Feasibility Study of an Owner-operator System for Producing Trichoderma reesei QM 9414 Cellulase

Carla M. Cass

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FEASIBILITY STUDY OF AN OWNER-OPERATOR SYSTEM FOR PRODUCING

*Trichoderma reesei* QM 9414 CELLULASE

BY

CARLA M. CASS

A thesis submitted
in partial fulfillment of the requirement for the
degree Master of Science
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FEASIBILITY STUDY OF AN OWNER-OPERATOR SYSTEM FOR PRODUCING

Trichoderma reesei QM 9414 CELLULASE

This thesis is approved as a creditable and independent investigation by a candidate for the degree, Master of Science, and is acceptable for meeting the thesis requirements for this degree. Acceptance of this thesis does not imply that the conclusions reached by the candidate are necessarily the conclusions of the major department.

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INTRODUCTION

Cellulosic wastes are the most abundant organic material on earth. Utilization of cellulose occurs through microorganisms by a process of hydrolysis to low molecular weight hexoses and pentoses. These are regarded as substrates for microbial production of food (single cell protein), ethanol (fuel alcohol) butanol and other fermentation chemicals.

Agricultural producers incur the largest volumes of these cellulosic wastes, therefore it is of economical importance that they be utilized. However, the owner-operator system for the utilization of in-situ produced cellulose for conversion to glucose has yet to be totally resolved.

Since the facility at SDSU is similar to that of an individual owner-operator, this study was organized to determine how effective scale-up from a laboratory scale production level to a rudimentary pilot plant would be. The second objective was to determine if a simpler enzyme assay could be developed to replace the commonly used assays which are time consuming and labor intensive. The method chosen was Pharmacia Fine Chemicals chromatofocusing which separates proteins by their respective isoelectric points. Thus, the enzyme complex can be analyzed for completeness.
LITERATURE REVIEW

AVAILABILITY OF CELLULOSE

Atmospheric carbon dioxide is transformed through photosynthesis into biomass in varying forms of vegetation (12, 13, 75, 80, 81). This makes cellulose the most abundant organic compound on the earth. Some of these forms of vegetation are produced for economical reasons; however, there is a large amount of cellulosic waste that is not currently utilized (12, 75, 80, 81). These occur in various forms including newsprint (8, 11, 38), pulp and paper (12, 13), agricultural residues (13, 54, 81) as well as urban and industrial refuse. Tsao, of the Laboratory of Renewable Resources Engineering at Purdue University, has estimated that agricultural residues alone currently constitute \(820 \times 10^6\) tons/year of material on a dry weight basis. Other economically important wastes include those derived from the urban sector \((130 \times 10^6\) tons/year\) and those of industrial origin \((60 \times 10^6\) tons/year\) basis (81). Availability of these wastes depends on their collection. Generally they are solid in nature and are scattered over large land surfaces (8, 13, 80, 81). Although they are widely distributed, their availability and low cost makes them an attractive alternative to other natural resources which are not annually renewable (75, 80, 81).

Cellulose cannot be used until it has been converted into glucose. After hydrolysis is complete, the glucose can be utilized in many industrial processes including production of single cell
protein (SCP) for animal feed; fermentation to ethanol, butanol, and citric acid as well as various other fermentation products (12, 13, 25, 40, 78).

Structure and Chemistry of Cellulose

Cellulosic wastes are composed of three components: cellulose, lignin and hemicellulose (12, 15, 60, 80, 81). Cellulose is a polymer of glucose. Lignin is a polyphenolic macromolecule. Hemicelluloses are polymers of sugar acids, mannose (hexose) and the pentose sugars xylose and arabinose (12, 60, 80, 81). Generally these components occur approximately in a 4:3:3 ratio. Soft wood, for example, contains 42, 28 and 25%, respectively, of cellulose, lignin and hemicellulose; however corn cobs which contains 40, 16 and 36% departs from this ratio (80, 81).

The cellulose molecule is a linear homopolymer of anhydroglucose units linked together by beta-1,4-glucosidic bonds (12, 60, 80, 81). The length of these molecules varies greatly from lambda-cellulose, which has fewer than 15 glucose monomers, to alpha-cellulose, which contains 10,000-14,000 glucose units per molecule (12, 35, 68, 80). Alpha-cellulose is quantitatively the most like native cellulose (12, 35, 68). Anhydroglucose monomers, within the cellulose polymer, exist in a chair conformation with the hydroxyl groups poised in an equatorial position and the hydrogen atoms in an axial position (65, 68). The chain rotates 180° from the longitudinal axis every other monomer (Figure 1) producing a linear
configuration which is unstrained and not hindered sterically (65, 68, 81). There also exist intrachain bonds between the hydroxyl group of carbon 3 and the ring oxygen of carbon 5 of consecutive glucose units (Figure 1) (65, 68). These polymers are arranged in an antiparallel manner so that the molecule is bound together, not only by strong covalent bonds and intrachain hydrogen bonding along the axis, but also by hydrogen bonding between glucose residues on adjacent chains (12, 60, 68, 81).

Cellulose polymers are aggregated into long slender bundles called fibrils (12, 81). Regions containing the highly oriented molecules are said to be crystalline; whereas, those possessing a lower degree of order are referred to as paracrystalline or amorphous (12, 60, 81). The amorphous region is more readily degraded when subjected to hydrolysis. After the amorphous cellulose has been converted, the highly ordered crystalline cellulose remains (8, 12, 13, 15, 40, 60, 68, 81). On the average, the distribution of cellulose species is 15% amorphous and 85% crystalline (68, 81).

The exact structure of cellulose has yet to be defined (12, 68, 80, 81). Several models exist; principally, based on two schools of thought. The first of these schools of thought provides models based on extended cellulose molecules (12, 80, 81). One such model of microfibril structure is that of Preston and Cronshaw (Figure 2) who have proposed that the microfibril consists of a rectangular crystalline core surrounded by a paracrystalline cortex (12).
Figure 1. Anhydroglucose units in the chain conformation (beta-1,4 glycosidic bond) within cellulosic polymers. — designates intrachain hydrogen bonding. (Redrawn from Whalen [84])

Figure 2. Models of microfibril structure. (A) Preston and Cronshaw; (B) Hess, Mahl and Gutter; (C) Manley. (Redrawn from Cowling and Brown [12])
According to Hess, Mahl and Gutter (Figure 2) the microfibril contains several elementary fibrils each of which contain 15-40 cellulose molecules that are segmented into crystalline and paracrystalline regions (12). The second school of thought includes models suggesting fibrils which are folded (12, 80, 81). Manely (Figure 2) proposes that the cellulose molecule is first folded into a flat ribbon which, in turn, is wound in a tight helix (12). The final model discussed here is that of Tsao. This theory suggests that as the 1,4-beta-glucosidic bonds are repeated, there occurs another beta bond which has a deflection of approximately 60° from the normal. After three successive deflected bonds, a loop is formed within the cellulose polymer which produces a 180° turn or fold. Based on thermodynamic considerations, it is proposed that the exposed, deflected, linkages are more susceptible to hydrolytic cleavage. Thus, this would make the amorphous region rich in loop bends with deflected beta bonds. After this region is hydrolyzed, the remaining polymer would be crystalline and more difficult to hydrolyze (80, 81).

The fibrils themselves are often surrounded by lignin (12, 15, 60, 80, 81). Lignin is a complex polymer of p-hydroxycinnamyl alcohols (12). It is virtually insoluble and remains after wood is hydrolyzed by oxidative degradation or acid hydrolysis (12). Presence of lignin must be considered when attempting to convert cellulose to glucose.
The other major component in cellulosic wastes is hemicellulose. It also occurs in cellulose at various levels (12, 15, 60, 68, 80, 81) and is composed of polymers of D-xylose linked in a beta-1,4 configuration. It also contains glycosidic side chains of mannose, galactose and arabinose (12, 68, 80, 81).

Consequently, the difficulty in obtaining rapid and complete hydrolysis of cellulose is due not only to innate chemical characteristics of cellulose, but also to the tertiary structures of the cellulosic materials (33, 80).

CELLULOSE CONVERSION

CHEMICAL/PHYSICAL TREATMENT: There are structural features of cellulosic residues which affect their hydrolysis to glucose (13, 15, 32, 40, 60). Various chemical and physical processes have been tested where cellulose-containing materials are partially or totally degraded. Generally these processes remove or weaken hemicellulose and lignin thereby exposing the cellulose and rendering it susceptible to attack (13, 30, 32, 40, 53, 54, 55). Further treatments swell crystalline cellulose, disintegrating its highly ordered arrangement so it is more accessible to hydrolysis (33, 68). Common treatments utilize mechanical disruption such as wet milling, simultaneous attrition, ballmilling and others (9, 30, 40, 53, 54) along with exposure to acids or oxidizing agents (13, 32, 33, 55). Of the chemical treatments, acid hydrolysis and alkalai degradation are the most prominent for pretreatment or direct conversion (12, 13, 28, 37,
Alkalai treatment (sodium hydroxide) has the greatest potential in a pretreatment protocol where it removes hemicellulose, exposing cellulose moieties (12, 13, 37, 75). Total decomposition by alkalaine methods produces many by-products other than glucose (24). Often these undesired products are detrimental to the overall process (24).

Acid hydrolysis has similar problems, but on a smaller scale (24, 32). This process occurs as the glucose molecule is protonated, splitting the glycosidic bond. During acid hydrolysis, amorphous cellulose is rapidly hydrolyzed initially, with the crystalline region being slowly hydrolyzed (24).

Methods of acid hydrolysis make use of either concentrated or dilute acids. Use of supersaturated solutions of hydrochloric acid results in numerous hemicellulose degradation products, considerable loss of sugar by degradation and high costs (25, 32, 33, 55). Dilute acid processes are not restricted by stringent economic considerations, but do have poorer conversion, hence lower product yields (32, 55).

Other chemical methods make use of solvents such as metalamine or metal-alkali complexes. Cadoxen, for example, is a colorless, odorless sovluent prepared by dissolving 5 percent cadmium oxide in 28 percent aqueous ethylenediamine (33). It has been shown to give high conversions of crystalline cellulose (33). However, it
appears not to be economically feasible for commercial use at this time (16, 33).

Disadvantages of chemical processes for conversion of cellulose to glucose include the necessity of using expensive, corrosion-proof equipment; high temperatures and hence added energy costs; difficult recovery and neutralization methods; and extended periods for conversion to glucose. They also produce degradation products other than glucose (25, 32, 33, 55). Consequently, enzymatic methods which economically produce glucose and virtually no additional degradation products under moderate operating parameters have gained widespread acceptance as desirable for industrial development (13, 25, 29, 33, 40, 60, 79, 85).

ENZYMATIC TREATMENT: Conversion of cellulosic residues to glucose, a process called saccharification, is facilitated by a multicomponent enzyme system known as cellulase (3, 4, 5, 6, 31, 45). This enzyme system results in the hydrolysis of cellulose to cellodextrins and eventually to glucose. Cellodextrins are watersoluble glucose oligomers bearing a low degree of polymerization. Therefore, enzymatic hydrolyses are advantageous in that there is little or no formation of undesirable by-products (33, 45).

The cellulase enzyme complex occurs naturally in insects, mollusks, protozoa, bacteria and fungi. Most of these sources are not applicable for commercial processing because cultural conditions are difficult for large scale production. Another problem associated
with many of these sources is the organism's inability to produce the complete enzyme complex. A major development requirement necessitates that the complete cellulase complex be produced; thus the organism's cellulase must be able to hydrolyze crystalline and amorphous forms of cellulose (45).

Various microorganisms have been tested by the U.S. Army's Natick Laboratory (Natick, MA) for production of the entire cellulase complex. Previous investigators determined that the fungus, \textit{Trichoderma viride}, produced the most complete, stable cellulase complex. The parent strain, \textit{T. viride} Qm6a, was mutated by irradiation. Strains which hyperproduced cellulase were selected for subsequent studies (2, 45). Among these variant strains are those designated QM 9123, QM 9414 and a mutant of QM 9414 designated as MCG 77. Investigators at Rutgers University also transformed Qm6a to produce the variants designated NG-14 and C-30. All of the hyperproducing stains of \textit{T. viride} were renamed \textit{Trichoderma reesei} (2).

\textbf{THE CELLULASE ENSEMBLE}

In 1950, Reese, Siu and Levinson proposed the $C_1-C_x$ concept for the cellulase activity (62). This two step model requires that first crystalline cellulose, or the $C_1$ component, must be attacked and degraded to the $C_x$ or amorphous component. The $C_x$ enzymes would then degrade the amorphous cellulose to its basic subunits (31, 62, 67). Reese, et al., further proposed that the $C_1$ component was an enzyme whose action was unspecified. The $C_x$ component was thought to
be composed of exo- and endoglucanase and a final component, beta-glucosidase. On further investigation it was determined that this $C_1$-$C_x$ concept was not valid (31, 62).

When it was shown that the $C_1$ enzyme is the exoglucanase and not an unspecified enzyme (5), Emert et al., (14) proposed a new hypothesis. This hypothesis states that within the ensemble containing both exo- and endoglucanases, it is the exoglucanases which initiates degradation of the crystalline form (14). This proposal has gained a broad degree of support based on experimental evidence (3, 4, 5, 15, 21, 60, 67, 74). Additional proposals suggest eliminating the $C_1$-$C_x$ nomenclature and replacing it by adopting the convention of using systematic names assigned by the Commission on Enzymes of the International Union of Biochemistry for each component of the cellulase system (14).

Isolation of these components from $T$. reesei cellulase has been accomplished (3, 5, 6, 21, 23, 31, 34). It was determined that the $C_1$ component is a beta-1,4-glucan cellobiohydrolase (EC 3.2.1.91) or exoglucanase (3, 5, 21). This glycoprotein degrades cellulose in an "exo" manner producing cellobiose as an endproduct (3, 5, 21, 31). At least four electrophoretically distinct beta-1,4-glucan cellobiohydrolases have been identified from Trichoderma. They occur in different glycoprotein forms of the same polypeptide and apparently have identical roles in cellulose degradation. These forms are
denoted as A, B, C and D. Form C possesses the highest affinity for crystalline cellulose (21).

The C\textsubscript{x} component was determined to be 1,4-glucan glucanohydrolase (EC 3.2.1.4) or endoglucanase. This is a glycoprotein which attacks cellulose in an "endo" manner producing free ends within the cellulose polymer (6, 23, 31, 34). A total of five different endoglucanases have been identified (5, 23). One of these has a low molecular weight (approximately 20,000) (23). This may be advantageous for access to a greater number of the cellulose fibers (5).

The final component of \textit{T. reesei} cellulase is beta-glucosidase (EC 3.2.1.21) or cellobiase. This component degrades cellobiose (beta dimer of glucose) produced during polymeric substrate degradation to glucose (4, 19, 31, 74). This is the only component of the ensemble which appears not to be a glycoprotein; rather, a single polypeptide (4). To date, only one form of beta-glucosidase has been described for the \textit{T. reesei} cellulase ensemble (4, 19, 31, 74).

The enzyme components work in a synergistic manner where endoglucanase initiates degradation by exposing new ends on the polymer. After this, the exoglucanase hydrolyzes the free ends to cellobiose, as well as aiding the endoglucanase activity (3, 6, 14, 23, 41, 67). The end product of this synergism, cellobiose, is
hydrolyzed by beta-glucosidase to produce two moles of glucose (3, 5, 41, 74).

CELLULASE PRODUCTION AND SACCHARIFICATION

PRETREATMENTS: Susceptibility of cellulosic wastes to enzymatic attack significantly depends on their structural features (13, 15, 32, 33, 54). These include: 1) the degree of swelling by moisture; 2) crystallinity; 3) molecular arrangement; 4) the content of associated material such as lignin and hemicellulose; and 5) the capillary structure of cellulose fibers (15, 81). This last feature is regarded as the most important since susceptibility of cellulose to hydrolysis is determined largely by the accessibility of its surface to cellulolytic enzymes. Consequently, direct physical contact between the enzyme molecules and the substrate (cellulose) is a prerequisite to hydrolysis (15, 29, 40, 81).

Another structural parameter important for hydrolysis is the degree of crystallinity of cellulose (15, 40, 81). It has been demonstrated that enzymatic digestion, and hence, saccharification efficiency of crystalline cellulose, is enhanced when the substrate is ground. This phenomenon occurs to a greater degree than when amorphous cellulose is subjected to similar treatment (8, 15, 60). This is presumed to be due to increased surface area and a decreased particle size rather than reduced crystallinity. Pretreatments to accomplish this are necessary for good enzymatic conversion (8, 15, 60). Alkaline swelling (13, 40, 54), acid pretreatment (32, 40),
solvent processing (33, 81), removal of lignin, irradiation, ball milling (40, 53) and simultaneous physical attrition (30) methods have all been used as pretreatments in an attempt to improve enzymatic degradation of cellulosic residues (8, 13, 15, 29, 30, 32, 33, 40, 53, 54, 60, 81).

CELLULASE PRODUCTION: The identity of the cellulose-derived substance that normally induces cellulase synthesis is not known (36). Inducers for the cellulase ensemble include lactose (2, 22, 42, 43) and sophorose (36, 42), as well as cellulose (2, 22, 36, 42, 43). It has been determined that sophorose is the best inducer (42) followed by cellulose and lactose (2, 63). Only low concentrations of sophorose can be used for active production as high levels inhibit cellulase formation (2, 36). This inhibition is believed to be due to the disaccharide, cellobiose, which is subsequently hydrolyzed to glucose resulting in catabolite repression of substrate. This low level of sophorose causes low enzyme yields which may be increased through pulse feeding in a continuous culture system. It has also been noted that cellulase production halts after the sophorose is completely utilized. This is due to the requirement, by T. reesei, for a constant source of inducer (36).

Since sophorose induces the production of the largest amount of cellulase, it is regarded as the material most like the natural inducer. Thus, cellulose which induces lower levels of cellulase is considered to serve as a substrate from which the natural inducer is
formed. However, even though sophorose is regarded as the best inducer, economic considerations require a less expensive substrate to be used for induction; specifically, cellulose or lactose (36).

Many other compounds have been studied to determine their ability to act as inducers, stimulators or activators, and enhancers of the cellulase production process (2, 28, 63). Some of these include additions of acetate, malate, alpha-ketoglutarate (22) or Tween 80 to the production medium. Tween 80 is believed to bring about some form of membrane response, allowing an enhanced release of enzyme; the actual mechanism, however, is unknown (61). The other compounds are precursors for protein synthesis.

Addition of trace elements to the culture medium of Mandels (37) has been shown to enhance cellulase production by T. reesei. Optimal results occur when cobalt, zinc, manganese and iron are added. These trace elements are not required for growth except on pure cellulose, but are required for enzyme production. The ion ratio of magnesium and calcium also appears to be important for cellulase production (43).

Mandels' production medium contains essential nutrients as follows: potassium, phosphorous, nitrogen, calcium and magnesium (37, 43). The best nitrogen sources are amino nitrogen from organic substrates and ammonium nitrogen (NH₄OH). Gupta, Das and Gupta (22) demonstrated that several nitrogen sources are superior in liquid medium (22, 75).
In addition to proper nutrients for optimal enzyme production, maintenance of pH is important (38, 75). Sternberg (75) showed that uncontrolled pH resulted in low production when the pH dropped to 2.5 inactivating the cellulase enzymes. For best yields it was determined that the pH should be maintained between 3.0 and 4.0 (38, 75).

Studies also show that ammonium hydroxide is an ideal base for controlling pH. These studies depict a pH profile which drops rapidly to 3.5, remains there for several days before an abrupt increase occurs (75, 76). This rise is due to the release of ammonia, mediated by the fungus, and originating from deamination of proteinaceous components. It has been shown that beta-glucosidase formation lags one to two days behind the rest of the components and coincides with the pH rise (76). In addition, studies have determined that much of the total enzyme is released into the medium under stationary phase conditions and on cell lysis (2, 38, 44, 75, 76).

SACCHARIFICATION: Optimal conditions for saccharification require a pH of 4.8 and a temperature of 50°C (39, 64). Saccharification efficiency is determined by measurement of reducing sugar, glucose, present after exposure of substrate to cellulase (39). The dinitrosalicylic acid (DNS) assay as modified by Miller is a colorimetric technique that results in the formation of an intensely colored complex that absorbs strongly at 550 nm when reducing sugars such as glucose are present (46).
Measurement of cellulolytic activity is difficult due to the complexity of cellulase (10). The methods most studies use, and which are considered standard by most investigators, were developed by Mandels and determine the activity of the individual components (37). The efficacy of these methods is subject to experimental error. Consequently, there are questions regarding whether the assays are accurate and precise (39). Thus, modifications of these methods, as well as development of new methods have been extensive and are widespread in the literature (20, 24, 37, 39, 50, 51).

Mandels' procedure is a filter paper assay and was developed at the U.S. Army Natick Research and Development Command (Natick, MA) (37, 39). This method required filter paper cut into 1x6 cm strips as a substrate which is subjected to the cellulase enzyme. The amount of enzyme activity is determined by a mathematical equation based on the degree of saccharification (glucose produced) of the filter paper (37).

Inadequacies of this particular assay include the cutting and curling of these filter paper strips which is tedious and time consuming. In addition, the susceptibility of this filter strip to degradation by the cellulase is great, resulting in a high degree of fiber production from paper processing necessitating the preparation of many dilutions by the investigator (10, 50).
A modification of the Mandels filter paper assay by Montencourt and Eveleigh, utilizes antibiotic disks in place of filter paper strips. This method eliminates much of the tedium associated with the former method. In addition, it dilutes the enzyme to produce reducing sugar values which fall within their standard curve (50). This procedure has been shown to reflect the actual enzyme activity when the dilution is in the range of 1:5 to 1:30. Unfortunately, the assay is labor and time intensive and there still remains a need for more specific and sensitive techniques for measuring enzyme activity as well as development of assays for all unfracti­tionated components (10).

PROCESSING FACTORS: T. reesei crude cellulase derived from hyperproducing strains QM 9414, MCG 77 and Rutgers C 30, were compared by Mandels et al. They determined that saccharification efficiency increased with dilution of the enzyme. It was also noted that cellulase produced when lactose was used as the inducer substrate was less effective in cellulose hydrolysis as more enzyme per gram of substrate was required to attain an equivalent level of conversion to glucose (41).

Other studies have shown that during cellulosic hydrolysis there is a substantial loss of enzyme due to its sorption to the resistant portion of substrate material (11, 27, 58). This phenomenon eventually limits the degree to which hydrolysis occurs (11, 27). Different substrates also affect the amount of adsorption.
For example, newsprint, which is highly processed cellulose, adsorbs more enzyme than solka floc, which is more like native cellulose. It has also been determined that the endoglucanases are preferentially adsorbed initially, while exoglucanases are preferentially adsorbed subsequently (11). This loss in enzyme increases the overall cost of hydrolysis resulting in decreased efficiency since the single largest expense of enzymatic hydrolysis is enzyme production (constituting approximately 50-60% of total costs) (11, 64, 85).

Process methods which have been studied include simultaneous saccharification/fermentation processes (56, 66), continuous culture methods (18, 57, 63), as well as pH cycling and temperature profiling (18, 52, 77). Simultaneous saccharification/fermentation has the advantage of glucose being fermented as it is produced. This mitigates problems associated with the feedback inhibition of cellulase production by glucose (56, 66). Continuous cellulase production seems to offer the most promising process method (18, 57, 63). The system developed by Ryu et al., (63) was most successful when contrasted with the process of Ghose and Sahai (18) which resulted in enzymes of low activities (18, 57). The Ryu et al., process utilized a fungal production vessel that fed directly into a separate enzyme production vessel. A production flow rate of 0.027 hours⁻¹ was maintained in their system (63).

Comparisons have been made between continuous and batch processes (18, 52). The Ryu et al., (63) system produced more enzyme
of higher specific activity and was sustained for a longer duration of operation when compared to batch processes (52).

The batch process of Mukopadhyay and Malik used temperature and pH profiling to attempt to increase enzyme yields. The idea in these studies was to shift the equilibrium states which are thought to be responsible for increased enzyme production (52).

CHROMATOFOCUSING

Chromatofocusing was first described by Sluyterman and associates (69, 71, 72, 79). They proposed that a pH gradient could be produced on an ion exchanger by taking advantage of the buffering action of the charged group resident on that exchanger. A pH gradient is formed internally as the eluting buffer titrates the exchanger. Thus, if a buffer at a lower pH is passed through the ion exchange column which is initially adjusted to a second, but higher pH, a pH gradient forms in the column. When this pH gradient is used to separate proteins bound onto the exchanger, the proteins are eluted in the order of their respective isoelectric points (pI). The potential value of this technique is great especially when studying solutions containing several proteins (69, 70) such as the cellulase ensemble.

The charge on an individual protein depends on its pI and the pH of the mobile phase. If the pH is less than its pI, the protein has a positive charge and is repelled from the anion exchanger. The
protein then migrates down the column in the eluting buffer until the pH of the buffer increases. This occurs as the distance from the top of the column increases and as the pH gradient changes. After the protein has traveled sufficiently down the column so that the pH is greater than its pI, the protein reverses its charge and binds to the ion exchanger. The protein will remain bound until the developing pH gradient causes the pH to drop below the pI of the protein. The protein then is carried by eluent buffer until the pH rises above the pI and the protein rebinds. This process is repeated until the protein emerges from the column at its isoelectric point. Proteins with different pI's will migrate different distances on the column before binding (69, 70).

This process of forming an internal pH gradient enables one to predict a focusing effect. If a protein is applied to the column it will migrate down the column in the eluent as far as its pI. The protein then moves slowly until it elutes off the column. If, during this process, a second sample of protein is applied, it will migrate down the column at the same velocity as the eluent until it meets the slower moving first protein. The two samples then proceed down the column to the bottom where they co-elute (69, 70).

Resolution in chromatofocusing is related to the width of the zone of protein as it elutes, and depends on several factors. To obtain well separated bands, it is best to have a fairly linear pH gradient. This is accomplished by having low buffer concentrations
which generate slight pH changes and good separation between peaks (70, 71, 72). The charge difference between the ion exchanger and the surrounding medium contributes to zone clarity in chromatofocusing. Finally, good technique in column packing improves resolution. Since chromatofocusing produces extremely narrow protein bands on separation, irregularities in column packing have a much more marked effect on resolution than can be detected with lower resolution techniques. If all of these factors are controlled to achieve optimal conditions, extremely narrow bands of 0.02-0.05 pH units may be obtained (71, 72).
EXPERIMENTAL APPROACH, RESULTS AND DISCUSSION

PILOT PLANT STUDIES

Enzymatic conversion to glucose of waste cellulose most frequently uses cellulase obtained from the mold *Trichoderma reesei* (13, 25, 29, 40, 41, 79). This fungus hyperproduces the complete ensemble composed of beta-1,4-glucan cellobiohydrolase (EC 3.2.1.91) beta-1,4-glucanohydrolase (EC 3.2.1.4), and beta-glucosidase (EC 3.2.1.21) (31, 67, 78).

Presently the limiting factor in conversion of cellulosic substrates is the cost of producing enzyme. Studies have shown that 50-60% of the cost of producing glucose is directly related to the efficiency of enzyme production (11, 64, 85). Since the cost of enzyme production is of importance, developing optimal production system(s) must be accomplished.

Production of *Trichoderma reesei* cellulase is accomplished by using a submerged culture technique with vigorous aeration to ensure maximal growth. The enzyme production cycle is indicated by observing a pH profile on cultivation of the organism (38, 43, 44, 64, 75, 76). As *Trichoderma reesei* grows, the pH of the medium will drop after 24-72 hours (38, 44, 64, 72, 75, 76). It must be maintained between 3.0-4.0 for optimal enzyme production and will rapidly rise at the end of cellulase production (38, 43, 44, 64, 75, 76).
To date, the most extensive work has been in the laboratory (bench-scale) by incorporating additives to the production medium (22, 38), pH cycling and temperature profiling as well as continuous culture processes (18, 52, 77). Thus, this study was to determine how effective scale-up to a rudimentary pilot plant would be.

MATERIALS AND METHODS

STOCK CULTURES: *Trichoderma reesei* strain QM 9414 was originally obtained from Dr. Mary Mandels, U.S. Army Natick Research and Development Command (Natick, MA) and is maintained as a part of the South Dakota State University (SDSU), Microbiology Department's stock culture collection.

This strain was cultivated and maintained on solid medium containing Vogel salts (82) supplemented with 1% (w/v) lactose. Plate cultures were stored at 4°C and transferred once every three to four weeks. Stocks of QM 9414 spores were retained as a back-up culture in sterile soil.

MAINTENANCE MEDIUM: The maintenance medium for QM 9414 was the Vogel salts solution supplemented with 1% lactose. A 50X stock solution was prepared by adding: 150 g Na$_3$Citrate·5 H$_2$O (or 117 g Na$_3$Citrate·2 H$_2$O), 250 g KH$_2$PO$_4$, 100 g NH$_4$NO$_3$, 10 g MgSO$_4$·7H$_2$O, 5 g CaCl$_2$·2H$_2$O, and 5 ml of Vogel's trace element solution to 700 ml of distilled water. This solution was diluted to one liter (82) and stored at 4°C.
Vogel's trace element solution was prepared as follows: 5 g citric acid monohydrate, 5 g ZnSO₄·7H₂O, 1.0 g Fe(NH₄)₂·6H₂O, 0.25 g CuSO₄·5H₂O, 0.05 g MnSO₄·4H₂O, 0.05 g H₃BO₃, 0.05 g NaMoO₄·2H₂O to 90 ml of distilled water, and brought to a final volume of 100 ml and stored at ambient room temperature (82). For solid medium, 20 ml of the 50X Vogel's solution was diluted to one liter and supplemented with 1% (w/v) lactose and 1.5% (w/v) agar.

PRODUCTION MEDIUM: Mandel's production medium was prepared; however, urea, proteose peptone and Tween 80 were omitted. A 50X stock solution of this medium was prepared by adding: 70 g (NH₄)₂SO₄, 100 g KH₂PO₄, 30 g MgSO₄·7H₂O, 3 ml of a 10% (w/v) CaCl₂ solution and 1 ml of trace elements solution to 700 ml of distilled water (37). This solution was diluted to one liter with distilled water (37) and stored at 4°C.

Trace elements solution for the production medium was prepared by adding: 5 g FeSO₄·7H₂O, 1.6 g MnSO₄·H₂O, 0.665 g ZnCl₂, 2.0 g CoCl₂ (or 3.75 g CoCl₂·6 H₂O) and 1 ml of concentrated HCl to 400 ml of distilled water. This solution was diluted to 500 ml with distilled water and stored at ambient room temperature. For enzyme production, 20 ml of the 50X stock solution was diluted to one liter (37) and supplemented with 1% (w/v) lactose.

ENZYME PRODUCTION AND SPORE INOCULUM: The spore inoculum for QM 9414 was obtained from a 7-14 day old maintenance medium plate culture. A 2 cm x 2 cm x 1 cm triangular section of inoculum
and agar was aseptically removed from the outer ring of the growth culture (84) as recommended by Stavy, Stavy and Galum (73). This was then washed to remove spores (84) using 1 ml of sterile Butterfield’s buffered phosphate diluent (26). A heavy spore suspension resulted and 0.4 ml of this was used as inoculum (84).

Butterfield’s buffered phosphate diluent stock solution was prepared by dissolving 34.0 g KH₂PO₄ in 500 ml of distilled water and adjusting the pH to 7.2 with approximately 175 ml 1N NaOH. Diluent was prepared by adding 1.25 ml of stock buffer solution to a one liter flask and bringing it to volume with distilled water and was sterilized by autoclaving (26).

PRODUCTION INOCULUM: The QM 9414 spore suspension was transferred into a 250 ml erlenmeyer flask containing 100 ml sterile production medium with 1% lactose. The flask was incubated at room temperature (28-30°C) on an NBS bench-top reciprocating shaker (New Brunswick Scientific, Edison, N. J.) at 200 rpm for 24-36 hours (84). At this time good mycelial growth was generally obtained and observed macroscopically.

Fifty milliliters of this culture was aseptically transferred as inoculum to 5 liters of sterile production medium containing 1% lactose in a 7.5 liter NBS Microferm Laboratory Fermentor (New Brunswick Scientific, Edison, N. J.). Agitation was set at 300-400 rpm, filtered, sterile air was bubbled at a flow rate of 2.0 l/min and the incubation temperature was kept at 28°C. The culture was
then incubated for 24-36 hours and checked for adequate mycelial growth. If a laboratory-bench scale experiment was to be performed, the culture was left in the fermentor for enzyme production and saccharification experiments; otherwise the culture was used to inoculate the plant fermentor.

The plant fermentor was a 325 liter (280 l working volume) stainless steel dairy culture vessel (Creamery Package, Chicago, IL) fitted with a stirring paddle, pH monitor/control (Electrofact-Control DataCorp., Minneapolis, MN or Chemtrix recorder, Hillsboro, Ore./Cole Parmer submersible electrode, Chicago, IL), heating/cooling coils and a filtered sterile air supply (Gast Manufacturing Co., Benton Harbor, Mich.). Air was bubbled into the fermentor using a stainless steel tube which had been bent to conform to the round slope of the vessel's bottom. Holes were drilled into this device to produce bubbles with a diameter of approximately 1.5 mm. A polyethylene reservoir provided 2N NH₄OH for pH adjustment via feedback and cellenoid control valves. Antifoam C (Dow Corning) was added as necessary to control foaming and temperature was recorded automatically but manually controlled.

The production medium was scaled-up to 280 l, by adding standard salts, trace elements, 1% lactose and tap water. The standard salts were technical grade; all other media used reagent grade chemicals. This medium was boiled for 30-40 minutes to sterilize it and microscope slide preparations were made to test for
contamination. Temperature was reduced to, and maintained at, 28°C for enzyme production. The 5 l fermentor culture (NBS Microferm) was used as inoculum (1.75% v/v) for the plant fermentor.

**SACCHARIFICATION:** The cellulose substrate employed for saccharification was Solka Floc SW-40 (Brown Co., Berlin, N.H.). Saccharification experiments were performed on both the laboratory and plant scale using in situ-produced cellulase. Solka Floc SW-40 is a purified form of alpha-cellulose derived from spruce wood. It is approximately 95% pure and ground to 40 mesh fibers (15, 40). Reducing sugars were measured by colorimetric methods using the dinitrosalicylic acid (DNS) assay procedure (46).

Laboratory saccharifications were performed in the NBS Microferm Fermentor using a 5% (w/v) slurry of Solka Floc SW-40 and crude unfiltered enzyme (50°C, 350 rpm). The pH was adjusted as necessary to 4.8 with 1M sodium citrate (pH 4.8) (37). Saccharification was generally carried out for a period of 4-5 days.

Pilot plant saccharification was facilitated by using the crude, unfiltered enzyme and Solka Floc SW-40 as substrate. A 5% (w/v) slurry was obtained by adding 14.52 kg of substrate to the crude enzyme. Agitation was set (400 rpm) and the temperature adjusted manually to 50°C. Adjustments of pH by addition of 1M sodium citrate buffer (pH 4.8) were made as necessary. The total period for saccharification was 2-4 days.
DNS ASSAY: The DNS method as modified by Miller was used to measure the amount of glucose, as reducing sugar, present. Samples were taken every 12-24 hours during saccharification and assayed in duplicate. A standard curve using anhydrous, reagent grade glucose was developed for each analysis (46). The range for this curve was 0.25-1.25 mg glucose/ml in increments of 0.25 mg.

Percent transmittance (550 nm) values for samples and standards were obtained spectrophotometrically (Spectronic 20, Baush and Lomb) and converted to absorbance (Absorbance = 2 - log_{10} %T). Absorbance vs. concentration (mg glucose/ml) plots were drawn for standards. A linear regression model was run on all analyses to determine the best fit line. Analysis of variance was computed to determine the variance between experiments.

RESULTS AND DISCUSSION

ENZYME PRODUCTION: Enzyme production was monitored by observation of the pH profile illustrated in Figure 3. The pH dropped rapidly after 24-72 hours and was maintained at 3.0 with addition of ammonium hydroxide. After several days the pH rose. This is attributed to the release of ammonium ions (44, 64, 76). Results observed in both the laboratory and plant scale experiments depict this type of profile (Figure 3). There was some variance in the amount of time it took for the pH to drop initially for some of the plant experiments, but they still followed the same basic pattern. These variances of time were probably due to the lack of control
Figure 3. Typical pH profile of a plant experiment.
over process parameters. For example, although temperature was monitored instrumentally, it was necessary to adjust it manually. Consequently, temperature fluctuated over the production period as much as 3-4°C. This is important since control of temperature to achieve optimal production is considered critical (44, 64).

Another variable was inoculum size. There is no rapid method for estimating inoculum size. Therefore, even though all experiments were treated alike, there was no way to ensure that the inoculum size was held constant from experiment to experiment. The greatest affect on the production cycle was probably the duration of a lag period which should be kept to a minimum if possible.

Saccharification was not initiated until the pH began to rise on its own (6-9 days). This increase in pH indicates the end of enzyme production as well as additional enzyme release from the cell wall on the organism's demise (2, 38, 44, 75, 76).

SACCHARIFICATION: Saccharification efficiency of the Solka Floc SW-40 substrate showed poor results for the pilot plant experiments when compared to a typical laboratory scale experiment (Figure 4). The pilot plant experiments showed an increase in saccharification for the first four trials. This suggested that the problems associated with scale-up and process control in the pilot plant were diminishing and overall improvement was being achieved. The final pilot plant experiment showed a decrease in saccharification efficiency which was most likely attributed to a small inoculum
Figure 4. Saccharification of Solka Floc SW-40 in a 5% w/v slurry.

- Concentration of reducing sugar as glucose
- Laboratory experiment
- Pilot plant experiments
- Saccharification period

Table:

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size. The NBS Microferm fermentor underwent mechanical difficulties during inoculum preparation. Its water pump failed, necessitating shutdown of all electrically controlled parameters including agitation and temperature control. Air flow was the only parameter which was not impaired. Thus, the inoculum size for the final trial was probably smaller than in the preceding experiments.

Statistical analyses were performed to determine if characteristics of saccharification efficiency differed significantly from trial to trial. An analysis of variance test established that there was a highly significant difference (0.01 level) between plant and laboratory experiments (Table 1). Furthermore, when a least significant difference statistical test was computed, it showed that trials 2 and 5 were the only two plant scale trials which were not significantly different from one another (Table 2).

These statistically significant differences between plant experiments as well as between the plant and laboratory experiments can be attributed to a variety of problems. The major problem was the need for improved process controls. Ideally, this production procedure should be controlled using a microprocessor device. This would eliminate error associated with manual operation of parameters and ensure that processes would be adjusted more frequently and possibly more economically. Other requirements necessary to optimize cellulase production require the use of proper equipment. Dairy
Table 1.

Variance analysis: Laboratory and Pilot Plant Experiments

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Variance Analysis: Pilot Plant Experiments

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\(^a\) Degrees of Freedom

** = Significantly Different F-test at the .01 level
Table 2.

Least Significant Difference Comparisons between Pilot Plant Experiments; #1-5

LSD value of 0.99 for comparison with experiment 1\textsuperscript{a}.
LSD value of 0.82 for comparisons with experiments 2-5\textsuperscript{a}.
Ranking of Means: experiments 4, 3, 2, 5, 1

<table>
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<th>Difference</th>
<th>Comparison</th>
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<td>(4) - (3)</td>
<td>= 1.34 ( \frac{1}{2} ) 0.82;*</td>
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<td>= 4.28 ( \frac{1}{2} ) 0.99;*</td>
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</table>

\textsuperscript{a} Plant Experiment 1 data contains two samples, whereas the remaining experiments contain four samples.

* = Significant Difference at the .05 level.

ns = Not Significant at the .05 level.
fermentors simply are not sufficiently sophisticated to produce the cellulase ensemble of proteins in the manner required industrially.

This study showed a low level of cellulase being produced at pilot plant levels, which is attributed to lack of parameter controls. Temperature controls is critical during saccharification. The optimum of 50°C was difficult to maintain manually, and probably lead to a drop in enzyme activity and saccharification efficiency (44, 64).

Saccharification periods varied from plant experiment to plant experiment as depicted in Figure 4. The time periods listed (Figure 4) were the amount of time in which maximum glucose was produced. All plant experiments showed a severe reduction after those times; this decrease was most likely due to contamination by bacteria and other microorganisms. The source for contamination could have been derived from the substrate, Solka Floc, which was not sterilized prior to its addition. Removal of samples from the fermentation tank could not easily be achieved aseptically. The lid, which covered the entire fermentor, has to be lifted up in order to withdraw a sample. Saccharification conditions (50°C, 4.8 pH) are not extreme enough to retard bacterial growth by the majority of organisms.

Another problem may have been associated with the product of conversion, glucose. This undoubtably provided an essential growth substrate for any of the contaminating organisms, including any
viable *Trichoderma reesei* cells. The total production period from enzyme production through saccharification was generally 1.5-2 weeks. Over this amount of time it's not surprising that contamination would occur; especially with rudimentary facilities. There was no contamination associated with the laboratory experiments suggesting that contamination of substrate is minimal and further support that rigorous process control is essential.
Chromatofocusing provides a unique chromatographic method for the separation of complex mixtures of proteins by developing a pH gradient within a column. Individual proteins migrate to a location in the column corresponding to their isoelectric points (pI) (69, 70). This method affords high resolution typically obtained by separations based on differences in isoelectric points, coupled with the high capacity of ion exchange techniques (69, 70, 71, 72). Peak widths can be in the range of 0.02-0.05 pH unit, and samples containing several hundred milligrams protein can be processed in one step (71, 72). This technique has the advantage of a self-generated pH gradient which eliminates the need for a gradient-making apparatus (69, 70). It also produces sharp, distinct bands while being easy to use (69, 70, 71, 72). These advantages made this procedure attractive for monitoring cellulase enzyme production over the standard filter paper methods.

MATERIALS AND METHODS

A Biorad (Richmond, CA) jacketed column (1x30 cm) was used with a bed volume of 25 ml. The stationary phase (gel) was Polybuffer Exchanger 94, PBE-94, (Pharmacia Fine Chemicals; Piscataway, NJ) for a pH range of 4-7. This was selected since cellulase is produced, and has the greatest activity, in this range. The eluent used was Polybuffer 74-HCl, pH 4.0 (Pharmacia Fine
Chemicals; Piscataway, NJ) with 0.025M imidazole-HCl (pH 7.4) used as equilibration buffer.

PBE-94 was dispersed in a small amount of imidazole buffer to form a slurry which was then degassed by boiling prior to column packing. Following this the slurry was poured into a column containing 2-3 ml of imidazole buffer. Then, imidazole buffer was passed through the column by gravity flow until equilibration was attained (58). Equilibration is when the pH of the eluate matches that of the eluent.

On equilibration, the column was calibrated using bovine cytochrome C (Sigma; St. Louis, MO). This is a highly colored and strongly basic (pI=10.5) protein repelled from the gel. If the column is properly established this protein will pass through as a distinct, intact, compact band (58). Once calibrated, the sample was applied. The packed column used in this study had a capacity of 350 mg of protein.

Sample was applied after 5-10 ml of eluent, (Polybuffer 74-HCl) had been loaded onto the column (58). The polybuffer forms an internal pH gradient separating individual proteins as they encounter their respective isoelectric points (69, 70, 71, 72). Eluate was collected in 8-10 ml aliquots using a fraction collector. Absorbance (280 nm) and pH were determined for each fraction obtained.
Samples from all successful plant experiments were analyzed in this manner along with standards. Standards used were 250 mg of beta-glucosidase extracted from almonds (Sigma; St. Louis, MO) and 300 mg of Trichoderma reesei cellulase complex (Sigma; St. Louis, MO). Beta-glucosidase extracted from almonds was selected for use as a standard since it is the purest form available. All chromatofocusing profiles were compared in an attempt to determine the enzyme production profile.

RESULTS AND DISCUSSION

The chromatofocusing profile of the T. reesei standard shows a fairly linear pH gradient with well separated protein peaks (Figure 5). The other standard used was beta-glucosidase; its profile is shown in Figure 6. It was not possible to run a linear regression on these pH gradients as the exact pH of the protein peaks (isoelectric points) must be determined. Otherwise, comparisons between the isoelectric points of the enzyme components of the standards with plant-produced cellulase would not be accurate.

The beta-glucosidase profile shows two relatively large peaks, one at pH 7.3 and the other at 4.8. Since these were the dominant peaks, they were chosen for further comparisons. The smaller peaks located between these are neither well defined nor large enough for comparison. They should be due to beta-glucosidase protein as the standard is the purest available, but there could
Figure 5. Chromatofocusing profile of 300 mg of Trichoderma reesei cellulase standard.
Figure 6. Chromatofocusing profile of 250 mg of beta-glucosidase.
also be some miscellaneous protein from the purification procedure. This is not to state that these proteins are absent in the other profiles, but since beta-glucosidase is only one of three major enzymes within the cellulase complex the resolution is not adequate for comparisons.

When comparisons of isoelectric points of the beta-glucosidase fraction (two peaks; pH 4.8 and 7.3) with *T. reesei* cellulase standard, it was shown that it contained peaks at pH 4.8 and 7.3 as well. The author speculates that these cellulase peaks were due, to some degree, to beta-glucosidase. This does not rule out the possibility that the other cellulase components, for which no standards are available, contribute to the total protein peak.

A similar comparison was made between the standards and plant-produced enzyme (Figure 7) profiles. After comparing isoelectric points of the beta-glucosidase dominant peaks (pH 4.8 and 7.3) with those of the plant-produced enzyme, the author contends that peaks appearing at 4.8 and 7.3 were attributed to the beta-glucosidase portion of the enzyme complex. Since the chromatofocusing profile of plant enzyme demonstrated few well separated peaks, further comparisons other than basic trends could not be performed. The basic profile of absorbance peaks within the *T. reesei* enzyme standard is similar to that of the plant-produced enzyme.
Figure 7. Typical Chromatofocusing profile of 10 ml of crude plant-produced cellulase.
The chromatofocusing profile of the plant experiment depicted low protein absorbance values for the cellulase produced. This was most likely associated with problems with the SDSU pilot plant system; specifically, poor control of process parameters. Temperature was adjusted manually and not controlled automatically which allowed for some variation in the culture temperature (4–5°C overnight). This was inconsistent with optimal enzyme production conditions (44, 64). Another problem was the lack of an adequate sampling method. The enzyme production tank did not lend itself to aseptic techniques. The entire top of the fermentor had to be raised to withdraw the sample. This enhanced the likelihood for contamination by enabling unwanted microbes access to the fermentor. However, this was probably not a great problem during enzyme production since a pH of 3.0 was maintained and would create environmental conditions where few microorganisms survive. It was a problem if the pH did not drop rapidly within 72 hours.

Additionally, the small protein peaks could be due to the use of 10 ml of crude, centrifuged enzyme as the protein concentration of these samples was not determined prior to application onto the chromatofocusing column. A Biorad (Richmond, CA) protein assay was attempted, but cellulase does not form an adequate color complex using this method because the intensely colored Coomassie Brilliant Blue complex fails to bind these proteins. A different technique for assaying protein should have been determined to assure that a standard or known amount of protein was applied to the column. For
low concentrations of proteins, samples should be concentrated by ultrafiltration, salt precipitation or some other method. It is evident that plant-produced enzyme should have been concentrated prior to chromatofocusing to ensure a good protein separation for comparison to the standards.

The most significant problem for immediate use of this technique to monitor enzyme production is the lack of available commercial standards for the exo- and endoglucanase glycoproteins. Techniques for separation and purification of these components is labor intensive, expensive and requires a great deal of time to produce very low quantities. Furthermore, there is no guarantee that these proteins would be stable for a sufficient period of time (M. Ladisch, personal communication). The fact that the endo- and exoglucanases exist as one of several forms in Trichoderma cellulase complicates matters even more. However, this technique has demonstrated how simple it is to separate complex, mixed protein samples by their isoelectric points and therefore has many other possible applications.
CONCLUSIONS

The first objective of this study was to determine how effective scale-up from a laboratory scale production level to a rudimentary pilot plant would be. It was determined that with the facilities at SDSU, which would be comparable to an owner-operator system, enzyme production is not feasible. The technical requirements for optimal enzyme production cannot be met with existing facilities and would require a substantial capital investment. Along with the proper equipment, adequate control of this equipment via a microprocessor-driven system would be necessary if the process is to operate smoothly without constant supervision as would be the case for an owner-operator setup. The level of technical understanding for enzyme production would require intensive training in microbiology. An alternative to production would be to purchase the enzyme, but presently this is not cost effective. Other conversion methods such as acid degradation are currently being compared, by other investigators, with enzyme hydrolysis at the community scale level. Perhaps after this comparison the most feasible method will be determined.

The saccharification process requires less technical understanding, but demands adequate control over process parameters. The substrates most likely used by the owner-operator would be in situ-produced agricultural wastes. For example, these might include wheat/oat stubble, wet/dry corn stover, and grain dust. Most of
these substrates require pretreatments as previously discussed (milling, delignification, etc.).

The second objective was to determine if a simpler enzyme assay could be developed to replace the commonly used assays which are time consuming and labor intensive. Chromatofocusing techniques are easy to use for separations of complex proteins, but do require some technical and analytical skills as well as expensive equipment. This is a major drawback for an owner-operator. This method does not suit itself presently for monitoring cellulase production since the component enzymes are not all available as commercial standards. Thus, it is impossible to determine if all components are being produced in the quantity required for saccharification. This process does lend itself towards many other applications of protein analysis.
LITERATURE CITED


