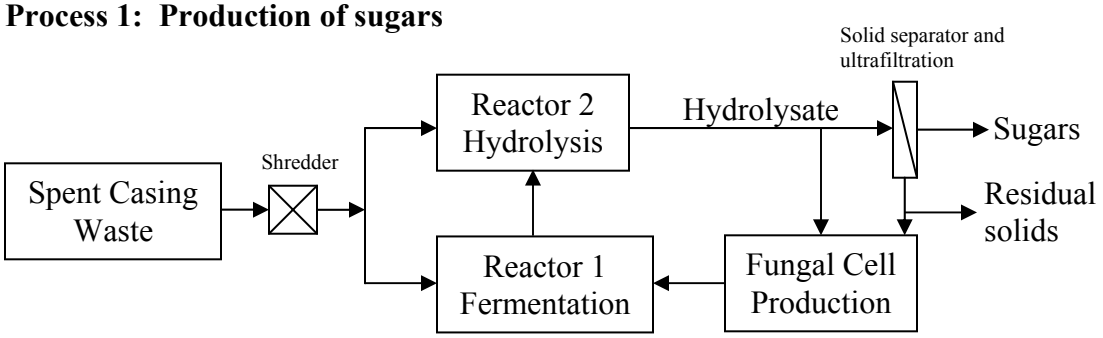
From both economic and quality standpoints, spent casing waste qualifies as an excellent source of waste cellulose. Two potential uses for the waste are to hydrolyze the cellulose into sugars or to use the spent casing waste as a substrate for the production of cellulase. Both alternatives could represent environmentally sound solutions to the waste disposal problem. In addition, they could offer significant savings to the meat processing industry, with potential for revenues from the production of useful byproducts.

Two potential process layouts will be considered in this work: Process 1 for the production of sugars and Process 2 for the production of cellulase enzymes (Figure 1.1). In Process 1, the cellulase enzymes will be produced in the first reactor by growing the fungi with a fraction of shredded spent casing waste generated in the plant. The cultured broth with the biomass (mycelium and residual casings) will be transferred into the second reactor for continuous enzymatic hydrolysis of the spent casing waste. Sugars and cellulase enzymes in the hydrolysate can be separated with ultrafiltration membranes so that the enzymes can be recycled and the energy-rich glucose can be bioconverted or refined for new products. Process 2 involves the production of cellulase enzyme using shredded spent casing waste. After the fermentation the biomass will be removed and recycled to the fermentation tank. The resulting solution containing the cellulase

enzymes can then be concentrated using ultrafiltration membranes. Disposal of residual solids which will build up during operation of both recycling processes will be treated at the on-site wastewater treatment plant.

The central hypothesis of this research is that bioconversion of cellulosic waste casing to sugars and/or cellulase enzymes could be an economic alternative to the current waste disposal process. To test this hypothesis, a series of extensive studies with the casing waste were conducted.

Process 1: Production of sugars



Process 2: Production of cellulase

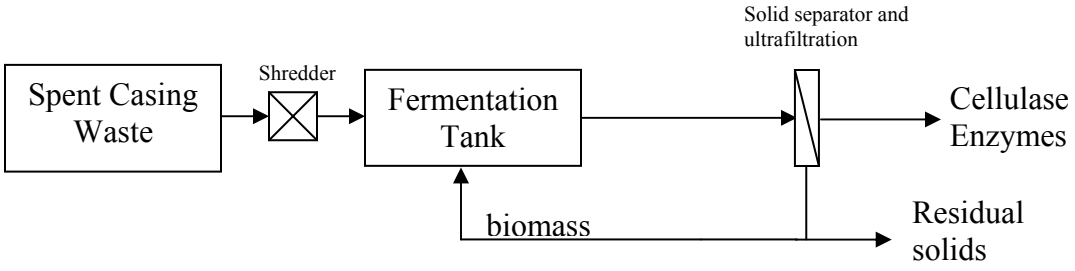


Figure 1.1. Batch processing options for complete biodegradation of spent casing waste from meat processing plants. In Process 1, produced cellulase enzymes are used for complete hydrolysis of spent casing waste into sugars. In Process 2, spent casing waste is used for the production of cellulase enzyme.

1.2 Research Objectives

The main goals of this research were to (1) determine hydrolysis efficiency for enzymatic hydrolysis of spent casing waste; (2) explore the use of spent casing waste as a substrate for the production of the cellulase enzyme complex in a laboratory scale fermentation reactor; and (3) determine the economic feasibility of producing value-added products from casing waste.

The hypothesis associated with each of the main goals is as follows:

1) Current commercial cellulase enzymes will be able to hydrolyze spent casing waste with high conversion efficiency. Cellulase enzymes should digest cellulosic casing waste, but methods to increase enzyme effectiveness are crucial for reduction of enzyme consumption and cost.

2) *Trichoderma reesei* RUT- 30 can metabolize cellulosic casing waste for synthesis of cellulase enzyme with similar hydrolytic capacity as commercial cellulase enzymes. Cellulosic material from the casing waste should be suitable as a substrate for the fungi, but the fact that the waste contains other chemicals could affect to some degree the cellulase production and its hydrolytic capacity when compared with commercial enzymes.

3) Deriving high-value products from the bioconversion of casing waste offers a new avenue of profitability that is more economical than current waste handling. If high quality and quantities of cellulase can be produced in Process 2, current high cellulase demand and prices could offset the capital and operational costs of the bioconversion process, making this new process economically feasible.

2 LITERATURE REVIEW

2.1 Spent Casing Waste

The waste management of spent frankfurter/sausage casing is a major problem for the meat processing industry due to the high costs involved in handling and disposal. Gentry et al. (1996) estimated that more than 30 million pounds of cellulose casings are generated in the U.S. annually. It is expected that the casing waste production is even higher considering the increased consumption of hot dogs since the 1996 estimate. In the State of Oklahoma, it is estimated that four BAR-S frankfurter/sausage plants, located in Clinton, Altus, Lawton, and Elk City, altogether produce an average of 15 tons of spent casing waste per day. The smallest plant processes 2 million pounds of sausages per week and generates four tons of spent casings per day. Due to the large quantity of solid waste produced, the waste-handling contractor takes a minimum of four trips per day to the landfill and the company currently pays $80 per load and $30 per ton of casing waste.

There exists a need for frankfurter plants to economically destroy the casing waste. In the past, a few alternatives were evaluated but none of them have been implemented. Compost is one alternative that could be used to break down the spent casing but physicochemical factors such as temperature, oxygen transfer, wet conditions, rodent control, and treatment space limitations make it impractical for on site treatment. Another alternative is combustion of the spent casing waste, but stringent Federal and State air quality regulations require the use of expensive air treatment devices for ash

removal before gas emission to the atmosphere. Neither alternative currently provides a cost-effective solution for the handling of the casing waste.

In an effort to find alternative uses for the spent casing waste, a few studies have been conducted in the past 10 years which target the reuse of spent casing as an energy-rich additive in feed for animals and the conversion of casings to sugars via enzymatic hydrolysis.

Gentry et al. (1996) conducted a study in which the spent casings were incorporated into ruminant diets since ruminant bacteria are able to break down lignocellulosic materials. Their findings showed that cellulose casings could partially replace forages in high fiber diets, but the quantity of casing supplemented in the feedstuff was limited by the high concentration of salt (NaCl) in the spent casings. Another study involved *in situ* experiments where spent casings inside a polyester bag were placed directly into the rumen of a fistulated cow (Sanders et al., 2000). Results showed that degradation of the casings by ruminant bacteria was less than 5%, indicating that ruminal enzymes could not efficiently hydrolyze the spent casing.

Recent work involving the enzymatic hydrolysis of spent casings showed great success in dissolving the casings with three commercial cellulase enzymes in batch and semi-continuous hydrolytic reactors (Viikari et al., 1998). The cellulase enzymes used were Econase CE (Alko), Spezyme CE (Genencor), Spezyme CP (Genencor), and β-glucosidase enzyme from Novozyme (Novozyme 188). β-glucosidase was fastened on a solid medium to enhance the conversion of soluble cellobiose to glucose. The results showed that with a casing loading of 100 g/L and enzyme loading of 30 IFPU/g casing,

(International Filter Paper Units, IFPU) the insoluble casing was degraded nearly 70% within 5 hours and more than 90% in 25 hours.

Sanders et al. (2000) also conducted studies to determine whether three commercial enzymes could break down the spent casings. In the experiment, they used a casing loading below 6 g/L and the enzyme dose was given as volume of commercial enzyme added and not by cellulase enzyme activity. The results showed that the commercial enzymes can degrade the casings and that pH and temperature are the most critical factors affecting casing degradation. They also found that particle size, pigmentation, and agitation did not affect the casing degradation. The names of the commercial enzymes used were not given.

2.2 Composition of the Spent Casing

The spent casing is mainly composed of regenerated cellulose that forms a rigid plastic-like texture. After being in contact with the meat during processing, the solid casing is hydrated and the texture becomes soft and flexible. The composition of the new and spent casing is shown in Table 2.1.

After processing, the spent casing waste contains more moisture than the new casing. The insoluble dry matter in the spent casing consists of cellulosic casing and minor fractions of meat from the process. The soluble dry matter portion in the unused casing is composed mainly of mineral oil and glycerin, whereas in the spent casing it is mainly soluble salts and fats. The fixed insoluble portion is mainly composed of the black ink on the exterior surface of the casing. The black ink stripes account for about 8% of the surface area of the casing.

The insoluble portion of the organic material accounts for 99% of the dry matter in both the new and the spent casing. It is this large fraction of organic material that is converted to sugars during enzymatic hydrolysis and is used as a substrate for the synthesis of cellulase enzymes during aerobic fermentation.

Table 2.1. Percent composition of the unused and spent casing.

Component	**New Casing**	**Spent Casing**
Moisture content	15	57
Dry matter	85	43
Insoluble dry matter	83	76
Volatile insoluble	99	99
Fixed insoluble	1	1
Soluble dry matter	17	24

Cellulosic materials are classified as native or non-native cellulose according to the origin of the cellulose compound. Native cellulose is synthesized by plants and is the most abundant organic compound known on Earth. Non-native cellulose contains processed cellulosic materials from plants and includes copy paper, newspaper, microcrystalline cellulose, carboxymethylcellulose, cellulosic casings, and many others.

The cellulose molecule is composed of highly stable homopolymer chains of up to 12,000 $\beta 1 \rightarrow 4$ linked monosaccharide glucose units. In both native and non-native forms, cellulose (polysaccharide) and its reduced form, cellobiose (disaccharide), are held

together laterally by intermolecular hydrogen bonds (Fengel and Wegener, 1984). The additive effect of the hydrogen bonds increases the rigidity of the cellulose molecule, making it insoluble and resistant to most organic solvents. The hydrogen bonds in the cellulose molecule can be cleaved effectively by the addition of strong acid or base chemicals or by enzymatic hydrolysis using cellulase complex enzymes. Cleavage of the H bonds by either of these mechanisms yields glucose compounds.

2.3 Cellulose Hydrolysis

The degradation of the cellulose to glucose can be accomplished by a chemical reaction know as a hydrolysis reaction, which involves the addition of water to the cellulose molecule. The two commonly used hydrolysis methods that can provide almost complete cellulose conversion into sugars are acid hydrolysis and enzymatic hydrolysis.

2.3.1 Acid Hydrolysis

Concentrated H_2SO_4 and HCl have been the traditional acids used to treat lignocellulosic materials for the production of ethanol. Although they are powerful agents for cellulose hydrolysis at elevated temperatures between 120 °C and 160 °C, concentrated acids are toxic, corrosive, and hazardous, and require reactors and mechanical equipment that are resistant to corrosion. In addition, the acid must be recovered after hydrolysis to make the process economically feasible (McMillan, 1994; Sivers and Zacchi, 1995). Another important fact is that the hydrolysate contains considerable amounts of toxic compounds due to the high temperature required (Szengyel

et al., 2000). Furthermore, pH neutralization is necessary for the downstream fermentation of glucose to biofuels or any other fermentable product. These process conditions require operation and management options that make acid hydrolysis a costly alternative for the degradation of spent casing waste.

2.3.2 Enzymatic Hydrolysis by Cellulase

Cellulase is composed of a group of highly specialized enzymes that act synergistically to hydrolyze cellulose to reducing sugars such as glucose and cellobiose (Figure 2.1). At least three major types of enzymes are involved in the hydrolysis process: (1) endoglucanase (EG or endo-1,4-D-glucanohydrolase) which attacks randomly different regions of the cellulose chain, creating free-chain ends which include cellobiose and glucose molecules; (2) exoglucanase or cellobiohydrolase (CBH or 1,4-β-D glucan cellobiohydrolase) which liberates cellobiose molecules from the free-chain ends of cellulose; and (3) β-glucosidase which hydrolyzes cellobiose to glucose (Coughlan and Ljungdahl, 1988).

β-glucosidase is under separate genetic control and is often not considered a part of the cellulase enzyme complex (Mandels et al., 1974). However, synergistic action of β-glucosidase is critical to remove cellobiose, the end-product inhibitor of cellulase enzymes. As shown in Figure 2.1, both reducing sugars cause inhibition; however, cellobiose is a much stronger inhibitor than glucose. Studies using cellulose concentrations between 10 and 400 g/L have found complete inhibition of cellobiohydrolase at cellobiose concentration of 0.01 to 0.1 g/L (Awafo et al., 1996). A reduction of β-glucosidase activity is noticeable at glucose concentrations above 30 g/L.

The degree of inhibition varies depending on the nature of the cellulosic material and the type of substrate used for cellulase fermentation (Gruno et al., 2003). In industrial application of commercial cellulase enzymes, the inhibition of β-glucosidase is reduced by the addition of extra β-glucosidase to the enzyme complex.

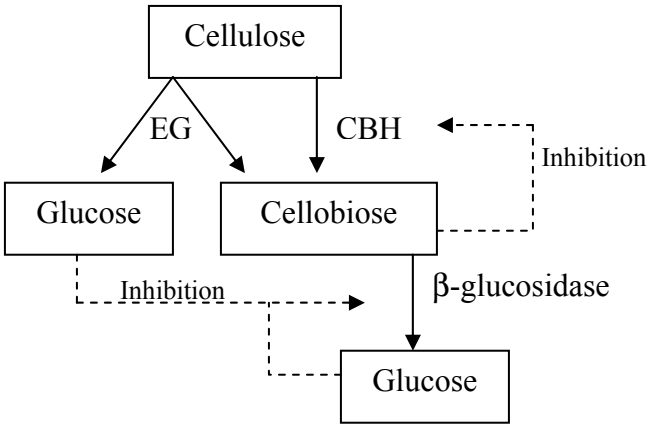


Figure 2.1. Cellulose hydrolysis by endoglucanase (EG), cellobiohydrolase (CBH), and β-glucosidase. Dashed lines indicates the end-product hydrolysis inhibition by glucose and cellobiose.

The utility cost of enzymatic hydrolysis is low compared to acid hydrolysis because enzyme hydrolysis is usually conducted at mild conditions (pH 4.8 and temperature between 45-50 °C) and does not have a corrosion problem (Duff and Murray, 1996). In addition, fewer byproducts are formed, resulting in a easily-fermentable sugar solution with good sugar yields (Szengyel et al., 2000). A drawback to this process is the fact that a considerable amount of cellulase enzymes are required per cellulosic material in order to obtain complete conversion into sugars. For an industrial application, such as the biomass-to-ethanol process, the cost of commercial cellulase enzymes constitutes about 60% of the total enzymatic hydrolysis process cost. The high level of cellulose purity that is required in order to induce cellulase formation in larger quantities during

fermentation is the main contribution to the high cost of enzyme production (Howard et al. 2003).

2.3.3 Improving Cellulase Enzyme Performance

The interaction of cellulase enzyme with cellulose requires three processes: adsorption of the cellulase onto the cellulosic surface, hydrolysis of cellulose to sugars, and desorption of cellulase. The overall reaction process performance of the cellulase enzyme with cellulosic materials can be improved by optimizing chemical and physical factors in the enzyme solution environment. Reaction conditions such as pH, temperature, mixing, and type of cellulosic substrate and size can be optimized to improve the rate of the enzymatic hydrolysis and the yield of glucose (Sun and Chen, 2000).

Researchers have found that the addition of surfactants during hydrolysis improves the conversion efficiency by modifying the cellulose surface properties and minimizing the irreversible binding of cellulase on cellulose (Eriksson et al., 2002). They also found that non-ionic surfactants were more effective than ionic surfactants in the enzymatic hydrolysis of steam-pretreated spruce, with Tween 20 and 80 producing the highest enzymatic conversion at low surfactant levels. Similarly, Kaar and Holtzapple (1998) found that Tween 20 improved the enzymatic efficiency of corn stover by 40% and it also prevented thermal deactivation of the cellulase enzyme. Alkasrawi et al. (2003) determined that the addition of 2.5 g/L of Tween 20 to steam-pretreated wood could lower enzyme loading by 50% without affecting the ethanol yield in simultaneous saccharification and fermentation processes.

2.4 Cellulase Production

Many bacteria and fungi can produce cellulase, however, the most widely used microorganism or commercial cellulase production is the filamentous fungus of the genera, *Trichoderma*, particularly *T. reesei*. The hyphal mode of growth gives a rigid mycelial structure able to penetrate into the solid substrates. The penetration occurs because the hydrolytic cellulase enzymes are excreted at the hyphal tips. The production of cellulase enzymes is non-growth associated. These enzymes consist of stable cellulase systems containing EG, CBH, and β-glucosidase (Jana et al., 1994 and Raimbault, 1998). Earlier strains of *T. reesei* produced low levels of β-glucosidase, which led to incomplete hydrolysis due to cellobiose accumulation (Figure 2.1). Over the years, more efficient cellulolytic mutant strains of *T. reesei* have been produced that synthesize high levels of cellulase enzymes and β-glucosidase (Table 2.2).

Table 2.2. Comparative cellulase productivity of *T.reesei* mutants (Jana et al., 1994).

T. reesei mutants	**Enzyme productivity IFPU/L-h**
QM 9414	30
MCG 77	33
MCG 80	37
RL-P37	41
NG 14	45
RUT- C30	**50**
D1-6	57
VTT-D-79125	70
D1-6/44	83

Trichoderma grows readily on most carbon sources, but cellulase is produced only when the fungus is grown on cellulose, on glucans of mixed linkage including the β(1-4), or on a few oligosaccharides (Mandels and Weber, 1969). They found that lactose (a β(1-4) galactoside) and sophorose (a β(1-2) glucoside) are the only known cellulase inducers that do not have a β(1-4) glucosidic linkage. The true inducers of cellulase for fungus grown on cellulose are the soluble hydrolysis products of the cellulose, especially cellobiose (Figure 2.2).

Cellobiose is an inducer of cellulase, and can also act as an inhibitor against the cellulase enzyme complex as described in Section 2.2.2. The addition of other rapidly metabolized carbon sources such as glucose or glycerol strongly represses cellulase formation. Oh et al. (2000) determined that the glucose repression to cellulase synthesis is noticeable at low glucose concentration from 0.20 to 0.30 g/L. Nevertheless, the cellulase synthesis may reappear after the glucose or other sugars has been consumed (Mandels and Weber, 1969).

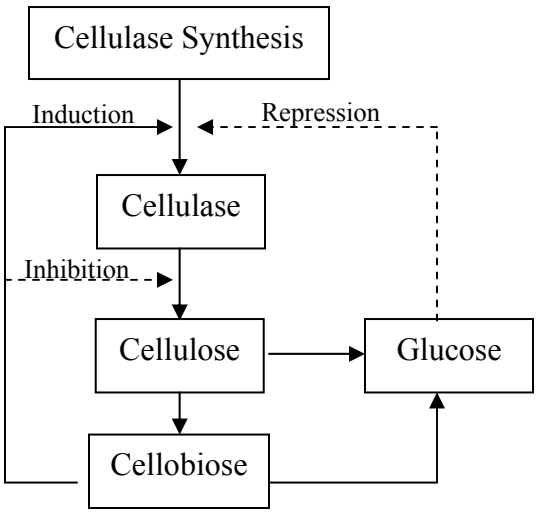


Figure 2.2. Synthesis of cellulase enzymes. Cellobiose acts as an inducer and inhibitor of cellulase production. Glucose concentrations above 300 mg/L cause a metabolic shift from cellulase synthesis to mycelial growth.

Large quantities of cellulosic enzymes with high activity are excreted by *T. reesei* when it is grown on a mineral medium supplemented with pure cellulose such as Solka Floc, cotton, Avicel or sulfite pulp. According to Esterbauer et al. (1991), the average cellulase and mycelium production is 0.25 g cellulase production/g carbon source and 0.25 g mycelial biomass/g carbon source. Obtaining these yields or higher on an industrial scale requires the constant supply of expensive crystalline cellulosic materials, which add a further high cost to the production of *Trichoderma* cellulase. Doppelbauer et al. (1987) reported that a cellulase production industry has an estimated cost associated with pure cellulose of $18.00/kg enzyme protein. Today market value of commercial cellulase enzymes rages approximately from $5 to $15/kg enzyme. The cost and range will very depending on the type of fermentation process used, type of microorganism, and enzyme purity.

Since the 1980s, a considerable amount of attention has been given to finding alternatives to reduce the cost associated with cellulase production. Studies have been done with low-value cellulosic materials as feedstuff for fermentation, strain improvement, optimization of culture conditions, and the characterization of cellulolytic enzymes at the biochemical and genetic level (Esterbauer et al., 1991). The original approach was to take advantage of the cellulosic portion found in lignocellulosic materials and cellulosic waste products as feedstuff for cellulase production. The use of these low-value cellulosic materials can significantly reduce (by 10 times) the cost associated with the raw materials (Doppelbauer et al., 1987). As a result, extensive studies with promising cellulase enzyme yields have been obtained in the fermentation of *T. reesei* RUT–C30 with different solid lignocellulosic materials and cellulosic waste

products such as wheat straw, bagasse, aspen wood, oak wood chips, willow, yellow poplar, waste paper sludge, waste newsprint, office paper, municipal solid waste, and banana waste (Reczey et al., 1996; Shin et al., 2000; Szengyel et al., 2000; Reddy et al., 2003). Soluble carbon sources such as lactose, xylose, and cheese-whey permeate have also been used for cellulase production but with only some strains of *T. reesei* (Mohagheghi et al., 1988; Cameron, 1996; Reczey et al., 1996).

The production of cellulase from lignocellulosic materials involves physicochemical pretreatment to reduce the material size and to remove lignin, which creates a barrier that prevents penetration of the enzymatic solution. This process is crucial because the hydrolytic enzymes from *Trichoderma* cannot degrade lignin (Howard et al., 2003).

Shin et al. (2000) evaluated the enzyme production of *T. reesei* RUT C-30 on various biomass substrates, including paper sludge (newspaper and office paper) and pretreated oak wood chips with diluted acid, ammonia, and steam explosion. Among the three cellulosic materials, the steam-exploded wood resulted in the highest cellulase activity during batch fermentation, with activity of 4.29 IFPU/ml in shake flasks and a bench scale stirred reactor.

Szengyel et al. (2000) conducted similar studies using different mixtures of fibrous cellulose and hemicellulose hydrolysate from steam-pretreated spruce as substrates for enzyme production. They also compared the capacity of the produced enzymes with two commercial enzymes. The maximum cellulase activity obtained was 0.79 IFPU/mL after 92 hours of incubation. They found that the high content of lignin and sugar degradation byproducts under higher pretreatment temperature and acid

catalyst conditions proved to be inhibitory to microorganisms. However, the conversion of cellulose to glucose with the produced enzymes was similar to that from commercial enzymes.

Cameron (1996) conducted an economic analysis of the utilization of *Trichoderma* cellulase with lactose and cheese-whey permeate as soluble substrates which produced a cellulase activity of 38 IFPU/ml. The economic analysis showed that the cost of lactose represents about 35% of the total operational cost. This is equivalent to $3.02 per kg of produced cellulase protein. In addition, it indicated that the unit cost for cellulase enzyme production and cellulase selling price was $31.08/kg and $48.90/kg, respectively. A significant reduction in total operational cost was obtained when lactose was substituted by cheaper cheese-whey permeate, decreasing the price per kg of carbon source by over 95 %. The production unit cost and the cellulase selling price were reduced to $4.74/kg and $4.75/kg, respectively.

The production of cellulase enzymes has been widely studied in submerged and solid-substrate fermentation (SSF) processes in the laboratory ranging from shake flasks to large volume reactors (Kim et al., 1997). Most industrial scale cellulase production utilizes the submerged method rather than the SSF due to ease of process control and liquid handling. In Japan, industrial cellulase is produced by SSF using the Koji process with wheat bran and steamed rice as the substrates (Esterbauer et al., 1991 and Raimbault, 1998).

Another factor that contributes to the high cost in the production of cellulase is maintaining an adequate dissolved oxygen level in the fermentation broth, which contains high concentrations of fungi and solid substrate. When the mycelia are growing and

propagating, the broth transforms from a Newtonian mixture to a non-Newtonian mixture over time, with varying rheological properties and high apparent viscosities (Weber and Agblevor, 2005).

Common types of aerobic reactors that have been studied for the production of cellulase are the stirred-tank, stirred-tank with microbubble, air-lift, and bubble column reactors (Wase et al., 1985; Kim et al., 1997; Weber and Agblevor, 2005). Stirred-tank reactors have been shown to cause shear problems, rupturing mycelial cells and deactivating cellulase enzymes (Wase et al., 1985). Kim et al. (1997) compared different types of reactors for the production of cellulase using ground rice straw as a substrate. They found better cellulase yields and productivity in a bubble-column and in an external-loop air-lift reactor than in a stirred-tank reactor due to the lower shear stress and higher oxygen transfer in solution.

Different substrate feeding modes (batch, fed-batch, and continuous) have been investigated with different types of solid and soluble substrates in an effort to overcome the long growth time. The nature of the substrate and loading levels typically dictate the selection of the feeding mode. For instance, continuous and fed-batch fermentation have resulted in higher cellulase yields and productivity than that achieved in conventional batch systems with cellulose, xylose, lactose, and cheese-whey permeate (Mohagheghi et al., 1990 and Esterbauer et al., 1991). But continuous fermentation with solid substrates is difficult and produces lower cellulase yields. Cellulose suspensions at concentrations above 8% (w/v) are very viscous and cannot be used in batch mode. However, in a fed batch mode, concentrations of up to 15% (w/v) have been tested, resulting in very high enzyme concentrations (Esterbauer et al., 1991).

3 EXPERIMENTAL MATERIALS AND METHODS

3.1 Methodology Overview

The overall goals of this study are to investigate the effect of cellulase enzymes on the conversion of spent casing waste to fermentable products and the production of cellulase enzymes from the fermentation of spent casing waste. The experimental setup includes two water bath incubators with orbital shaking capabilities to treat all the experimental samples under the same temperature and mixing conditions. Also, a 2.5 L bench scale bubble column reactor was designed for the cellulase production studies with different spent casing loadings. All the chemicals used were of analytical grade.

Different experimental designs were used for the enzymatic hydrolysis and the cellulase production experiments, as described in the next sections. For the hydrolysis experiments the analysis was conducted to address two main areas of interest, (1) screening of commercial enzymes and (2) optimization of the efficiency in enzymatic conversion of the spent casing. Performance comparisons were based on the total sugar yield and the percentage of spent casing converted into soluble sugars. For the cellulase enzyme production the performance was based on cellulase activity, cellulase yield, and productivity. All experimental work was conducted at the Advanced Technology Research Center, Bioengineering Laboratories, Biosystems and Agricultural Engineering Department, Oklahoma State University.

3.2 Source of the Cellulosic Casing Waste

The spent casing waste used in this study was obtained from BAR-S (Clinton, OK). This meat processing company has four plants in Oklahoma that specialize in the production of frankfurters, specialty sausage, and sliced luncheon meat. Raw spent casings were collected as produced and packed into a styrofoam cooler for overnight delivery. Upon receipt, casings were transferred to a freezer and stored at –3 °C for future use. Prior to the experiments, they were dried at 50 °C and cut with scissors into sections of 1.5 cm x 1.5 cm, to fit into the opening of the experimental 40-ml glass vials. The company also provided new, unused casings, which were treated similarly to the spent casings. The casings did not receive any pretreatment before either the enzymatic hydrolysis experiments or the cellulase production experiments. Figure 3.1 shows the spent casing (SC) and the new casing (NC). The black ink stripes on the casing are used to identify the presence and size of casings in the sausage process lines. An examination of the surface of the casing was performed using a Scanning Electron Microscope (SEM) in Figures 3.2 and 3.3. These photographs showed that the surface of the casing is very smooth with no porosity to embed the cell wall structure of the fungi. Figure 3.3 clearly showed the black pigment of iron dioxide applied on the surface of the casing.

Figure 3.1. Spent casings (SC) and new casings (NC).

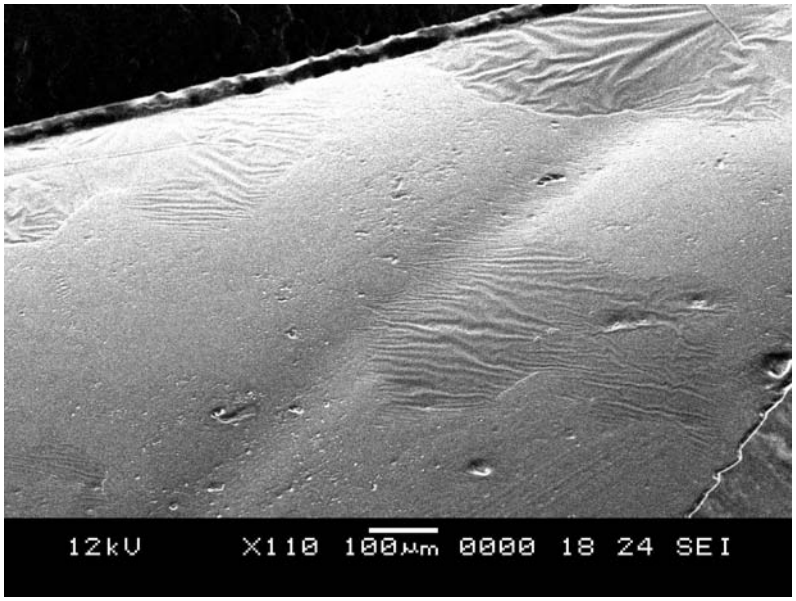


Figure 3.2. SEM photograph of a clear region of the spent casing.

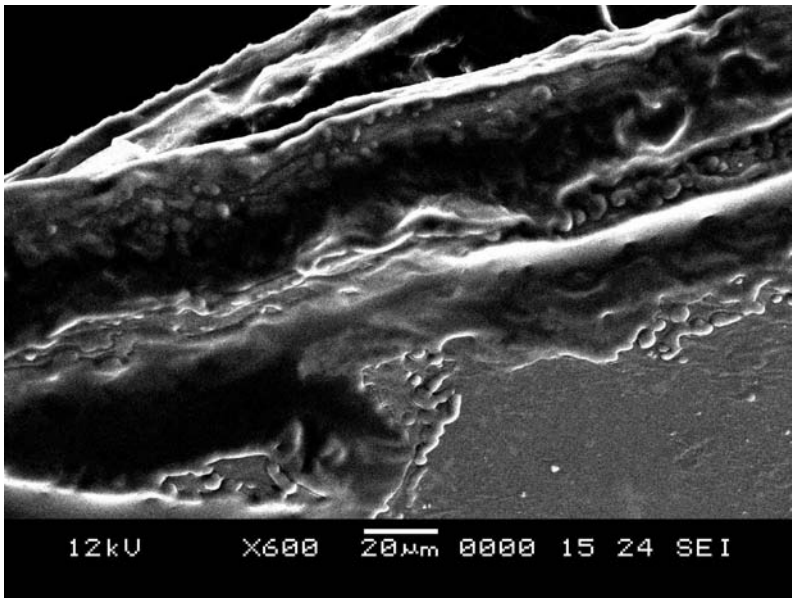


Figure 3.3. SEM photograph of a section of the spent casing with the black pigment.

3.3 Characterization of the Commercial Cellulase Enzymes

Six commercial cellulase enzyme solutions were donated by various manufacturers to test their ability in degrading the spent casing waste (Table 3.1). Upon arrival, they were stored in the refrigerator and handled according to the manufacturer's recommendations. Typical applications for these enzymes include the textile industry, baking, filter cleaning, fruit juice production, wine production, and animal feed. The cellulase activity of all the commercial enzymes was standardized following the International Union of Pure and Applied Chemistry (IUPAC) protocol and reported as International Filter Paper Unit (IFPU) per ml. Because this procedure is complicated and long, most cellulase enzyme manufacturers have adapted their own standard for determination of cellulase activity. Some of them correlate cellulase activity to solution viscosity, others simply use soluble substrates such as carboxymethylcellulose.

Table 3.1. Commercial cellulase enzymes tested.

Commercial Enzyme	Manufacturer	Suggested Use Range	
		pH	Temperature, °C
Fibrilase	IOGEN	4.0 – 4.6	40 – 60
ViscoStart	DYADIC	4.0 – 5.0	45 – 60
CelluPract	BIOPRACT	4.0 – 5.0	40 – 55
Multifect GC	GENECOR	2.7 – 5.7	35 – 70
Multifect XL	GENECOR	4.5 – 5.0	45 – 60
Multifect CL	GENECOR	3.5 – 5.5	45 – 80

3.4 Enzymatic Hydrolysis of the Spent Casing

The hydrolytic reaction studied here involved the mixture of an insoluble substrate (spent casing) with a soluble cellulase enzyme in a 75 mM sodium acetate buffer at the optimum pH. This heterogeneous substrate with enzyme combination limited the collection of periodic samples from a single vial because after the collection of sample aliquot the amount of cellulase enzyme available to react with substrate was reduced; therefore, only one sample at a given hydrolytic time was collected from a single vial.

All the hydrolysis experiments were performed in glass vials (40 ml) with agitation and temperature controlled by the orbital shaker water bath (Figure 3.4). The incubator temperature was calibrated within the temperature range required for the

experiments. A temperature datalogger from Campbell Scientific (Model 21X) was used to record the temperature data every 5 minutes.

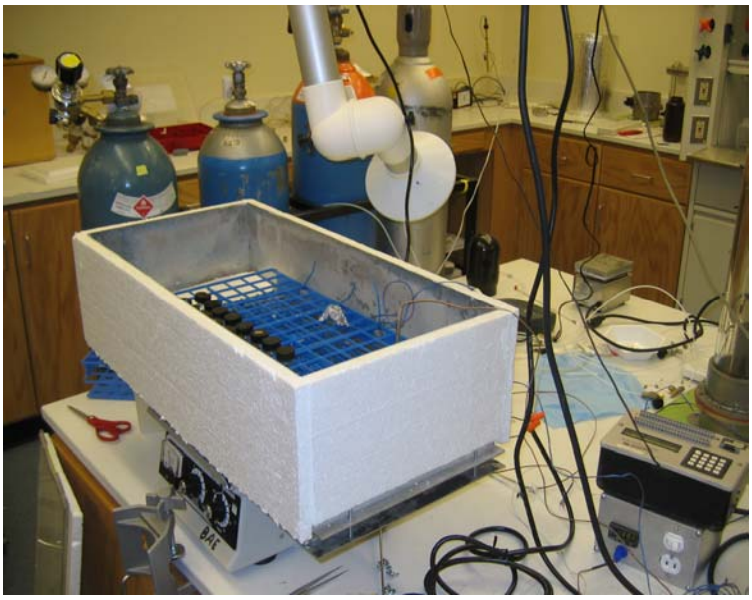


Figure 3.4. Orbital shaker water bath for the hydrolysis experiments. Mixing and temperature capabilities are from 0-300 rpm and 25 to 65 °C, respectively.

A diluted enzymatic solution was prepared from the commercial enzyme using a 75 mM acetate buffer so that 1.0 ml of solution contained the cellulase activity (IFPU) per gram of spent casing required for each enzymatic treatment. All treatment vials received the same amount of spent casings (0.25 grams dry weight) with different buffer volumes to adjust for the casing loading. The enzymatic solution was added to each vial once the mixture of buffer and casings was acclimated to the desired reaction temperature.

Controls consisting of enzyme without the spent casing, spent casing without the enzyme, and samples with known amounts of sugars were run in parallel with each

enzymatic treatment. Duplicates from each treatment and controls were incubated on the orbital shaker water bath.

In all the hydrolysis experiments, a 1.5 ml sample aliquot from the 40-ml vials was transferred to a 1.5-ml plastic microcentrifuge tube placed in an ice-water bath. The 40-mL vials with the remaining sample were also transferred to an ice-water bath for later analysis of total suspended solids.

3.4.1 Effect of pH

The enzyme manufacturers provide pH and temperature values at which the enzyme activity is highest. However, they recommend that these two process parameters be evaluated under the hydrolytic conditions to be used in order to obtain the optimum enzyme activity. The pH optimization experiment was conducted by preparing seven buffers each with 75 mM sodium acetate and the pH adjusted to 3.8, 4.2, 4.4, 4.6, 4.8, 5.0, and 5.4 using a solution of 25% (w/w) NaOH. The enzymatic hydrolysis was carried out at 50 °C for 6h using a casing loading of 25 g/L and an enzymatic loading of 30 IFPU/g casing. Optimum hydrolytic pH was obtained from the highest percent of casing conversion within the range tested.

3.4.2 Cellulase Enzyme Screening

The six commercial enzymes were tested to compare their hydrolysis efficiency with spent casing at optimum conditions. The enzyme screening was based on the percentage conversion of casing and on the cost per mass added at the highest casing conversion. Some manufacturers reported the cost of the enzymes, which ranged from $5

to $15 per kg of enzyme. The variation in this cost is because the commercial cellulase enzymes are produced by the fermentation of different strains of *T. reesei* with expensive substrates (solka floc, cotton, avicel, and lactose). These differences also cause a variation in the resulting proportion of cellulase enzyme (endoglucanase and exoglucanase) and β-glucosidase. In many cases, manufacturers supplement the cellulase production batch with additional β-glucosidase in order to provide higher glucose yields for the customers.

For enzyme screening, the experimental setup included spent and new casings to determine whether the presence of chemical compounds on the spent casing, such as salts and fat, affected the production of sugars. This experiment was performed using the optimum pH obtained from the previous experiment at temperature of 35 °C and 50 °C. The spent casing and new casing loading was 25 g casings/L and enzyme loading was 20 IFPU/g casing. Samples from each enzyme treatment were removed for sugar and suspended solids analysis at 0, 6, and 24 h.

The two commercial enzymes with the highest percent conversion in the screening experiment were further investigated to determine the optimum temperature, agitation, enzyme loading, and casing loading.

3.4.3 Effect of Temperature and Agitation

For the temperature experiment, the temperature of the orbital shaker water bath was set at 30, 40, 50, and 60 °C for 6h at 100 rpm. The casing and enzymatic loadings were the same as those used in the pH experiment. The hydrolysis time of 6 hours was chosen to replicate hydrolysis conditions from previous experiments.

Mixing of the enzyme-substrate solution increases hydrolytic activity of the enzyme. However, too much agitation can reduce the activity or even destroy the enzyme. Because of the plastic-like texture of the spent casing during the early stages of hydrolysis, a slight increase in the agitation could create turbulent zones between sections of the casings, resulting in a decrease in the cellulase activity. In order to test the effect of agitation in this experiment, the speed of the shaker water bath was set to 0, 50, 100, and 200 rpm at 50 °C for 6 h with a spent casing loading of 25 g/L and an enzyme loading of 30 IFPU/g casing.

3.4.4 Effect of Substrate and Enzyme Loading

A series of trials with the shaker and vials was conducted to determine the casing loading range. It was observed that at casing loading below 12.5 g/L, the water level inside the controlled temperature container was too high and it spilled over during low mixing at 50 rpm. The water level was high in order to cover the volume inside the vials. When the casing loading was above 50 g/L, the buffer solution was not enough to cover the casing. Cellulase enzyme loadings were based on the substrate mass and they were within the typical range used in hydrolysis studies.

Optimum dose of enzyme and casing loading was determine by the maximum percentage of conversion of spent casing in the least hydrolytic time. The amount of casing added was 12.5, 25, and 50 g casing/L at enzyme loadings of 10, 20, 30, and 40 IFPU/g casing at 50 °C. Samples were removed for sugar and suspended solids analysis at 0, 4, 8, and 24 h.

3.4.5 Effect of Mechanical Mixing on the Degradation of the Spent Casing

In order to ensure that adequate mixing enhance casing conversion, a 2-L stirred reactor was built and was operated with a working volume of 1.0 L. Two experiments were conducted with casing loadings of 25 and 50 g/L with a Fibrilase enzyme solution containing 30 IFPU/g casing at 50 °C and 100 rpm. Dry and chopped spent casing was transferred to the reactor and it was mixed with 75 mM sodium acetate buffer solution with pH of 4.8. After temperature acclimation the concentrated commercial enzyme was added to the reactor to start the hydrolysis. The temperature was controlled using a heating blanket around the reactor glass body (Figure 3.5). Samples were withdrawn from the top of the reactor and the clean supernatant was analyzed for sugars at the following hydrolysis times of 0, 4, 6, 8, 12, and 24 h. Solids analysis was performed at the end of the hydrolysis.

Figure 3.5. 2-L bench scale stirred reactor for enzymatic study.

3.4.6 Effect of Ionic and Non-ionic Surfactant

Eleven surfactants were screened for their ability to improve enzymatic hydrolysis of the spent casing. These surfactants were selected based on (1) the polar hydrophilic region in the molecules, which is either ionic or nonionic; (2) various industrial applications such as food, textile, and pharmaceutical; and (3) the solubility, especially the high critical micelle concentration which decreases the chance of forming organic precipitates.

The ionic and non-ionic surfactants evaluated in this study are given in Table 3.2. Tweens are a series of food grade nonionic surfactants derived from sorbitan ester and were obtained from ICN Biomedicals. Tweens 20 and 80 are the only Tweens reported in hydrolysis studies. Anionic surfactants are widely used in detergents and are mainly formed either by a mixture of sodium and fatty acids (soaps) or sodium and a sulfonate group. Sulfonate surfactants are commonly used in solutions containing high concentration of salts. Cationic surfactants have a more specific industrial application since most of them contain petroleum-based derivatives. Antifoam B (Sigma) is a surfactant solution commonly added to a fermentation culture to prevent foam formation. This antifoam is widely used in industrial fermentation and it has been used in cellulase production. The other surfactants used for the study were obtained from Sigma.

The surfactant screening experiment was conducted to evaluate the improvement in casing conversion when surfactant loadings of 2 or 8 mg/g casing were compared with a control with no surfactant. These loadings were within the range reported in studies of enzymatic hydrolysis with lignocellulosic materials. A stock solution of 50 g/L was prepared for each surfactant and it was diluted with sodium acetate buffer. The

respective stock solution volumes for each loading were transferred to the hydrolysis flask containing a casing loading of 25 g/L. In this experiment, the commercial enzyme Fibrilase was used with an enzyme loading of 10 IFPU/g casing. The hydrolytic reaction was conducted for 6h at 50 °C. After 6 hours, samples were taken for sugar analysis.

It was observed from the surfactant screening results that among all the surfactants, the non-ionic Tween surfactants had the greatest casing conversion at both surfactant levels. A further study with the Tweens was conducted to compare the effect of surfactant loadings on the casing conversion efficiency. The surfactant concentrations evaluated were 8, 20, and 40 mg of Tween/g casing mixed with a casing loading of 25 g/L and an enzyme loading of 30 IFPU/g casing at 50 °C for 6 h. Samples at the end of the hydrolysis were analyzed for sugars.

Table 3.2. Ionic and non-ionic surfactants.

Surfactants	**Type**
Tween 20, 40, 60, 80, and 85	Non-ionic
Oleic acid (OA), sodium dodecylsulfate (SDS), oletinsulfonate sodium salt (OS)	Anionic
lapyrium chloride (LC), isostearamidopropyl morpholine lactate (ISML), diethylmonium chloride (DEC)	Cationic
Antifoam B	Non-ionic

3.5 Cellulase Production from Spent Casing

Cellulase production by the RUT-C30 mutant of the fungus *Trichoderma reesei* was studied on cellulosic spent casing waste. It was expected that *T. reesei* would have normal growth with this waste since its main constituent is regenerated cellulose. However, no studies have been conducted using a waste with similar physical properties or similar levels of salts, oils, and fats in the production mixture. The plastic-like texture of the casing can produce more friction between fungi cells resulting in poor mycelia attachment and consequently low cellulase production. High levels of salts in the fermentation broth can affect the cell osmotic pressure, protein solubility, and the dissolved oxygen concentration. The fungi can possibly assimilate oils and fats as carbon sources for the synthesis of biomass but not for cellulase production.

The experimental setup was designed to simulate the conditions existing in the two processes presented in Figure 1.1. The main objective was to optimize the cellulase production by increasing the spent casing loading and determine the hydrolytic capacity of the newly produced enzyme. According to Process 1, the sugars produced from the enzymatic hydrolysis of the spent casing will be used as growth media since it contains the nutrients and sugars needed for fungi growth. This growth method will be compared with the adaptation culture normally used for inoculation. For Processes 1 and 2, the casing loading experiments were conducted in fermentation flasks and the bench scale reactor. A drawback in the shake flask fermentation is that neither the pH nor the oxygen can be adjusted to optimum levels while the fermentation is taking place. The fungi can die due to low levels of dissolved oxygen but not due to pH changes during growth. The bench scale reactor was designed to simulate optimum growth and cellulase conditions, such as, pH, oxygen, temperature, and agitation.

T. reesei RUT-C30 was obtained from the microbial collection of ATCC (56765). It was cultured on potato dextrose agar plates and incubated at 28 °C until sporulation occurred (Figure 3.6). After seven days, spores were suspended on 10 % sterile glycerol, then 1.0 ml was aseptically transferred into 75 cryovials and stored at -70 °C as a stock culture.

The frozen stock cultures were grown in a two-stage pre-inoculum culture growth following the methodology and medium formulation described by Mohagheghi et al., (1990). The first stage involves the growth of the fungi in a solution containing 1% (w/v) glucose and the second stage involves the growth and adaptation of the fungus with the spent casing for the synthesis of cellulase enzymes. Table 3.3 summarizes the media compositions used for the mycelia growth and cellulase enzyme production.

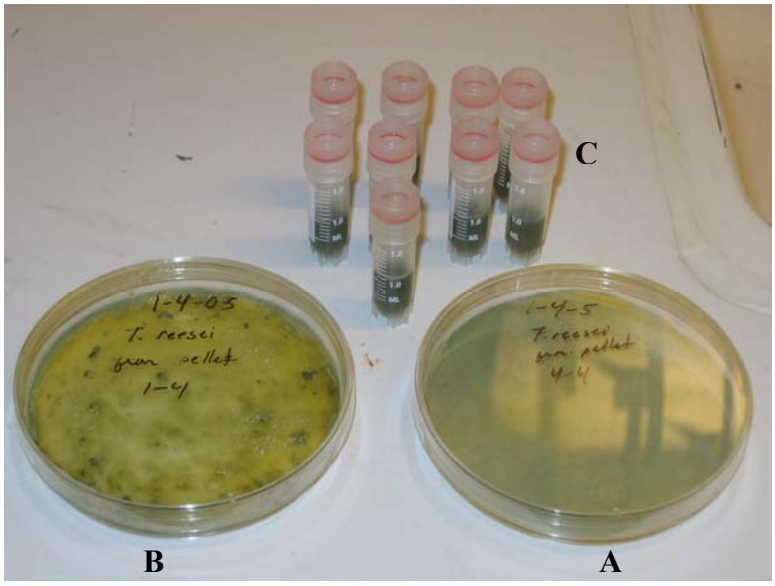


Figure 3.6. Preparation of stock cultures of *T. reesei* RUT-C30. Cultures were streaked on potato dextrose agar (A). Spores were suspended on 10% sterile glycerol (B). A 1.0 mL of the suspension was placed in 1.5 mL sterile cryovials (C). The green pigment indicates the presence of spores (sporulation).

Table 3.3. Composition of media for growth and cellulase production by *T. reesei*. Stage 1 is growth, Stage 2 for growth and adaptation with casing, and Stage 3 for cellulase production.

Component	**Stage 1**	**Stage 2**	**Stage 3**
Basal Salts, g/L			
$(NH_4)_2SO_4$ (ammonium sulfate)	1.4	1.4	1.4,4.4,8.8
KH_2PO_4 (potassium phosphate)	2	2	2
$CaCl_2 \cdot 2H_2O$ (calcium chloride dihydrate)	0.4	0.4	0.4
$MgSO_4 \cdot 7H_2O$ (magnesium sulfate)	0.3	0.3	0.3
Trace minerals, mg/L			
$FeSO_4 \cdot 7H_2O$ (ferric sulfate heptahydrate)	5	5	5
$MnSO_4 \cdot 4H_2O$ (manganese sulfate tetrahydrate)	1.6	1.6	1.6
$ZnSO_4 \cdot 7H_2O$ (zinc sulfate heptahydrate)	1.4	1.4	1.4
$CoCl_2 \cdot 6H_2O$ (cobalt chloride hexahydrate)	3.7	3.7	3.7
Carbon source			
Glucose, % (w/v)	1		
Spent Casing, % (w/v)		2	2.5,5,7.5
Protein and N supplement			
Corn steep liquor (CSL), mL/L	15	15	15
Antibiotics			
Penicillin and Streptomycin (5 mg/ml stock solution), mL/L		2	2
Miscellaneous			
Tween 80, mL/L		0.2	0.2
Antifoam (as needed), mL/L			0.1

Stock cultures were thawed and the contents added to a 250-ml baffled shake flask containing 50 ml of Stage 1 growth medium. Flasks were incubated at 28 °C and

200 rpm in an orbital shaker water bath until mycelia formation (Figure 3.7). After 3 days, 5 % (v/v) of the mycelia was transferred to a 250-ml baffled shake flask containing 100 ml of Stage 2 medium and incubated at 28 °C for 9 days (Figure 3.8). Mycelia grown in this flask served as the inoculum for the shake-flasks and the batch fermentation studies.

Once the flasks and fermentation reactor contained the corresponding amounts of spent casing, basal salts and corn steep liquor, then the solution pH was adjusted to 4.8 with 4.0 M NH_4OH or 2.0 M H_3PO_4 before autoclaving. Filter-sterilized glucose, Tween 80, and trace metals were added to the medium with a syringe. The initial amount of $(NH_4)_2SO_4$ was adjusted to start with a carbon to nitrogen ratio close to 8 (Tholudur et. al., 1999).

Duplicate flasks for each experimental condition were run in parallel and 2.50 ml samples from each flask were transferred to 13-mm weighted glass test tubes, centrifuged, and stored in the refrigerator at 4 °C for later analysis.

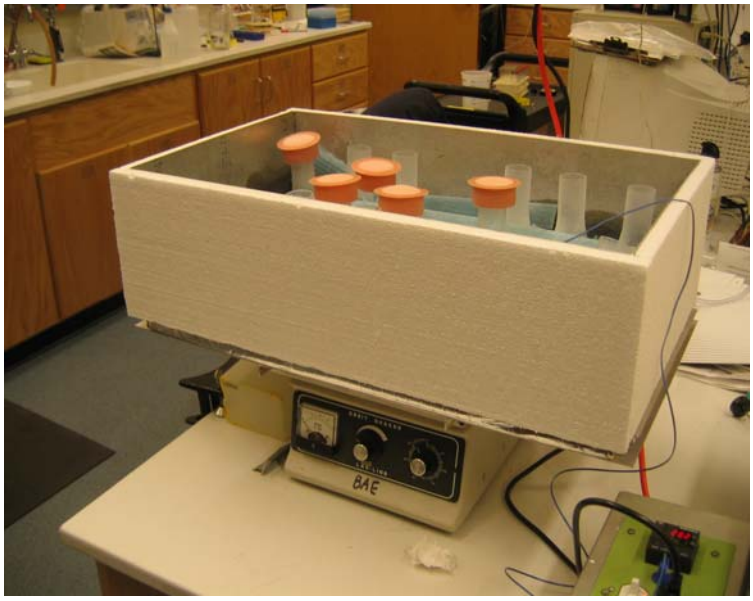


Figure 3.7. Designed water bath incubator with orbital shaking capabilities. Temperature was adjusted to 28 °C with a PID controlled.

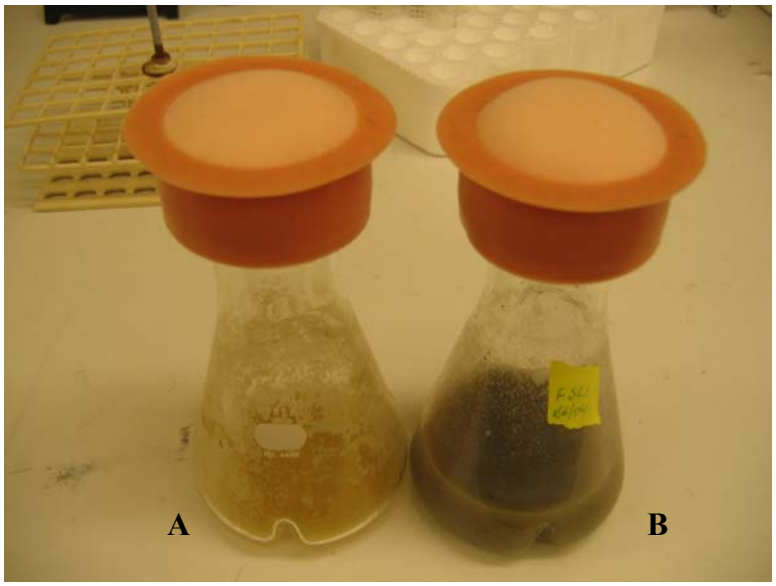


Figure 3.8. Fungi growth flask on 1% (w/v) glucose at 3 days (A) and fungi growth and adaptation flask on 25 g/L of spent casing at 9 days (B).

3.5.1 Shake-Flask Studies

A series of cellulase production experiments were conducted using 250-ml baffled shake flasks containing 100 ml of medium and 5% (v/v) of inoculum from the growth and adaptation culture. The first experiment was performed to produce cellulase enzymes with 25 and 50 g/L of spent casing. Controls consisting of growth medium alone and without the spent casing were run in parallel with the cellulase production flasks. No pH control was used in the flask studies. Duplicates of each flask were incubated under the same growth conditions and samples were withdrawn at 0, 2, 4, 6, 8, 10, 12, and 15 days of fermentation. Samples were analyzed for soluble protein, pH, total solids, and cellulase activity.

The second experiment was conducted to determine the effect of the hydrolysis temperature (50 °C) on the growth of the fungi. This condition would exist if all the culture from Reactor 1 (Figure 1.1) were added to the hydrolysis reactor. If the main objective of Process 1 is to maximize the glucose yield, then it is important to avoid glucose consumption by the fungi in the hydrolysis reactor. Growth studies were performed at 28 °C (control) and 50 °C with 1% (w/v) glucose and inoculated with 5% (v/v) from 3 days mycelium culture. The shaker water bath used for the hydrolysis experiment was modified to accommodate the shake flasks. A plastic cover was placed on top of the insulated incubator to minimize water evaporation. Samples from each flask were collected at 0 and 72 hours of incubation.

In the third experiment, the performance of the inoculum adaptation on cellulase production was evaluated by growing the fungi in different media containing glucose, spent casing, and spent casing hydrolysate as the carbon source. To conduct this

experiment, a series of growth flasks was prepared so that the product of each was used for the following experiment (Figure 3.9).

First, the produced cellulase enzyme was used for hydrolysis of the spent casing, then the hydrolysis product, or the hydrolysate, became the medium for growth and adaptation, and finally, this culture was used to inoculate the fermentation flask. If the fungus is adapted on the hydrolysate containing the reducing sugars and cellulase enzyme, the growth phase could be shortened and similar cellulase yields would be obtained as the mycelia grown with spent casing.

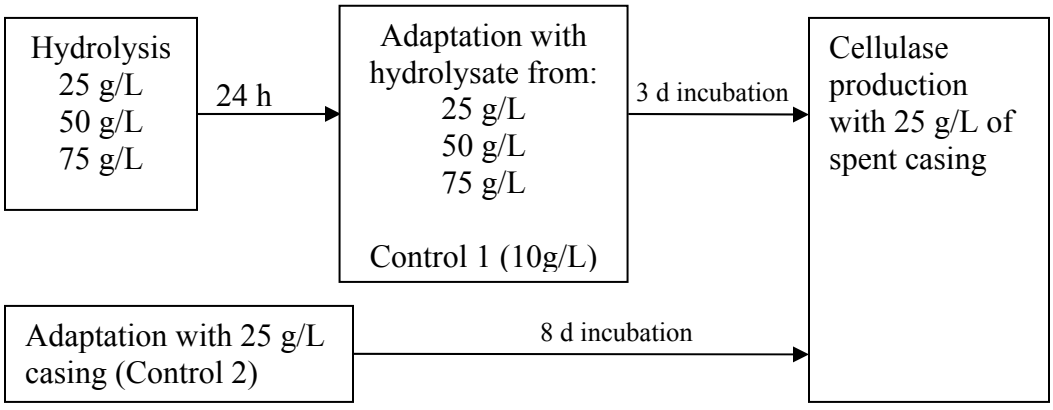


Figure 3.9. Experimental set up for the cellulase production from different inoculum cultures.

3.5.2 Bench Scale Fermentation Studies

Submerged cultivation of *T. reesei* was carried out for cellulase enzyme production in a designed 2.5-L bench-scale bubble column reactor connected to a Bioflow 110 Advance Fermentation Kit (NBS Scientific, New Brunswick, NJ). The reactor was equipped with pH, oxygen, and temperature probes controlled by an integrated PID controller and data acquisition (Figure 3.10). Small size bubbles were

formed when the pressurized air passed through a sintered glass filter with an average pore size of 20 μm, located at the top of the air inlet chamber. The fermentor was prepared by adding dried and chopped spent casings from the top, concentrated basalt salts with CSL (Stage 3 medium), and water. This mixture had a pH of 4.30 and it was increased to 4.8 by adding 3.0 M NH_4OH. After autoclaving, the reactor was allowed to cool down to 28 °C before adding the antibiotic, filter-sterilized Tween 80, and concentrated trace metal solutions. The inoculum constituted 5% (v/v) of the total working volume of 1.5 L. The pH was maintained at 4.8 with 3.0 M NH_4OH and 2.0 M H_3PO_4. A heating blanket and a PID controller were used to maintain a constant 28 °C temperature in the reactor. The agitation rate of the culture varied from 3 rpm during early growth stage to 40 rpm during the production stage. A gear motor with a speed controller was used for mixing to replace the motor that came with the BioFlow which has a minimum speed of 50 rpm. The dissolved oxygen was maintained above 10% of air saturation using a mixture of filter-sterilized air and oxygen. The air was continuously supplied to the reactor at various rates ranging from 0.2 to 1.2 vvm (volume of gas/volume of reactor per min) and it was supplemented with oxygen when the DO level was below 10%. Two calibrated air and oxygen flow meters were installed to monitor the flow rates. A 1:20 Antifoam B sterile solution (Sigma) was added to the reactor as needed to control foaming.

Three substrate-loading experiments were conducted in triplicate, each having a spent casing amount of 25, 50, and 75 g/L. Samples were collected at the top, middle, and bottom of the reactor every 24 hours for a period of 9 days.

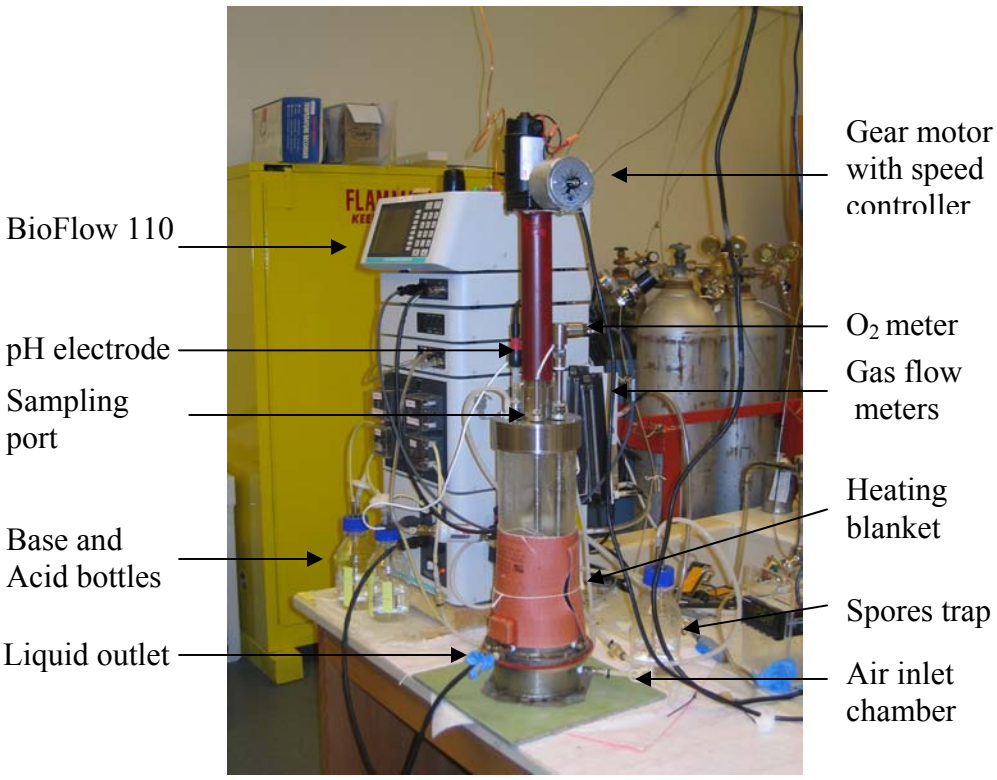


Figure 3.10. Bench scale bubble column reactor.

3.6 Analysis

3.6.1 Sample Preparation

Hydrolysis samples were centrifuged at 3,500 rpm for 10 minutes at 4 °C using a Sorvall model Legend RT. Then, 0.75 mL of the supernatant was used for the sugar analysis and the rest of the volume was stored in the freezer. Samples collected for the sugar analysis were diluted in 3.0 mL of DI water, boiled for 5 min to stop the enzymatic reaction, and centrifuged at 3,500 rpm for 10 min to precipitate the denatured protein. Clean supernatant was analyzed for soluble sugars.

The 2.5 mL of culture samples in the test tubes were centrifuged at 3,500 rpm for 10 minutes at 4 °C using a Sorvall model Legend RT. The samples were diluted with DI water 20 times for protein analysis and 5 times for sugar analysis and ammonia.

3.6.2 Enzyme Activity and Soluble Protein

The cellulase and β-glucosidase activities in the commercial and produced cellulase enzymes were measured by the Filter Paper Unit (IFPU) assay and the β-glucosidase or cellobiase (CB) assay, respectively. These assays are recommended by Ghose (1997) and are approved by the International Union of Pure and Applied Chemistry (IUPAC). The filter paper assay measures the release of reducing sugars from a 50 mg Whatman No. 1 filter paper (1 x 6 cm) in 60 minutes in a mixture of 0.5 mL of enzymatic solution and 1.0 mL of 50 mM citrate buffer with pH of 4.8 incubated at 50 °C. One international unit of filter paper activity (IFPU) is the amount of enzyme that produces 1 μmol of "glucose" (reducing sugars as glucose) per min. The cellobiase assay measures the release of glucose from 15.0 mM cellobiose in 30 minutes in a mixture of 1.0 mL of enzymatic solution and 1.0 mL of 50 mM citrate buffer with pH of 4.8 incubated at 50 °C. One international unit (IU) of cellobiase is the amount of enzyme which forms 2 μmol per min of glucose from cellobiose. The reducing sugars and glucose released were determined by the dinitrosalicylic acid method and the glucose oxidase assay (Stanbio Laboratory, Boerne, TX), respectively (Ghose 1987). The activity of the cellulase and β-glucosidase is expressed as IFPU/ml and IU/ml, respectively. Released proteins were measured by the modified Lowry protein assay kit (Pierce Chemical Company, Rockford, IL) using bovine serum albumin as a standard.

The produced cellulase enzyme yield (Y_c) and productivity (P_c) were calculated as follows,

$$Y_c = \frac{IFPU}{SC}$$

$$P_c = \frac{IFPU}{T}$$

where, IFPU is the cellulase enzyme concentration (IFPU/ml), SC is the initial spent casing concentration (g/L), and T is the total fermentation hours at each sample point.

3.6.3 Solids Analysis

Total suspended solids for the enzyme hydrolysis experiments were determined by filtering with glass fiber filters with a pore size of 1.2 μm and drying overnight at 105 °C. Solids were determined as the difference in filter weights before and after the analysis.

3.6.4 Sugar Analysis

Soluble sugars were analyzed by Ion Chromatography (IC) using a Dionex DX-600 equipped with a CarboPac MA1 anion-exchange column and a pulsed amperometric detector (Dionex, Sunnyvale, CA). The mobile phase was 700 mM NaOH with a flow rate of 0.40 ml/min at room temperature.

Data from the solids analysis and sugar analysis were used to calculate reducing sugars (RS) and the percent conversion of spent casing as follows:

$$RS = G_t + C_t$$

$$SCC = \frac{RS}{SC_o} * 100$$

Where, RS is the concentration of reducing sugars at time t (mg/L), G_t is the concentration of glucose at time t (mg/L), C_t is the concentration of cellobiose at time t, (mg/L), SCC is the spent casing conversion to sugars (%), and SC_0 is the initial concentration of spent casing (mg/L).

3.6.5 Ammonia and Cation Analysis

Samples for ammonia and sodium were analyzed by IC using a Dionex DX-600 equipped with a CarboPac MA1 anion-exchange column and a conductivity detector (Dionex, Sunnyvale, CA). The mobile phase was 30 mM H_2SO_4 with a flow rate of 1.0 ml/min at room temperature.

3.6.6 Fungal Cell Mass

The use of insoluble substrate leads to difficulties with accurate determination of cell mass in the presence of other insoluble components (Tholudur et al. 1999). Mohagheghi et al. (1990) and Jana et al. (1994) estimated the mycelium dry weight using a correlation factor of 0.37 g protein/g dry weight, but this factor varied over the range 0.29-0.42, depending on the stage of growth. Recent studies on cellulase production with complex insoluble substrates used a near-infrared (NIR) spectrometric technique to estimate fungal cell mass (Sáez et al., 2002). In this experiment, fungal cell mass was estimated following the methodology used by Mohagheghi et al. (1990). A 2.5 mL sample was transferred to a pre-weighted 13-mm glass test tube and centrifuged at 3,500 rpm for 10 minutes. The clear supernatant was saved for later analysis. The pellet was rinsed with DI water and centrifuged again. The pellets were rinsed 3 times with a total

water volume of 18 ml. After drying the pellets under vacuum at 50 °C for two days, the test tubes were weighed to determine the total solids. Protein extraction was performed by soaking the pellet overnight with 2.0 mL of 2.0 N NaOH, then vortex mixing to suspend the mycelium cells. Samples were heated in a boiling water bath for 10 min. After cooling at room temperature, the samples were centrifuged and the clean supernatant was used for mycelium protein analysis with the Pierce Lowry method. Mycelium dry weight was estimated indirectly using the correlation factor of 0.37. The solid residues were determined by the difference of total dry weight and mycelium dry weight.

The exponential increase in the mycelium dry weight was used to determine the specific growth rate (μ),

$$\mu = \frac{\ln(M_2) - \ln(M_1)}{t_2 - t_1}$$

where M is the mycelium dry weight (g/L) at time t (h) during the exponential mycelium growth.

3.7 Statistical Analysis

Duplicate flasks were set up for all the enzymatic hydrolysis experiments and cellulase production experiments in the shake flasks. The average values from the duplicate experimental flasks are presented in the Figures and Tables. All the results from each analysis were tested for standard deviation, and the coefficient of variation did not exceed 5%. Triplicate runs were conducted in the bench scale reactor with casing loading of 25 and 50 g/L and only one run was attempted with a casing loading of 75 g/L.

For each run, triplicate samples were taken and average values are presented in Figures and Tables. The resulting coefficient of variation was less than 5%.

4 RESULTS AND DISCUSSION

4.1 Enzymatic Hydrolysis Experiments

The use of cellulase enzymes to break down insoluble cellulose produces soluble cellobiose and glucose sugars. These two sugars were the only sugars produced during the hydrolysis of the spent casing. It was noticed that the control flasks containing spent casing showed a small amount of glucose (1 mg/g casing) from meat processing residual ingredients, and it was subtracted from the glucose in the hydrolysate.

4.1.1 Commercial Enzyme Activity

For consistency and comparison among six commercial cellulase enzymes and the produced cellulase enzyme, the cellulase and β-glucosidase enzyme activities were measured by the recommended method (IUPAC). The enzyme activity of the concentrated commercial enzymes is given in Table 4.1, along with the ratio of β-glucosidase-to-IFPU. Both enzyme assays were performed in triplicate with coefficient of variation of less than 2%.

Commercial soluble enzymes are processed to high concentrations, resulting in very high cellulase and β-glucosidase activities. The enzyme range for cellulase activity with Whatman paper as substrate was 64 to 96 IFPU/ml and for β-glucosidase activity with CMC as substrate was from 9 to 46 IU/ml.

Table 4.1. Comparison of the enzyme activity of the commercial cellulase enzymes.

Commercial Enzyme	Cellulase Activity, IFPU/ml	β-glucosidase, IU/ml	$\frac{\beta\text{-glucosidase}}{\text{cellulase}}$
Fibrilase	84	35	0.42
ViscoStart	96	31	0.32
CelluPract	83	30	0.36
Multifect GC	72	27	0.38
Multifect XL	76	9	0.12
Multifect CL	64	46	0.72

These data clearly indicate that there is a significant variation in the complete cellulase system in commercial cellulase enzymes. This variation could be caused by the difference in fermentation methods, carbon substrates, or microorganisms used for cellulase production. For example, Multifect XL is produced by a genetically modified strain of *Trichoderma reesei* to produce large quantities of xylanase and is used as a processing aid to modify xylan polymers. This strain modification affected the cellulase enzyme complex formation and caused a lower production of β-glucosidase. Based on the evaluation of cellulase enzymes developed by Awafo et al. (1996), complete cellulase systems with high enzymatic hydrolysis rates have a ratio of β-glucosidase to IFPU equal to 1. This indicates that these commercial enzymes have a low hydrolytic capacity and can accumulate cellobiose during the hydrolysis of spent casing. To avoid cellobiose accumulation, the commercial enzymes have to be supplemented with β-glucosidase.

This result also shows the importance of standardizing the enzyme loadings based on cellulase activity and not as protein mass.

4.1.2 Effect of pH

All enzymatic reactions are affected by the pH and temperature of the process. Hydrolysis of spent casings was conducted at 7 different pH levels, with the range of values taken from manufacturer-suggested ranges (Figure 4.1). The resultant optimum pH for the hydrolysis of the spent casings with each of the six commercial enzymes fell between 4.2 and 4.8 (Table 4.2). The results showed that the pH of the medium affected the percent of casing conversion because the pH causes an effect on the rate of enzymatic hydrolysis. The curve response obtained in Figure 4.1 is a typical curve for the effect of pH on enzymes. Enzyme activity increased with an increase in the pH until reaching a maximum activity, after which the activity decreased as the pH continued to increase. Figure 4.1 also shows that there appears to be a variation in the pH sensitivity among the six commercial enzymes. The commercial enzymes Fibrilase, Multifect XL, and CelluPract resulted in less variation in casing conversion than the other three enzymes in the pH range tested. The optimum pH was then used for further enzymatic studies with each commercial enzyme.

4.1.3 Cellulase Enzyme Screening

Six commercial enzymes were evaluated for their ability to hydrolyze the new and the spent casings at a casing loading of 25 g/L and enzyme loading of 20 IFPU/g at pH within their optimum range (Table 4.2). The hydrolytic response was determined based on the percent of casing conversion at 6 and 24 hrs using a reaction temperature of 50°C (Figure 4.2) and 35 °C (Figure 4.3). The two types of casings (new and spent) were used to observe possible inhibition from the spent casing. A comparison between the two casings indicates that there appear to be no chemical inhibitors in the spent casing that

affect the overall enzymatic hydrolysis process. In fact, the rate of hydrolysis with the spent casing was greater than with the new casing at both temperatures. The lower conversion rate for the new casing could be caused by the mineral oil and glycerin present in the new casing. It is possible that a portion of the enzyme complex is interacting with the oil, resulting in lowered enzyme activity.

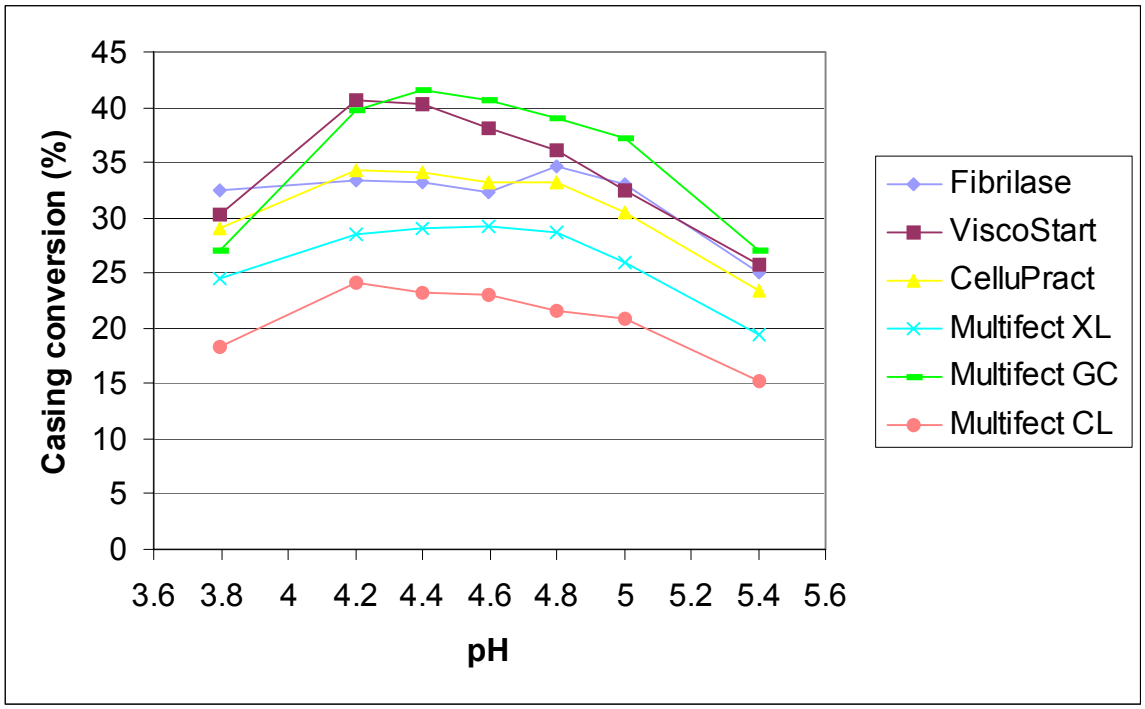


Figure 4.1. Effect of pH on spent casing hydrolysis at 50 °C for 6 h using casing and enzyme loadings of 25 g/L and 30 IFPU/g casing, respectively.

Table 4.2. pH used for experimentation with the commercial cellulase enzymes (taken from approximate optimum range).

Commercial Enzyme	Optimum PH
Fibrilase	4.8
ViscoStart	4.4
CelluPract	4.4
Multifect GC	4.4
Multifect XL	4.6
Multifect CL	4.2

Among the six commercial enzymes, Fibrilase, ViscoStart, and Multifect GC resulted in 75% to 85% casing conversion with the new casing at 50 °C in 24 h. When using the spent casing, Fibrilase and ViscoStart produced about 90% casing conversion followed by Multifect GC with 75% conversion. With the spent casing, the response of Multifect XL was reduced from 75% to 52%. Among the six enzymes, Multifect CL and CelluPract enzymes produced the least amount of casing conversion at 35 °C and 50 °C in 6h and 24 h. At 35 °C the percentage of casing conversion with the new casing using Fibrilase and Multifect GC was approximately 28% and 37%, respectively. For the spent casing, it was about 33% for these enzymes. The order of response in the percent conversion among the six enzymes at 50 °C differed somewhat from that obtained at 35 °C. Fibrilase and Multifect GC enzymes were selected for further studies because they produced higher percent conversion of casing at both temperature ranges and because their cost is the lowest among all the commercial enzymes.

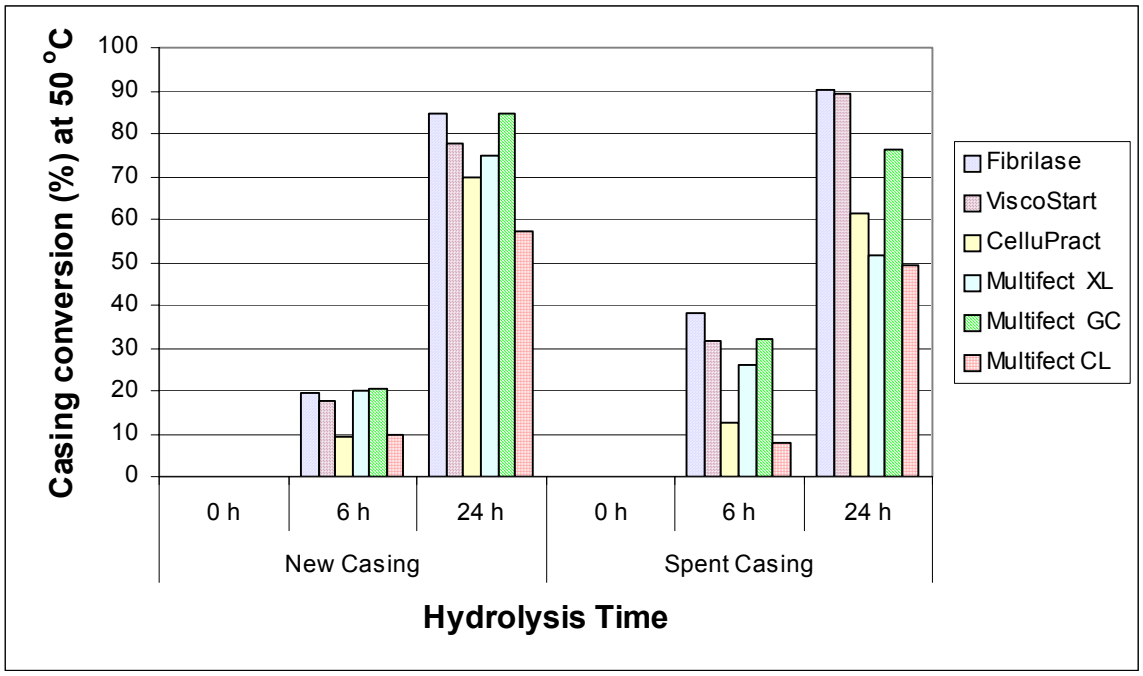


Figure 4.2. Hydrolysis of new and spent casing using commercial enzymes with a casing loading of 25 g/L and an enzyme loading of 20 IFPU/g casing for 6 h and 24 h at 50°C.

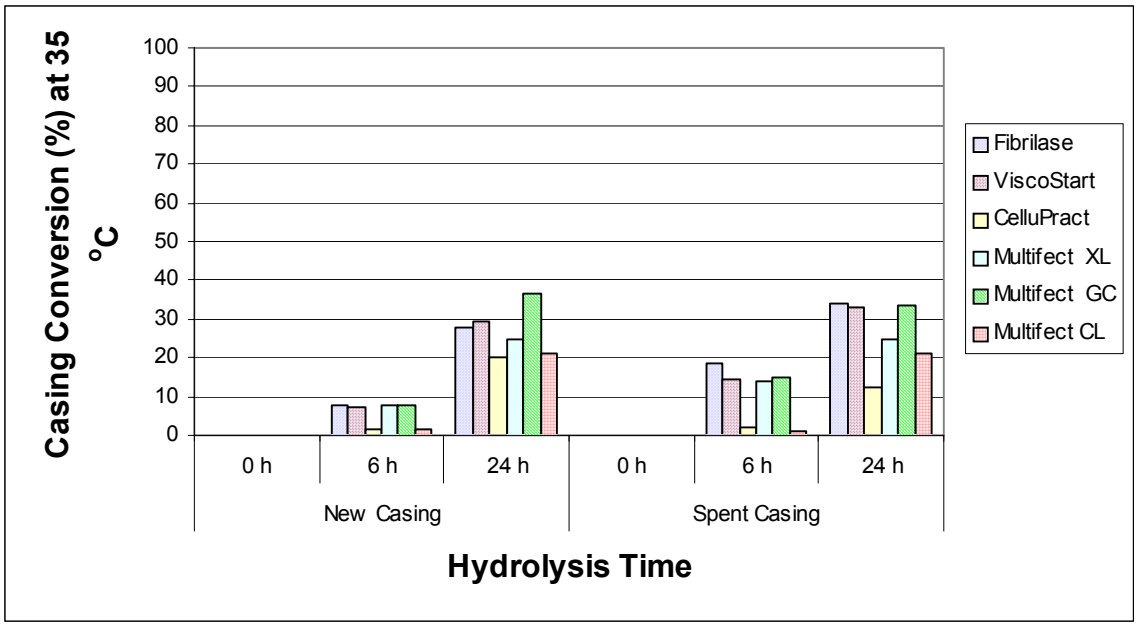


Figure 4.3. Hydrolysis of new and spent casing using commercial enzymes with a casing loading of 25 g/L and an enzyme loading of 20 IFPU/g casing for 6 h and 24 h at 35°C.

4.1.4 Effect of Temperature and Agitation

The effect of temperature on casing hydrolysis using Fibrilase and Multifect GC commercial enzymes with spent casing was also tested (Figure 4.4). The results showed that the casing conversion increased with an increase in temperature until reaching an optimum, at approximately 50 °C. At 30 °C the casing conversion for Fibrilase and Multifect GC was about 4% and at 50 °C the conversion increased to 36% and 37%, respectively. Above 50 °C the casing conversion decreased due to a decrease in enzyme activity. It can be seen that with each ten-degree increase from 30 °C to 50 °C the casing conversion increased by about a factor of four using the same enzyme dosage (30 IFPU/g casing). The results also showed that at high temperature (60 °C) the Multifect GC enzyme performed better, indicating that it is more thermally stable than Fibrilase.

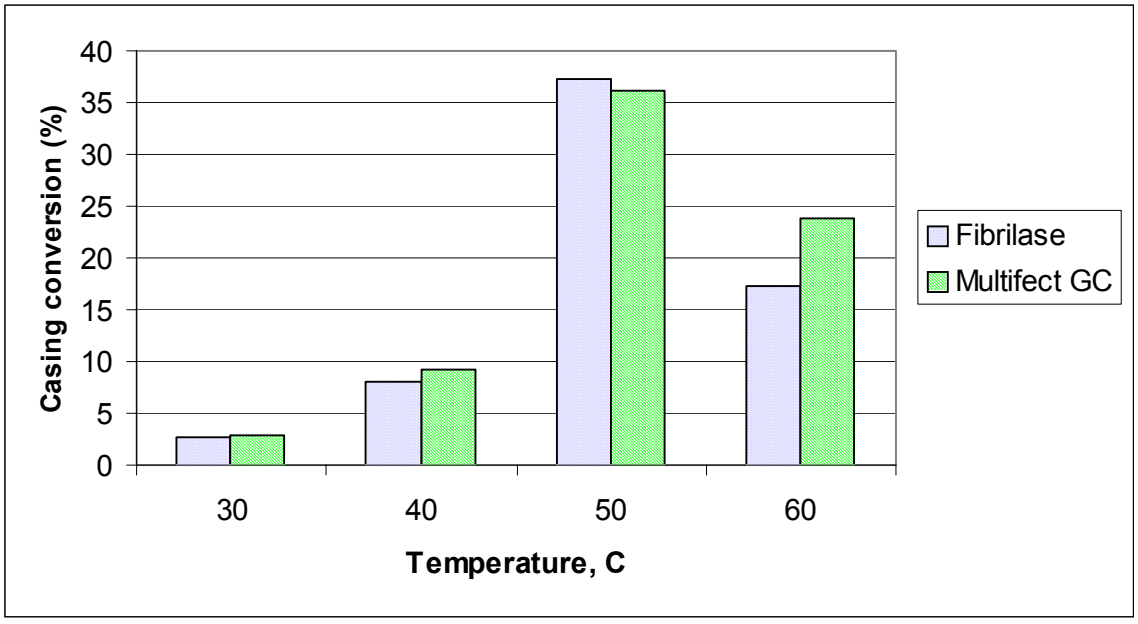


Figure 4.4. Effect of temperature on the sugar yield with a casing loading of 25 g/L and an enzyme loading of 30 IFPU/g casing at 6 h.

The effect of agitation on the casing conversion was evaluated with four speeds (50, 100, 150, and 200 rpm) and two casing loadings (12.5 and 25 g/L) using a Fibrilase enzyme loading of 30 IFPU/g casing at 50 °C for 8 hours (Figure 4.5). The samples exposed to agitation had slightly higher casing conversion than those without agitation. However, the improvement was not that great, with maximum differences of 6% and 9% for casing loadings of 12.5 and 25 g/L, respectively. It can also be observed that above 100 rpm there was little, if any, improvement in conversion. In fact, the conversion decreased slightly when the speed increased from 100 to 200 rpm with a casing loading of 12.5 g/L. This result suggests that an agitation of about 100 rpm should be sufficient to enhance the enzymatic hydrolysis of the spent casing. Because agitation will add a significant cost to the process it may be beneficial to study the effect of intermittent mixing at 100 rpm.

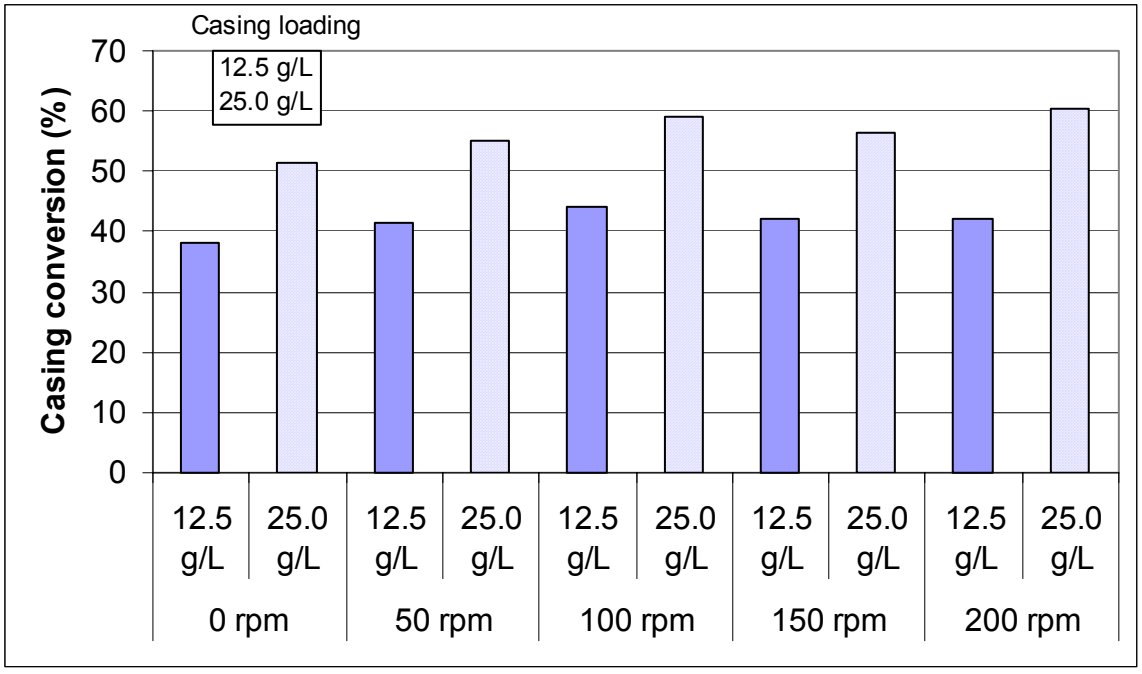


Figure 4.5. Effect of agitation on the reducing sugars with casing loadings of 12.5 and 25 g/L, and Fibrilase enzyme loading of 30 IFPU/g casing at 8 h.

4.1.5 Effect of Substrate and Enzyme Loading

Different doses of the commercial enzymes (Fibrilase and Multifect GC) were used in combination with different spent casing loadings at a temperature of 50°C in the vials (Figures 4.6 and 4.7). In comparison of these figures, it can be observed that the response of both enzymes is very similar.

It can also be seen from the figures that as the concentration of enzyme increases, the percent of casing conversion also increases. With an enzyme loading of 10 FPU/g casing the percent conversion was between 70 and 80%, and it went up by about 5 % for every 10 unit increase in enzyme activity between 10 and 30 IFPU/g casing. No significant increase was observed between 30 and 40 IFPU/ g casing. The percent conversion also increased as the substrate concentration increased from 12.5 to 25 g/L and it decreased at 50 g/L. This response is in agreement with those obtained by previous studies which utilized cellulase enzymes for the hydrolysis of pure cellulosic materials and lignocellulosic biomass (Ramos et al., 1993; Krishna et al., 1998; Gruno et al., 2004; Kadam et al., 2004). They have reported decreases in enzymatic hydrolysis rates at high substrate concentration due to cellobiose accumulation. Ramos et al. (1993) repeated the hydrolysis experiments and supplemented cellulase preparation with β-glucosidase and noted a significant increase in the hydrolysis rate with approximately 90% conversion.

Figure 4.8 shows that the conversion with a casing loading of 12.5 g/L for all the enzyme loadings after 8 hours was lowered than that obtained for 25 g/L and 50 g/L of casing. Poor mixing and enzyme dilution at a casing loading of 12.5 g/L likely caused a reduction in the casing conversion. The vials with a casing loading of 25 g/L received more buffer volume (20 mL) than the other two casing loadings, causing a dilution of the

enzyme, which was added, based on casing mass. It was also observed that during the hydrolysis reaction the casing was sitting on the bottom of the vials without adequate mixing. Nevertheless, it can be seen from Figures 4.6 and 4.7 that a casing conversion between 65% and 76% was obtained after 24 hours.

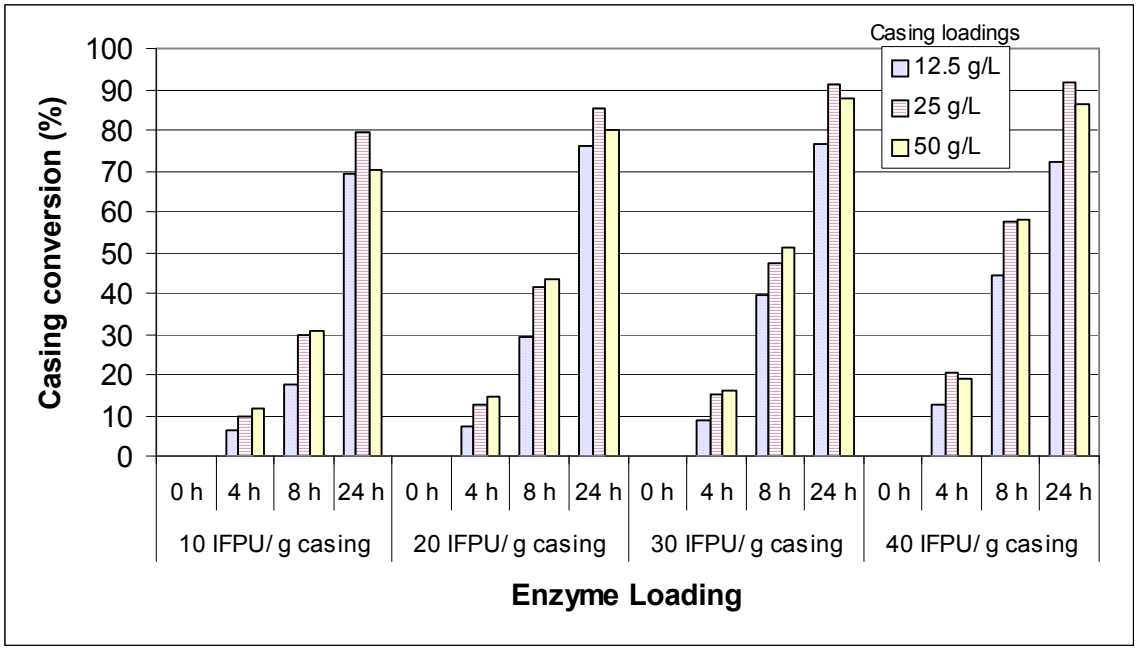


Figure 4.6. Effect of casing and enzyme loading on the conversion of spent casing to sugars by Fibrilase enzyme at 50 °C.

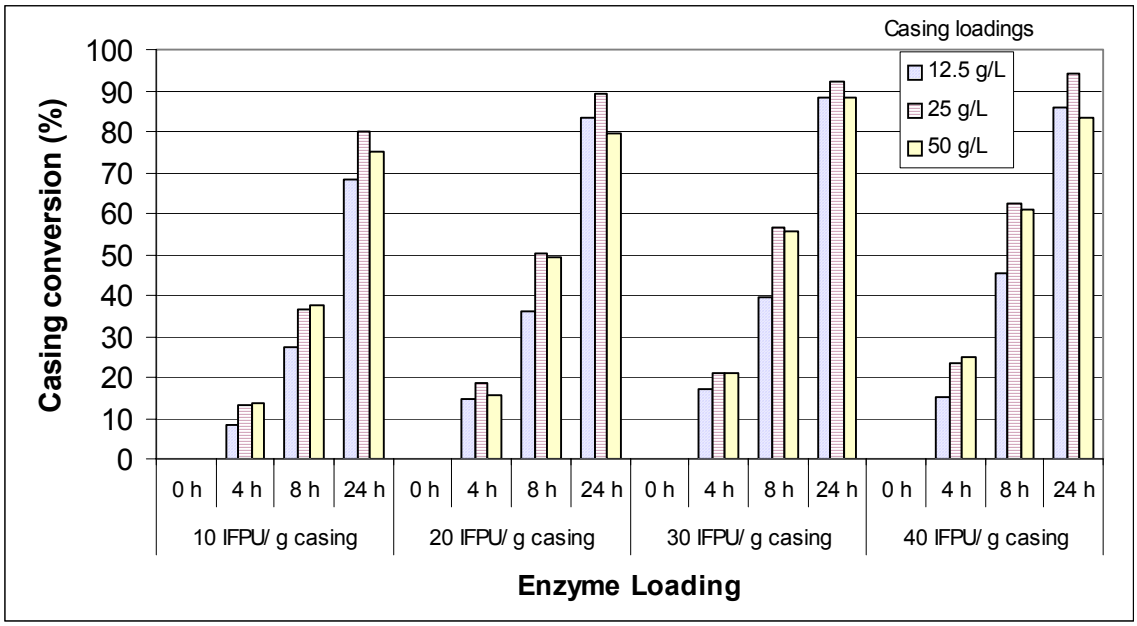


Figure 4.7. Effect of casing and enzyme loading on the conversion of spent casing to sugars by Multifect GC enzyme at 50 °C.

It can also be seen that conversion rate increases fairly linearly with enzyme dose at all substrate loading levels. However the casing conversion is not proportional to the enzyme loading, for instance, twice the amount of cellulase enzyme did not produce twice the amount of sugars. This cellulase response has been observed in other enzymatic hydrolysis studies and in the cellulase activity assay (Mandel et al. 1975, Ghose, 1987).

The spent casing hydrolysis was also performed on a 2-L bench scale stirred reactor to determine if the casing conversion can be improved when the casing is mechanically mixed rather than orbital mixing of the vial. The reactor was loaded with 25 and 50 g/L of casing, Fibrilase enzyme loading of 30 IFPU/ml, and a working volume of 1.0 L at 50 °C and 100 rpm (Figure 4.9). It can be observed that the casing conversion for these loadings is much less, with about 48 to 50% in the vials versus 67 to 69% in the

mixed reactor after 8 hours of hydrolysis time. It can also be seen in the hydrolysis reaction in the vials and the reactor that a casing conversion reduction started to show at casing loading of 50 g/L possibly due to accumulation of sugars at high casing loadings.

As shown in Figure 4.10, the cellobiose concentration was very high compared to the inhibition concentration of 0.1 g/L reported by Awafo et al. (1996). It appears that the concentration at which cellobiose inhibition occurs depends on the cellulase enzyme systems and type of substrate reaction with the enzyme. Another possible inhibitor is glucose, but the glucose concentration was maintained below the inhibition level of 25 g/L at 8 hr with the casing loading of 50 g/L and above enzyme loading of 20 IFPU/g casing (Figure 4.11).

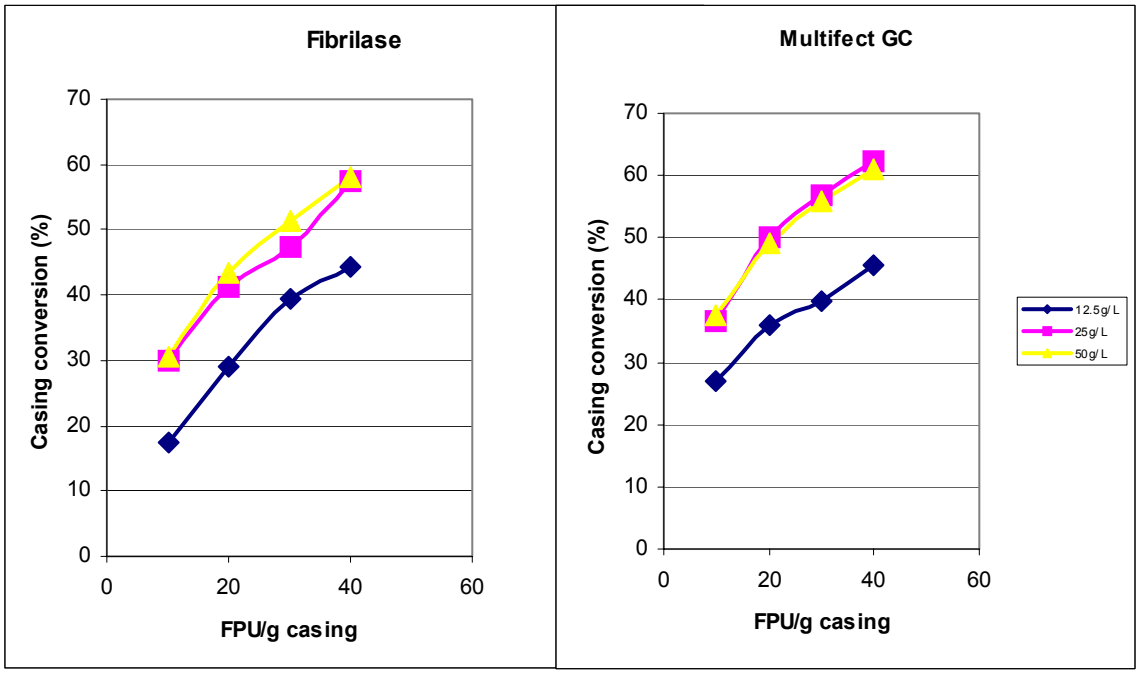


Figure 4.8. Effect of enzyme loading on the conversion of spent casing to sugar in 8 h at 50 °C.

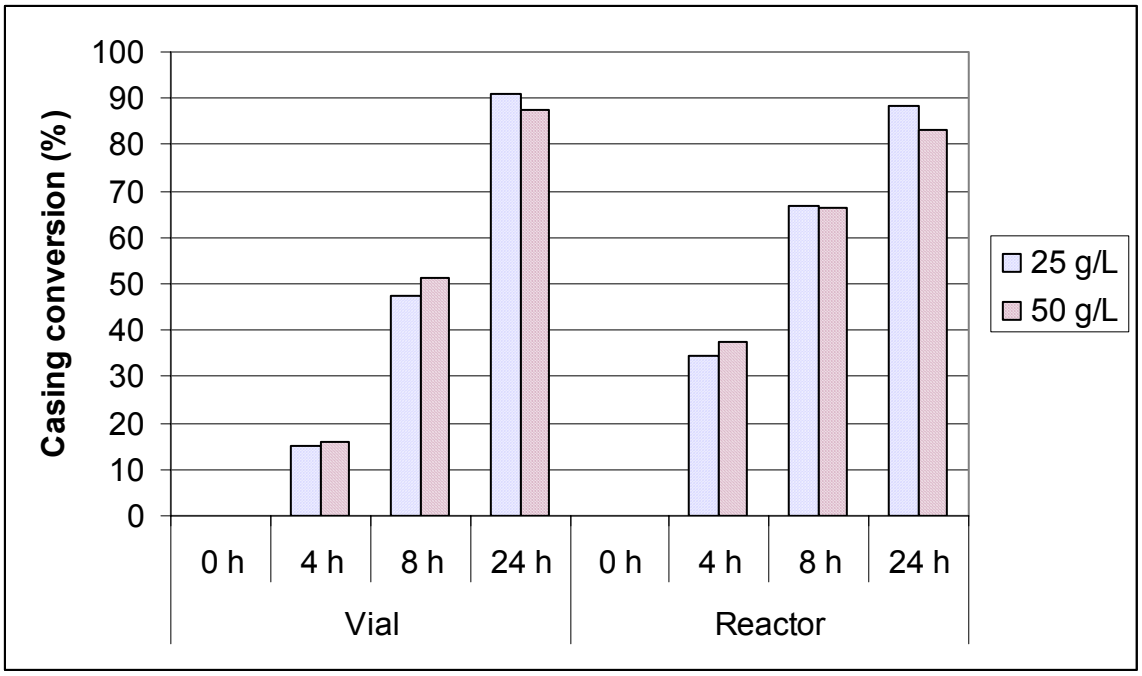


Figure 4.9. Effect of casing loading on the conversion of spent casing in a 2-L stirred reactor with a Fibrilase enzyme loading of 30 IFPU/g casing at 50°C and 100 rpm.

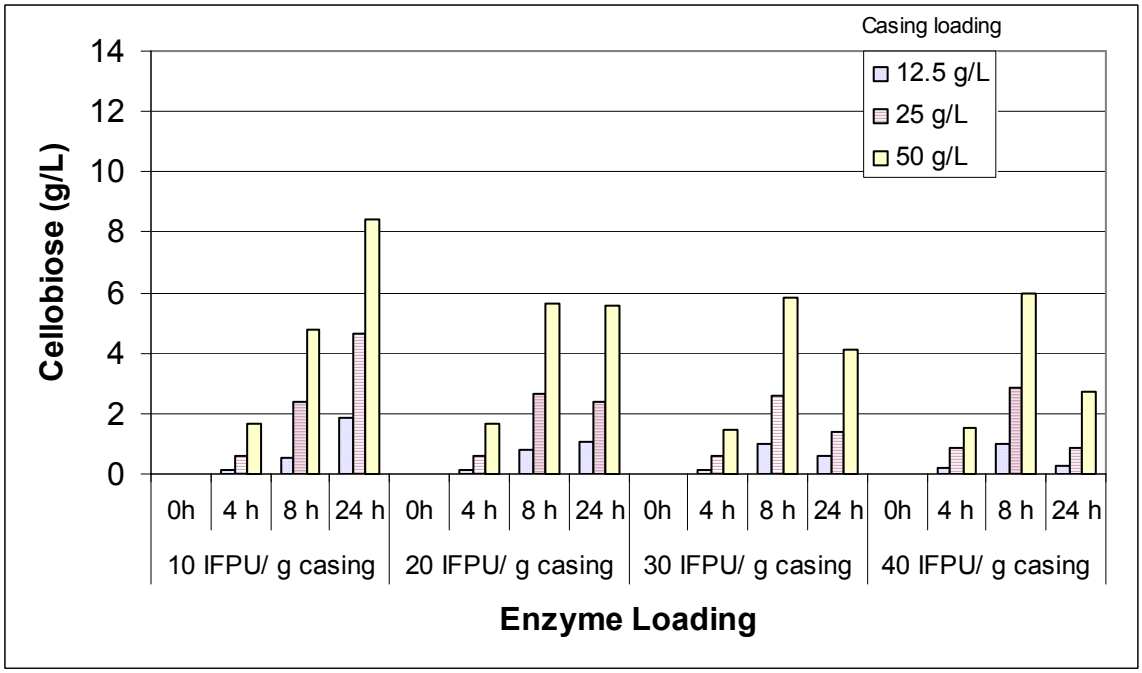


Figure 4.10. Cellobiose concentration during enzymatic hydrolysis of spent casing with Fibrilase enzyme at 50°C.

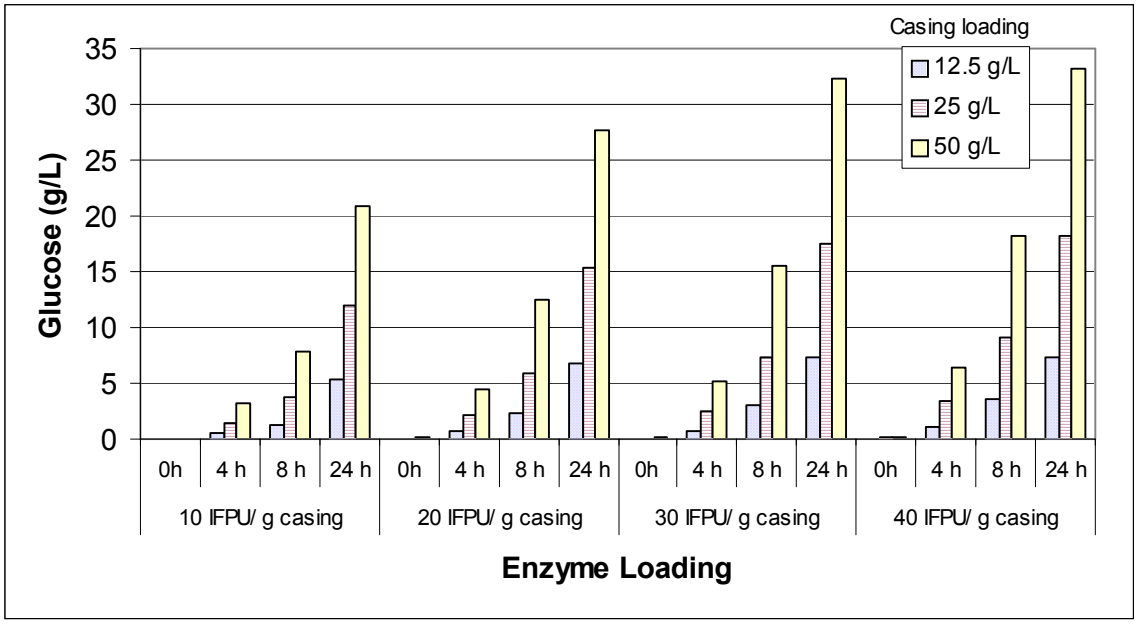


Figure 4.11. Glucose concentration during enzymatic hydrolysis of spent casing with Fibrilase enzyme at 50°C.

It can be observed from Figures 4.10 and 4.11 that the concentration response of cellobiose with different casing and enzyme combinations is different from glucose. Glucose concentration increased with casing and enzyme loading whereas cellobiose reached a plateau at about 6 g/l at 8 hours and then decreased after 24 hours for all enzyme loadings above 10 IFPU/g SC. A comparison between the cellobiose concentration at 8 hours and 24 hours at the different enzyme loadings showed that at 10 IFPU/g casing the cellobiose concentration increased by 70%, at 20 IFPU/ g casing there was no increase in concentration, and at 30 and 40 IFPU/g casing the concentration decreased by 29% and 55%, respectively. In comparing figures 4.10 and 4.11, it appears that cellobiose concentration plateaued whenever glucose concentration was greater than 10 g/L, indicating that there may be a glucose threshold concentration of 10 g/L that is causing a reduction in the activity of β-glucosidase and cellobiohydrolase. This does not

affect the overall production of glucose because it can also be produced by endoglucanase via a separate pathway as described in Figure 2.1.

4.1.6 Effect of Surfactants

The addition of surfactants, especially non-ionic surfactants, has been shown to improve the enzymatic hydrolysis when used with lignocellulosic materials. In this experiment, 12 surfactants (ionic and non-ionic surfactants) were added to the hydrolytic solution to evaluate their ability to enhance the enzymatic hydrolysis of spent casing waste. The surfactant treatments were compared using a casing loading of 25 g/L, 10 IFPU/g of Fibrilase enzyme, and two dosages of surfactant (2 and 8 mg surfactant per g of dry casing) for 6 h at 50 °C (Figure 4.12). The results show that non-ionic surfactants (Tweens and Oleic Acid) have a greater positive impact on the enzymatic hydrolysis of the spent casing than the anionic and cationic surfactants. The casing conversion increased by 3% with a surfactant loading of 8 mg/g for all the Tweens except Tween 80. Also it was observed that with these Tweens, the increase in surfactant dose resulted in an increase in percent conversion. With the Tween 20, both surfactant loadings had similar casing conversion of 26%. It can also be seen in this figure that the ionic surfactants affected the enzymatic hydrolysis and the response worsened at higher surfactant loadings. The enhancement in cellulase hydrolysis by non-ionic surfactants is in agreement with results obtained by Ericksson et al. (2002). Inhibitory effects have been observed with cationic and anionic surfactants (Sun and Chen, 2002).

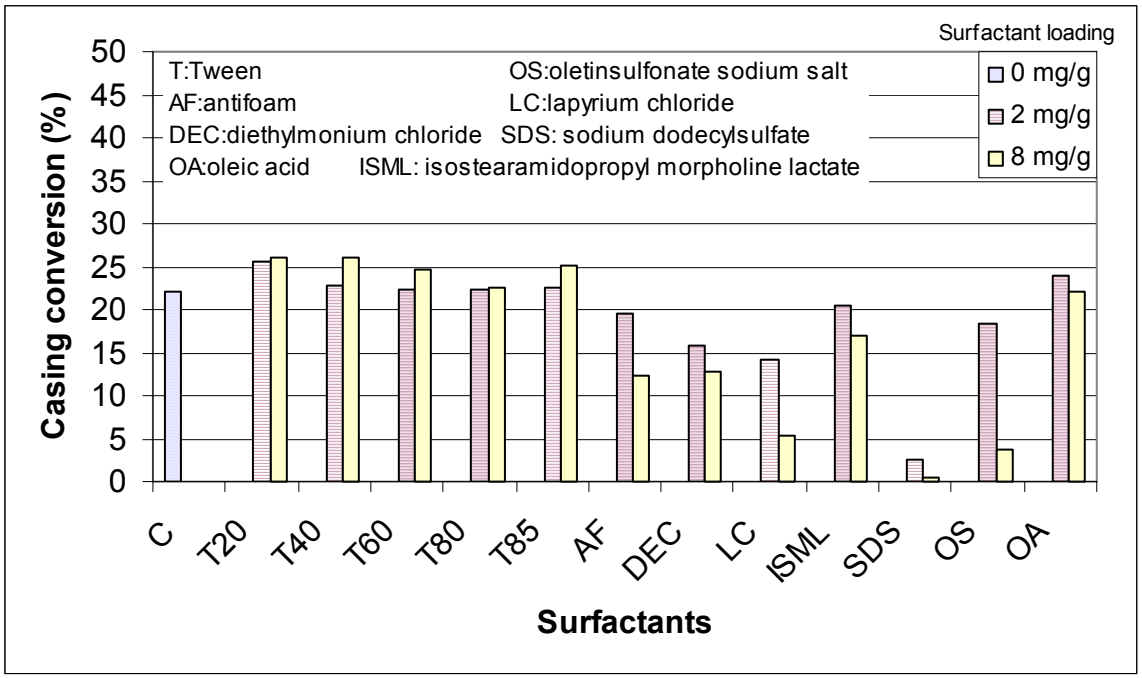


Figure 4.12. Effect of ionic and non-ionic surfactants on the enzymatic hydrolysis of 25 g/L of spent casing with an enzyme dose of 10 IFPU/g for 6 h at 50 °C.

A second study was conducted to evaluate the effect of all the Tween surfactants with a higher dose of enzyme (30 IFPU/g) on the enzymatic hydrolysis of 25 g/L of spent casing for 6 hrs. Results from the Tween screening and loading experiment are shown in Figure 4.13. The Tween surfactants enhance the enzymatic hydrolysis of the spent casing when compared with the Tween-free control. Among the five Tween surfactants, Tween 20 and 85 produced the maximum conversion at a surfactant loading of 40 mg Tween/g casing followed by Tween 40, 80, and 60. When Tween 60 and 80 were added at 40 mg Tween/g casing the results showed some inhibitory effect. The addition of Tween 20 and 85 at this surfactant loading improved the casing conversion by 17% and 15%, respectively. The effect of the Tween loading showed that only a small amount of Tween is required to obtain an enhancement in the enzymatic hydrolysis before reaching

surfactant inhibition. Other studies have found conversion improvement of 63% when a mixture of lignocellulosic biomass with cellulase was treated with 5 g/L of Tween 20, five times more than the maximum amount tested in this experiment (Eriksson et al. 2002). An economic study must be conducted to determine if adding more surfactant is more feasible that increasing the enzyme loading for a particular application.

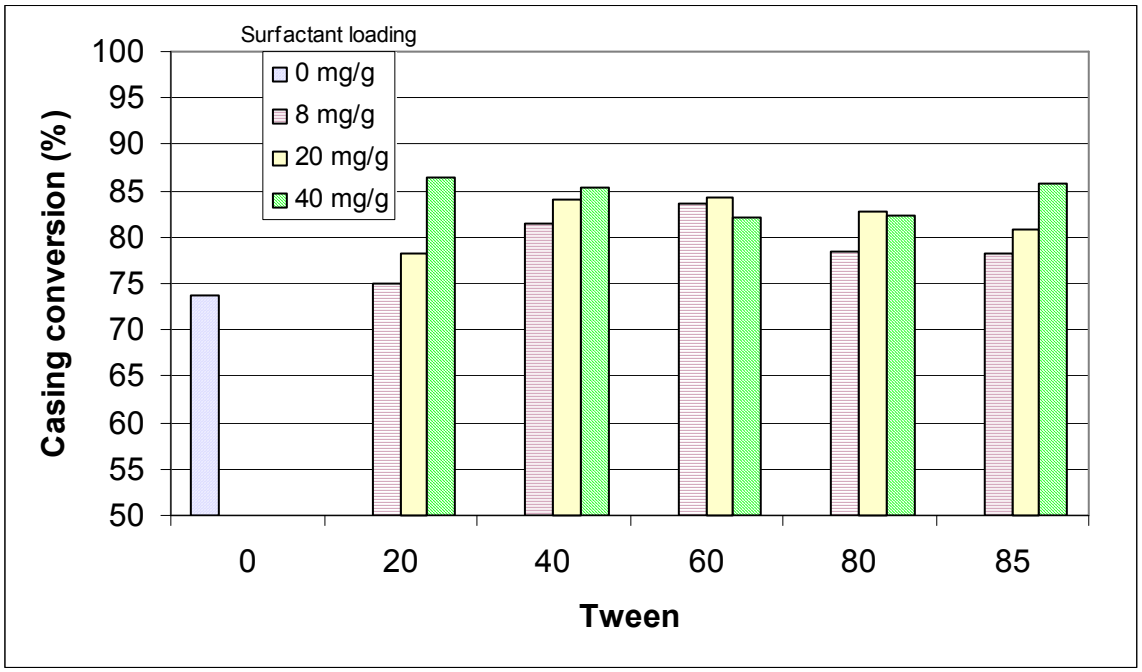


Figure 4.13. Screening of Tween surfactants for enhancement of the enzymatic hydrolysis of 25 g/L of spent casing with an enzyme dose of 30 IFPU/g for 6 h at 50 °C.

4.2 Cellulase Production from Spent Casing in Shake Flask

The production of hydrolytic cellulase enzymes was evaluated using spent casing waste as a carbon source for the growth of the fungi *T. reesei*, mutant RUT-C30 using bath cultures in shake flasks. The raw spent casing waste was dried at 50 °C for 3 days

then chopped before each study. The fungus was then grown and adapted to the spent casing using Stage 2 medium as described in Section 3.5.

Concentration of total solids and solid residues during the time course of the fermentation was not determined because the pieces of the solid spent casings were too big to fit through the broken tip opening of the plastic pipette. Cellulase activity in the control flask containing Stage 1 medium (Control) was not detected. All experimental cultures were conducted in duplicate and the average values were reported.

The first attempt at cellulase production from casing waste was carried out by growing the fungi with two casing loadings of 25 and 50 g/L. This experiment was conducted to determine whether chemical materials present in the waste would interfere with the growth and cellulase production. The following two experiments were based on the cellulase production scenario in Process 1 (Figure 1.1), in which the culture containing the hydrolytic enzyme was fed into the hydrolysis tank and that a portion of the hydrolysate is used for fungi growth and adaptation. The second experiment was conducted to see whether or not the fungus could grow at the hydrolysis temperature of 50 °C. Finally, a series of adaptation experiments was conducted to determine if the hydrolysate can be used as growth medium and if the resulting culture has the same inoculum properties to produce the same or better cellulase yields obtained from traditional adaptation cultures.

4.2.1 Effect of Casing Loading

The effect of increased levels of casing waste was studied in batch casing loadings of 25 and 50 g/L at 28 °C for a period of 15 days. The cellulase activity and

mycelium mass when grown on spent casing waste is shown in Figure 4.14. Two flasks were run in parallel for each loading with data variability of less than 5%. These results show that *Trichoderma reesei* RUT-C30 was able to assimilate to growth conditions with the spent casing waste and to produce cellulase enzymes. The growth response and cellulase activity with a casing loading of 25 g/L was superior to that at a casing loading of 50 g/L. After inoculation, the mycelia threads remained suspended for one day then started forming small clots of about 2 mm diameter. The culture and insoluble casing was uniform at 150 rpm. For the next two days, the number of clots and mycelia threads increased, resulting in a more viscous solution. The solution became thicker in the following 2 days as the clots disappeared and the insoluble casing began degrading. Within 6 days most of the clear sections of the casing were consumed. At this time, the mycelia mass reached its maximum level of 10 g/L and cellulase activity was measured at 0.15 IFPU/ml. After this time the mycelia concentration slowly decreased and the solution became thinner, likely because of autohydrolysis.

As the casings were hydrolyzed, more cellulase enzymes were produced and activity increased very rapidly, reaching a maximum level of 0.87 IFPU/ml and a cellulase productivity of 3.6 IFPU/L-h after 10 days. This sharp cellulase increase is also due to desorption of the enzymes from the hydrolyzed casing into solution. The enzyme production ceased once the casing was consumed. It was observed that sections of casing containing the black ink were still present in the culture, indicating that they were more recalcitrant to degradation than the clear sections of the casing.

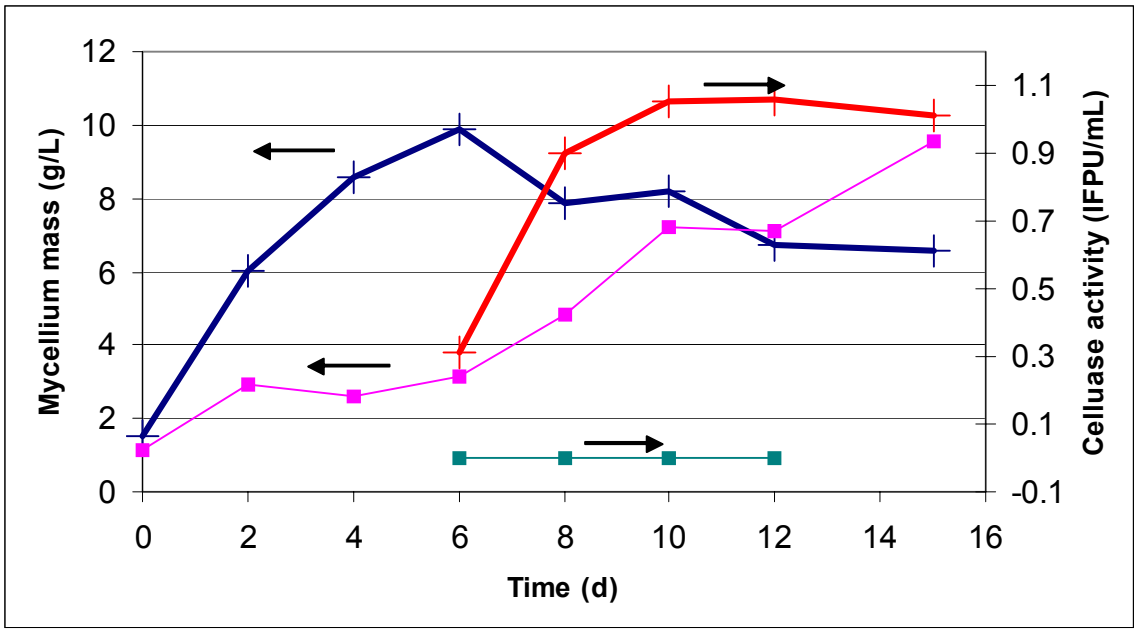


Figure 4.14. Effect of spent casing loadings of 25 g/L (▬) and 50 g/L (—) on the mycelia growth and cellulase activity of *T.reesei*, RUT-C30 at 28 °C and 150 rpm.

The fungi did not grow well and no cellulase was detected with a casing loading of 50 g/L. After inoculation, this culture also formed small clots but fewer suspended mycelia were observed. The mycelia and clots were embedded with the films of casing and they were trapped due to the slow internal mixing at 150 rpm. After 3 days of incubation, these cultures had a foul odor that persisted for 5 days. It was evident that the insoluble casing was not allowing the diffusion of oxygen and the low dissolved oxygen caused a septic condition. However, it was observed that the upper layer of spent casing was slowly decomposed. The pH had a steady slow decrease compared to the drop in pH with 25 g/L of casing, which reaffirms that the metabolic growth was affected.

As the fermentation reached completion, the casing was broken down by the cellulase enzymes and the liquid-like solution contained fungi biomass with very small black clots. The composition of the solids analysis (mycelia mass and residues) at the end of the run is given in Table 4.3. The reduction in total casing solids for the casing

loading of 25 g/L and 50 g/l was 93% and 68%, respectively. The fraction of total solids composed of mycelium mass was estimated based on mycelial protein using a correction factor of 0.37 (Section 3.6.6), resulting in 79% and 37%. The solids residues were calculated based on the difference between total solids and mycelium mass and resulted in 21% and 63%, respectively. If we assume that all the residues came from the casing material, then 93% of the waste with a lading of 25 g/L was metabolized for the production of biomass and cellulase.

Table 4.3. Solids composition of the culture after 15 days with casing loadings of 25 and 50 g/L and 28 °C.

Casing loading, G/L	**Total solids, g/L**	**Mycelia mass, G/L**	**Residual solids, g/L**
25	8.32	6.56	1.76
50	25.6	9.56	16.0

Figure 4.15 shows that during the time course of the fermentation in the flask, metabolic activity caused the pH to drop from 4.8 to 2.8 and the soluble protein to increase from 2.8 g/L to 5.4 g/L for the 25 g/L casing loading. During the adaptation period, the fungi metabolized the spent casing, consumed soluble proteins, and excreted organic acids resulting in a decrease in the pH and soluble protein. As cellulase enzyme appeared in the medium, the soluble protein increased. The pH slightly went up after growth stopped, possibly due to assimilation of the organic acids.

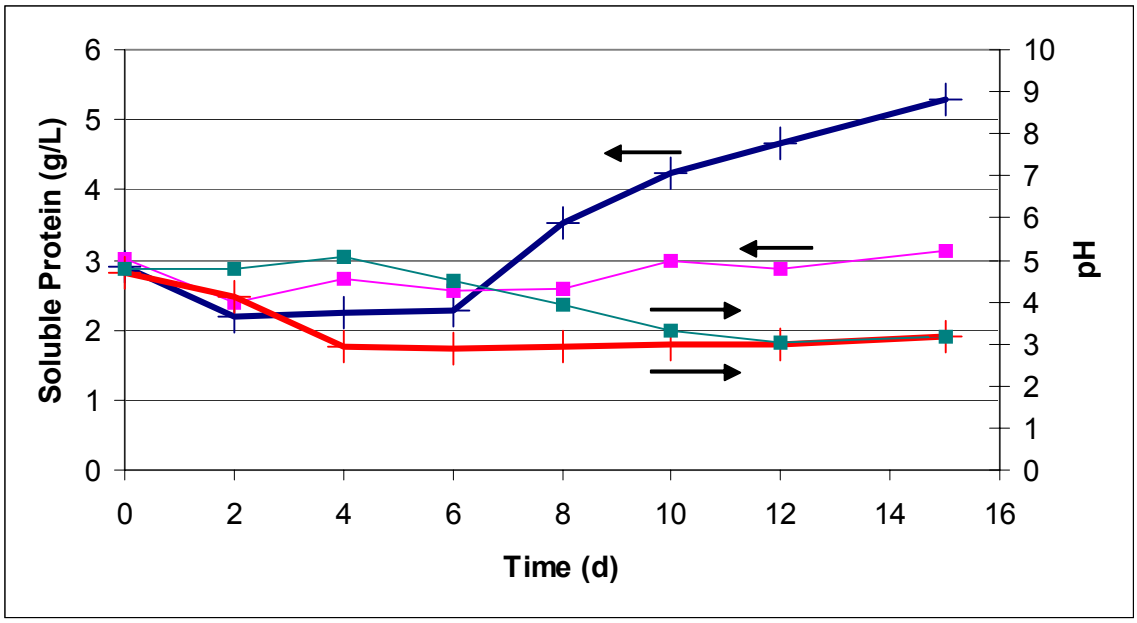


Figure 4.15. Response of soluble protein and pH during the growth and cellulase production by T. reesei RUT-C30 with casing loadings of 25 g/L (—) and 50 g/L (—).

4.2.2 Effect of Hydrolysate Temperature on T.reesei growth

The results obtained from the effect of the hydrolysis temperature on the growth of *T. reesei* RUT-C30 are shown in Figure 4.16. A comparison between the total suspended solids after 3 days of incubation at 28 °C and 50 °C indicates that the fungi cannot grow at the hydrolysis temperature of 50 °C. This would have some advantage in the proposed Process 1 (Figure 1.1). Culture containing the cellulase can be added to the hydrolysis tank without solid separation because the fungi will not grow at the hydrolysis temperature. Another benefit of the growth repression in the hydrolysis reactor is the conservation of high levels of sugars in the hydrolysate, which can be transformed into high value products instead of being consumed by the fungi.

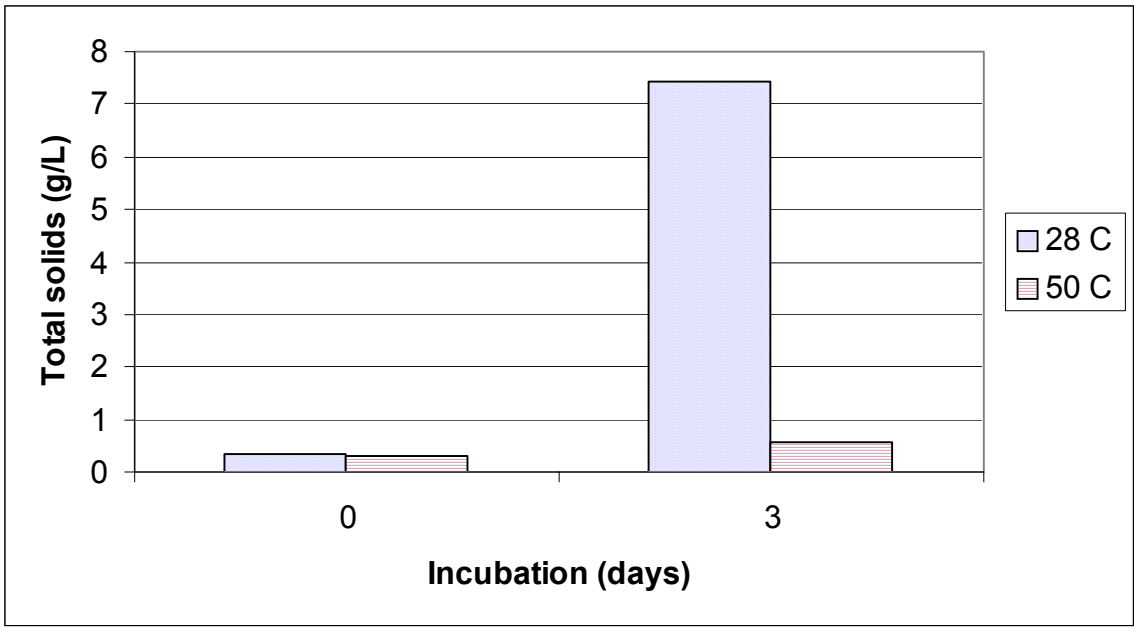


Figure 4.16. Effect of incubation temperature on the growth of *T.reesei* RUT-C30 with 1% (w/v) glucose after 3 days of incubation.

4.2.3 Adaptation of T.reesei to Spent Casing Hydrolysate

Three spent casing loadings (25, 50, and 75 g/L) were mixed with produced enzyme for a total volume of 100 ml at 50 °C for 24 hours. The spent casing hydrolysate was used to grow the fungi without the addition of growth nutrients. Figure 4.17 shows the reducing sugar (glucose and cellobiose) consumption by *T. reesei* with spent casing hydrolysate. After 24 hours, the hydrolysate without any treatment and a control containing 1% (w/v) glucose were inoculated with 0.5 ml of stock culture. It can be observed in this figure that the variation in the initial concentration of sugars is due to the difference in the casing loadings used in the hydrolysis experiment. The fungi metabolized sugars in the range of 10 to 53 g/L, however the rate of consumption decreased as the concentration of sugars increased. The hydrolysate from the 50 and 75

g/L spent casing loadings had an increase in sugar concentration due to the hydrolysis of casing residues.

The reducing sugars for the control (10 g/L) and the hydrolysate with 25 g/L were completely consumed in 3 and 5 days, respectively. When the casing loading was above 50 g/L the reducing sugars were consumed after eleven days.

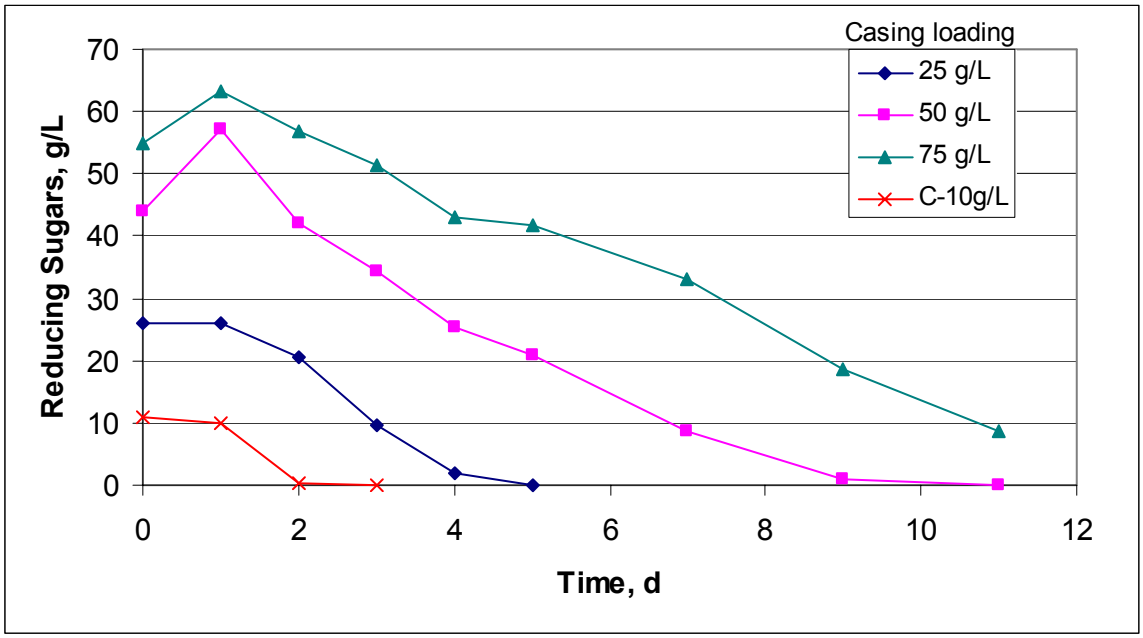


Figure 4.17. Time course of reducing sugars consumption in the inoculum culture flasks with spent casing hydrolysate from 25, 50, and 75 g/L of casing and a control with 1% (w/v) glucose.

After 3 days of incubation, 5 mL of the mycelia culture was used to inoculate the flasks containing 25 g/L of casing with Stage 2 media. Figure 4.18 shows the effect of adaptation culture grown with glucose, hydrolysate, and spent casing on the production of cellulase enzyme. The result shows that cellulase can be produced regardless of the type of adaptation conditions used in the inoculum culture. Within the three hydrolysate

inoculums, the 25 g/L gave the highest cellulase production of 1.01 IFPU/ml followed by 50 g/L and 75 g/L with 0.84 and 0.78 IFPU/mL, respectively. The quantity of residual glucose present in the inoculum will increase the growth stage of the fungi. Once the glucose is consumed, cellulase production will be detected in the production flask. A comparison of the control with spent casing and the 25 g/L hydrolysate resulted in almost the same cellulase activity with 1.06 and 1.01, respectively. It appeared that the hydrolysate solution contains the necessary nutrients and carbon source that the fungi need for adaptation and metabolic induction of the cellulase enzymes.

It can be observed in Figure 4.19 that the fungi utilized both glucose and cellobiose carbon simultaneously, glucose to support the growth and cellobiose to induce cellulase enzyme production. The utilization of hydrolysate as adaptation media will be more cost effective than the conventional adaptation method because the incubation period for the hydrolysate is 3 days rather than 8 days with the casing, and from a process handling perspective, it is more simple to pump and mix liquid in a reactor than solid spent casing waste.

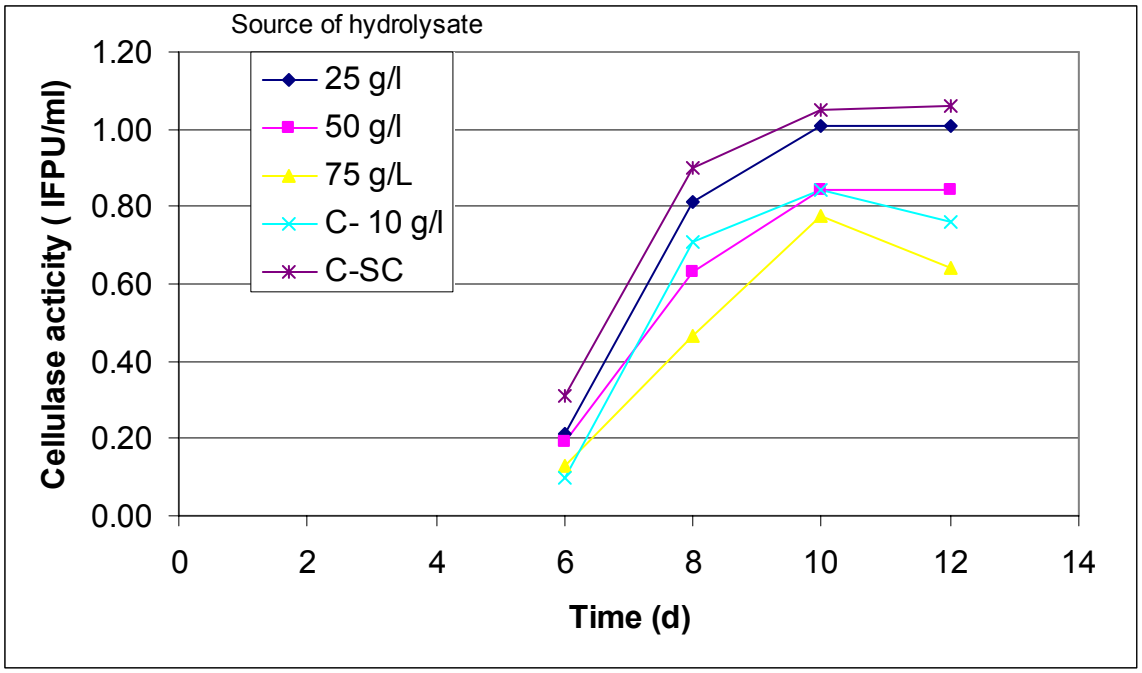


Figure 4.18. Effect of inoculants adapted with spent casing hydrolysate on the cellulase enzyme activity. Control (C) with 10 g/L glucose and 25 g/L spent casing (SC).

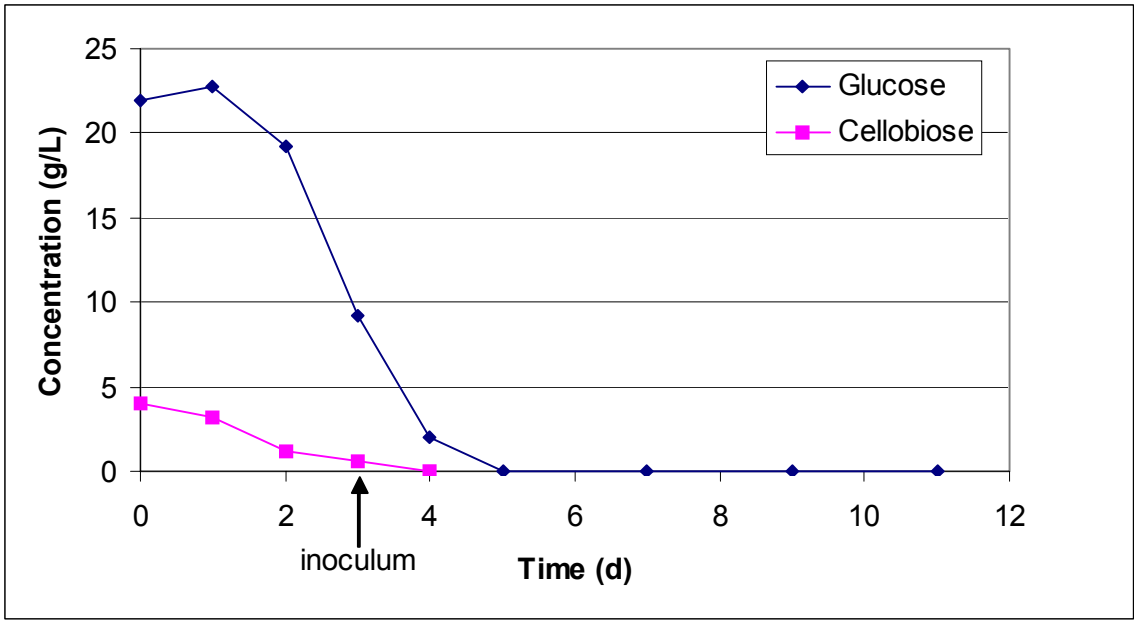


Figure 4.19. Utilization of glucose and cellobiose in the hydrolysate for growth and induction of cellulase by *T. reesei* RUT-C30. Hydrolysate from 25 g/L of spent casing.

4.2.4 Test Performance for the Bubble Column Reactor

Before cellulase production experiments were performed, a series of test runs were conducted to determine the growth performance of the fungi with spent casing in the bubble column reactor. Initial problems were centered around difficulties in maintaining the solid spent casing submerged and uniformly mixed during the time course of the fermentation. Based on observations from the flask studies, the culture solution underwent several changes, starting with a heterogeneous mixture of solid casing and media, then the culture turned viscous as the mycelia mass increased, and finishing with a more homogeneous and liquid-like culture mainly composed of mycelia mass. In order to have uniform mixing, different mixing devices were tested with the installation of a perforated disk to maintain the casing in solution (Figure 4.20).

A prototype perforated disk was built using a stainless steel plate with ¼ inch diameter holes and a silicone gasket around the disk to avoid contact of the steel with glass. The vaned and rod impellers created mixing zones with several different regions without continuous mixing. A consistent mixing during the time course of the fermentation was accomplished by using a combination of three straight impellers with the perforated disk. Figure 4.21 A was taken after four hours of inoculation and shows the casing being kept under the disk. Figure 4.21 B was taken after 9 days and it shows the arrangement of the impellers, biomass clogs, and foam attached to the mixing shaft and temperature probe.

A test was also conducted to determine the performance of the pH, DO, and temperature sensors. The response of the pH controller was monitored by adding a small amount of 4 M NH_4OH from the bottom of the reactor and recording the response time

and the change in pH. This test was conducted at various stages of the fermentation to account for the change in culture viscosity. A similar procedure was used for the DO probe, except that pure oxygen was mixed with air. The results showed that the pH and DO response time varied with the time course of the fermentation. In order to maintain the pH close to 4.8, the pump flow rates on the acid and base pumps were set to 0.2 ml/hr. Nothing was adjusted for the DO, since it was not used as an internal process control. The DO was maintained above 10 % of saturation by manually controlling the air and oxygen flow. The reactor temperature was continuously maintained at the set value of 28 °C.

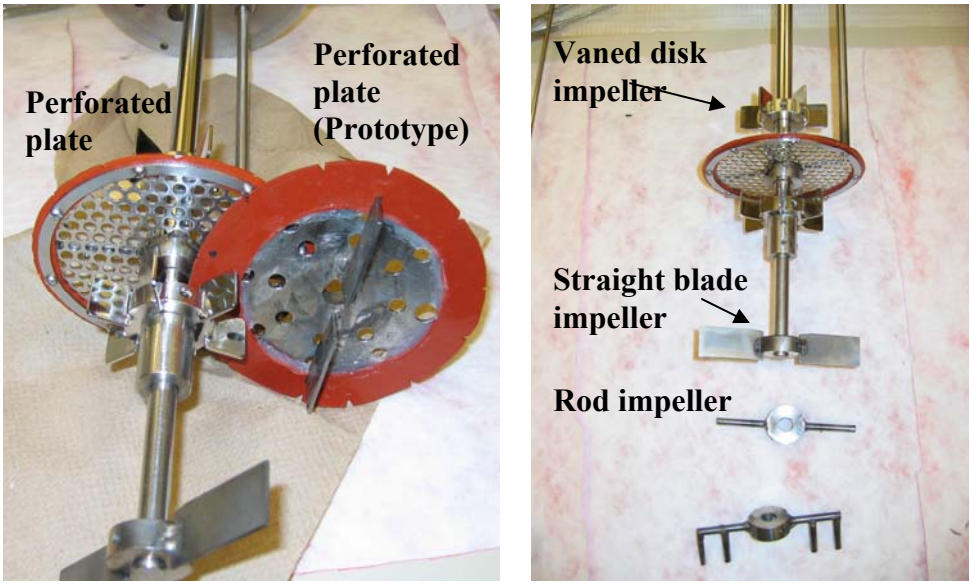


Figure 4.20. Implements tested to improve mixing of solid spent casing in submerged fermentation.

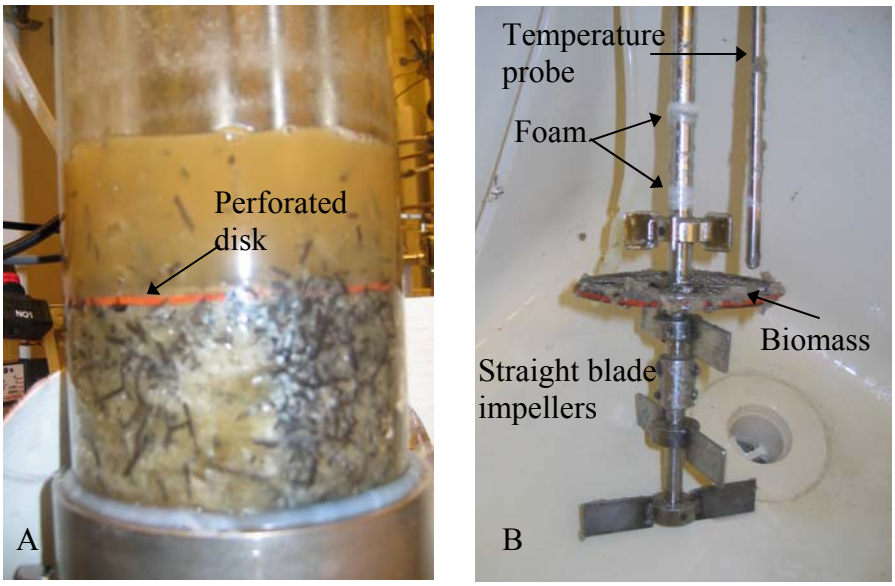


Figure 4.21. Perforated disk used for the bench scale fermentation (A) and mixing impeller arrays (B).

4.2.5 Cellulase Production in the Bubble-Column Reactor

The effect of spent casing loading on the production of cellulase enzyme was studied with a 2.5 L bubble-column reactor in batch fermentation with loading levels of 25, 50, and 75 g/L of casing. The reactor was prepared by filling with dry casing and Stage 3 media in DI water, and autoclaved for 50 min. Once the reactor's temperature was 28 °C and with stable DO level, the remaining minerals and Tween-80 were added along with 5% (v/v) inoculum for a start-up volume of 1.5 L. The pH was maintained at 4.8 ± 0.05 and temperature at 28 °C. The agitation speed was 10 rpm for 4 - 6 days depending on the loading, and then increased to 45 rpm until completion of the run. The aeration was manually adjusted to between 0.2 and 1.2 L/L-min depending on the percent level of saturation. The first experiment was conducted with 25 g/L of casing followed

by 50 g/L and 75 g/L. Between each run, the reactor apparatus was disinfected with a solution containing 10% Clorox, and the sintered glass filter was cleaned with concentrated sulfuric acid.

The results from the production of cellulase enzyme in the bubble-column reactor with different loadings of spent casing are shown in Figures 4.22 to 4.26. The figures contain reproducible profiles of the cellulase activity, productivity, mycelium mass, and soluble protein during the time-course of the fermentation for each run with *T. reesei* culture. The cultivation period for each batch was 8 or 9 days.

The highest cellulase activity (1.3 IFPU/ml) and productivity (7 IFPU/L-h) was obtained after 7 days using a spent casing loading of 50 g/L (Figures 4.22 and 4.23). The cellulase activity with a casing loading of 50 g/L was significant different ($\propto = 0.025$, $p > 0.001$) from that of 25 g/L of casing as seen in Figure 4.22. The maximum cellulase activity and productivity with a casing loading of 75 g/L was 0.81 IFPU/ml and 3.8 IFPU/L-h at 9 days and with a casing loading of 25g/L was 0.77 IFPU/ml at 6 days and 5.1 IFPU/L-h at 5 days. The cellulase activity for all the loading levels was within the values obtained in other studies using waste paper and biomass as substrate with *T. reesei* RUT-30 culture (Silvia et al., 1995; Reczey et al., 1996; Hayward et al., 1999; Shin et al., 2000).

Compared with the cellulase activity of 1.05 IFPU/ml at 10 days with 25 g/L of casing obtained in the flask studies, the production in the bubble-column reactor was 24% less. However, the productivity in the reactor was about 50% higher than that obtained in the flask study (4.4 IFPU/L-h). The build-up and accumulation of foam in the headspace of the reactor may have caused the decrease in cellulase quantifications.

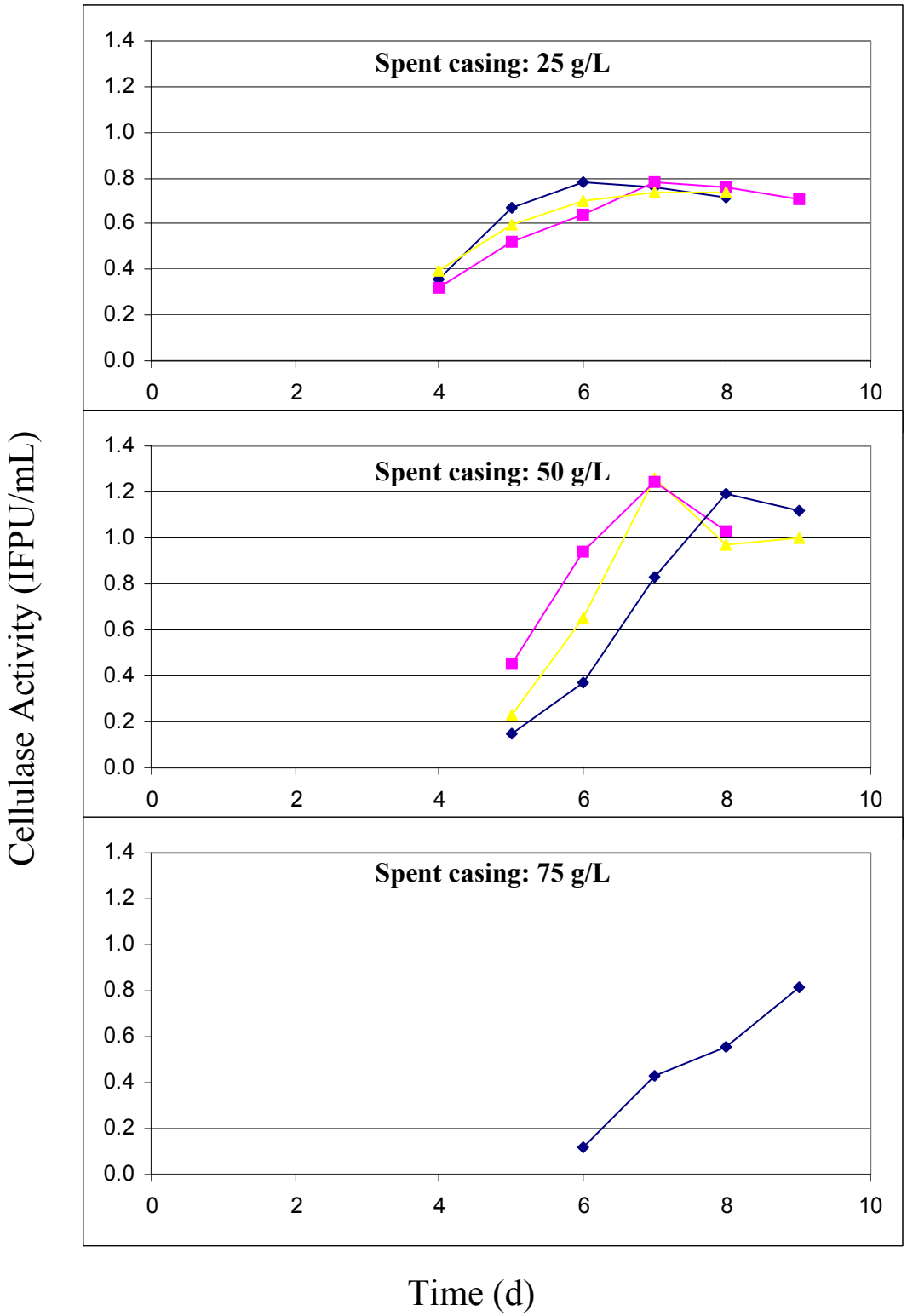


Figure 4.22. Cellulase activity of *T. reesei* RUT-C30 grown in a batch fermentation with spent casing loadings of 25, 50, and 75 g/L in a bubble-column reactor with pH of 4.8 and temperature of 28 °C.

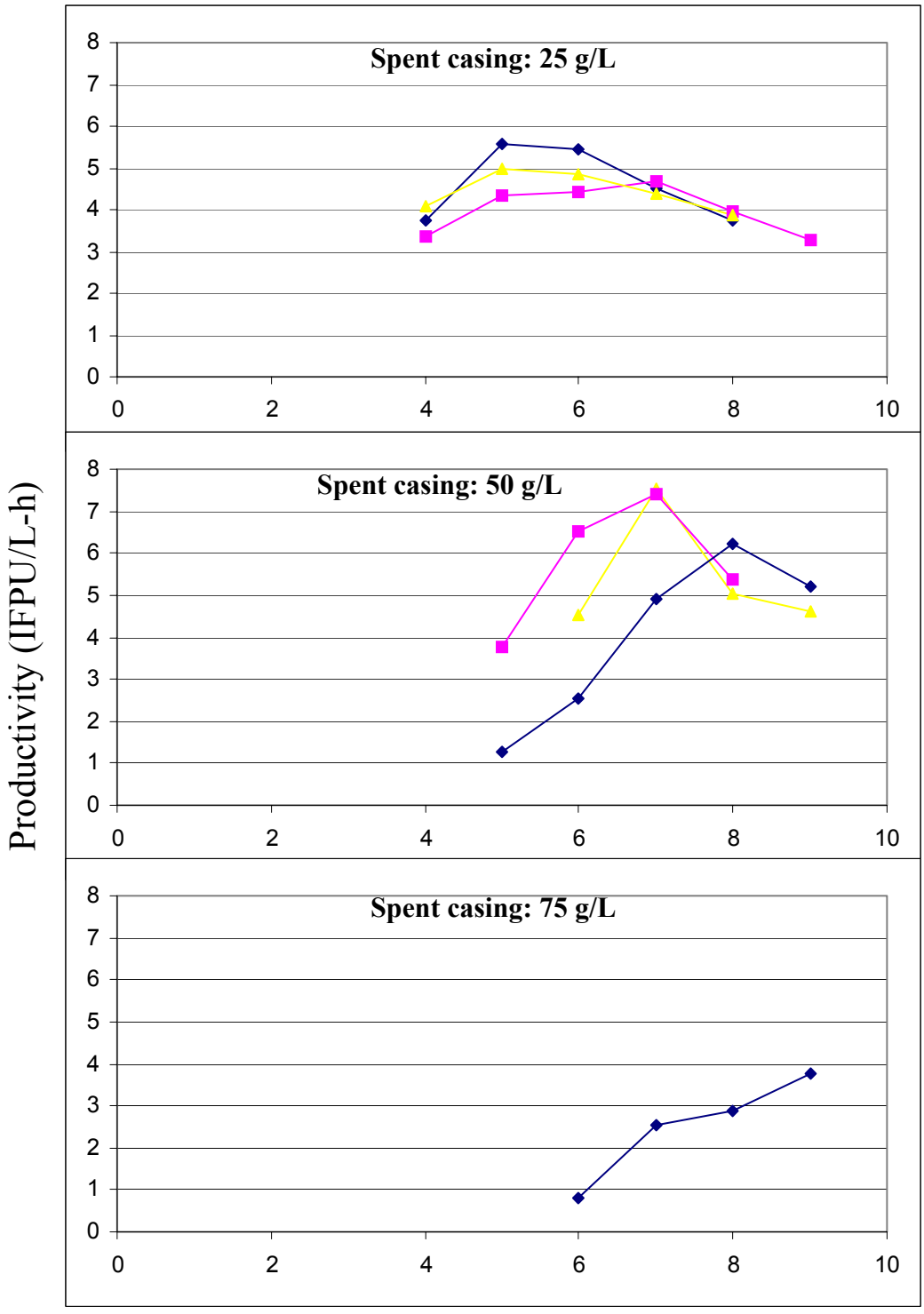


Figure 4.23. Cellulase productivity of *T. reesei* RUT-C30 grown in a batch fermentation with spent casing loadings of 25, 50, and 75 g/L in a bubble-column reactor with pH of 4.8 and temperature of 28 °C.

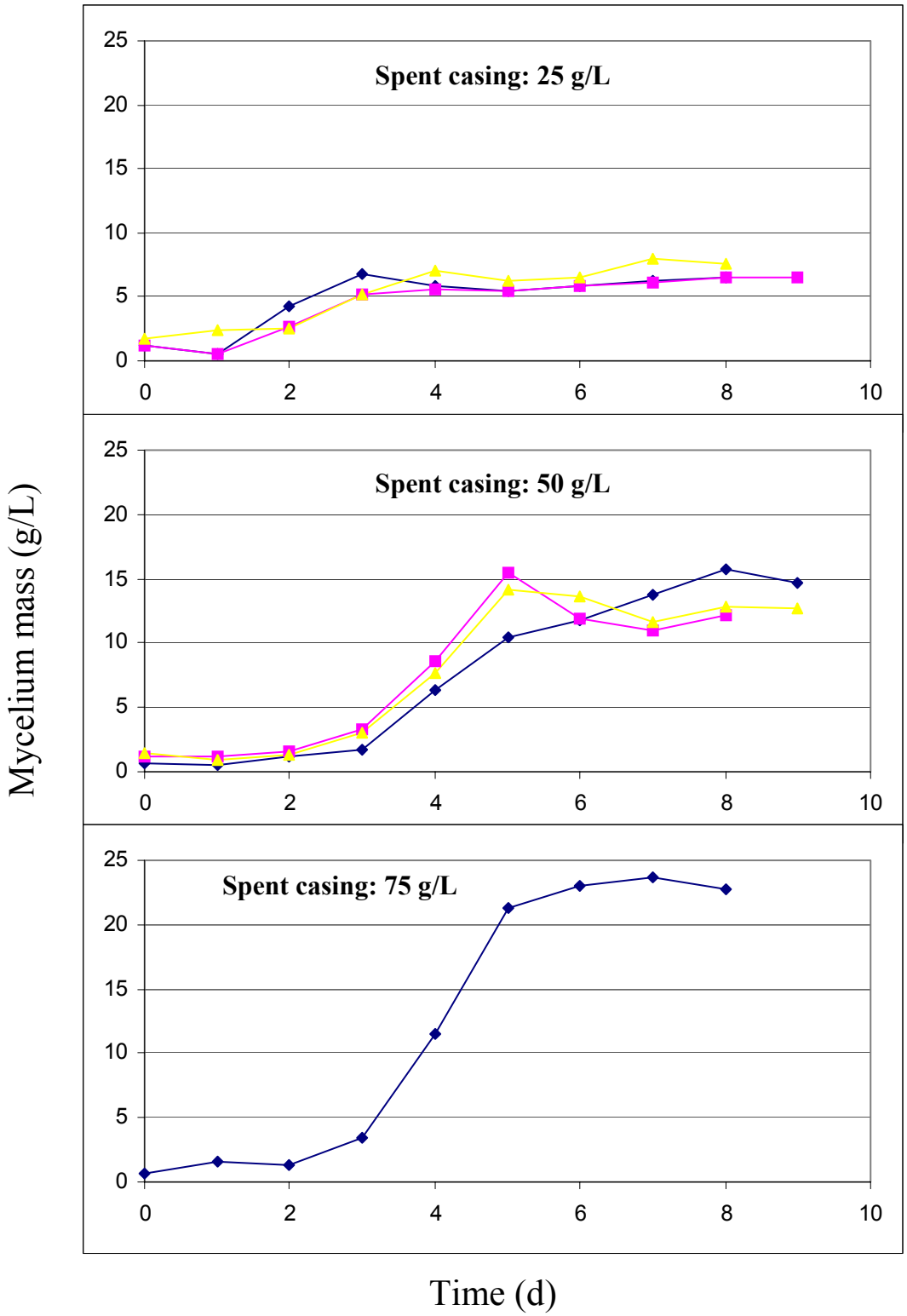


Figure 4.24. Mycelium mass produced by *T. reesei* RUT-C30 grown in a batch fermentation with spent casing loadings of 25, 50, and 75 g/L in a bubble-column reactor with pH of 4.8 and temperature of 28 °C.

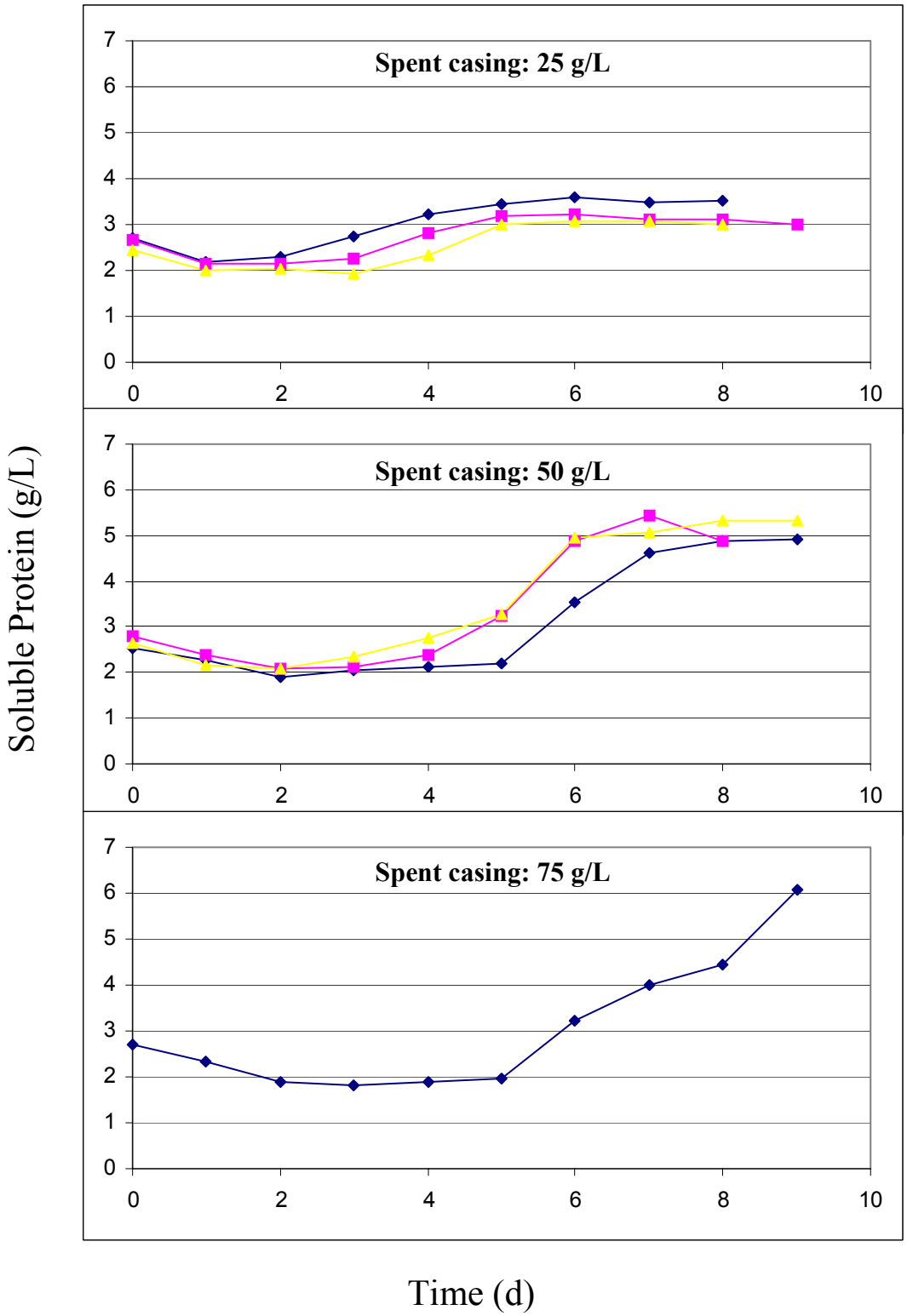


Figure 4.25. Soluble protein produced by *T. reesei* RUT-C30 grown in a batch fermentation with spent casing loadings of 25, 50, and 75 g/L in a bubble-column reactor with pH of 4.8 and temperature of 28 °C.

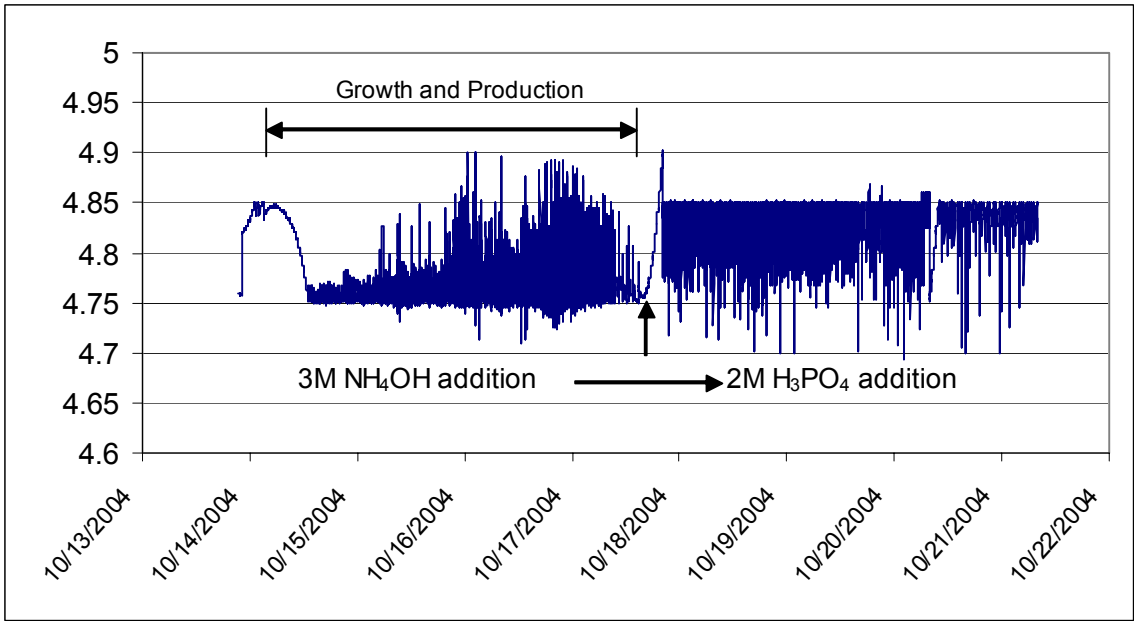


Figure 4.26. Time course of the pH during the production of cellulase by *T. reesei* with a casing loading of 25 g/L at 28 °C.

Also, the problem of foaming became more severe as the casing loading increased, despite the addition of antifoam solution. The productivity in the reactor was higher because the fungi were growing in a controlled pH and DO environment, with the pH maintained at 4.8 and the DO above 10 % of air saturation throughout the time course of the fermentation.

It was expected to obtain the highest cellulase production at the casing dose of 75 g/L, but due to process difficulties the overall production performance at the high loading was lower than at 25 g/L of casing (Figure 4.22). Maintaining a DO level above 10% after the fourth day was the main problem faced with the casing loading of 75 g/L because the culture became too viscous. Several operational alternatives were performed to increase the DO, however the DO level remained too low, with recorded values below

2% for 3 days. During this period the agitation speed and airflow were gradually increased from 42 rpm to 100 rpm and from 1 lpm to 2.5 lpm and the air was supplemented with 30 % (v/v) oxygen. These operational changes were not sufficient to increase the DO above 8%. Due to the high mixing and airflow, more foam was produced and antifoam added. In one night, the foam containing fungi mass came out from the reactor through the gas outlet and was dissolved in the spore's water trap located downstream from the gas outlet. The trap solution contained suspended cells and this solution (100 ml) was added to the reactor to recover the fungi cells and cellulase. During the sixth day, the sample was very thick and difficult to collect. As the culture became more thin the DO gradually increased and operational parameters went back to normal operating conditions. Despite this operational difficulty, the culture produced 0.81 IFPU/ml of cellulase. Due to this operation problem, the remaining comparisons were conducted using the casing loadings of 25 g/L and 50 g/L.

The mycelia growth phases were determined from the dry mycelium analysis (Figure 4.24) and by the changes in the pH. Rapidly digested glucose present in the media from the spent casing and corn steep liquor caused a steady mycelium growth during the apparent lag phase, which lasted between 1 day for the casing dose of 25 g/L and 2 days for the other two doses. In this period fungi growth was observed but adhesion of the fungi on the casing was visible making it difficult to quantify the biomass. The growth during this phase agreed with the rapid consumption in protein (Figure 4.25) and the addition of 3 M NH_4OH (Figure 4.26).

In the log phase, the mycelial thread-like mass rapidly propagated and the culture viscosity increased to a maximum followed by a slow growth phase in which the mycelial

mass decreased and the solution became thinner. The specific growth rate for the three casing levels was calculated during the exponential mycelium growth and in increasing casing loading order was 0.04, 0.03, and 0.04 h^{-1}. It was observed that most of the casing was consumed by the time the mycelial mass reached the maximum level. The end of the growth phase could also be determined by monitoring the pH of the reactor. As shown in Figure 4.26, during the growth phase the pH was maintained at 4.8 ± 0.1 by automatically adding 3M NH_4OH, then at the end of the growth phase it switched to 2 M H_3PO_4, indicating that the casing has been consumed.

A comparison between the mycelium growth (Figure 4.24) and the cellulase activity (Figure 4.22) shows that the enzyme production started near the end of the exponential growth phase and reached the maximum activity during the stationary phase. At the end of the run, both the mycelium mass and cellulase activity slightly decreased, possibly due to autohydrolysis and adhesion of enzymes to spores, respectively. Sporulation was noted in the culture by observing a dark greenish color development.

The response of the cellulase production could be correlated with the soluble protein (Figure 4.25). The soluble protein, which includes cellulase enzymes, decreased during the lag phase and gradually increased as the fungi started growing and producing hydrolytic enzymes. When the time course of protein and enzyme activity is compared, it can be seen that the protein increases parallel to the mycelium mass with about a two-day lag and that the protein assay detected the production of the cellulase enzyme before the cellulase assay. A comparison of the soluble protein and the mycelium mass (Figure 4.24) shows that the time it took the protein to reach the maximum level was after the mycelium mass reached the highest level. This validated that the production of cellulase

is non-growth associated. However, metabolic cellulase synthesis suggests that the cellulase production is growth associated because the fungi rely on glucose for growth and energy. In fact, the continuous production of cellulase activity after growth could result from the detachment of the enzyme from the insoluble materials.

Because the soluble protein at the end of the batch is mainly composed of hydrolytic enzymes, some researchers have reported cellulase performance based on soluble protein. Another benefit to that approach is that the protein analysis is more accurate and less cumbersome to perform than the cellulase assay.

Table 4.4 shows a comparison of the characteristics of cellulase systems produced with the three casing loading levels. The average ratio of β-glucosidase to cellulase activity in increasing order of loading was 0.31, 0.32, and 0.21, respectively. Even though these ratios are lower than a value of 1.0 recommended by Awafor et al. (1996) for complete cellulase enzymes, they are within the range obtained with commercial enzymes. This table also shows that higher casing loadings had a negative effect on the cellulase yield. The cellulase yield with a loading of 50 g/L and 25 g/L was 25 IFPU/g casing and 31 IFPU/g casing, respectively. It is possible that at high cellulose concentration, the rate of glucose and cellobiose production is higher than the rate of utilization, which will lead to accumulation and cellulase enzyme repression.

Esterbauer et al. (1991) mentioned that a temperature shift from 30 °C during growth to 28 °C for production could improve the cellulase yield. The high temperature may help in maintaining a lower glucose concentration during the early growth stages and also shorten the lag phase by increasing the rate of glucose production. Similar

results involving the effect of high substrate levels on cellulase yield were reported by Hayward et al. (1999).

The solids composition of the fermentation broth at the end of each run is shown in Table 4.5. The end product contains solids composed of mycelia and spent casing residues. The casing residues contained fractions of casings with the black ink, indicating that the black pigment of iron dioxide offers some type of resistance to the mycelium attachment and to the cellulase enzyme. The percent of solid casing reduction, in order of increasing dose, was 88 %, 90 %, and 93 %, respectively, with an average mycelium yield of 0.27 g/g casing.

Table 4.4. Comparison of enzyme production by *T. reesei* RUT-C30 with three levels of spent casing loadings.

Casing loading, g/L	**Cellulase activity, IFPU/ml**	**β-glucosidase, IU/ml**	**Ratio IU/IFPU**	**Cellulase yield IFPU/g SC**	**Productivity, IFPU/L-h**
	0.78	0.20	0.26	31	5.6
25	0.78	0.25	0.32	31	4.7
	0.74	0.27	0.36	30	5.0
	1.3	0.41	0.32	25	7.5
50	1.2	0.38	0.32	25	7.4
	1.2	0.37	0.31	24	6.2
75	0.81	0.17	0.21	11	3.8
Average					
25	0.77	0.24	0.31	31	5.1
50	1.3	0.39	0.32	25	7.0

Table 4.5. Comparison of insoluble materials in the fermentation broth at the end of the batch.

Casing loading, G/L	Total solids, g/L	Mycelia mass, g/L	Residual solids, g/L	Mycelium yield, g/g SC	Residues yield, g/ g SC
	9.6	6.5	3.1	0.26	0.12
25	9.5	6.5	3.0	0.26	0.12
	10	7.6	2.6	0.30	0.10
	21	15	6.6	0.30	0.13
50	16	12	3.6	0.24	0.07
	18	13	5.1	0.26	0.10
75	28	23	5	0.31	0.07
Average					
25	9.7	6.9	2.9	0.27	0.11
50	18	13	5.1	0.27	0.10

4.3 Hydrolytic Capacity of Produced Enzyme

The produced enzyme from the bubble-column reactor with a casing loading of 50 g/L (run 2) was selected to determine the hydrolytic performance of the produced enzyme with four casing loadings and to compare the conversion efficiency with the commercial enzyme. The commercial enzyme ViscoStart was selected for this study because it has a similar ratio of β-glucosidase to cellulase activity as the produced enzyme.

At the end of the fermentation run, the thin culture was easily removed from the reactor through a 0.25-inch outside diameter plastic tube connected to a peristaltic pump. The agitation remained on while the culture was transferred to a 2-L flask. The culture was then centrifuged at 5,000 rpm for 15 min and the clean supernatant along with the solids were stored in the refrigerator at 4 °C. Figure 4.27 shows the resulting solution containing the produced cellulase enzymes with a cellulase activity of 1.2 IFPU/ml. The

commercial enzyme has a cellulase activity of 96 IFPU/ml and it was diluted to 1.2 IFPU/ml with 75 mM sodium acetate buffer.

The effect of produced enzyme on the concentration of spent casings is shown in Figure 4.28. The produced cellulase contains all the hydrolytic enzymes complex required to break down the spent casing waste into sugars. The spent casing dose of 25 g/L was completely hydrolyzed to sugars in 24 hours, followed by 50 g/l in less than 48 hours and 75 g/L in 48 hours. The spent casing conversion above 75 g/L showed a lower hydrolysis response after 24 hours possibly due to a reduction in the cellulase enzyme loading as the mass of casing increased.

A comparison of the hydrolytic potential between the produced enzyme and the commercial enzyme is presented in Figure 4.29. In 24 hours, the spent casing was completely hydrolyzed with both enzymes, whereas 55 % was hydrolyzed with the filter paper. It is evident that the spent casing waste is composed of pure cellulose that is easily and rapidly hydrolyzed to sugars. The filter paper is also composed of pure cellulosic material but a fraction of the cellulose is in a crystalline form, which tends to be more resistant to cellulase. The response of the produced enzyme with the spent casing waste was slightly higher than the commercial enzyme. Other researchers have found similar hydrolytic improvement when cellulase enzymes produced from lignocellulosic materials were used against the same substrate (Esterbauer et al., 1991; Ortega et al., 2001). It is possible that when the fungi have been grown with a cellulosic material, they excrete the right proportions of hydrolytic enzymes that are necessary to break down the cellulosic material. Ortega et al. (2001) found that the best cellulosic conversion occurred when the substrate is degraded at the same concentration used during the enzyme induction. The

casing loading for production was 50 g/L and for hydrolysis was 25 g/L, and this may explain why the response was slightly better than the commercial enzyme.

Figure 4.27. Celluase produced with *T.reesei* with 50 g/L of spent casing waste during batch fermentation at 28 °C for 7 days.

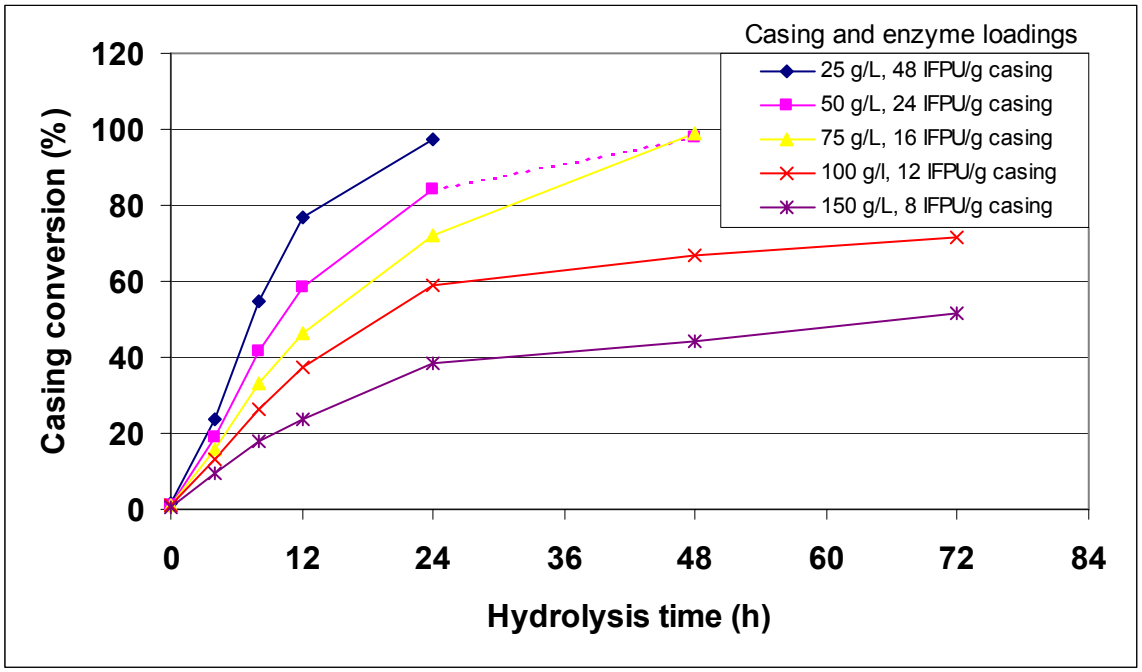


Figure 4.28. Effect of casing and enzyme loadings on the casing conversion using produced enzyme with activity of 1.2 IFPU/ml.

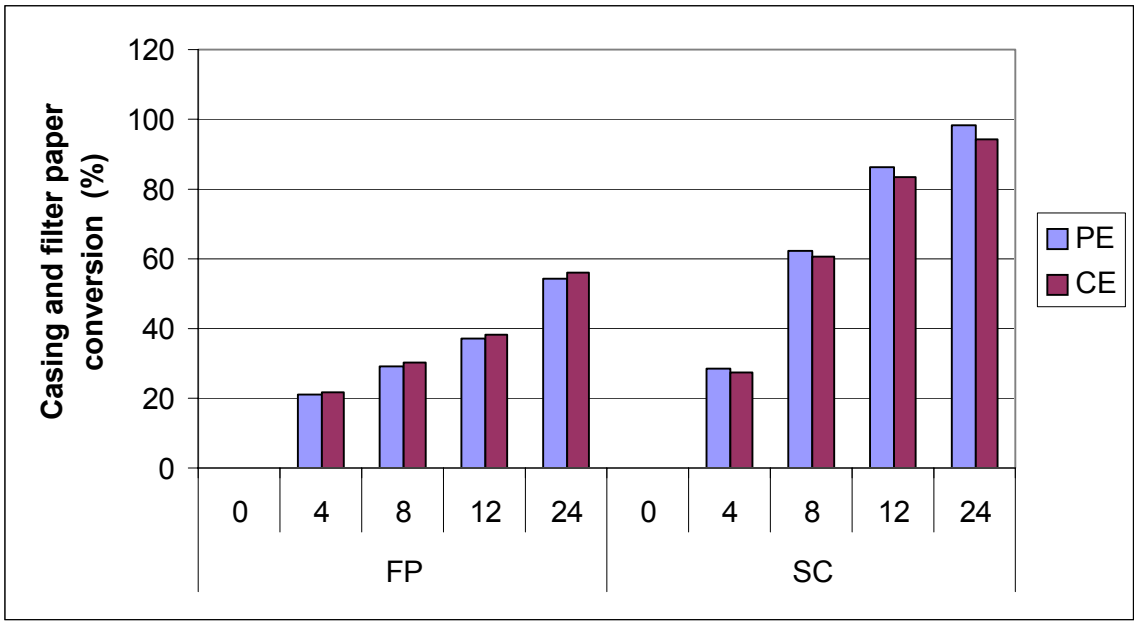


Figure 4.29. Hydrolytic potential of the produced enzyme (PE) and ViscoStart commercial enzyme (CE) on 25 g/L of spent casing (SC) and filter paper (FP) for 24 hours at 50 °C.

5 ECONOMIC ANALYSIS

This study demonstrated that spent casing waste can be used to produce value added products such as glucose from enzymatic hydrolysis and cellulase enzymes from submerged fermentation. It is envisioned that the meat industry would prefer to generate an asset from the casing waste rather than treat it as a waste handling operational cost, as is currently done.

Both glucose and cellulase products have potential marketing possibilities in many industries. The overall goal of the feasibility study was to determine the technical and economic viability to develop a bioconversion process that generates glucose and cellulase at the existing Bar-S facility. Once the process was shown to be technically feasible, a rough economic evaluation was conducted. The economic evaluation considered the production cost, capital cost, market price for glucose and cellulase enzyme, and cost savings from actual waste handling practice.

5.1 Current Market Value of the Product

The annual U.S. raw and refined sugar prices average 22 and 25 cents per pound, respectively (USDA, 2004). The oversupply of sugar production around the world contributes to the low profitability that the sugar industry is facing. Currently the low glucose prices are forcing this industry to explore possible opportunities for adding value to the glucose product, by means of bioprocessing of chemicals and enzymes (Godsahll, 2003).

The produced glucose can be used in value-added products, such as a supplement for local animal feedlots or as a starting point for many fermentation products such as alcohols and organic acids. Another possibility is the on-site digestion of the sugars into biogas, which could then be used for production of heat and electricity.

The development of cellulase enzymes with similar hydrolytic capacity as commercial enzymes is a novel approach to adding value to the casing waste. Cellulase enzyme prices are about 10 times that of sugar, with prices for concentrated cellulase enzymes ranging from $2.30 to $6.80 per pound. There is a potential market for these enzymes in existing industries such as detergent and textile manufacturers, with annual world wide estimated markets of $150 million and $462 million, respectively (Hettenhaus, 2004). Another potential market, which demands large quantities of cellulase enzymes, is the lignocellulose-to-bioethanol industry with a cellulase requirement of 4.9×10^4 IFPU/gallon of ethanol and annual estimated market of $400 million. The bioethanol industry is expected to grow worldwide as a consequence of the reduction in the dependence on fossil fuel, the desired reduction of CO_2 emissions, which contributes to global warming. At present, the bioethanol process is not considered economical due to the high production cost, with a major cost contributor being the cellulase enzyme. Several economic analyses of bioethanol processes have been conducted, with results showing that enzymatic cellulose hydrolysis is the major cost driver in bioethanol production, with cellulase enzyme production cost ranging from 40 to 60 % of the total bioethanol production cost (Howard et al., 2003; Ruth, 2003). Other studies have shown that cellulase cost needs to be reduced by at least a factor of 10 to make the process economically feasible (US-DOE-NREL, 2004).

5.2 Conceptual Process Design Layout

To assist in the selection of the most profitable process with the utilization of spent casing waste, two conceptual process designs were developed using the operational parameters obtained in this research, and an economic analysis was performed on each process. The preliminary design assumed that the plant operates five days per week and that daily production of casing waste was used to generate either sugars (Process 1) or cellulase enzymes (Process 2) as output products. There are four unit operations in Process 1, identified as: casing size reduction, cellulase production, enzymatic hydrolysis, and downstream product purifier (Figure 5.1). In Process 2 the hydrolysis process is omitted resulting in three unit operations: casing size reduction, cellulase production, and downstream cellulase purifier (Figure 5.2). Table 5.1 shows the design values used to develop a conceptual layout of both processes.

In the Process 1 (Figure 5.1), the spent casing is fed into the process where it is shredded, ground, and mixed with water in the slurry tank. Size reduction pretreatment improves conversion efficiency and facilitates the process of transporting the casing to the treatment reactors. Water is added at the hopper to wash out the casing from the shredder and grinder. While rapid mixing occurs in the slurry tank, half the volume is pumped to the hydrolysis tank, which already contains the culture with produced cellulase. The pH of the mixture is adjusted with 4 N H_2SO_4 and 4 N NaOH. The hydrolysis reactor is heated to 50 °C and remains at this temperature for a hydrolysis time of 24 hours with continuous agitation at 100 rpm. The cellulase enzymes present convert 84% of the spent casing to glucose.

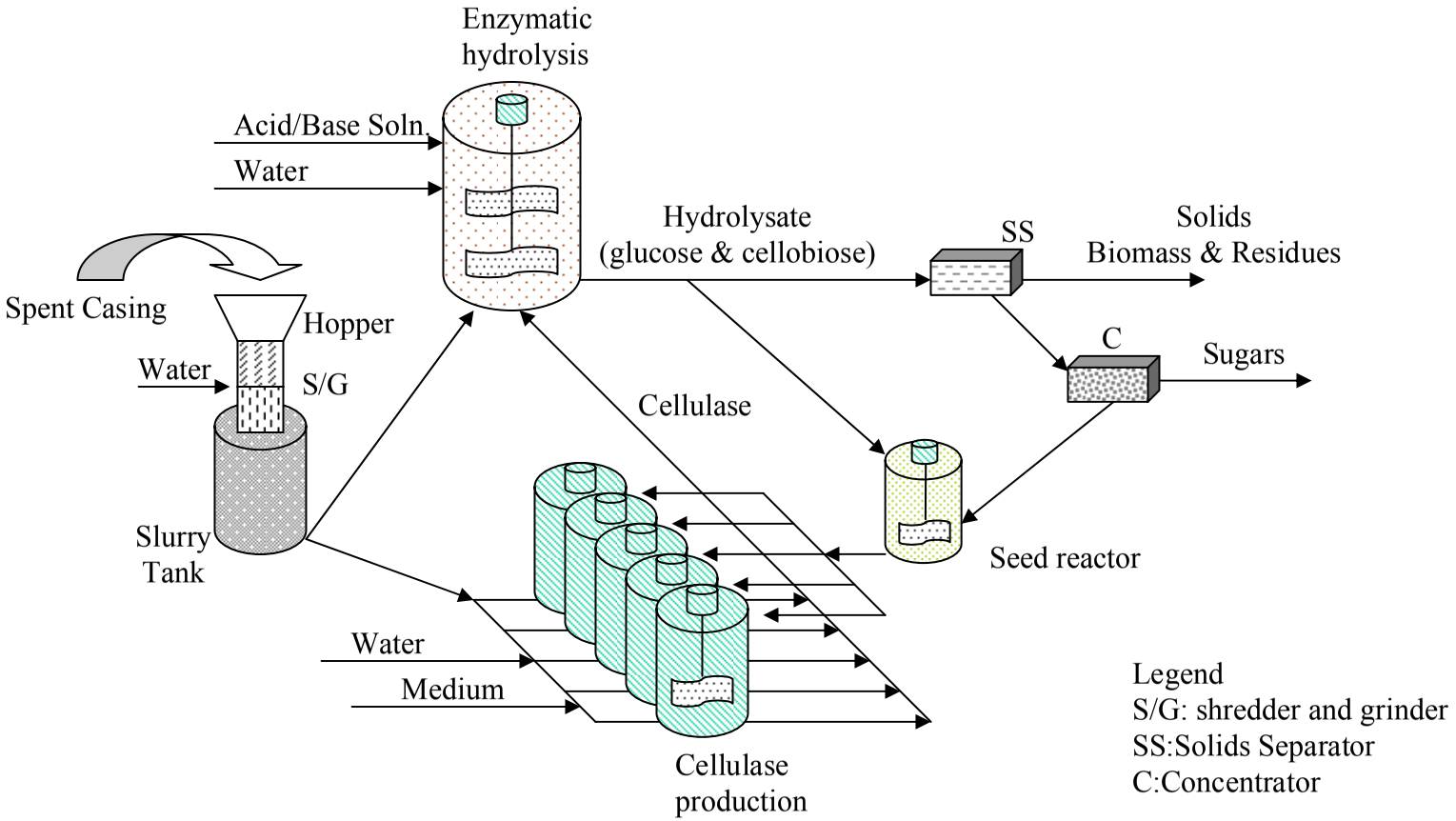


Figure 5.1 . Process 1: Hydrolysis and cellulase production

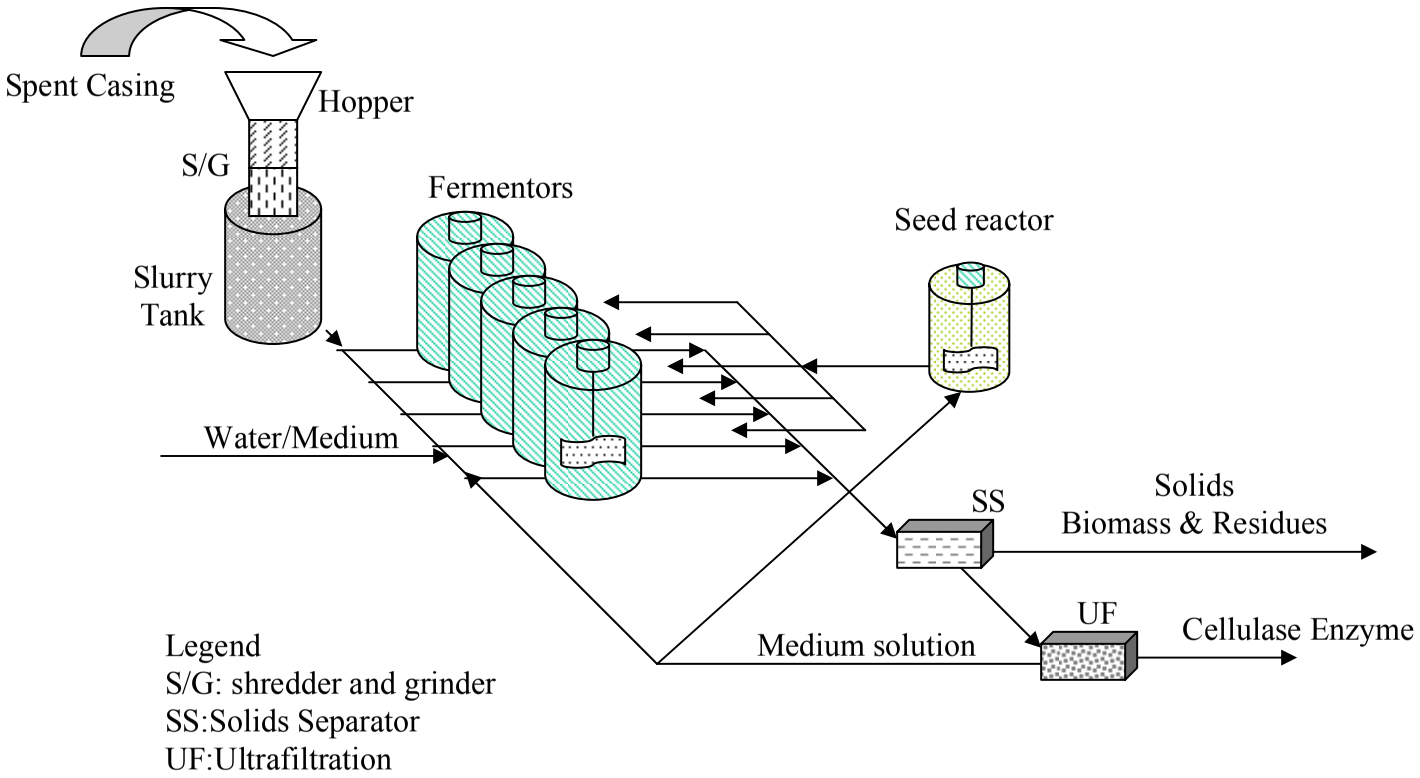


Figure 5.2 Process 1: Hydrolysis and cellulase production

The remaining volume in the slurry tank is pumped to the cellulase production reactor. The inoculum (5%, v/v), along with other nutrients, is added to the reactor and mixed with the slurry. The reactor temperature is held at 28°C for 6 days with an average airflow rate of 0.4- vvm and variable agitation from 10 to 50 rpm depending on the viscosity of the culture. The pH is adjusted to 4.8 using concentrated NH_4OH and H_3PO_4.

Table 5.1. Process parameter values for spent casing hydrolysis and cellulase production.

Parameter	**Design Value**
Spent casing as produced ton/d, (kg/d)	4 (3629)
Casing Hydrolysis	
Casing loading, g/L	25
Enzyme loading, g/L	25
Hydrolytic efficiency, %	100
Glucose yield, g/g	0.84
Glucose production, kg	440,771
Cellulase Production	
Casing loading, g/L	25
Cellulase yield, IFPU/g SC	31
Produced activity, IFPU/ml	0.78
Batch length, d	6
Cellulase Production, 10^6 IFPU	3064

The hydrolysate leaving the hydrolysis reactor contains soluble sugars and approximately10% of insoluble matter (casing residues and biomass). A small portion of the hydrolysate is used in the seed reactor to grow and adapt the fungi. The solution is then filtered with a belt press to remove the insoluble matter. The resulting solution is passed through a concentrator and the concentrated sugars are sold as a product. The annual glucose production from this process is estimated to be 440,771 kg.

When reviewing the conceptual design of Process 2, it can be seen that with the exception of the hydrolysis operation in Process 1, the overall process design is very similar. In Process 2, a fermentor is fed with the daily production of spent casing where it is held for 6 days for cellulase production. The downstream processing is different. Here, the resulting enzymatic solution from the belt press is concentrated by ultrafiltration membranes with a minimum cut off of 10,000 Daltons. The permeate from the ultrafiltration is recycled to lower the cost associated with water and culture medium, which are only added to the reactor as needed. The annual cellulase activity from this process is estimated to be 3064×10^6 IFPU.

5.3 Capital Cost Estimate

Several assumptions were used to analyze the projected capital cost for the two processes. These assumptions were: 1) The company already owned the land and existing facility space, 2) process water from the plant is recycled in the bioprocessing facility, 3) sufficient steam capacity to support this process is available from the main plant, 4) existing conveyors to transport the casing out of the plant were retrofitted to fit in the hopper.

Some of the major pieces of equipment required in each unit operation of these two processes are the same. The estimated equipment price was obtained from different suppliers in the U.S. Tanks and reactors were custom designed using stainless steel. Only the equipment contributing significant capital cost was included in the analysis. These are listed in Table 5.2, which includes the capacity of the equipment, number of units required, and pricing data for total quantity of equipment.

5.3.1 Operational Cost Estimate

An energy balance was performed to estimate the annual operating cost of the equipment. The following assumptions were used in the analysis: 1) electricity costs were $0.05/kW-h, 2) processed water was recycled from the production plant, 3) nutrients are recycled and added only when needed, 4) the cost for spent casing as feedstock material to the process is zero, 5) variable speed gear motors were used for culture mixing and ½ of the calculated HP at full load was used for estimating the operating cost.

Currently the company pays approximately $115,000 per year for casing waste handling and disposal in landfills. With the installation of the new process, this operational cost is transformed into savings and therefore they were considered as a negative operational cost in the economic analysis. Table 5.3 presents the estimated equipment operating costs.

Table 5.2. Major equipment costs for glucose (Process 1) and cellulase production (Process 2).

Equipment	Qty	Process 1	Process 2
Pre-treatment			
Shredder/Grinder (75 HP)	1	150,000	150,000
Slurry Tank (10,000 gal)	1	15,000	15,000
Mixer (100 HP)	1	6,000	6,000
Centrifugal pump (3 HP)	1	1,500	1,500
Casing Hydrolysis			
Stainless Steel Tank (25,000 gal)	1	30,000	
Mixer (75 HP)	1	5,000	
Centrifugal pump (3 HP)	1	1,500	
Cellulase Production			
Reactors w/ diffusers,			
Process 1 (10,000 gal)	5	75,000	125,000
Process 2 (25,000 gal)	5		
Mixers			
Process 1 (50 HP)	5	17,500	25,000
Process 2 (100 HP)	5		
Air Compressor (400 HP)	1	200,000	200,000
Air filter	2	4,000	4,000
Centrifugal pump (3 HP)	5	7,500	7,500
Seed Reactor			
Reactor w/diffuser (5,000 gal)	1	10,000	10,000
Mixers (75 HP)	1	5,000	5,000
Centrifugal pump (3 HP)	1	1,500	1,500
Downstream Processing			
Belt Press Filter (75 HP)	1	80,000	80,000
UF Membrane (100 HP)	1	100,000	100,000
Centrifugal pump (5 HP)	1	1,500	1,500
Cost of unlisted equipment (20% of total)		153,700	146,400
Total Equipment Purchase Cost		922,200	878,400

Table 5.3. Annual operational costs for major equipment in Process 1 and Process 2 ($/yr).

Equipment	Qty	Operating time Hrs/day	Process 1	Process 2
Pre-treatment				
Shredder/Grinder (75 HP)	1	4	3,233	3,233
Mixer (100 HP)	1	1.5	1,212	1,212
Centrifugal pump (3 HP)	1	1	48	48
Casing Hydrolysis				
Heating		24	11,091	
Mixer (50 HP)	1	24	12,931	12,931
Centrifugal pump (3 HP)	1	1	48	48
Cellulase Production				
Heating		24	10,993	10,993
Mixers Process 1 (50 HP) Process 2 (125 HP)	5	24	 90,515	 226,287
Air Compressor Process 1 (200 HP) Process 2 (400 HP)	1	24	 72,412	 144,823
Centrifugal pump (3 HP)	5	1	48	48
Seed Reactor				
Mixers (75 HP)	1	24	9,051	9,051
Centrifugal pump (3 HP)	1	1	48	48
Downstream Processing				
Belt Press Filter (75 HP)	1	6	4,849	4,849
UF Membrane (100 HP)	1	6	6,465	6,465
Centrifugal pump (3 HP)	1	1	48	48
Equipment Operating Cost			222,992	420,084
Savings (waste handling)			-115,000	-115,000
Total Equipment Operating Cost			**107,992**	**305,084**

The feasibility of each process was evaluated using a Feasibility Assessment Template developed for the Ag Marketing Resource Center by agricultural economists Phil Kenkel and Rodney Holcomb, Oklahoma State University. The feasibility of the process was measured by three profitability indicators: payback period, net present value, and internal rate of return. Market values for glucose and cellulase of $0.25/lb and

$2.27/lb were used for the feasibility calculation. Other financial values considered in the feasibility template as shown in Table 5.4. Table 5.5 shows economic feasibility results for the two processes at current conditions. It can be seen that for the initial production values and assumptions used in the study, neither process was economically feasible. The current oversupply of glucose causes the glucose market to be low. For Process 1, even if the process is optimized so that all the spent casing is converted into glucose, the internal rate of return will remain zero.

Table 5.4. Other inputs related to financial structure for economic feasibility template.

Financial Expenses	
Percent Financed	50.00%
Long Term Interest Rate	7.50%
Loan Term	10
Working Capital	$0
Short Term Interest Rate	6.00%
Property Tax as % of Prop and Plant	0.50%
Income Tax Rate	30.00%
Payroll Information	
Salary	$ 50,000
Benefits	$15,000
Payroll Tax to Salaries	5.00%
Retirement Tax to Salaries	15.00%
Employee INS Tax to Salaries	10.00%
Wage Inflation	1.00%
Other	
Expense Inflation Rate	1.00%
Maintenance as % of Plant & Equip	3.00%
Insurance as % of Plant & Equip	2.00%
Discount rate for NPV calculation	8.00%
Equipment depreciation (MACRS with half year convention),	7 yrs

Table 5.5. Economic feasibility results from the production of glucose and cellulase.

Products	Cellulase Activity, IFPU/ml	Price $/lb	Production	Payback period, years	Net present value, $	Internal rate of return, %
Process 1 Glucose	0.78	0.25	440,771 kg	>10	(866,522)	0
Process 2 Cellulase	0.78	2.30	3064×10^6IFPU	>10	(2,302,198)	0

The economic feasibility of the cellulase production with casing waste (Process 2) could produce significant profitability if higher cellulase market values and existing cellulase optimization techniques are incorporated in the analysis. A sensitivity analysis was conducted to determine the effect of changing certain costs on process fesibility. This analysis assessed the influence of cellulase price, initial product volume, and process optimization by changing the value of one variable and maintaining constant the other values during each analysis. The following cost variable ranges were evaluated from the known baseline: price of the cellulase from $5 to $10 per kg, cellulase activity from 0.78 to 2.2 IFPU/mL, and a 20% reduction in operational cost. A cellulase production of 2.2 IFPU/ml was calculated from previously reported cellulase production yields of 350 IFPU/ g carbon by *T. reesei* CL-847 (Esterbauer, 1991). The cellulase activity can be even higher if optimum operating conditions are used in combination with a hyperproducing fungi strain. The operational cost was decreased by 20% assuming that optimized fermentation operations such as intermittent mixing and aeration showed no effect on the cellulase productivity. The results from the sensitivity analysis are shown in Table 5.6

Table 5.6. Sensitivity analysis of the market value, cellulase activity, and operational cost on the profitability of cellulase production (Process 2) with spent casing waste.

Parameter adjusted					
Cellulase activity, IFPU/ml	Price, $/kg	% reduction in operational Cost , %	Payback period, years	Net present value, $	Internal rate of return, %
0.78	5	0		(2,302,198)	
0.78	7	0		(1,647,912)	
0.78	10	0		(677,763)	
1.50	5	0		(823,165)	
1.50	7	0	7	435,234	18
1.50	10	0	2	2,301,135	54
2.20	5	0	6	614,262	22
2.20	7	0	2	2,459,778	56
2.20	10	0	1	5,196,233	102
0.78	5	20		(1,870,610)	
0.78	7	20		(1,216,324)	
0.78	10	20		(246,175)	
1.50	5	20		(391,577)	
1.50	7	20	5	866,822	27
1.50	10	20	2	2,732,723	61
2.20	5	20	4	1,045,849	31
2.20	7	20	2	2,891,365	64
2.20	10	20	1	5,627,820	110

The sensitivity analysis shows that cellulase activity and sales price have a significant impact on the economic feasibility. An increase by a factor of two in the cellulase production (1.50 IFPU/ml) and cellulase price ($10/kg) resulted in an internal rate of return of 54 % with a payback period of 2 years. A further increase is possible if the operational costs are reduced by 20%, resulting in an internal rate of return of 61%.

The produced cellulase enzyme can compete with current commercial enzymes, since it was determined from the cellulase production study that these enzymes have similar hydrolytic capacity and β-glucosidase-to-IFPU ratio as commercial enzymes. Therefore, with some process optimization it should be possible to develop a profitable process for production of cellulase enzyme from casing waste.

6 CONCLUSIONS AND RECOMMENDATIONS

6.1 Conclusions

It has been determined in this project that the spent casing waste can easily be broken down and converted to valuable organic products by treating the waste with enzymes in a submerged aerobic fermentation. Conclusions related to each main goal are as follows:

1) **Determine hydrolysis efficiency for enzymatic hydrolysis of spent casing waste.**

Enzymatic hydrolysis of spent casing waste was successfully performed with commercial cellulase enzymes with more than 80% casing conversion in 24 hours when using a cellulase loading of 20 IFPU/mL and casing loadings of 25 and 50 g/L at a temperature of 50 °C and 100 rpm. Among the different treatments tested, the variable which most affected enzymatic hydrolysis was temperature, followed by pH, enzyme loading, substrate loading, and mixing.

2) **Explore the use of spent casing waste as a substrate for the production of the cellulase enzyme complex in a laboratory scale fermentation reactor.**

The spent casing waste contains the essential cellulosic component used by the fungi as insoluble inducing substrate for cellulase production in batch submerged

culture in shake flasks and bench scale bubble column reactor, producing a cellulase activity of 0.83 and 0.78 IFPU/ml with a casing loading of 25 g/L. A comparison of the produced enzyme with commercial enzymes shows that the produced cellulase enzyme has identical or superior enzymatic capacity in hydrolyzing the casing waste.

3) Determine the economic feasibility of producing value-added products from casing waste.

The productivity of glucose and cellulase enzymes obtained under the experimental conditions tested indicates that neither bioconversion process is economically feasible. However, the economic analysis demonstrated that if the process of cellulase production is optimized, it could develop significant profit for the meat industry with potential internal rates of return greater than 50%.

The following specific conclusions were drawn from this study:

1. Spent casing waste can be easily hydrolyzed to glucose and cellobiose by cellulase enzymes in less than 24 hours.
2. All six commercial enzymes were able to hydrolyze the casing. Multifect GC and Fibrilase were the two most efficient commercial enzymes with more than 80% degradation in 8 hrs.
3. Among the different treatments tested, the variable which most affected enzymatic hydrolysis was temperature, followed by pH, enzyme loading, substrate loading, and mixing.

4. Concentration of glucose above 10 % (w/w) reduces the spent casing conversion, with more impact at higher casing concentration.
5. The addition of surfactant during hydrolysis significantly increased the bioconversion rate, with Tween 20 showing a 20% improvement.
6. *Trichoderma reesei* RUT-C30 metabolizes the spent casing waste for growth and cellulase enzyme production in submerged fermentation.
7. A casing concentration of 25 g/L can produce a cellulase activity of 0.78 IFPU/ml and cellulase yield of 31 IFPU/g casing in 6 days at 28 °C.
8. Casing loadings above 50 g/L are difficult to operate due to high culture viscosity and dissolved oxygen limitation.
9. The average ratio of β-glucosidase to IFPU/ml of the produced enzyme was 0.31 and it was close to the ratio of commercial cellulase enzyme (0.32 to 0.42).
10. The hydrolytic capacity of the produced enzyme was similar to the commercial enzyme.

6.2 Recommendations for Future Research

In order to improve the efficiency and overall economic viability of the proposed process, the following areas of additional process optimization are recommended:

1. Screen different *T. reesei* strains using spent casing waste as substrate to increase cellulase yields.

2. Optimize cellulase production by changing operational process conditions such as intermittent mixing and aeration, temperature profile, size reduction, and growth media.
3. If casing size can be reduced, compare bubble column reactor with conventional stirred reactor.
4. Use anaerobic solid-state fermentation with *Clostridium thermocellum* to overcome the viscosity problem at high casing loadings.
5. Conduct trials to determine rate and efficiency of cellulase concentration using ultrafiltration system.

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