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PILOT PLANT PRODUCTION OF CELLULASE BY Trichoderma reesei QM9414  
AND THE EFFECT OF DIMETHYL SULFOXIDE ON  
CELLULASE PRODUCTION BY T. reesei MCG77

BY

PAUL J. WHALEN

A thesis submitted  
in partial fulfillment of the requirements for the  
degree of Master of Science  
Major in Microbiology  
South Dakota State University  
1983

PILOT PLANT PRODUCTION OF CELLULASE BY Trichoderma reesei QM9414  
AND THE EFFECT OF DIMETHYL SULFOXIDE ON  
CELLULASE PRODUCTION BY T. reesei MCG77

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

Approximately  $15-20 \times 10^{10}$  tons of organic plant substance is produced on earth per annum. Half of this material is cellulose (95). Much of this cellulosic material is highly resistant to breakdown due to other plant components such as lignin and hemicellulose which together with the cellulose contribute to the structural integrity of the plants (58). Thus these materials find relatively limited uses in comparison to their abundance. Cellulosic materials are utilized by ruminants as an energy source and in lumber and pulping applications but commercial cellulose processing to produce glucose or glucose derived products is not currently performed.

The abundance of cellulosic materials make them attractive as a cheap substrate for industrial applications. However, the conversion of these materials is greatly limited by other protective constituents such as lignin and hemicellulose (65, 77) and by the arrangement of the cellulose molecules themselves (20, 90).

In the past, processes for hydrolysis of cellulose have been primarily based upon the use of acids (105, 131). These processes proved to be uneconomical. More recent methods have concentrated on the use of microbial cellulases usually in conjunction with pretreatment of the cellulosic materials. The advantages of the enzymatic methods lie in the moderate temperatures for conversion, the noncorrosive nature of the process and quantitative conversion to the desired end product-- glucose (5).

The best microbial cellulase producer is the fungus Trichoderma reesei (70). Trichoderma reesei mutant strains hyperproduce a complete cellulase complex capable of attacking crystalline cellulose and reducing it to glucose (70). The T. reesei cellulase complex is composed of:  $\beta$ -1,4-glucan cellobiohydrolases, endo 1,4- $\beta$ -glucan glucanohydrolases and a  $\beta$ -glucosidase (135).

Production of T. reesei cellulase is conveniently accomplished in aerated, submerged culture (63). Trichoderma reesei fermentations on cellulose substrate go through a drop in pH with a subsequent rise at the end of the fermentation (118). The rise in pH can be used as a general indicator of when to harvest the enzyme. Optimum yields of enzyme require that the acidic phase of the fermentation be maintained between pH 3.0 to 3.5 (118). Yields (in units activity/ml) of enzyme increase with an increase in the cellulose level employed in the production medium (120). The maximum level is approximately 8% w/v cellulose. Viscosity is a problem in media greater than 8% w/v.

The cost of enzyme production is the limiting factor for an enzymatic conversion process. The system of Wilke, Yang and Von Stockar (134) resulted in an enzyme production cost of \$0.05-\$0.06/lb. of glucose produced or about 60% of the cost of production. Optimization of cellulase production systems for increased yields are therefore of paramount importance to reduce the cost of enzymatic cellulose conversion.

Increased yields and production rates of cellulase have been reported for the use of various additives to the production medium (41,

96, 106) and from advanced culture techniques such as continuous processes (34, 83, 100). This study involves the production of T. reesei cellulase in a rudimentary production system employing used dairy equipment. The potential for enzyme stimulation by addition of dimethyl sulfoxide to the production medium is also investigated.

## LITERATURE REVIEW

Sources and Quantities of Cellulose

Cellulose is the world's most abundant organic compound, annually replenished and representing a vast natural resource (21, 45, 65). The most abundant natural sources of cellulose, in descending order of magnitude, are: 1) the stems of woody angiosperms or hardwoods, 2) the stems of woody gymnosperms or softwoods, 3) the stems of monocotyledons or grasses, 4) the non-lignified parenchyma cells of most leaves, and 5) certain non-lignified or partially lignified fiber such as the seed hair of cotton and bast fiber of flax (21). The general composition of hardwoods is 40-55% cellulose, 24-40% hemicellulose, and 8-25% lignin. The softwoods are similar with a cellulose content of 45-50%, hemicellulose of 25-35%, and lignin from 25-35%. Grasses such as bamboo, wheat, rice, etc. are 25-40% cellulose, 25-50% hemicellulose, and 10-30% lignin. The non-lignified parenchyma cells of leaves are 15-20% cellulose, with 80-85% pectin and hemicellulose. Cotton and bast fiber are 80-90% cellulose, 5-20% hemicellulose, and little or no lignin. The bulk of available cellulose in nature is derived from the hardwoods, softwoods and grasses and is thus heavily lignified material (21).

Cellulosic materials are available in many forms in nature and in many more forms of processed materials such as newspaper, sawdust, pulp slurries and residue, etc. (4, 5, 21). Much of the material is considered waste, finding little industrial use and, in some cases, representing a disposal problem (86). Projections of the MITRE, Corp.

to 1985 estimate U.S. terrestrial biomass resources of cellulose as residue to be approximately 681 million tons in dry ton equivalent (DTE) (130). Subdivisions of this material include forest residue (175.4 million DTE), mill residue (28 million DTE) and agricultural, silviculture and crop residue (477.6 million DTE) (130).

Collection methods reflect the true availability of these materials for potential use. For example, of the approximately 477 million DTE of agricultural residue, approximately 78 million DTE is estimated as usable via current collection methods (127). Ninety percent of this material results from corn residue and small grains crops where harvest methods permit collection (127). Over one-half of the gross U.S. production of paper and paperboard which is recyclable but not recovered resulted as wastepaper in 1973 (121). With proper collection and separation methods this material (33 million tons) represents another potential cellulose source.

When cellulose is hydrolyzed to its basic constituent, glucose, many potential uses evolve. Practically all commercial microbial fermentations use glucose for such products as single cell protein, alcohol, citric acid, gluconic acid, ascorbic acid, etc. (5, 103).

### Cellulose Structure and Chemistry

Cellulose composes the skeletal constitution of all higher plants (45). It is the principle component of the cell walls of wood (16). The purest form of native cellulose occurs in the seed hair of cotton (16, 45, 58, 109). When raw cotton undergoes light treatment with dilute alkali, is washed and bleached, the residue consists of

almost pure cellulose. Because of this cotton serves as a reference material for cellulose investigations (16). The material resulting from rigorously purified wood is essentially identical to cotton in chemical nature (16, 58).

Cellulose is a linear polymer of anhydro-D-glucose in  $\beta$ -1,4 linkage (16, 58, 101, 109, 131). The length of cellulose molecules in a fiber varies over a wide range from lambda-cellulose, containing less than 15 glucose units, to alpha-cellulose with as many as 10,000-14,000 glucose units/molecule (21). Alpha-cellulose is alkali resistant cellulose and corresponds quantitatively to pure cellulose (131). We shall be concerned with alpha-cellulose in this paper.

The molecular weight of native cellulose is  $> 1.5 \times 10^6$  (21, 58, 109). Browning (16) points out that the term "cellulose" is used with two distinct meanings: 1) as a chemical definition as described above and 2) in a generic sense to describe preparations (existing or potential) in which the principle and common constituent is defined according to the first interpretation. Cellulose in nature exists in various states of purity from 90% for cotton to about 45% of typical wood cell walls (20). Analytically, cellulose can be distinguished from other constituents by: 1) its insolubility in water and organic solvents, 2) from hemicelluloses by its insolubility in aqueous alkaline solutions and 3) from lignin by its relative resistance to oxidizing agents and susceptibility to hydrolysis by acids (16).

In the cellulose chain molecule, the anhydroglucose units adopt the chair conformation with the hydroxyl groups in the equatorial and

the hydrogen atoms in the axial positions (95, 101, 109). Every other chain unit is rotated 180 degrees around the main axis (109). This results in an unstrained linear configuration, minimizing steric hindrance (109). It has been found that the hydroxyl group of carbon #3 forms an intrachain bond with the ring oxygen of carbon #5 of the next glucose unit (Fig. 1) (90, 95, 101, 109).

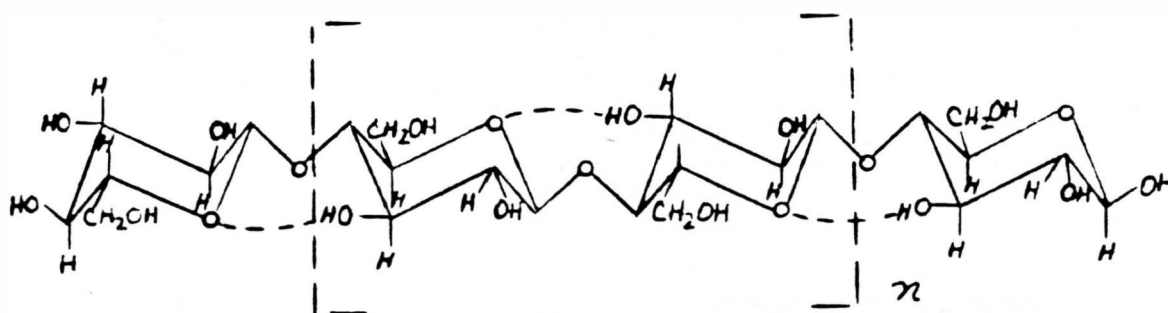


Figure 1. Chair conformation of anhydroglucose units in cellulose polymer ( $\beta$ -1,4 glycosidic bond). ----denotes intrachain hydrogen bonding.

The cellulose molecule is thread-like, existing as fibrils which are organized bundles of parallel cellulose chains (58, 90, 95, 101, 109). The molecular arrangement of the fibrils by interchain hydrogen bonding is highly ordered. An x-ray diffraction pattern is obtainable for this highly ordered or crystalline cellulose (16, 90, 95, 101, 109).

The structure of the native cellulose microfibrils and their size is subject to controversy. Several models exist (see Fig. 2). According to Ranby (90), the microfibril consists of a rectangular cross section with a crystalline core resulting from the orientation of the microfibril with their lattice planes well aligned parallel with the flat surface of the lamellae, reflecting the tangential plane of



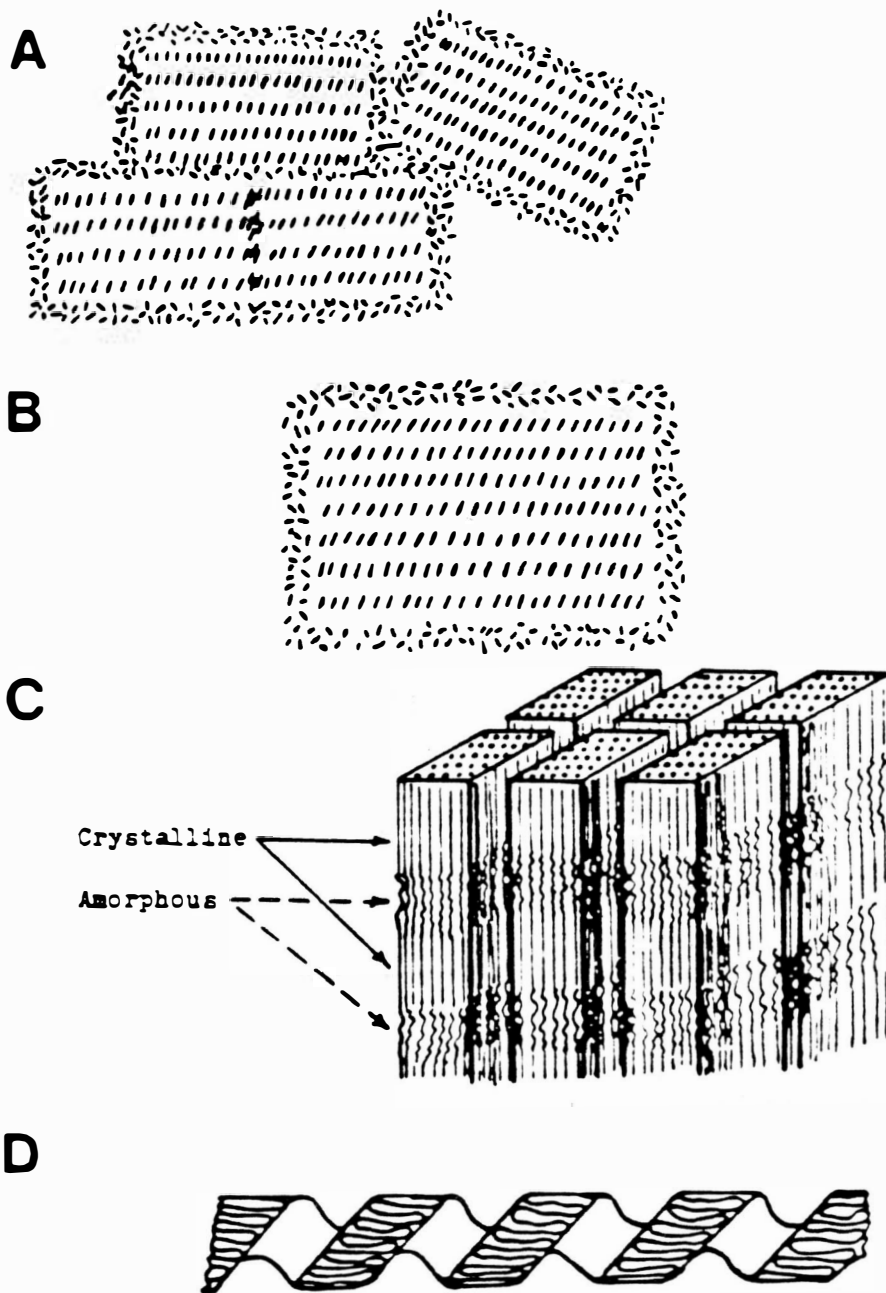


Figure 2. Models of microfibril arrangement. (A) Ranby--cross sectional view (90); (B) Preston and Cronshaw--cross sectional view; (C) Hess, Mahl and Gutter--perspective; and (D) Manley. (Redrawn from Ranby [90] and Cowling and Brown [20]).

the cell wall or membrane where they occur. This, according to Ranby, is the preferred orientation of the microfibrils. The surface layer surrounding the microfibrils is disordered to display a natural discontinuity that occurs on the surface layer of the microfibrils. This orientation effect was first found by x-ray diffraction and confirmed by infrared absorption measurements. Further, this model portrays the microfibrils overlapping or extending into one another. No preferred number of fibrils (and therefore size) is noted for the microfibrils (90).

A somewhat similar model is proposed by Preston and Cronshaw as reviewed by Cowling and Brown (20). This model projects a rectangular cross section with the crystalline core surrounded by a paracrystalline or amorphous outer region. The amorphous region is the area of occurrence for hemicellulose and lignin. A microfibril size of 50 x 100 Angstroms is proposed (20).

Cowling and Brown (20) also reviewed the two following models of Hess, Mahl and Gutter, and that of Manley.

In the Hess, Mahl and Gutter model, the microfibril displays packets of elementary fibrils containing 15-40 cellulose molecules. The fibrils are segmented linearly into regions of crystalline and amorphous areas, thus displaying regions of less well ordered cellulose molecules along the length of the microfibril. The cross sectional dimension is a minimum of 35 Angstroms in this model (20).

The final model for consideration is that of Manley in which the microfibril is a flat helix where the cellulose chains are folded to form a flat ribbon which is wound spirally. Upon mechanical

disruption the helix collapses to a flat ribbon (20, 90). The infrared spectra for interchain hydrogen bonding does not support a helical model nor does the density of the fiber wall agree with a cylindrical helical model (20). Manley indicates that the helix may be deformed to meet these requirements by flattening of the helix. The forces required to accomplish this are discounted by Ranby as unlikely to occur. A microfibril diameter of 30-40 Angstroms is projected for this model (20, 90).

Native cellulosic materials contain other constituents such as hemicellulose, lignin, waxes, pectin and certain nitrogenous substances (20). In cotton these materials occur in the cuticle and primary wall layers. In wood these materials may extend to the middle lamella, decreasing in quantity towards the inner layer (20). Hemicellulose content in wood varies from 20-40% (21). The principal component in hemicellulose is D-xylan, polymers of D-xylose in  $\beta$ -1,4 configuration with side chains of arabinose, mannose and galactose (20, 58, 131).

Lignin is a complex, three dimensional polymer formed from p-hydroxycinnamyl alcohols (20). The composition varies according to source and is further complicated by covalent bonding to certain hemicellulose (20, 131). Lignin in plants is an almost insoluble substance (20). It is isolated by hydrolysis with acids or oxidative degradation of all other components in wood, the resulting insoluble material being lignin (131). Material free of lignin is termed "holocellulose." The term "lignin" refers to a mixture of substances that have similar chemical composition but may have structural differences. The aromatic

nature of lignin has been established (131). Lignin is located principally in the layer of material between individual wood cells called the middle lamella (15). This is important when considering extraction and conversion of cellulose.

### Cellulose Treatments and Processes

As stated previously, the composition of various cellulosic materials varies widely with regard to source and treatment. Typical pulped products such as newspaper and municipal solid waste are approximately 60-70% cellulose (27, 65, 134). Raw or untreated sprucewood is 41% cellulose while processed or bleached spruce sulphite is approximately 85% cellulose (128). The degree of pretreatment influences the accessibility of cellulose to hydrolyzing processes (25, 56, 65, 66, 77, 78, 95, 99, 126). The pretreatment of cellulose or cellulosic materials deals primarily with factors preventing exposure of cellulose to the hydrolyzing agent. These factors are lignin and hemicellulose which seal the cellulose from the hydrolyzing agent and, the crystalline, highly ordered cellulose which in itself is inaccessible (90) due to its stereochemistry. Generally, treatment with various acids, bases or metal-amine complexes, in combination with extensive milling and heat, result in the removal of the layering components. In addition, upon hydrolysis crystalline cellulose swells disrupting its highly ordered arrangement creating areas of accessibility for breakdown into its constituents (4, 5, 15, 44, 51, 56, 98, 105, 109, 126). The nature of the swelling action is a result notably of the alkali agent breaking uronic esters functioning as crosslinks in layering substances such as xylans

(126). The cellulose itself swells in aqueous sodium hydroxide. The swelling is a transformation from Cellulose I to Cellulose II due to intramicellar and intermicellar swelling (109, 126). Cellulose I is the native, highly ordered crystalline cellulose exhibiting strong hydrogen bonding and thus inaccessibility. Cellulose II is the result of alkali (15% sodium hydroxide) treatment, washing and subsequent drying of Cellulose I (109, 131). Cellulose II reflects greatly weakened hydrogen bonding and thus a shift in the bond angles of the micelle (109). Tarkow and Feist (126) in their work with hardwoods treated with alkali reported that the resulting material exhibited greater water sorbtion, lesser tensile strength when wet and greater accessibility of the cellulose chains. Although alkali treatment results in increased reactivity of cellulose, it is incapable of completely solubilizing cellulose (109). The change in the cellulose as a result of dilute alkali treatment occurs with little loss in weight (126).

Pulp Processes. The processes utilized in pulp and papermaking are primarily concerned with obtaining a comparatively high content of cellulose in the final product (15, 131). Therefore, methods involved are basically directed at the removal of lignin and hemicelluloses. Sulfite, alkaline, mechanical and chemi-mechanical methods are used (15). In addition to removal of lignin and hemicelluloses, these processes modify cellulose fibers resulting in increased specific surface, degree of swelling, and flexibility (15). The conversion of pulped material in general, and especially that material resulting from Nicolet sulfite pulp (sulfurous acid agent) and from Kraft pulp (caustic

and sodium sulfate agents), have been shown to be excellent for exposing cellulose to hydrolyzing agents (4, 5).

Various methods of physical and chemical treatments are used to aid in or directly reduce cellulose to glucose. Methods such as acid hydrolysis, alkaline degradation, irradiation, thermodegradation, ball milling, etc. are prominent among the methods investigated. Bikales and Segal (14) provide an excellent review of several of these processes. Millet, Baker and Satter (72) concluded that the most successful techniques for effecting drastic crystalline disruption of cellulose were electron irradiation and vibratory ball milling. These authors also noted the impracticality of these two methods of pretreatment due to the high energy input demanded.

Acid and Alkali Processes. Of the chemical methods for either pretreatment or for direct conversion to glucose, acid hydrolysis and alkali degradation have received the most attention.

Acid processes for conversion are generally preferred over alkaline methods due to the degradation products of the latter which result in further complication of a system based on these techniques (44). Notably, alkali (sodium hydroxide) processes result in high organic acid content. Alkali processes also limit conversion capability by chain stabilization (98). Alkali treatments do, however, display high potential when used as a pretreatment process for the exposure of cellulose and removal of hemicellulose (25, 63, 65, 126).

~~Acid~~ hydrolysis techniques result in similar problems, but not nearly as extensive. The main degradation product is furfural formed

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from the pentose sugars of the hemicellulose (44, 105, 131). The mode of hydrolysis (addition of water to split the glycosidic bond) of cellulose by acid is the protonation of the glucose molecule (44, 105, 131). The rate of hydrolysis of the glycosidic bond at the nonreducing end of the cellulose chain is 50% higher than that of the bond at the reducing portion (44). The amorphous cellulose in an acid hydrolysis is hydrolyzed first and at a much more rapid rate than crystalline cellulose. Crystalline cellulose is hydrolyzed slowly but linearly thereafter (44, 105, 131).

It is believed that the stereochemistry of the crystalline region, as well as the microfibril conformation, play a part in the kinetics of crystalline cellulose hydrolysis (29, 44). The review by Sharples (105) points out that physical abnormalities (due to mechanical or chemical pretreatments) increase the accessibility of crystalline cellulose and assist hydrolysis.

Wenzl (131) describes the use of supersaturated (42-45%) hydrochloric acid in a consecutive action type of cellulose hydrolysis. First concentrated acid is used and recovered, then a dilute acid hydrolysis is performed by boiling in water. Yields from this method resulted in 61% conversion to glucose with 76% of the total carbohydrate content of the wood recovered in the form of a 30% solution of simple sugars. Hemicellulose degradation products were noted as a disadvantage to this method. Wenzl further notes that due to these degradation products, the reverse procedure of the above method is preferred, that is, to

remove the hemicellulose prior to crystalline hydrolysis using the supersaturated acid. Hydrolysis of wood which has not been delignified is inhibited by the coating or sealing action of lignin (131).

Dilute acid hydrolysis is a more economical type of system because of lower equipment costs and less neutralization is required. However, pilot studies of dilute acid hydrolysis methods have resulted in low sugar yields and could not be operated economically (105, 131). Two basic problems are inherent in the acid hydrolysis of cellulose: 1) poor conversion yields of glucose result when convenient dilute acid solutions are employed and 2) stronger acid solutions result in higher conversion to sugar but requires expensive neutralization procedures and considerable sugar degradation occurs (105).

Recently, acid hydrolysis processes employing extruders have been reported for the conversion of cellulose to sugars (24, 87, 112). The extrusion processes employ high temperatures, high shear and dilute acid concentrations (approximately 1%). Cellulose to sugar conversion yields are comparable to the acid procedure described by Wenzl (131) however the extrusion process results in a much lower concentration of sugar in the final product (12% sugar vs. 30% sugar).

Solvent Processes. Numerous solvents are capable of solubilizing cellulose. These solvents range from metal-amine complexes involving cobalt, zinc, nickel and cadmium, to metal-alkali complexes containing copper, iron or nickel (51). Jayme (51) has reviewed the use of these solvents, one of which--cadoxen--has recently resurfaced as having great potential in cellulose conversion. The process is commonly referred to as the Tsao process and is based upon the use of



cadoxen. This process formed the premise of the Purdue University projections (127) for fuel alcohol production from biomass in the United States.

Cadoxen is a complex obtained by dissolving cadmium oxide (5%) or cadmium hydroxide in 28% aqueous ethylenediamine solution (51). Cadoxen is a very strong base and is believed to dissolve cellulose by forming a cellulose-cadoxen complex combined with splitting of hydrogen bonds within the crystalline areas (51). Recovery of cadmium oxide and ethylenediamine from cadoxen-cellulose solutions can be facilitated by simple refluxing (51, 56). These components of cadoxen may then be re-used in making subsequent solutions. Ladisch, Ladisch and Tsao (56) of Purdue University reported high conversion of crystalline cellulose as well as agricultural residues to glucose upon treatment with cadoxen prior to saccharification using enzymatic hydrolysis. Commercialization of such a process has been criticized by Flickinger (30) as being capital intensive for equipment and recovery technology.

Enzymatic Processes. Of the chemical processes for hydrolysis discussed above, all require expensive corrosion-proof equipment, high temperatures, extended periods of time and/or methods of recovery. In addition, these methods result in reduced yields and the presence of degradation products. Enzymatic methods have the potential of resulting in a quantitatively higher yield of product from the substrate, with no degradation products, and at moderate temperatures of conversion (5).

Cellulase Enzyme Sources. Insects, molluscs, protozoa, bacteria, actinomyces and fungi produce cellulases (70). Some of these sources, such as insects and molluscs, are inherently prohibitive for

commercial production of cellulase. Others may impede production due to conditions of growth or cellulase formation (70). The basis upon which an organism is characterized as truly cellulolytic is whether the enzyme produced by the organism is capable of attacking the crystalline portion of cellulose (97).

Reese, Siu and Levinson (97) proposed the  $C_1-C_x$  concept of cellulase systems in 1950. The original  $C_1-C_x$  concept held that for complete cellulase activity an organism must be capable of producing an enzyme which attacks the highly ordered crystalline cellulose. This activity was designated the  $C_1$  component and was used to differentiate enzyme complexes of various organisms. Thus, for a complete cellulase complex an organism must have the ability to hydrolyze the crystalline cellulose ( $C_1$  activity) and the amorphous cellulose ( $C_x$  activity).

U.S. Army Natick Laboratory has screened and tested various microorganisms for their production of a complete cellulase complex and has determined that the fungus, Trichoderma viride is the most reliable producer of a stable cellulase (70). Their selection was made after testing over 100 other strains and closely related organisms producing similar complexes. Yields from the others were lower than that obtained from T. viride. The parent strain isolated in the South Pacific in the 1940's was designated T. viride QM6a (7). Strains which produced higher activities of cellulase were derived from QM6a by mutation. Strains QM9123 and QM9414 were selected by subjecting strain QM6a to linear irradiation. The most recent Natick mutant, strain MCG-77, was isolated in 1978 after ultraviolet irradiation and Kabicidin treatment from a QM9414 intermediate strain, TKO41. The Rutgers mutants NG14 and C30

were isolated after ultraviolet irradiation and nitroso-guanidine treatment of the QM6a strain (7). These hypercellulase producing strains (including QM6a) were later renamed Trichoderma reesei (33).

### The Cellulase Complex

The original  $C_1$ - $C_x$  cellulase complex as postulated by Reese et al. (97) in 1950 presumed a two-step hydrolysis mode whereby the " $C_1$ " component effected a change in crystalline cellulose that allowed the " $C_x$ " components to further degrade or hydrolyze the cellulose to its subunits. No characterization of the enzymes was available at the time and the existence of " $C_1$ " was postulated on indirect evidence (135).

As research on the T. reesei cellulase complex continued, isolation of the components resulted in three major categories of enzymes. 1) The " $C_1$ " component has been identified as a  $\beta$ -1,4-glucan cellobiohydrolase (EC 3.2.1.91), a glycoprotein which reacts with cellulose in "exo-" fashion resulting in cellobiose as the end product (10, 12, 28, 39, 40, 43, 135). At least four electrophoretically distinct  $\beta$ -1,4-glucan cellobiohydrolases have been identified from Trichoderma cellulases (28, 40). These forms, termed A, B, C, and D, have been determined to be different glycoprotein forms of the same polypeptide with identical roles in cellulose degradation (40). Form C has the highest affinity for crystalline cellulose, is the most predominant form containing higher carbohydrate levels (mannose, largely [40]), and forms a slightly more active cellulase system when combined with other fractions of the complex (28).

II) The "C<sub>x</sub>" component is a 1,4- $\beta$ -glucan glucohydrolase (EC 3.2.1.4), a glycoprotein which attacks cellulose and cellooligomers in "endo-" fashion resulting in new free chain ends (13, 28, 42, 43, 107, 135). Shoemaker and Brown (107) isolated four endo 1,4- $\beta$ -glucan glucohydrolases designated I, II, III and IV. These investigators qualified the different specific reaction properties for endoglucanases II, III and IV from Trichoderma reesei. It was noted by these authors that the specific activities of the endoglucanases increased with the length of the cellooligosaccharide substrate, and that endoglucanase IV had some transglycosidase activity (107). In addition, Hakansson et al. (42) isolated an endoglucanase with a molecular weight of about 20,000. The earlier work of Berghem, Pettersson and Axio-Fredriksson (13) indicated that the higher activity of this low molecular weight endoglucanase resulted from its smaller size which may allow it access to a greater portion of the cellulose fibers.

Thus the "C<sub>x</sub>" portion of the cellulase complex is composed of at least five endoglucanases.

III) The  $\beta$ -glucosidase (EC 3.2.1.21) or cellobiase component of the cellulase complex of Trichoderma reesei degrades cellobiose resulting in glucose (11, 28, 117, 119, 135). Berghem and Pettersson (11) concluded that this enzyme is composed of a single polypeptide chain and probably not a glycoprotein. In accordance with the terminology of Emert et al. (28), the term  $\beta$ -glucosidase is used here to designate the cellulase enzyme components where  $\beta$ -glucosidase is the enzyme functioning to degrade cellobiose produced during polymeric

substrate degradation. Only one  $\beta$ -glucosidase has been observed in T. reesei cellulases (28).

As the research on the nature of the cellulase complex continued, the basic concept of the  $C_1$ - $C_x$  hypothesis became controversial. Two predominant views prevail with " $C_1$ " at the heart of each.

In the first view, Reese and Mandels (93) as reviewed by Wood and McCrae (135) maintain that " $C_1$ " is a prehydrolytic factor, conditioning the crystalline cellulose to allow subsequent attack and conversion by the " $C_x$ " components (Fig. 3).

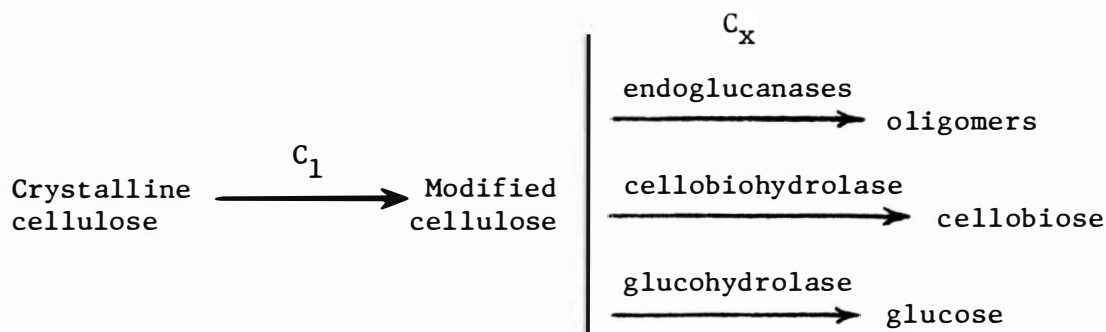


Figure 3. Modified  $C_1$ - $C_x$  hypothesis of E. T. Reese (135).

In the second view, Emert et al. (28) utilizing the individual components of the cellulase complex as presented above, propose an opposing hypothesis. This model maintains that the " $C_1$ " component is the  $\beta$ -1,4-glucan cellobiohydrolase. In this hypothesis they argue that in an enzyme complex containing both "exo-" and endoglucanases, it is the endoglucanases which are responsible for initiating the attack. This hypothesis, therefore reverses the order of the " $C_1$ - $C_x$ " concept propounded by Reese.

The hypothesis of Emert et al. (28) enjoys a great deal of experimental evidence and support (10, 11, 12, 29, 39, 40, 43, 91, 104, 117, 135). Emert et al. (28) also propose that the "C<sub>1</sub>-C<sub>x</sub>" catch-all term be eliminated in favor of the systematic names assigned by the Commission on Enzymes of the International Union of Biochemistry for the individual components of the cellulase complex.

In this light, the second hypothesis of Emert et al. (28) suggests that reference to the cellulase "complex" or "system" is in nature a true function indicating synergism. In this synergistic action the endoglucanases initiate the attack exposing new chain ends to the cellobiohydrolase for exoglucanase activity at the site which aids the endoglucanase action on the crystalline cellulose (10, 13, 28, 39, 42, 66, 104, 107, 135). The product of the cellobiohydrolase, cellobiose, is removed by the  $\beta$ -glucosidase thereby reducing end product inhibition of the cellobiohydrolase (10, 12, 39, 43, 66, 117, 119).

Trichoderma reesei enzyme culture filtrates also contain arabinase and xylanase (pentosanases) (55, 80, 125) as well as alpha-amylase (68, 79, 125).

#### Factors Affecting Enzymatic Hydrolysis

Electron micrographs of enzymatic degradation of cellulose reveal a phenomenon of breakdown in which the cellulose particles are reduced to finer particles or are fragmented (29, 54, 60, 91). Berg and Hofsten (8) used electron microscopy to examine the growth of T. reesei (viride) on cellulose. Their study revealed that the fungus appeared to grow in directions parallel to the laminar structure of the

fibers. This is interesting in that the enzyme attacks in a manner that reduces the crystallite in width while the length remains constant (29). Rautela and King (91) postulate that enzymatic action along the crystallite would result in fragmentation because of binding to the surface, anticipating that this bonding would weaken interstructural bonding and result in smaller crystallites being freed. Presumably this process continues until extensive reduction in size is realized.

Enzymatic cellulose degradation requires physical contact between the substrate and the enzyme. Thus, structural features of the cellulosic material are integral to the degree of susceptibility to degradation. These factors include: 1) the water sorbtion capability, 2) the crystallinity, 3) the molecular arrangement, 4) the content of associated material such as lignin and 5) the capillary structure of the cellulose fibers (29, 122).

The influence of one factor over the others in relation to rate and extent of hydrolysis has been a subject of investigation. The work of King (54) showed that the hydrolysis (solubilization) rate was directly proportional to the surface area of the substrate. Stone et al. (122) in a similar study suggested hydrolysis as a function of capillary dimension of the cellulosic substrate. The accessibility of the cellulose to the cellulase enzymes, at a molecular weight of around 50,000 (29), was assumed to be assisted by increased surface area in these postulations. Fan, Lee and Beardmore (29) dispute the importance of surface area and emphasize the effects (or limitations) of crystallinity. In this work, they thoroughly document the hydrolytic increase in relation to percent crystallinity. They attribute the slowdown of

hydrolysis to the "inactive" fraction of the crystalline cellulose, noting that surface area at this point (96 h of hydrolysis) is greater than at initiation. They conclude that surface area is not limiting and therefore the decrease in hydrolysis rate is not due to this factor. They attribute the decrease in hydrolysis to the fine structural order of the cellulose confirming that the amorphous portion is hydrolyzed more rapidly. This work points to the importance of the fine structural order of the crystalline cellulose as the predominant factor affecting enzymatic hydrolysis. The authors recommend investigation into pretreatments directed towards altering the crystalline arrangement rather than treatment as a simple function of increasing the surface area.

Physical Treatments. Studies aimed at altering the crystalline structure of cellulose indeed do show significant increases in percent hydrolysis. Kelsey and Shafizadeh (53) used shaker flask studies in which simultaneous physical attrition and enzymatic hydrolysis of the cellulose was obtained by adding glass beads to the hydrolysis mixture. This method was compared to vibratory ball milling pretreatment. The simultaneous attrition, wet milling method (53) resulted in higher initial hydrolysis rates and twice as much hydrolysis as the ball milled samples. Lignocellulosic materials also responded to wet milling procedures (using stainless steel beads) by an increase in hydrolysis of three times that of ball milling. Significant increases in conversion using newsprint were also noted using the stainless steel beads. The underlying feature of simultaneous wet milling-hydrolysis is the continued exposure of new surfaces and irregular cellulose molecules via mechanical breaking of the cellulose (53). The enzyme is thus capable



of reacting with cellulose molecules otherwise inaccessible due to high orientation of the crystallites. The major drawback of this process is the inactivation of the enzyme due to the grinding action of the wet milling. Neilson, Kelsey and Shafizadeh (78) in a subsequent study using a bench-scale attritor felt that this type of inactivation played a significant role in the leveling off of the saccharification at approximately 70% conversion.

Another method of disrupting the crystalline structure of cellulose is multiple passes through a two roll compression mill. Ryu et al. (99) used this method in working with a two-phase kinetic model for cellulose hydrolysis. X-ray diffractograms of highly crystalline cellulose (Avicel) passed up to 35 times through the mill had a lower level of crystallinity. Corresponding saccharification of Solka Floc SW-40 treated in a similar manner matched the decrease in crystallinity to an increase in conversion to glucose. It is interesting to note that multiple passes resulted in a decrease in surface area of all cellulosic materials used. This was attributed to the collapse of the capillary structure of the materials via compression (99).

Paper mill wastes and chemical pulps represent materials which have the highest susceptibility to enzymatic conversion (4, 5). Such materials have been exposed to extensive processing both mechanical and chemical. The resulting material has a highly refined cellulose content and a more open physical structure (4). Pulping procedures such as beating cause fibrillation and swelling (4). Fibrillation causes individual fibrils to loosen and raises the small fibrillae on the surface of the fibers thus affording accessibility to the enzyme (4). Such

materials become more resistant upon drying and require extensive milling (ball milling) to restore their susceptibility (4, 5). Drying of cellulosic materials induces recrystallization and increases resistance to breakdown (29, 122).

Chemical Treatments. While the methods of physical treatments by wet milling and compression milling are significant, alternate methods (primarily chemical) can not be discounted. Use of cadoxen pretreatment on agricultural residue (lignocellulosic materials) has resulted in up to 99% conversion of the cellulose to glucose and reducing sugars (56). These results are impressive given the natural higher resistance of these materials (cornstalks, bagasse, alfalfa, fescue and orchard grass). Alkali pretreatment has resulted in a 2.3- to 6.8-fold increase in hydrolysis of similar materials (bagasse, wheat straw, cornstalks, rice husks) (25). Alkali treatment of newspaper was noted as the most successful method of increasing the susceptibility to hydrolysis by Mandels, Hontz and Nystrom (65).

The above mentioned studies were limited to a 1 to 5% concentration of substrate. This low concentration is a result of the difficulty of working with material which has a low bulk density (65). Katz and Reese (52) in an experiment to investigate the potential of cellulose conversion utilized high enzyme concentrations to produce a solution of greater than 30% glucose. The aim of the demonstration was to achieve slurries of glucose equivalent to that obtained in the starch industry.

Kinetics. Kinetics of hydrolytic catalysis by the cellulase complex of T. reesei have been studied by numerous authors (18, 49, 81,

92, 99). The models generally propose or illustrate mathematically a means of understanding and/or predicting cellulose degradation taking into account factors such as end product inhibition, enzyme-substrate adsorption/inactivation, reversion products, etc.

Enzymatic Hydrolysis Process Factors. Significant loss of enzyme is incurred in cellulose hydrolysis due to adsorption of enzyme on that portion of the substrate which resists further breakdown (18, 48, 84). Hydrolysis eventually becomes limiting due to this adsorption and a lack of reaction due to the unavailability of sites (18, 48). Castanon and Wilke (18) note the differences in enzyme affinity or release in later periods of hydrolysis as a function of the type of substrate used. For example, newspaper retains or adsorbs more enzyme than purer cellulose forms such as Solka Floc. They also found that the  $C_x$  enzymes (endoglucanases) of the cellulase complex were preferentially adsorbed initially in the hydrolysis of newspaper. The  $C_1$  (exocellobiohydrolase) was preferentially adsorbed later in the hydrolysis. Enzyme production represents about 60% (18) of total process costs in the system of Wilke et al. (133). Castanon and Wilke (18) note the importance of the ability to recover enzyme which may reduce enzyme process costs by 20 to 40% (18).

Much of the expense of enzymatic cellulose conversion is attributed to the large quantities of enzyme required to produce substantial concentrations of sugar solutions (as glucose) (66). The addition of  $\beta$ -glucosidase (cellobiase) substantially reduces the cellulase requirement by reducing the end product inhibition effect (66, 117, 119).

18, 66, 117, 119

Aspergillus phoenicis QM329 (Natick) is a high yielding  $\beta$ -glucosidase producer used to supplement T. reesei cellulase (119).

Application of crude enzyme preparations for hydrolysis has been employed in systems such as the simultaneous saccharification/fermentation process (27). In this process on-site enzyme production supplies cellulase to a pretreated cellulosic slurry and is fermented at 40°C. The combined fermentation and saccharification, according to the authors, affords a built-in removal system (yeast) for feedback inhibition products (27). A similar method on a lab scale involved employment of Saccharomyces cerevisiae at 30°C with good results (102). The above methods are a compromise of the optimum fermentation temperature for the organism used and the optimum temperature (50°C) and pH (4.8) for enzymatic hydrolysis using T. reesei cellulase (6, 63, 65, 114).

Investigations by Mandels et al. (66) on cellulase filtrates (crude enzyme) under use conditions compared the three best T. reesei cellulase producers--QM9414, MCG77 and Rutgers C-30. They reported an increase efficiency in saccharification using diluted enzyme. These authors also noted that lactose cultured cellulase was less effective in hydrolysis, requiring more units/gram substrate to produce equivalent sugar concentrations. In an earlier paper, Reese and Mandels (94) reported factors affecting stability of trichoderma cellulase, noting that shaking during hydrolysis and the use of certain preservatives detrimentally affects hydrolysis. Bacterial contamination (specifically Bacillus coagulans) directly affected the C<sub>1</sub> or crystalline activity (Avicelase) of the cellulase. The C-30 mutant cellulase with added  $\beta$ -glucosidase was the most stable cellulase.

## Cellulase Production

Cellulase Inducers. Cellulase production by T. reesei is induced by lactose (7, 41, 67, 68), sophorose (61, 67, 79), as well as cellulose. Lactose induces cellulase at a level of less than one-half that of cellulose (7, 100). Mandels, Parrish and Reese (67) found sophorose, 2-0- $\beta$ -D-glucopyranosyl-D-glucose, to be the most active inducer of cellulase for T. reesei and is specific for this organism. They discovered that sophorose occurs as a contaminant in reagent grade glucose at a level of 0.0058%. The relative activities of cellobiose, lactose and sophorose are 1, 20 and 2500 based upon one  $C_x$  unit induced by 1, 0.05 and 0.0004 mg of sugar respectively (67). Nisizawa et al. (79) found a distinct inhibition of cellulase production at higher levels of sophorose. They attribute this to catabolic repression from glucose derived by  $\beta$ -glucosidase action on the substrate. Thus, sophorose is active only at low concentrations (maximum activity at  $1 \times 10^{-3}$  M), and therefore produces low enzyme quantities (79). Lowenberg and Chapman (61) found that pulse feeding of sophorose at designated intervals was effective in obtaining higher cellulase levels. These authors note that the cessation of cellulase production coincides with the complete disappearance of inducer (sophorose). This trait was believed to be due to T. reesei's requirement of a constant source of inducer (61). Lowenberg and Chapman (61) state that because sophorose promotes cellulase formation much more rapidly than does cellulose, it is closer to the natural inducer than is cellulose, and, that cellulose serves as a substrate from which an inducer can be formed.

Because of the application demand for feasible processes, cellulase must be produced in quantities and activities suitable for direct use and as cheaply as possible. For these reasons cellulose has been and is the logical substrate for production. In addition, cellulose based media do provide the constant inducer source previously mentioned as a requirement in cellulase production (61).

Growth Inhibitors. Other compounds have been studied as inducer, stimulators/activators and enhancers of cellulase production. Cellulase production has been suggested as being inversely related to growth rate (7, 50, 100). Studies employing known growth inhibitors in small to trace quantities seem to show increased cellulase activity (106, 124). Stranks and Bieniada (124) employed phenethyl alcohol (PEA) as a growth inhibitor in experiments including several known cellulase producers. They noted significant increases in cellulase production using PEA (0.03% v/v) with Myrothecium verrucaria. Trichoderma reesei (viride) was least affected by addition of PEA (124). The experiments used 1% glucose as the substrate.

Shirkot et al. (106) found that small concentrations (0.1 to 0.4 ppm) of dithiocarbamates (fungicides) dramatically increased the yield of Avicelase (crystalline activity) and carboxymethyl cellulase (CMCase-amorphorous activity). The  $\beta$ -glucosidase activity was also increased in 8 days but was greatly diminished in 12 days (106). The authors suggest that by use of these fungicides high yields of Avicelase and CMCase may be preferentially obtained from T. reesei cultures. /

Stimulators/Activators. Gupta, Das and Gupta (41) found that additions of acetate, malate and alpha-ketoglutarate to a basal medium

plus 1% cellulose as a substrate increased cellulase yields from T. reesei. It was also found that ascorbate increased enzyme yield. The most interesting result of this study was the marked increase (two-fold) in enzyme yield when a combination of acetate and ascorbate at 0.1% each was employed. The authors were unable to explain this phenomenon.

Enhancement and Trace Elements. The inclusion of surfactants such as Tween 80 (polyoxyethylene sorbitan monooleate) in cellulase production media has been found to enhance enzyme yield (96). The method of enhancement is postulated as being a favorable membrane response in releasing the enzyme (96, 125). Thus, permeability of the membrane is suggested as a possible factor in enzyme yield although the authors note that the mechanism may be more complex since release or leakage of other enzymes is not encountered in all cases (96).

In addition to the standard nutrients such as carbon, nitrogen, potassium and phosphate which are provided in T. reesei production media (63), Mandels and Reese (68) determined that the magnesium and calcium ion ratio played a significant role in obtaining optimum cellulase yields. Their study (68) also revealed that certain trace elements aid maximal enzyme production. Cobalt was the only trace element active alone and best results are obtained with additions of iron, manganese, zinc and cobalt (68). Trace elements are required for enzyme production but not for growth except on pure cellulose (68). Metal ions do not play a role in the activity of Trichoderma reesei cellulase (68).

Nitrogen Sources. Cellulase production media employ the essential growth elements of potassium, phosphorous, nitrogen, calcium and magnesium as per Mandels and Reese (68). Trichoderma species in general grow best with amino nitrogen, ammonium ion ( $\text{NH}_4\text{OH}$ ) as the next best source, followed by urea and nitrate (22). Gupta, Das and Gupta (41) found a combined source of nitrogen to be superior in a liquid medium. A trace element supply of iron, manganese, zinc and cobalt is provided as recommended by Mandels (63). The Mandels method (63) supplies proteose peptone at one-tenth of the cellulose level employed. Yeast extract can be substituted for the proteose peptone (62). Use of Tween 80 is optional (63).

Trichoderma reesei Strain Variations. The hyperproducing cellulase strains vary in their response to inducing substrates and fermentation conditions (7, 125). Andreotti et al. (7) made a comparative study on the Natick and Rutgers mutants and found that Rutgers (Rut) C-30 was the highest producer on 6% ball milled cellulose pulp and the Rut NG-14 was the highest producer on 6% compression milled cotton. MCG77 (Natick) was the superior producer on lactose with Rut C-30 slightly below it on 6% lactose medium (7). The other strains produced low levels of enzyme on 6% lactose.

Glucose represses cellulase synthesis in T. reesei (33). MCG77, Rut NG-14 and Rut C-30 have the advantage of resistance to catabolic repression (33, 74, 125). In particular, MCG77 and Rut C-30 have the ability to recover full cellulase producing capacity after growth on or introduction of glucose (33, 74). Rut NG-14 does so to a lesser extent (74). This characteristic allows the convenient propagation of heavy



inoculums for these two strains on glucose medium for subsequent cellulase production (33, 125).

### Production Methods

Sternberg (118) investigated the pH optimum, nitrogen requirements and an increased cellulose level for improved enzyme yields. Uncontrolled pH resulted in dramatically reduced enzyme activity in media with greater than 0.75% w/v cellulose levels (118). He found that the best yields could be obtained by controlling the pH during the fermentation to not less than 3.0 or greater than 4.0. A controlled pH of not less than 3.5 favored higher  $\beta$ -glucosidase activity. Without pH control the pH in 2% w/v cellulose medium would drop to 2.5 diminishing the yield by inactivation of the enzyme.

Sternberg and Dorval (120) used ammonium hydroxide to control pH in a lower salt content medium employing cellulose levels of 2 to 8% w/v for high cellulase yields. In both studies (118, 120), a pH profile with a fairly rapid drop to the control level (pH 3.5), holding for several days, and a subsequent rise is described. The pH rise is attributed to ammonia release by the fungus (120). Sternberg (118) notes that  $\beta$ -glucosidase formation lags 1 to 2 days behind cellulase production coinciding with the rise in pH. Maximum enzyme yields were obtained on 8% cellulose although yields at 6% cellulose were only slightly lower (120). Sternberg and Dorval (120) mention the necessity of using a high inoculum level (20% v/v) for the 8% cellulose level.

Other production methods for enhanced cellulase production involve mixed culture fermentation of T. reesei and a yeast (82), pH

cycling and temperature profiling (76, 125) and continuous culture methods using T. reesei (34, 83, 100).

Peitersen (82) added an inoculum of either Candida utilis or Saccharomyces cerevisiae 24 to 32 h after initiating cellulase production using T. reesei QM9123 (Natick). He notes the earlier appearance of cellulase due to the competitive uptake of glucose by the yeast. The cellulosic material was not further utilized by use of the yeast culture (82).

Mukhopadhyay and Malik (76) employed a production strategy in a batch operation whereby they varied the temperature and the pH during the fermentation. They used higher temperatures during the growth phase. They also cycled the pH during the acid phase of enzyme production by radically adjusting from the control point (pH 3.0) to above pH 5.0, repeating this procedure once the pH fell to the control point again. The authors claimed an increase in yield using this strategy and believed that a shift in states of equilibrium and the different conditions produced during the fermentation were responsible for this increase.

The most promising and productive cellulase production methods are the continuous processes. Peitersen (83) employed a cellulose medium stream of 0.5 to 1.0% w/v cellulose feeding a single stage reactor on a continuous basis. The results were a low enzyme activity product, 10 to 30% the activity of a batch production, and a production value of 12-13 International Units (IU)/l/h.

Ghose and Sahai (34) used a single stage reactor with cell recycle which resulted in cellulase of 1.0 to 1.2 IU/ml depending upon

the nitrogen source employed. This system produced 30 IU/l/h using T. reesei QM9414 (Natick).

Ryu et al. (100) reported the most successful and informative continuous cellulase production system. These authors employed the T. reesei strain MCG77 with lactose as the inducer/carbon source. They operated the process for 1350 h on a continuous basis. This process involved the use of a seed or biomass production vessel feeding into an enzyme production vessel. They determined the optimal enzyme production rate of the second vessel to be  $0.027 \text{ h}^{-1}$ . At this rate a production value of 90 IU/l/h was obtained. Ryu et al. (100) project the theoretical enzyme productivity to be approximately 300 IU/l/h from this system based on biomass concentrations obtainable in the process.

Comparison of production of batch and continuous processes have been made by Ghose and Sahai (34) and by Mukhopadhyay and Malik (76). Mukhopadhyay and Malik (76) infer that the method they employ of controlled temperature profiling and pH cycling in batch mode is competitive if not superior to the system of Ryu et al. (100). In actuality a comparison of the two systems reveals that the system of Ryu et al. (100) utilizes 56% of the fermenter volume required by Mukhopadhyay and Malik (76) and produces 14.5% more enzyme as IU/h in a respectable concentration (5.6 IU/ml). This is a result of the activity of the enzyme produced, the volume or retention time employed, and the total production time involved. In addition, the system of Ryu et al. (100) sustained an operating time well beyond that which a single batch system is capable of achieving without duplication of facilities (i.e., 1350 h [100] vs. 170.5 h [76]).

The results of Ryu et al. (100) suggest that the second stage or enzyme production stage should be maintained at zero or negative growth rate. They further relate that this implies that the cells in the second stage are under conditions of resting cells or slight degeneration. This is significant in that much of the total enzyme realized in a production system is released into the medium under stationary phase conditions and lysis, later in the production profile (i.e., after 48 to 72 h) (8, 9, 33, 69, 118, 120).

#### Assessing Cellulase Enzymes

Methods of determining cellulase activity are compounded by the complex nature of this enzyme (17). Mandels (63) compiled methods generally accepted as indicative of expressing the activities of the various components which make up the cellulase enzyme. These methods are subject to some degree to the individual investigator's interpretation of whether they truly reflect what he wishes to illustrate and his faith in the method employed (64). As a result, a multitude of methods and modifications of methods (quantitative and qualitative) can be found in the literature (1, 2, 3, 19, 36, 37, 38, 59, 63, 64, 73, 74, 75, 85, 108, 111, 123).

Mandels, Andreotti and Roche (64) maintain that the filter paper assay (units as FPU or IU) developed and employed by U.S. Army Natick Research Laboratories fulfills the requirements for expression of a complete cellulase being relatively fast, simple, reproducible and quantitative. However, modifications of even this assay have been proposed and employed (37, 74, 75). An excellent example is the

modification employed by Montencourt and Eveleigh (74). These authors employed the method of Mandels et al. (64) except they diluted the enzyme to obtain reducing sugar values which would fall within their standard curve limits (74). This modification was very useful for the large number of assays required in this study. It readily points out the tedium and exertion required to perform the method of Mandels et al. (64) when high numbers of samples are encountered. These authors also point out that the values resulting from their modification reflect higher than actual yields (74).

Canevascini and Gattlen (17), in a comparative study of various cellulase assays, found that for T. reesei the effects of dilution in the filter paper assay of Mandels et al. (64) related reasonably well to the actual activity in dilutions of the enzyme from 1:5 to 1:30. They further assert the problem of comparing studies in cellulase activity and conclude that the most specific, sensitive method is the viscometric method for estimation of endo-cellulase activity. They also note the continued lack of an assay which can quantitate the exo-cellulase (cellobiohydrolase) component in unfractionated culture filtrates.

#### Dimethyl Sulfoxide

As previously mentioned, various inducers (7, 41, 61, 67, 68, 79), growth inhibitors (106, 124), stimulators/activators (41) and enhancers (68, 96) have been employed in the search to improve cellulase yields from T. reesei strains. At least one method (Tween 80 addition) is postulated as favorably affecting the release of enzyme through the

cell membrane (96, 125). Such manipulations via cell treatment or stimulation leads to a search for other similarly acting chemicals and compounds not unlike that encountered in the search for a solvent capable of solubilizing cellulose (i.e., cadoxen, metal amine complexes, etc.) (51, 56).

One potential candidate is the aprotic solvent (89) dimethyl sulfoxide (DMSO). DMSO is noted for its use as a therapeutic agent and as a carrier for its ability to penetrate the skin in topical applications (132). Weyer (132) reviews some medical and clinical applications of DMSO in this capacity. Technical applications of DMSO involve: cold solubilization of starch, solubilization of nitrocellulose, solubilization of lignin and hemicelluloses, a solvent for proteins, use in the manufacture of sugar esters of fatty acids, as a cryogenic agent, as a carrier liquid for herbicides, insecticides, and bactericides, influencing the uptake of transition metals in plants, and other applications (46).

Franz and Van Bruggen (32) investigated the mechanism of the penetrating action of DMSO using frog skin as a representative tissue membrane. In their study they found a three-fold increase with sodium ion and a six-fold increase with chloride ion in the rate of influx through the membrane in the presence of 2.5% DMSO. Nonelectrolyte materials (urea, mannitol and sucrose) also moved across the membrane more rapidly in the presence of DMSO (32). These authors noted that no permanent change in the membrane was effected by 5% DMSO, requiring only a rinse and the membrane returned to normal parameters. Rammler

(88) found that DMSO has a stimulatory effect on several enzyme catalyzed hydrolytic reactions.

Closer to the application of DMSO on microorganisms is the study by De Bruijne and Van Steveninck (23) in which a yeast (Saccharomyces cerevisiae) was used to study the phenomenon of asymmetrical influx and efflux of DMSO through the yeast cell membrane. They determined that DMSO penetrates the yeast cell membrane with simple first-order kinetics; no active transport is involved in the process. These authors found a discrepancy between growing yeast cells and mature or resting stage yeast cells. In growing cells the rate of influx approximately equaled the rate of efflux from the cell (23). In resting cells the rate of efflux was significantly higher than the rate of influx (23). They suggest that this may be due to a membrane structural and functional change during the life cycle of the yeast cell (23).

DMSO has a stimulatory effect on vegetative growth of a number of bacteria, yeasts and molds. Herschler (47) found that low levels (20-500 ppm) of DMSO added to conventional growth or production media effectively stimulated vegetative cell growth while depressing spore formation. He found that the addition of DMSO increased the yield and/or decreased the production time of desirable microbial metabolites such as enzymes, alcohols, polysaccharides, etc. Concentrations above 3% by weight of DMSO have a biocidal or biostatic effect. Aerobic processes (antibiotic fermentations, sewage treatment, etc.) respond better to DMSO addition than anaerobic processes (47).

The purpose of this study was to investigate the production of Trichoderma reesei cellulase at pilot plant scale employing used dairy equipment. Basic production methods and requirements for production were investigated as well as the limits of this system.

The effects of dimethyl sulfoxide on a higher mutant of T. reesei (MCG77) were also investigated in laboratory shaker flask studies.



## MATERIALS AND METHODS

Stock Cultures

Trichoderma reesei strains QM9414 and MCG77 were obtained from Dr. Mary Mandels, U.S. Army Natick Research and Development Command, Natick, MA.

Strain QM9414 was maintained on Potato Dextrose Agar (BBL) slants. Strain MCG77 was maintained on a 1.5% agar slant containing Vogels salts (129) supplemented with 1% Solka Floc BW200 cellulose (Brown Co., Berlin, NH), 5.0 micrograms/l biotin, 0.5% yeast extract and 0.5% glycerol (62). Slant cultures were stored at 4°C and transferred once per month.

MCG77 deteriorates with several passes on alternate media (62) therefore a more dependable stock culture reservoir was desired. Soil stocks were made for spore storage for both QM9414 and MCG77.

Soil Stock Procedure. A good loamy soil was dried for 5 days at 60°C in a convection oven and screened. Approximately 3 g of soil was transferred to each 16 x 150 mm test tube and cotton plugged. The tubes plus soil were autoclaved for 20 min at 121°C, 15 psi steam, fast exhausted and dried. The medium was then returned to the convection oven (60°C) for 48 h. The test tubes plus soil were cooled to room temperature and the contents of one tube transferred aseptically to 100 ml of a 1% glucose nutrient medium and incubated at 30°C for 48 h. The nutrient medium was checked for growth microscopically.

For spore inoculum the original MCG77 culture was transferred once to the MCG77 maintenance medium to minimize any deterioration that

might be incurred by multiple passes. QM9414 was also cultured on the MCG77 maintenance medium. The organisms were cultured in petri plates (25 ml medium/plate) for maximum surface exposure and a heavy spore cover. By inoculating in the center of the petri plate an outward progressing ring of mycelium and spores formed in 7 to 10 days at 30°C.

A 2 x 2 x 1 cm triangular section of agar and culture was aseptically removed from the outer ring of the culture and placed in a sterile petri dish for washing. Spores were washed off the section using 0.5 ml of sterile distilled water and a 1 ml pipet. A heavy spore suspension resulted of which 3 to 5 drops were aseptically transferred to the soil medium. The clot or ball that formed upon addition of the spore suspension to the dry soil was broken up by mixing with a sterile pasteur pipet which had the capillary end flame sealed. The spore suspension was mixed thoroughly with the soil for even distribution and the tubes left at room temperature for 5 days before storing at 4°C. Stock cultures were in triplicate for each strain.

The soil stocks allowed returning to the spores from the first pass of the original MCG77 culture avoiding possible mutation of this strain from passing on agar media. Transfer of the soil stock to the maintenance medium may be facilitated with a loopful of soil as inoculum. An active culture of MCG77 strain was maintained for spore inoculum by successive passing on the maintenance medium for two to three transfers before returning to the soil stock for new inoculum. The QM9414 culture was passed successively with no apparent deterioration. The soil stock was used when the transfer sequence was interrupted by extended periods of time between experiments.

## Media

MCG77 Maintenance Medium. The MCG77 maintenance medium used the Vogels salts solution (129) according to the formula of Dr. Gallo (62).

A 50X stock solution of the Vogels salts was prepared by adding to 700 ml distilled water: 150 g  $\text{Na}_3$  Citrate  $\cdot$   $5\text{H}_2\text{O}$  (or 117 g  $\text{Na}_3$  Citrate  $\cdot$   $2\text{H}_2\text{O}$ ), 250 g  $\text{KH}_2\text{PO}_4$ , 100 g  $\text{NH}_4\text{NO}_3$ , 10 g  $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ , 5 g  $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$  and 5 ml each of the Vogels trace element solution and biotin solution. The medium was diluted to 1 liter and stored at room temperature.

The Vogels trace element solution was prepared by adding to 90 ml distilled water: 5 g Citric acid monohydrate, 5 g  $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$ , 1.0 g  $\text{Fe}(\text{NH}_4)_2(\text{SO}_4)_2 \cdot 6\text{H}_2\text{O}$ , 0.25 g  $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$ , 0.05 g  $\text{MnSO}_4 \cdot 4\text{H}_2\text{O}$ , 0.05 g  $\text{H}_3\text{BO}_3$ , 0.05 g  $\text{NaMoO}_4 \cdot 2\text{H}_2\text{O}$ . The solution was diluted to 100 ml with distilled water.

The biotin solution was prepared by adding 5 mg biotin (Sigma) to 100 ml of 50% ethanol and distilled water.

Addition of the 50X Vogels solution was 20 ml per liter of ~~medium~~ plus 0.5% yeast extract, 0.5% glycerol, 1% BW200 cellulose and 1.5% agar. After autoclaving, the medium was allowed to cool until it began to gel before pouring petri plates or setting slants to maintain suspension of the cellulose.

The cellulose substrate employed was Solka Floc SW-40 or BW200 (Brown Co., Berlin, NH). Solka Floc is a purified form of alpha-cellulose derived from spruce wood of approximately 95% purity in 40 mesh (SW-40) or 200 mesh (BW200) fibers.

QM9414 Production Medium. The QM9414 production medium was prepared as described by Mandels (63) omitting the urea and substituting yeast extract for proteose peptone. The QM9414 production medium contained per liter: 1.4 g  $(\text{NH}_4)_2\text{SO}_4$ , 2.0 g  $\text{KH}_2\text{PO}_4$ , 0.3 g  $\text{CaCl}_2$ , 0.3 g  $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ , 2 ml Tween 80 and 1 ml trace element solution.

The trace element solution for the QM9414 production medium contained: 495 ml distilled water, 5 ml concentrated HCl, 2.7 g  $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$ , 0.77 g  $\text{MnSO}_4 \cdot \text{H}_2\text{O}$ , 0.83 g  $\text{ZnCl}_2$  and 0.55 g  $\text{CoCl}_2$  for a total volume of 500 ml. The concentration of trace elements in the final medium is 1 ppm of iron, 0.5 ppm of manganese, 0.8 ppm of zinc and 0.5 ppm of cobalt.

Cellulose as Solka Floc SW-40 or BW200 was added at 0.75 to 4% w/v. Yeast extract was added at a level of two-tenths the cellulose concentration employed.

MCG77 Production Medium. The MCG77 production medium contained the same concentrations of nitrogen, potassium, phosphate and calcium as the QM9414 production medium but utilized higher levels of magnesium and trace elements (100).

The MCG77 production medium contained per liter: 1.4 g  $(\text{NH}_4)_2\text{SO}_4$ , 2.0 g  $\text{KH}_2\text{PO}_4$ , 0.3 g  $\text{CaCl}_2$ , 0.6 g  $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ , 2 ml Tween 80 and 1 ml trace element solution. The salts for this medium were prepared in a 50X solution containing per liter: 70 g  $(\text{NH}_4)_2\text{SO}_4$ , 100 g  $\text{KH}_2\text{PO}_4$ , 30 g  $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$  and made to volume with distilled water. The solution was stored at 4°C. Addition of the 50X solution was 20 ml per

liter of medium. Calcium was added as a 3 ml aliquot of a 10% w/v  $\text{CaCl}_2$  solution per liter of medium.

The MCG77 trace element solution contained: 495 ml distilled water, 5 ml concentrated HCl, 5.0 g  $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$ , 1.6 g  $\text{MnSO}_4 \cdot \text{H}_2\text{O}$ , 0.665 g  $\text{ZnCl}_2$  and 2.0 g  $\text{CoCl}_2$  for a total of 500 ml. The concentration of trace elements in the final medium is 2.0 ppm of iron, 1.0 ppm of manganese, 0.64 ppm of zinc and 1.82 ppm of cobalt.

Cellulose as Solka Floc BW200 was added at a 1.0% w/v level. Yeast extract was added at a level of two-tenths the cellulose level (or 0.2% at the 1% cellulose level).

### Production

Inoculum. Spore inoculum for both the QM9414 and MCG77 was obtained from 7- to 14-day-old maintenance medium petri plate cultures. Spores were washed as in the soil stock culture procedure except 1 ml of distilled water was employed and 0.4 ml of the spore suspension was used as inoculum. Agar-culture sections from the outer ring of the petri plate culture were selected for washing according to Stavy, Stavy and Galum (115).

QM9414 Inoculum. QM9414 spores were transferred into 250 ml erlenmeyer flasks containing 100 ml sterile QM9414 production medium with a 0.75 to 1.0% cellulose level. The flasks were incubated at 29 to 30°C on a NBS Bench-Top reciprocating shaker (New Brunswick Scientific, Edison, NJ) at 250 rpm for 24 to 48 h until good mycelial growth was obtained. Growth was checked by microscopic examination and indicated by a drop in pH from 4.8 to < 3.0. The culture was

aseptically transferred to a sterile 250 ml blender jar and homogenized for 30 to 60 seconds on an Osterizer blender. Fragmentation of the culture in this manner is known to increase the efficiency of a fungal inoculum (31).

Two hundred ml of the fragmented culture was used to inoculate 5 l of sterile QM9414 production medium containing 1% cellulose in a 7.5 l NBS MicroFerm laboratory fermenter. Filter sterile air was set at 2.0 l/min, agitation at 250-400 rpm, and temperature control at 28°C. The culture was incubated for 24 to 48 h and checked in the same manner as the shaker culture. Pilot plant runs utilized the 5 l NBS MicroFerm culture as inoculum. Only QM9414 was run at pilot plant scale.

The pilot plant fermenter was a 757 l stainless steel dairy culture vessel (Creamery Package Corp., Chicago, IL) equipped with agitation, pH monitor/control (Electrofact-Control Data Corp., Minneapolis, MN), heating/cooling coils and filter sterile air supply. A 19 l polyethylene reservoir provided 2N  $\text{NH}_4\text{OH}$  for pH adjustment via pH feedback and cellonoid control valves. The pH control was frequently manual. Antifoam C (Dow Corning) was added as needed manually.

Scale-up of the QM9414 production medium to the working volume of 190 l was accomplished by adding to tap water: the standard salts, yeast extract (as Milbrew DBY series #1, Amber Laboratories, Juneau, WI) and 0.75-4% w/v cellulose (Solka Floc). These ingredients were boiled for 10 min in the vessel and cooled to 10°C. This procedure was repeated and the medium sampled for contaminants. The trace elements and Tween 80 additions were autoclaved separate from the medium and

added prior to inoculation. The temperature was adjusted to and maintained at 28°C for production.

The 5 liters of culture propagated in the NBS MicroFerm provided a 2.6% v/v inoculum to the pilot plant batch. An NBS fermenter culture was run in parallel to the pilot culture by addition of fresh sterile medium to the NBS fermenter and reinoculated with a similar inoculum volume. Figure 4 illustrates the method employed.

MCG77 Inoculum. Spore inoculum for the MCG77 experiments was obtained in the same manner as the QM9414 procedure. The MCG77 mycelial inoculum was obtained by incubating the spores in 250 ml erlenmeyer flasks containing 100 ml MCG77 production medium using 1% lactose instead of cellulose. The lactose MCG77 medium provided a clear, amber-colored solution in which a contaminated culture would often appear as off-colored, grayish mycelium allowing macroscopic observation of culture quality. Contamination was rarely encountered. Incubation conditions and culture treatments were identical to the QM9414 procedure.

For the dimethyl sulfoxide (DMSO) experiment, six 1 liter erlenmeyer flasks containing 500 ml each of sterile MCG77 production medium at 1% cellulose (BW200) were inoculated with 5 ml/flask of a fragmented 24- to 48-hour-old MCG77 culture. DMSO levels were 0 ppm (control), 50 ppm, 100 ppm, 200 ppm, 300 ppm and 400 ppm using filter sterile DMSO. The flasks were incubated on an NBS Gyrotory shaker with environmental chamber at 28-30°C and 300 rpm. The cultures were checked for contaminants via Gram stain at 24 h. The pH was checked by aseptically removing 2 ml of culture using a sterile 10 ml wide

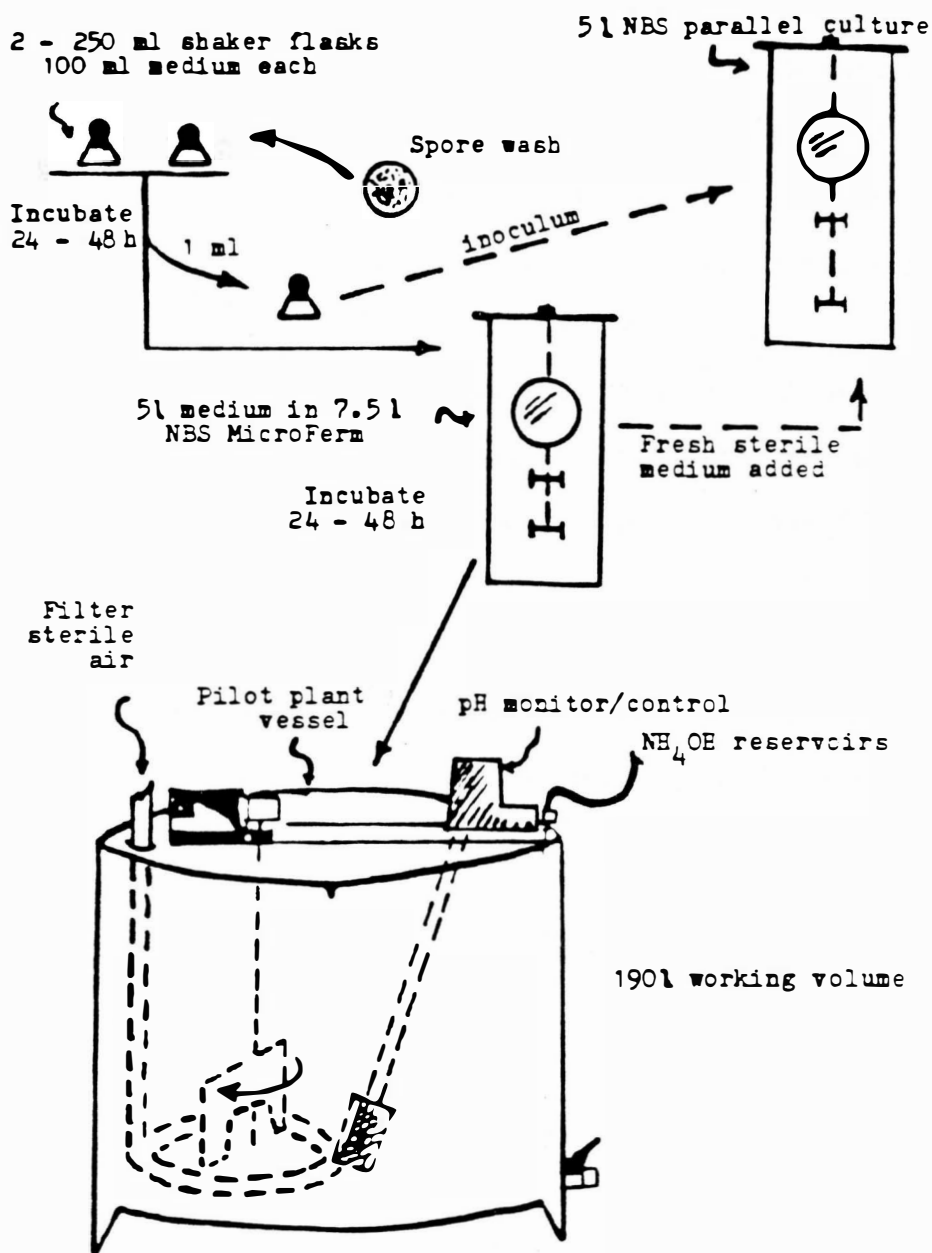


Figure 4. Method of pilot plant inoculum preparation for cellulase production using *T. reesei* QM9414.



bore pipet and transferring to an 18 x 150 mm test tube for pH measurement to the nearest 0.01 unit with a Corning model 130 pH meter. The pH was monitored by measuring samples taken at approximately 24 h, then at 36, 42, 48, 60, 72, 74, 76, 78, 80, 84, 90 and 96 h. The pH was adjusted to  $> 3.0$  to  $< 3.5$  by addition of 6.5 N  $\text{NH}_4\text{OH}$  dropwise to the culture. Ten samples for enzyme assay were drawn at 48, 60, 72, 74, 76, 78, 80, 84, 90 and 96 h. For enzyme samples, 5 ml of culture was removed, centrifuged at high speed on a lab centrifuge, the supernatant fluid collected in 13 x 100 mm test tubes, capped with parafilm and refrigerated at  $4^\circ\text{C}$  until assayed.

For the whey-cellulose experiment, Amber SWP, series 1, demineralized whey product (Amber Laboratories, Juneau, WI) was used as a source of lactose. This product contained 86.5% lactose, 9.5% protein, 1.0% ash, and 3.0% moisture.

### Saccharification

Saccharification of finely ground newspaper as a representative waste material was performed on lab and pilot plant scale by employing enzyme produced on the project. The newspaper was 63.5% cellulose as analyzed by the forage fiber procedure. Reducing sugars were measured by the dinitrosalicylic acid (DNS) method.

Laboratory saccharifications were performed in the NBS Gyrotory shaker with environmental chamber at  $50^\circ\text{C}$  and 250 rpm. The pH was maintained at 4.8 by 0.05 M sodium citrate buffer as diluent or by adjustment of the enzyme using 1 M sodium citrate buffer (63).

The pilot plant saccharification was accomplished by harvesting approximately 170 l of cellulose culture fluid produced in Run #2 using strain QM9414. This fluid was crude filtered through a dairy sock filter (Kendall D-577) to remove mycelium and transferred to a 1300 l dairy pasteurization vessel (Girton Dairy Eq., Millville, PA) equipped with agitation (40 rpm) and temperature control. The enzyme was assayed at 2.6 IU/ml and was diluted for saccharification 70:30, crude enzyme solution to water. A 5% w/v newspaper slurry was obtained by adding 12.15 Kg of finely ground newspaper to the diluted enzyme. Temperature control was set to maintain 50°C and a pH of 4.8 was obtained by addition of 750 ml of 1 M sodium citrate buffer (pH 4.8). The total volume was 243 l and total time of saccharification was 24 h.

### Assays

Glucose as reducing sugar was measured by the dinitrosalicylic acid (DNS) method as modified by Miller (71). A standard curve using reagent grade anhydrous glucose was prepared with each analysis. The range for the standard curve was 0.25 to 1.25 mg glucose/ml in increments of 0.25 mg for 5 points. The standard curve was plotted as absorbance vs. mg glucose/ml from spectrophotometer readings (Spectronic 20, Bausch and Lomb). The line of best fit for the curve was calculated using the method for 5 points in the A.O.A.C., 13th edition, section 42.200.

The cellulase enzyme was measured using the filter paper assay for complete cellulase according to Mandels et al. (64) as modified by Montencourt and Eveleigh (74). Whatman #1 filter paper cut into 1 x 6

cm strips (50 mg) was the substrate. The strips were coiled and placed in 18 x 150 mm test tubes and 1 ml of 0.05 M sodium citrate buffer (pH 4.8) was added. The enzyme was diluted with the sodium citrate buffer to anticipate a reducing sugar value within the standard curve range. Two dilutions per enzyme sample were assayed in triplicate. The enzyme activity in IU/ml was derived from the dilution which fell within the 0.5 to 1.0 mg/ml reducing sugar range used by Montencourt and Eveleigh (74). This method results in higher apparent activity than the method of Mandels et al. (64) but is a more convenient method of assaying the enzyme since less dilutions are involved when a large number of samples are to be assayed (74). In the event of both dilutions resulting in reducing sugar values falling in the 0.5 to 1.0 mg range, the corresponding IU/ml activity was averaged.

One-half ml of enzyme solution was added to the tubes and incubated for 1 h at 50°C in a circulating water bath. The reaction was stopped by addition of 3 ml DNS reagent and the tubes placed in a boiling water bath for 5 min. The tubes were allowed to cool and the solution diluted to 20 ml with distilled water. The tubes were inverted several times for mixing and read on the spectrophotometer at 550 nm for % transmittance (%T). Percent T was converted to absorbance and the glucose produced (as reducing sugars) read from the standard curve plot. The reducing sugar value from the average of the triplicates was corrected for residual glucose in the enzyme by assaying enzyme blanks for reducing sugars. Units of enzyme were calculated from the equation:

(mg glucose x dilution) x 0.185 = FPU/ml (Filter Paper Units/ml)  
or IU/ml (International Units/ml).

The absorbance readings from the spectrophotometer were subjected to analysis of variance procedure (116). The yield relationships for each dilution were also checked by analysis of variance.

The cellulose content of the newspaper was determined by the forage fiber analysis method (35).

## RESULTS AND DISCUSSION

Pilot Plant Runs Using QM9414

Enzyme Production. The pH profile of T. reesei in submerged culture cellulase production is used as an indicator of growth and cellulase production in cellulose based media (118, 120). Growth is indicated by the rapid drop in pH after inoculation and cellulase activity correspondingly rises with the pH in the later stages of fermentation. The pH rise can thus be used as an indirect means of determining when to harvest the enzyme.

The 190 l pilot plant runs at 0.75, 2.0 and 4.0% cellulose produced different pH profiles (Fig. 5). The principle differences are the time required for the pH to drop to the control point of 3.0 and the time required in achieving the subsequent pH rise. These differences are most apparent in the 4.0% cellulose culture medium. This medium displayed a visible increase in viscosity as growth ensued. This factor is believed to have impeded oxygen transfer and proper mixing in the production vessel. Although the viscosity change and mixing problems were not as apparent in the 2% cellulose medium runs, these factors no doubt contributed to the longer production time and to the resulting yields in the pilot plant vessel.

The design of the pilot plant vessel is not optimal for oxygen transfer. The height (or depth) of the working volume of the vessel is exceeded by the diameter. This factor does not allow for extended contact of the air with the medium (113). Higher cellulose levels in the production medium resulted in dead zones due to the improper mixing

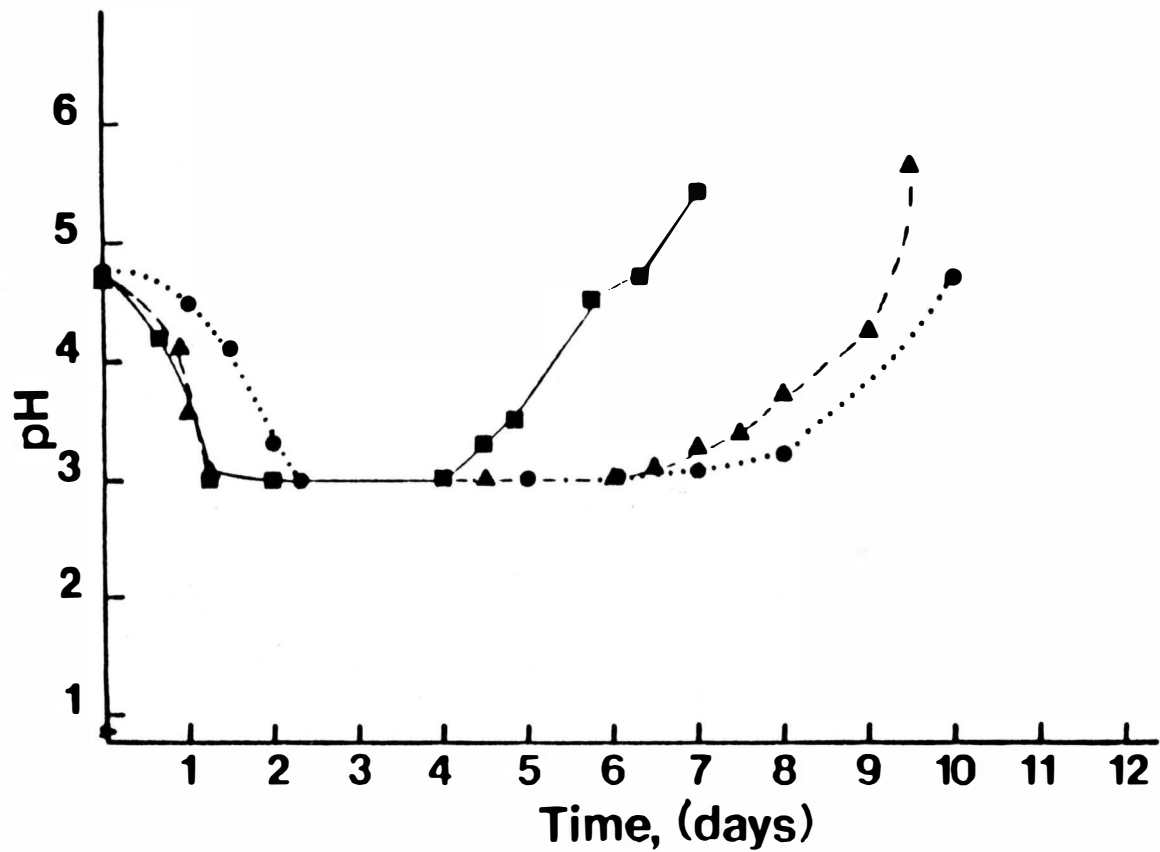


Figure 5. Pilot plant pH profiles for submerged culture cellulase production using *T. reesei* QM9414 on QM9414 production media at 0.75% w/v cellulose (■), 2% w/v cellulose (▲), and 4% w/v cellulose (●).

in the vessel. The advantages of optimal fermenter design are seen when a comparison is made of the NBS lab fermenter run in parallel to the pilot plant (Table 1). The NBS parallel culture shows a higher enzyme activity in a shorter time in all cases for the 2 and 4% cellulose media.

Variation in the pH profile of the pilot plant runs from those reported by Sternberg and Dorval (120) are due to the inoculum levels employed. Sternberg and Dorval (ibid.) used a 10% v/v inoculum vs. the 2.6% inoculum level available in this study. The above authors encountered difficulties in the 8% cellulose medium similar to those observed in this work for the pilot plant runs at the 4% level. Sternberg and Dorval (120) remedied the lag and low production in the 8% cellulose medium by increasing the inoculum level to 20%. In the pilot plant runs using the 4% cellulose medium in the present study, the low yields realized in Runs #4 and 5 could not be attributed entirely to low inoculum levels since a respectable enzyme activity was obtained in the parallel NBS lab fermenter for these runs (Table 1). The yield results obtained in the NBS lab fermenter on the 4% cellulose level do not indicate that there was a deficiency in nutrients or in essential trace elements of the medium.

The progression of improved yields recorded in Table 1 for the 4% cellulose medium pilot plant runs was the result of an improved aeration method and an increase in the rate of aeration. The improved yield in Run #6 at the 4% cellulose level shows no advantage over the yield obtained at the 2% cellulose level in Run #2 and indeed the 4% level required more time to achieve a comparable enzyme activity. Pilot

Table 1. Production data for pilot plant batch runs using T. reesei QM9414 in 190 l production media at different cellulose levels.

Run	% Cellulose	pH Control point	Pilot plant yield	NBS parallel yield	Time	Pilot plant production rate
			IU/ml	IU/ml	days	IU/l/h
1	0.75	3.0	1.46	--	7	8.69
2	2.0	3.0	2.08 2.60	2.21	6 10	14.44 10.83
3	2.0	3.5	1.32 1.79	1.76	8 12	6.88 6.22
4	4.0	3.0	0.93	2.64	10	3.88
5	4.0	3.0	1.03 1.16	3.56	8 10	5.36 4.83
6	4.0	3.0	2.21 2.64	--	11 14	8.37 7.86



plant Run #2 produced an enzyme activity comparable to that obtained by Sternberg and Dorval (120) at the 2% cellulose level. However, when production is related in terms of IU/l/h the Sternberg and Dorval study showed in an enzyme production rate of 21.6 IU/l/h compared to a rate of 10.8 IU/l/h for this study. The difference is attributed to the difference in inoculum levels employed (10% vs. 2.6%) allowing Sternberg and Dorval (ibid.) to achieve the same activity in 5 days vs. 10 days for the pilot plant run reported here (Table 1).

Saccharification. The saccharifying ability of the cellulase enzyme was investigated employing finely milled newspaper in a 5% w/v solids slurry. Laboratory shaker flask saccharifications were performed using dilutions of a 0.79 IU/ml cellulase produced in the NBS lab fermenter on 1% cellulose QM9414 production medium. Figure 6 shows the saccharification curves for the diluted vs. undiluted enzyme. The undiluted enzyme shows a higher initial hydrolysis rate at the 4 h time period. In 24 h however, all enzyme solutions achieved a comparable level of hydrolysis. Thus, enzyme efficiency is greater using diluted enzyme. Mandels et al. (66) found a similar result in a study comparing diluted enzyme solutions on newspaper slurries. No explanation was offered for this reaction. Dilution may lessen any enzyme-enzyme inhibition between the cellulase complex components. Such a response has been noted in dual enzyme systems where one enzyme has a higher affinity for a coenzyme which is necessary for both enzymes and their concerted action on the substrate (26).

The 70:30 dilution, enzyme to diluent, was used as a guide for the pilot plant saccharifications of a 5% w/v newspaper slurry. Crude

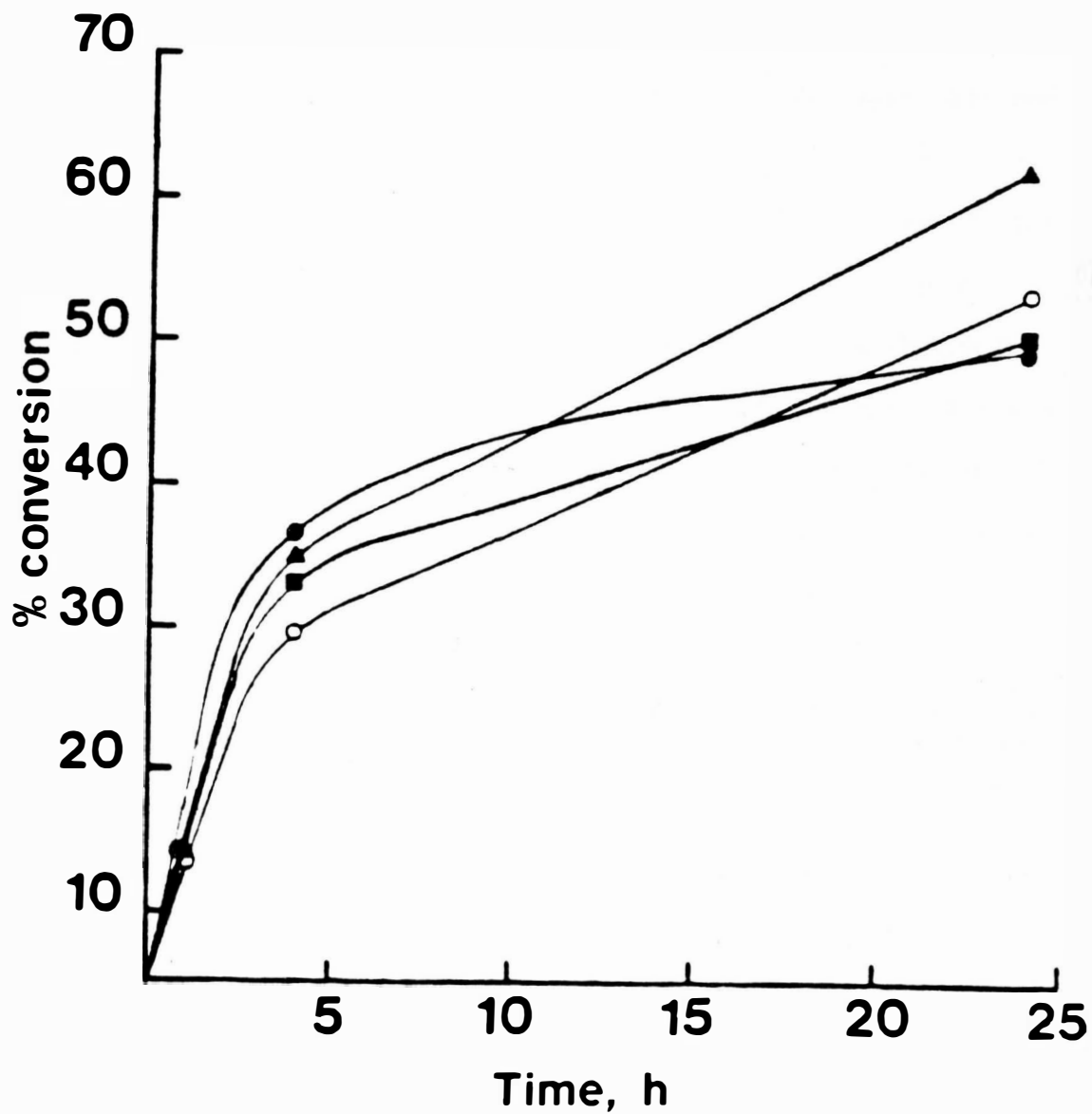


Figure 6. Laboratory conversion of a 5% w/v newspaper slurry using dilutions of a 0.79 IU/ml *T. reesei* cellulase. Dilutions (enzyme:0.1 M sodium citrate buffer) were: 90:10 (○), 80:20 (■), and 70:30 (▲) and undiluted crude enzyme (●). The saccharification was performed at 50°C, pH 4.8 and 250 rpm.

filtered enzyme was extended by dilution 70:30 and operated under the conditions for saccharification for 24 h. The results are shown in Table 2. Conversion of the cellulose portion of the newspaper obtained in the pilot plant was comparable to that obtained in the lab in 24 h. The slightly higher pilot plant saccharification is probably due to the higher cellulase activity of the enzyme employed in the pilot plant (2.6 IU/ml vs. 0.79 IU/ml in the lab) and to the reduced agitation in the pilot plant (40 rpm vs. 250 rpm in the lab). Reduced agitation may have favored higher conversion in the pilot plant since shaking has been found to be detrimental to saccharification of cellulose using T. reesei cellulase (94).

#### Lactose/Cellulose Induction by MCG77

The MCG77 strain of T. reesei is capable of producing cellulase induced by lactose (7, 41, 67, 68). Whey is an abundant source of lactose and a combined cellulose-whey substrate may have potential processing attributes attractive in cellulase production relating to the viscosity of the production medium. Figure 7 shows the results of an experiment in which demineralized whey powder (Amber Laboratories, Juneau, WI) was employed as a source of lactose. The whey powder was used at a level which resulted in a 1% lactose content. By virtue of the protein content of the whey powder, a protein concentration of approximately 0.1% is obtained at a 1% lactose concentration in the medium. This is in agreement with the recommendation of Mandels (63) for the synthetic medium requirements for cellulase production using T. reesei. The whey powder medium was tested with and without the

Table 2. Pilot plant saccharification of finely ground newspaper in a 5% w/v slurry. Total volume was 243 l containing a 70:30 enzyme to water dilution of a 2.6 IU/ml crude enzyme filtrate produced in pilot plant Run #2. Saccharification was performed at pH 4.8 and 50°C under constant agitation (40 rpm).

Saccharification time	% Glucose as reducing sugar	% Conversion*
h		
15	1.9	59.84
24	2.2	69.29
24	2.45	77.16
24	2.3	72.44
Avg. for 24	2.32	72.965

\*% conversion is based upon the % cellulose of the newspaper--63.5% cellulose.

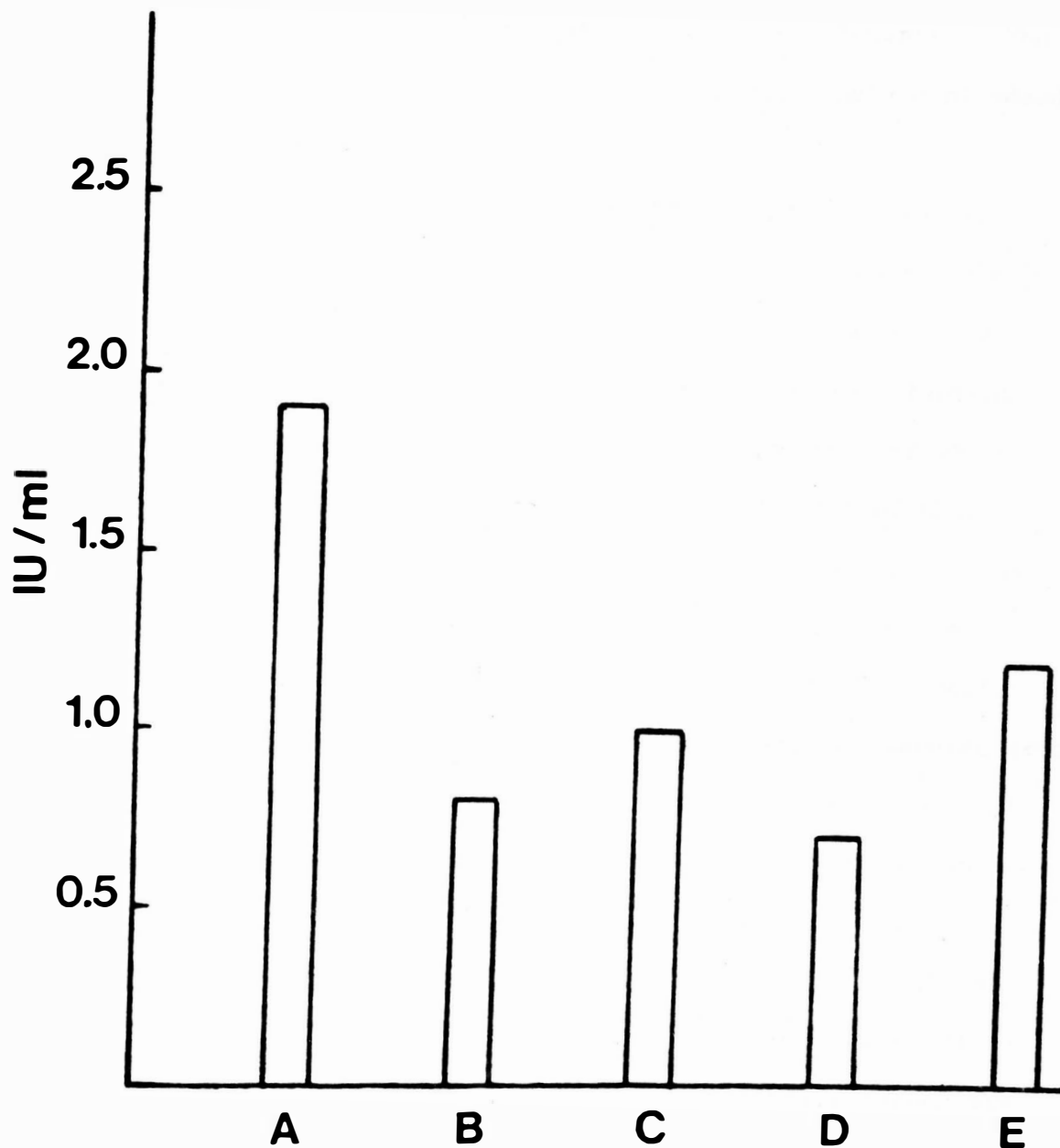


Figure 7. Cellulase production by *T. reesei* MCG77 on: (A) 1% cellulose MCG77 production medium; (B) whey powder for a 1% lactose content; (C) whey powder (1% lactose) plus supplements; (D) whey powder (0.5% lactose) and 0.5% cellulose; and (E) whey powder (0.5% lactose) and 0.5% cellulose plus supplements. Supplements are the salts and trace elements as in the MCG77 production medium.

additional salts and trace elements (referred to as supplements). The medium relied upon the whey protein of the whey powder instead of added proteose peptone.

The supplemented whey powder medium produced higher enzyme activity/ml than the unsupplemented whey powder medium. This would be expected if the whey powder is lacking in the balanced magnesium and calcium ion content or is deficient in the established trace element requirements for enzyme production (63, 68). The supplemented whey-powder medium produced approximately one-half the activity of that obtained on the standard cellulose production medium. This is in good agreement with the results reported for lactose induced cellulase (7, 100). The unsupplemented combination of 0.5% lactose and 0.5% cellulose medium yielded the lowest IU/ml. This could again be expected with the lack of the required supplements and in consideration of the yield obtained on the unsupplemented whey powder medium. Addition of supplements and proteose peptone for a protein concentration of 0.1% in the combined 0.5% lactose-0.5% cellulose medium gave the best results of the test media. The fact that the above combination was not equal to the standard cellulose medium is due to the lower inducing capability of the lactose fraction. Mandels et al. (66) found that lactose induced cellulase had 50% less saccharifying capability than cellulase induced by cellulose. This lower saccharifying power does not favor the use of cellulase produced with lactose as the sole inducer.

### Dimethyl Sulfoxide Experiment

Statistical. The statistical analysis of the absorbance readings (in triplicate) and the yield data (in duplicate) were not statistically significant for the Montencourt and Eveleigh modification (74) of the cellulase assay. For example, in the DMSO Runs #1 and #2, the reducing sugar values obtained for the 48, 60 and 72 h sample times correspond closely to the 0.5 to 1.0 mg/ml reducing sugar range. The analysis of variance of the absorbance readings had a  $P < 0.016$  for Run #1 and a  $P < 0.019$  for Run #2. However, the yield data obtained from these assays were not statistically significant ( $P > 0.05$ ).

Further discrepancies were noted for this method of assay from the statistical analysis of the data. In Run #1, the reducing sugar value for the 72 h sample of the 300 ppm DMSO test flask at the 1:10 dilution was 0.84 mg/ml. The reducing sugar value of the 1:20 dilution was 0.53 mg/ml. The probability factor for the absorbance readings at the 1:10 dilution was  $< 0.0162$  and for the 1:20 dilution was  $< 0.0076$ . The yield value obtained was 1.56 IU/ml at the 1:10 dilution and 1.95 IU/ml at the 1:20 dilution for an average of 1.755 IU/ml. The individual activity of the dilutions differ from their average by 11.1% and the yield data for Run #1 at 72 h were not statistically significant.

Enzyme activities in the literature have been reported as the mean values of triplicates (106) or other multiples (17) for a mean value to represent activity. Canevascini and Gattlen (17) in their evaluation of the filter paper assay using diluted enzyme reported a variance, in that, "constant values were never reached, but to a good approximation, the actual activity could be reasonably well deduced from

that [activity] obtained with the most diluted enzyme sample [1:30]." The selection of the 0.5 to 1.0 mg/ml reducing sugar range by Montencourt and Eveleigh is similar to the 2.0 mg/ml assay point used by Mandels et al. (64). The latter maintains that the 2.0 mg/ml assay point is best because it reflects the complete cellulase complex more accurately. This is the converse of the findings of Canevascini and Gattlen (17) previously cited above. This can be expected when attempting to represent an enzyme complex in terms of one unit of measurement.

Thus the assay method of Montencourt and Eveleigh (74) can be used to measure the cellulase activity to a good approximation. The resulting IU/ml are slightly higher than those obtained using the method of Mandels et al. (74). Strict interpretation of the reducing sugar range proposed by Montencourt and Eveleigh does not exhibit confidence in the method according to the analysis of variance procedure. Consequently, this study used the average of the yield values obtained when both dilutions fell within the 0.5 to 1.0 mg/ml reducing sugar range. Thus the values for the IU/ml are the result of the mean value of the yields in triplicate and, where averaged, the mean value of six (6) yield values.

Runs. The times for sampling in the DMSO experiment were determined from previous trial runs. These trial runs showed a change in activity and pH at approximately the 72 h period. Therefore sampling was increased to once every 2 h from 72 h to 80 h inclusive in an attempt to illustrate a more intimate view of enzyme formation during this period.



Three runs were performed comparing the pH profiles and enzyme activity of a control flask without DMSO and test flasks at 50, 100, 200, 300 and 400 ppm DMSO. The composite enzyme activities and pH profiles for DMSO Run #1, #2 and #3 are shown in Figs. 8 and 9, 15 and 16, 22 and 23 respectively. Separate comparison of each DMSO test flask to the control flask illustrating the enzyme activity profile and pH profile are shown in Figs. 10 to 14 inclusive (Run #1), Figs. 17 to 21 inclusive (Run #2) and Figs. 24 to 28 inclusive (Run #3).

Consistencies are apparent in the pH profiles and the enzyme activity profiles for all three runs. First, the pH profile for all flasks consistently undergoes a leveling off or slight drop at or around the 72 h sampling. This appears to coincide with the increased enzyme activity around this time period. This phenomenon was apparent in the control and test flasks. The pH rise was not directly related to enzyme activity. The effect of the DMSO on the pH profile was (with few exceptions) an increase in the rate of ammonium ion release (118) and thus an advanced pH profile when compared to the control. This phenomenon can be compared to the increased rate of efflux effected by DMSO on resting cells of yeast (23). The DMSO may affect the T. reesei membrane in a similar manner and at a similar phase in the life of this organism thereby facilitating efflux of ammonium ion resulting in the advanced pH profile. This effect can be observed with the lowest level of DMSO addition (50 ppm) but was more consistent at the 100 ppm level.

The other consistent characteristic is the fluctuation in the enzyme activity which occurs from 72 h on in the fermentation. All runs displayed a biphasic enzyme increase and decrease. A similar

observation was made by Shirkot et al. (106). In their study they noted that a dramatic decrease in the  $\beta$ -glucosidase activity occurred between 8 and 12 days of the fermentation. They suggested that this effect was somehow related to the dithiocarbamates employed in this study and could be exploited to result in an enzyme complex favoring the Avicelase (crystalline activity) and the CMCase (amorphous activity).

In the present work, overall enzyme activity was determined by using the filter paper assay. No differentiation of the cellulase complex was attempted. Nonetheless, the  $\beta$ -glucosidase component is known to contribute greatly to the overall saccharifying ability of the T. reesei cellulase complex (119). This component is an integral part of the cellulase complex and the extent to which it is present would influence the total activity as measured by the filter paper assay. Thus, in consideration of the effect noted in the work of Shirkot et al. (106), it appears that the fluctuations noted in the present work are due to the  $\beta$ -glucosidase component. This is further supported by the appearance of this component in the fermentation as characterized by Sternberg (118). However, the present study shows the activity fluctuations are a natural occurrence since they are observed in the control flasks as well as the DMSO test flasks. It is possible that the  $\beta$ -glucosidase is in a bound form and subsequently releasing during this portion of the fermentation. The ability to obtain a preferentially Avicelase-CMCase complex, as suggested by Shirkot et al. (ibid.), is not likely. This is probably a natural evolution or maturation of the enzyme complex in the medium in the presence of residual cellulose and related polymeric forms. The pH profile

throughout the latter phase of enzyme production also favors the enzyme-substrate reaction as the pH approaches the optimum for the enzyme (pH 4.8). Action on the residual cellulose would naturally result in products (cellooligomers and cellobiose) which would allow the binding of the  $\beta$ -glucosidase component and explain the activity phasing observed.

The DMSO affected the amount of 6.5 N ammonium hydroxide required to maintain the pH between the control points of pH 3.0 to 3.5 during the acid phase of the fermentation. Table 3 shows the total ammonium hydroxide added for each flask. The control consistently required 1.0 ml while the DMSO test flasks generally required 1.05 to 1.15 ml. Qualitatively, the DMSO flasks began foaming earlier in the fermentation requiring addition of the antifoam. The DMSO flasks also resulted in a darker amber fluid than the control flask.

Run #1 (Figs. 8 to 14) shows a substantial difference between the control and DMSO flasks in both the enzyme activity profile and the pH profile. The subdued pH rise for the control flask in this run (Fig. 9) indicates a possible oxygen deficiency. Similarities in pH profiles can be noted between the 4% cellulose pilot plant run (Fig. 5) and DMSO Run #1 in the control flask. No restriction of any of the flasks' cotton plugs was noticed during the course of sampling for this run. However, a similar pH curve did result in Run #2 (Fig. 16) which was due to a mycelial plug or seal forming around the cotton plug of the 300 ppm DMSO flask. In this case the plug was obvious and was replaced when noticed at the 36 h pH sampling time. No similar condition was observed for the control flask of Run #1.

Table 3. Ammonium hydroxide required per flask/run for pH control to between pH 3.0-3.5 for the DMSO experiment.

Flask	Total volume of 6.5 N NH <sub>4</sub> OH (ml)		
	Run #1	Run #2	Run #3
Control	1.0	1.0	1.0
50 ppm	1.15	1.0	1.1
100 ppm	1.15	1.0	1.1
200 ppm	1.15	1.1	1.05
300 ppm	1.1	0.9*	1.1
400 ppm	1.15	1.1	1.1

\*Inhibition due to oxygen deficiency.

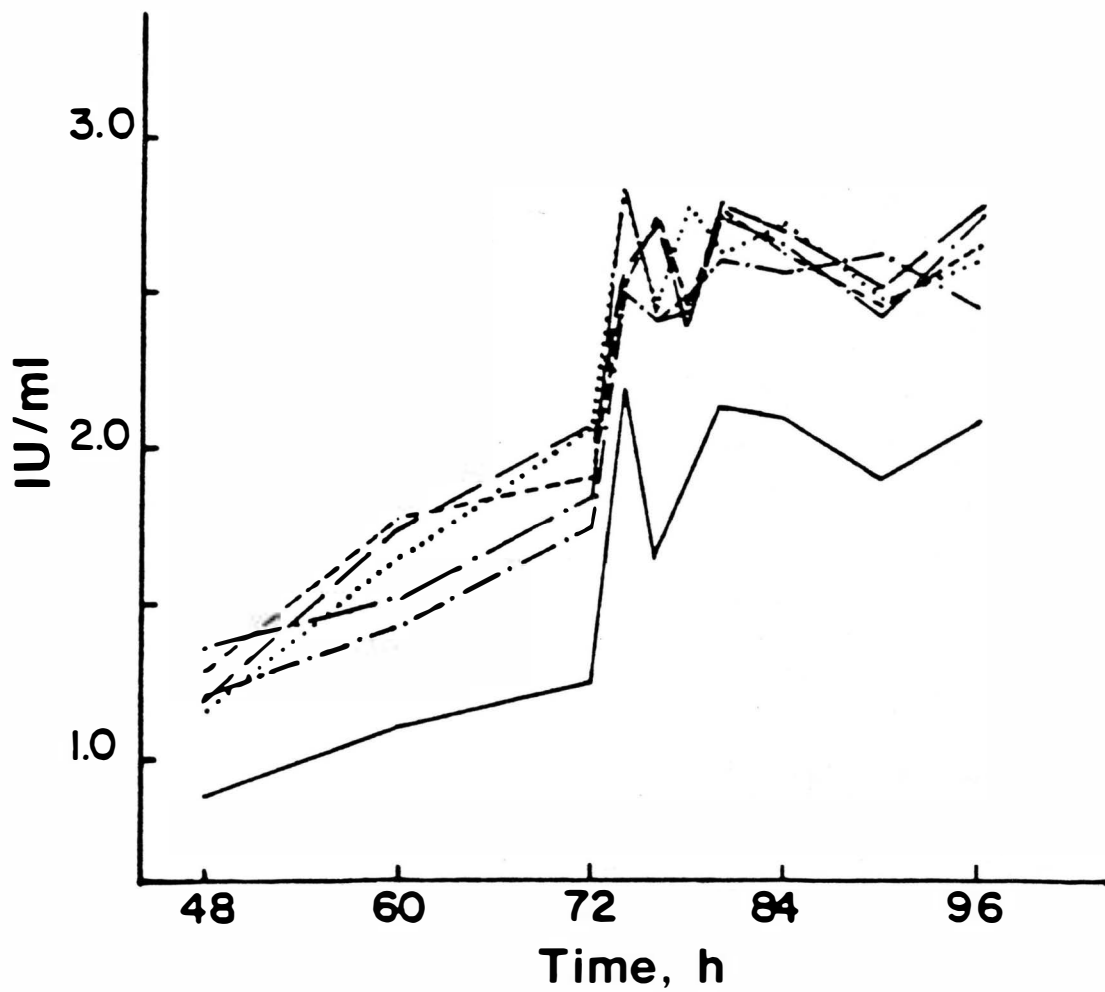


Figure 8. Composite of activity (IU/ml) vs. time for Run #1 of the DMSO test additions and the control without DMSO. Control —; 50 ppm DMSO ---; 100 ppm DMSO - - -; 200 ppm DMSO .....; 300 ppm DMSO - · -; and 400 ppm DMSO — · —.

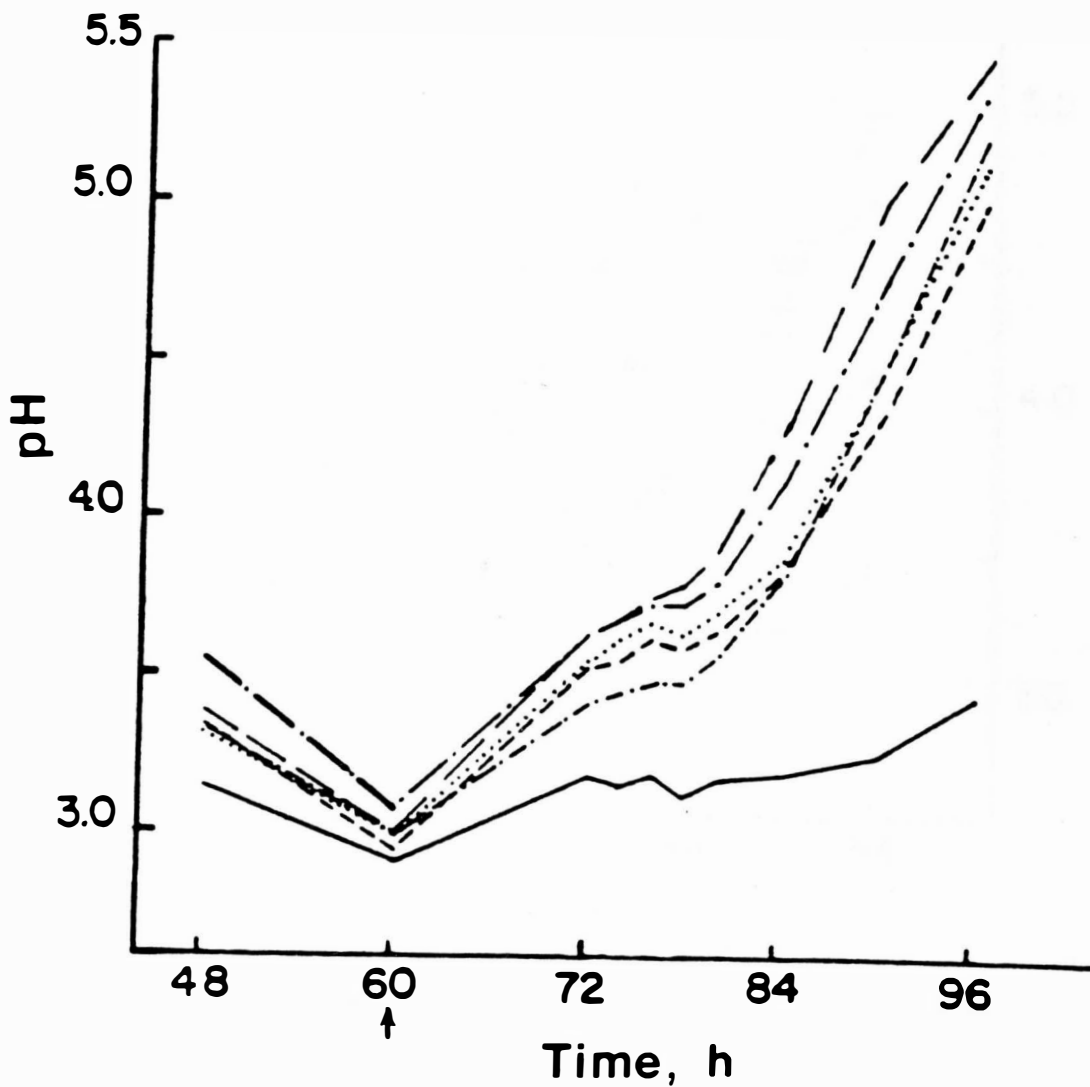


Figure 9. Composite of pH vs. time for Run #1 of the DMSO test additions and the control without DMSO. Control —; 50 ppm DMSO ---; 100 ppm DMSO — —; 200 ppm DMSO ·····; 300 ppm DMSO - · -; and 400 ppm DMSO — · —. ↑ indicates final pH adjustment with 6.5 N  $\text{NH}_4\text{OH}$ .

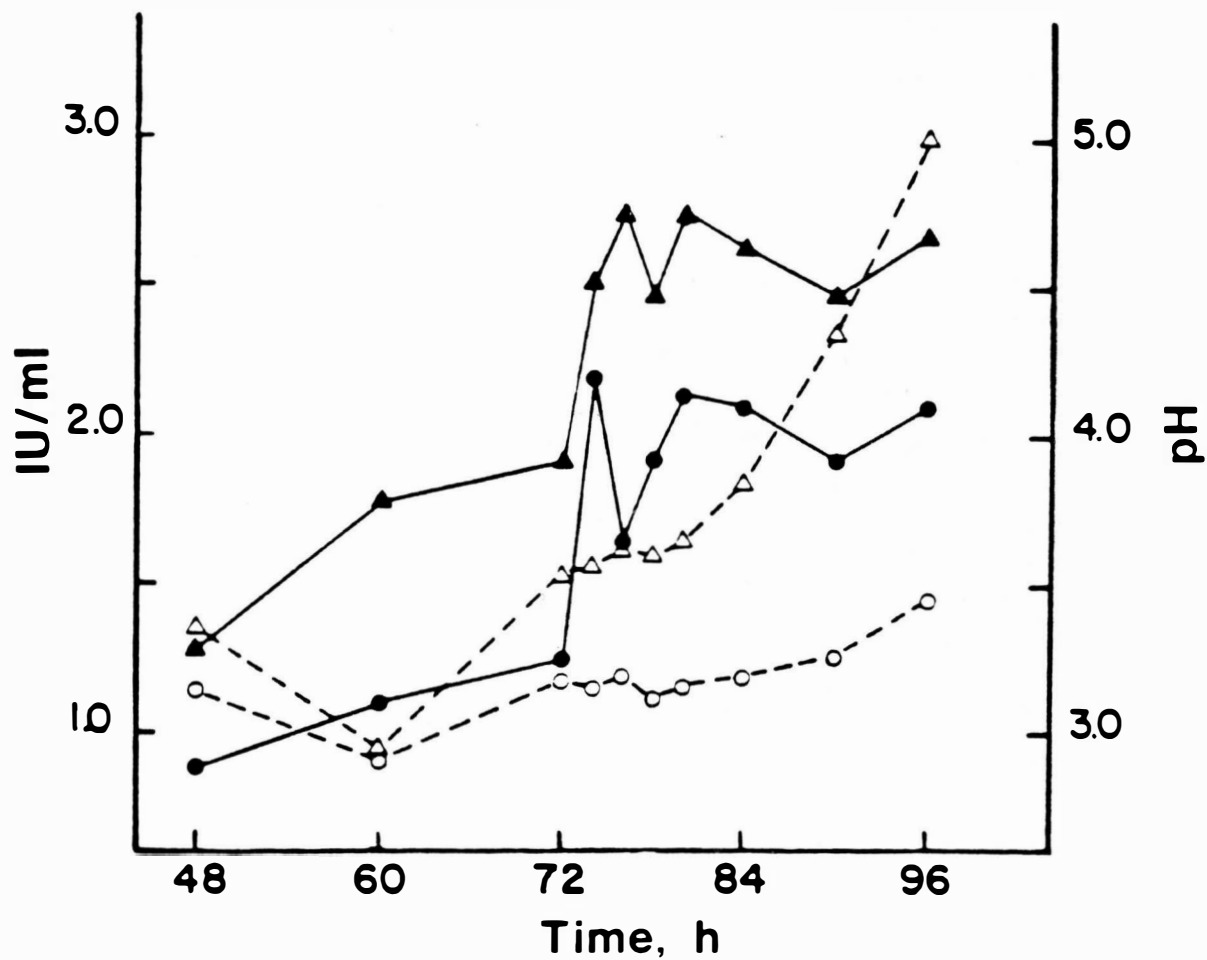


Figure 10. Run #1. Comparison of pH and IU/ml vs. time of the 50 ppm DMSO test flask to the control without DMSO. Control, pH -  $\circ$  -, and IU/ml -  $\bullet$  -; 50 ppm DMSO test flask, pH -  $\triangle$  -, and IU/ml -  $\blacktriangle$  -.

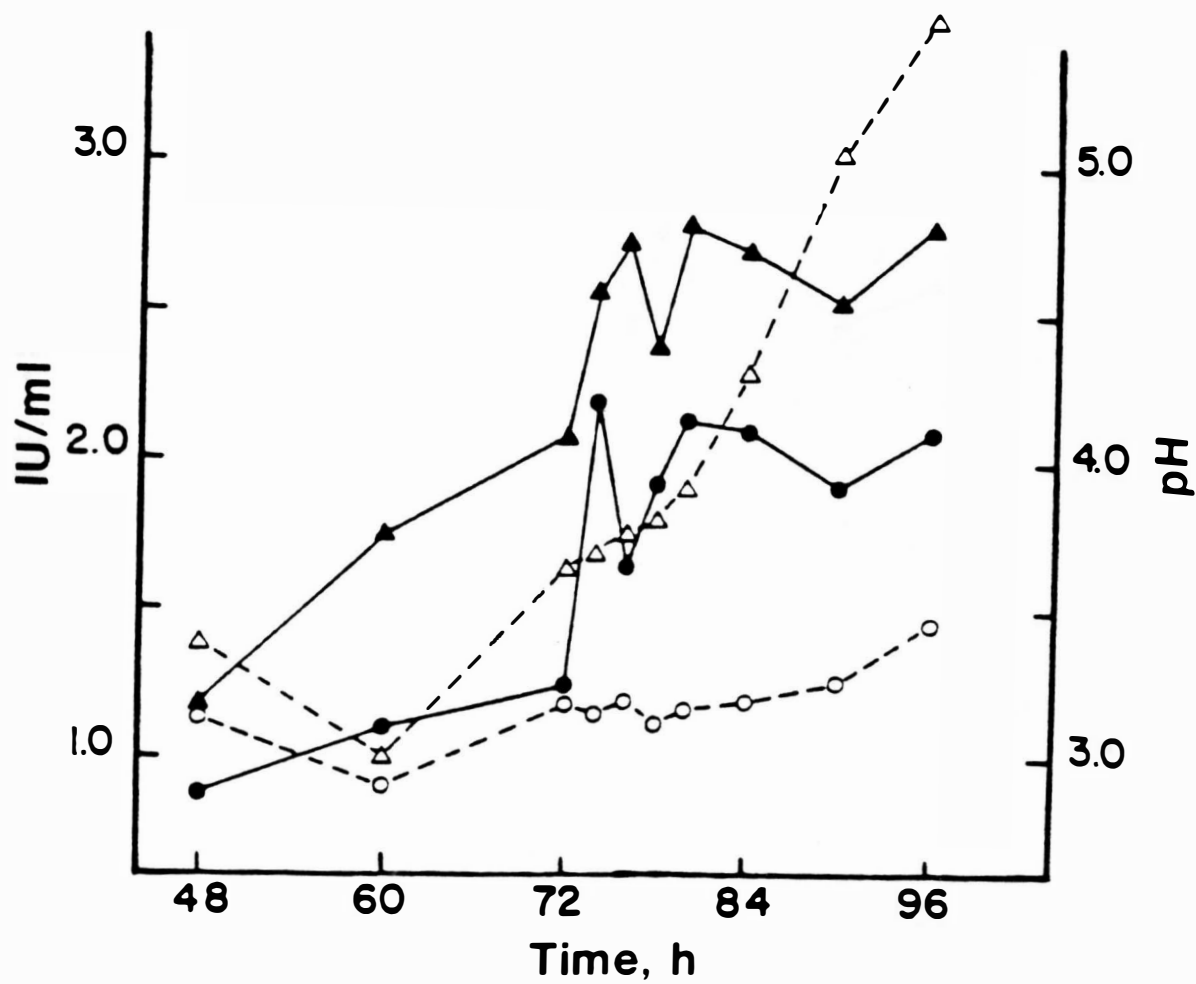


Figure 11. Run #1. Comparison of pH and IU/ml vs. time of the 100 ppm DMSO test flask to the control without DMSO. Control, pH - ○ -, and IU/ml - ● -; 100 ppm DMSO test flask, pH - △ -, and IU/ml - ▲ -.



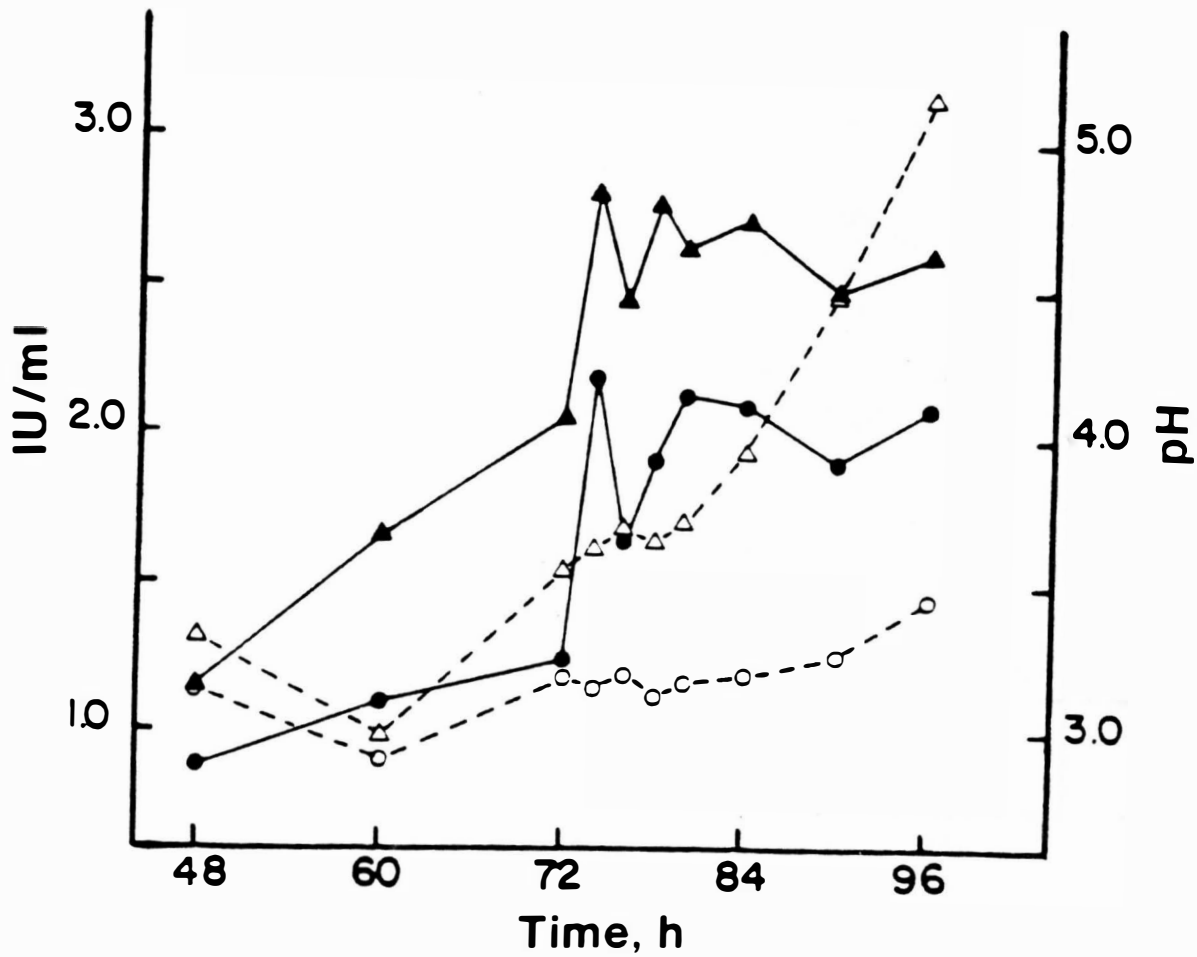


Figure 12. Run #1. Comparison of pH and IU/ml vs. time of the 200 ppm DMSO test flask to the control without DMSO. Control, pH -○-, and IU/ml -●-; 200 ppm DMSO test flask, pH -Δ-, and IU/ml -▲-.

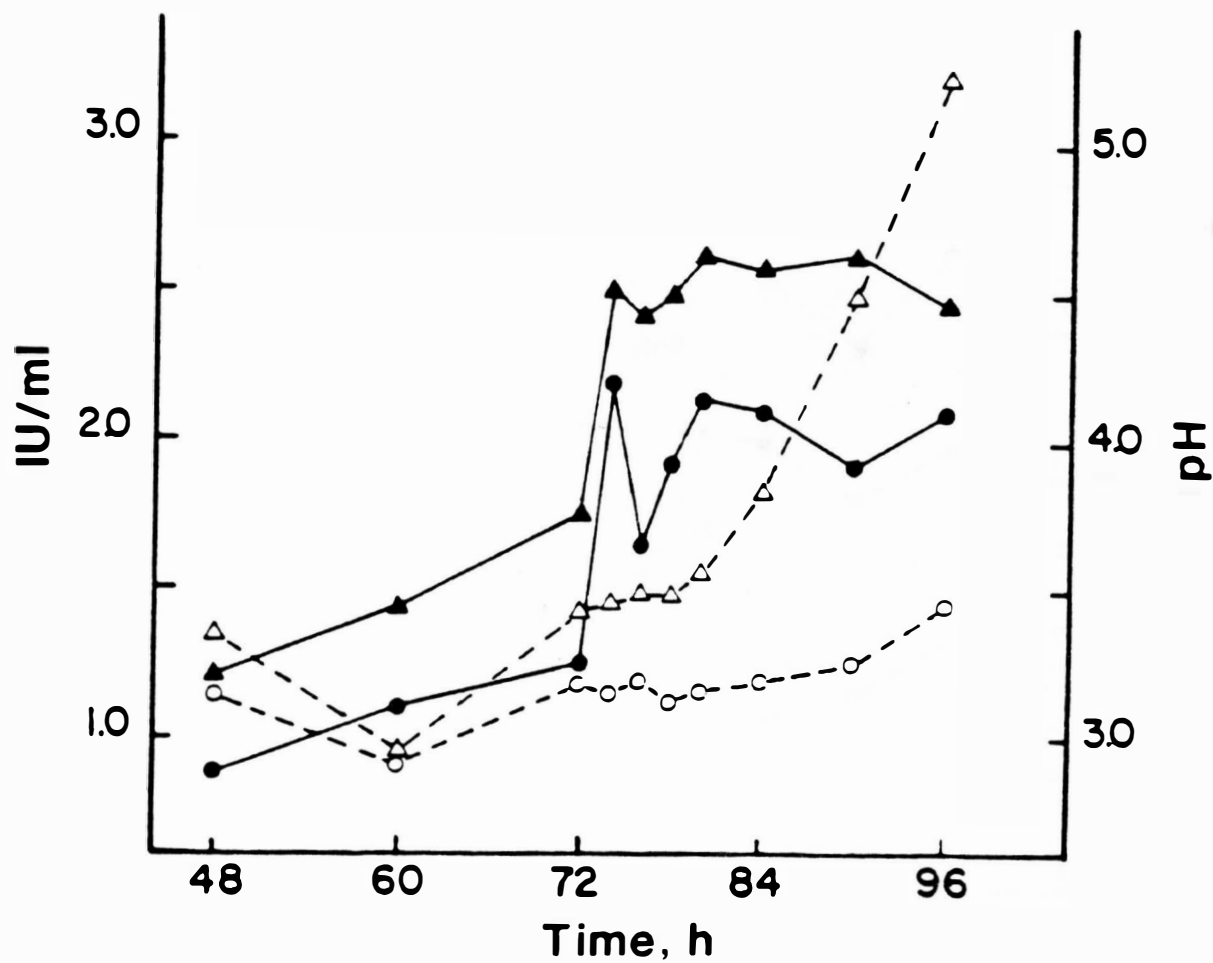


Figure 13. Run #1. Comparison of pH and IU/ml vs. time of the 300 ppm DMSO test flask to the control without DMSO. Control, pH -○-, and IU/ml —●—; 300 ppm DMSO test flask, pH -△-, and IU/ml —▲—.

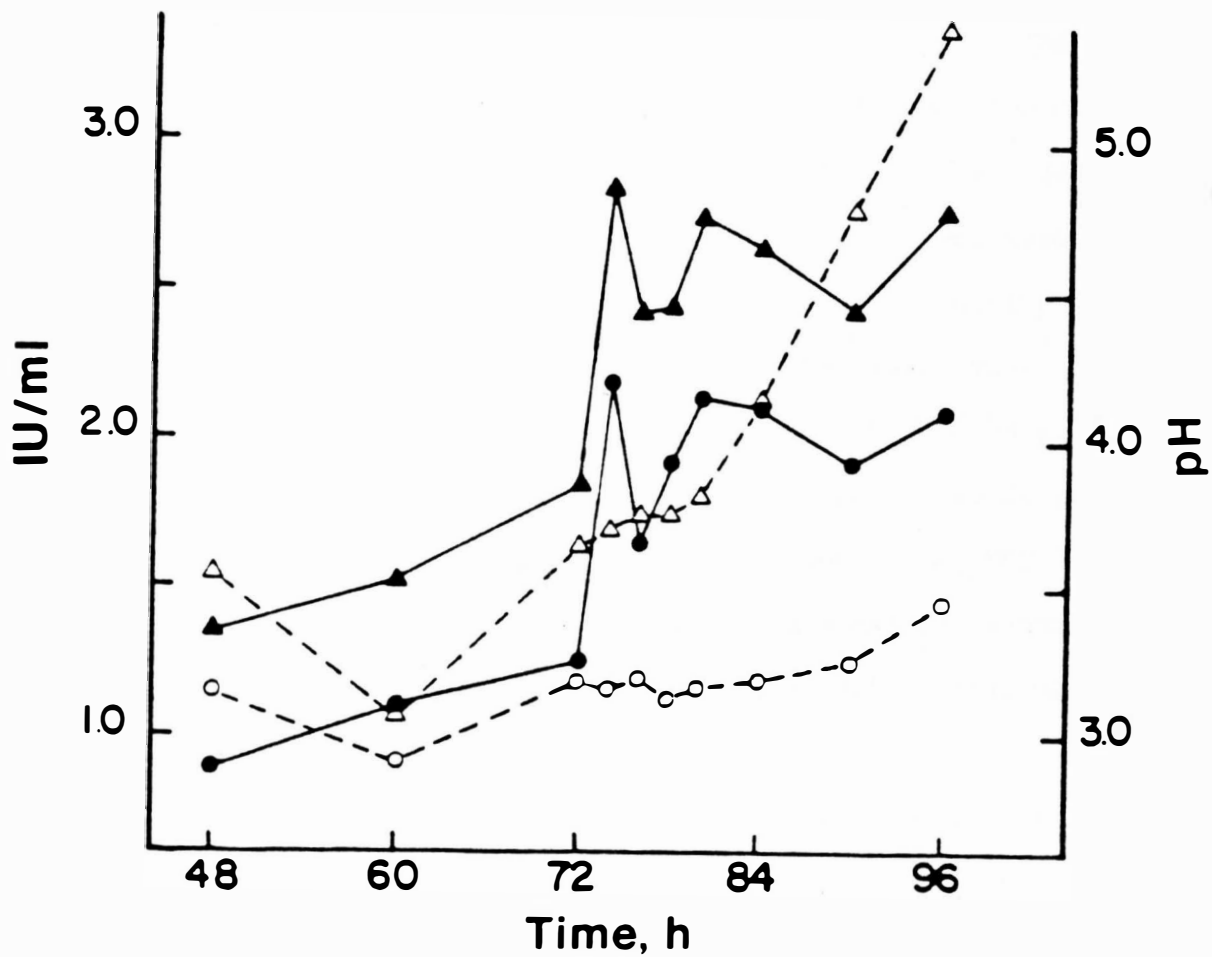


Figure 14. Run #1. Comparison of pH and IU/ml vs. time of the 400 ppm DMSO test flask to the control without DMSO. Control, pH -○-, and IU/ml -●- ; 400 ppm DMSO test flask, pH -△-, and IU/ml -▲- .

When considered collectively, the runs do not show a clear-cut advantage in yield for the DMSO additions. In Run #1, Figs. 8, and 10 to 14 inclusive, all of the DMSO test flasks substantially exceeded the activity attained by the control flask. The DMSO additions showed an increase in final enzyme activity over the control ranging from a low of 17.6% at the 300 ppm level to 32.8% and 32.1% at the 100 and 400 ppm levels respectively. The 300 ppm level gave an increase of 25.2% at the 90 h sampling and was in a declining phase at the final 96 h sampling (Figs. 8 and 13). Thus the lower value in the final sample. Despite the indication of an anomaly by the pH profile of the control flask, the enzyme yield from the control was in good agreement with other values obtained on 1% cellulose using MCG77. As previously mentioned, the pH profile indicated a possible oxygen deficiency although nothing was observed which could confirm this during the run.

Run #2, Figs. 15, and 17 to 21 inclusive, showed the least difference between the control and the DMSO test flasks in relation to enzyme activity. Only the 400 ppm DMSO addition gave an increase in enzyme activity at the final 96 h sampling. The enzyme activity from the 400 ppm test flask was 7.6% higher than the control. The 300 ppm DMSO test flask was definitely deprived of oxygen during the early phase of incubation by a mycelial seal forming on the cotton plug. When Run #2 is observed at the 90 h sampling point, the DMSO test flasks show a gain in enzyme activity over that of the control. The 90 h samples show an increased enzyme activity of 9.3% for the 200 ppm DMSO addition and 11.6% for the 400 ppm. At this point even the oxygen

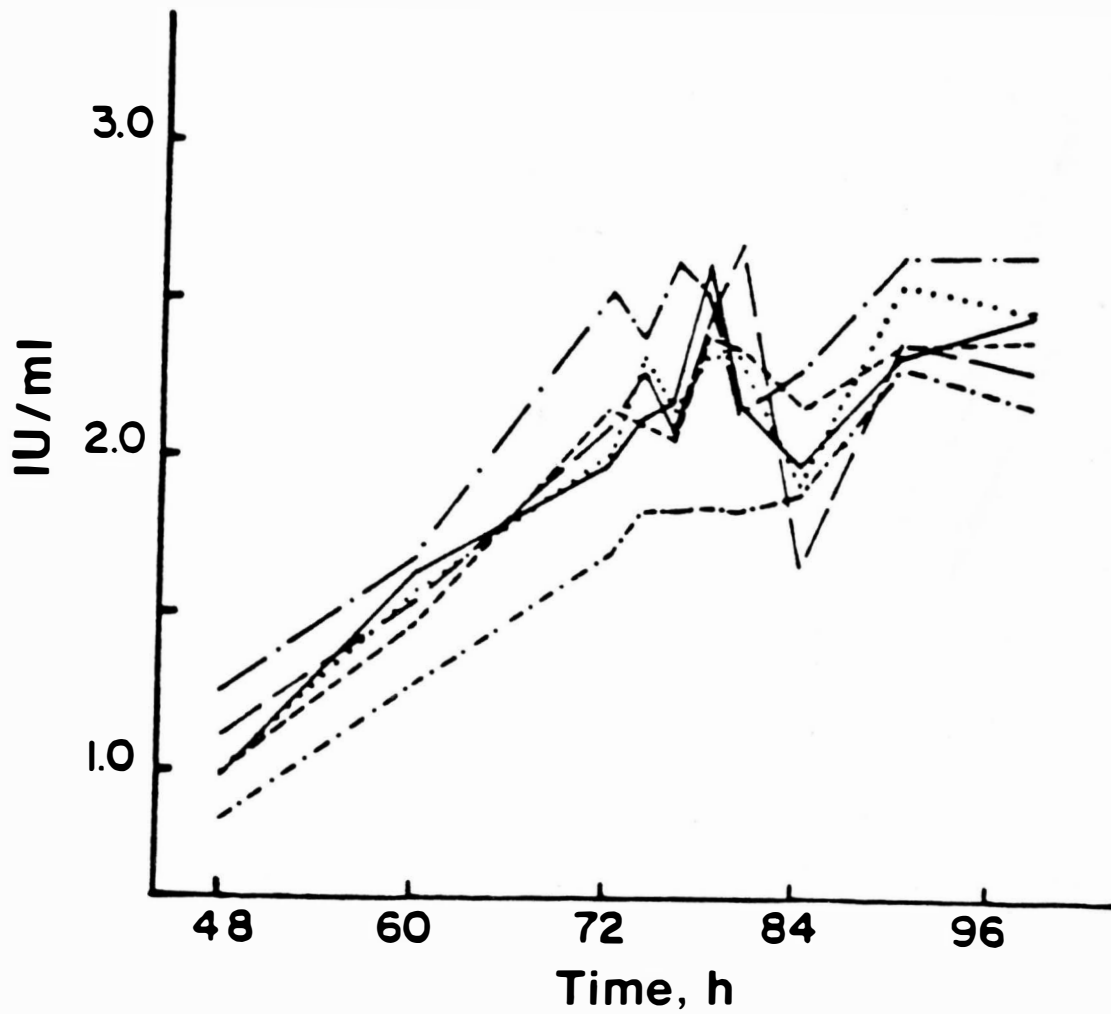


Figure 15. Composite of activity (IU/ml) vs. time for Run #2 of the DMSO test additions and the control without DMSO. Control — ; 50 ppm DMSO - - - ; 100 ppm DMSO — — ; 200 ppm DMSO ..... ; 300 ppm DMSO - · - ; and 400 ppm DMSO — · —.

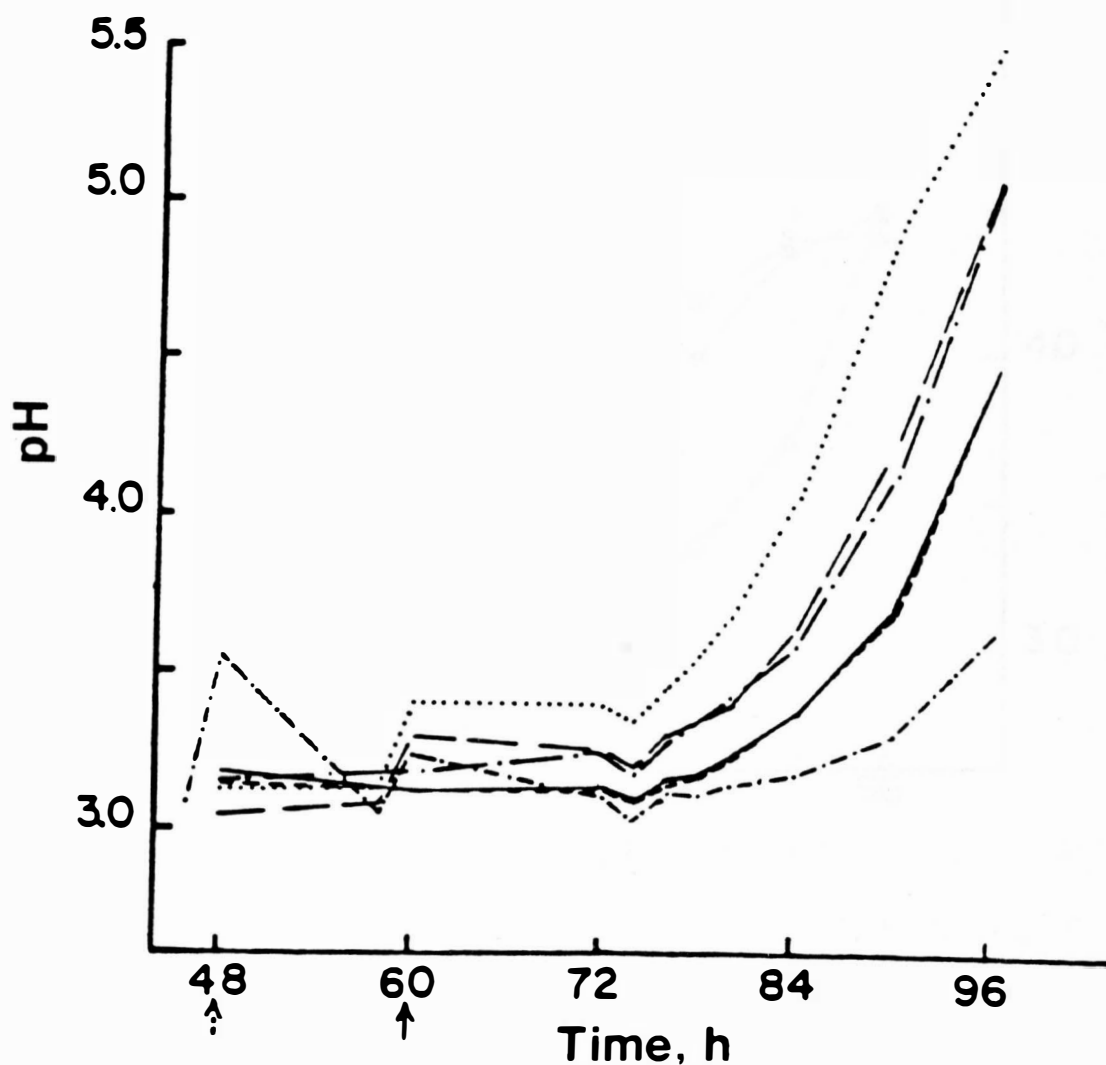


Figure 16. Composite of pH vs. time for Run #2 of the DMSO test additions and the control without DMSO. Control —; 50 ppm DMSO ---; 100 ppm DMSO -.-; 200 ppm DMSO .....; 300 ppm DMSO -.-.-; and 400 ppm DMSO -.-.-.-. ↑ indicates final pH adjustment with 6.5 N  $\text{NH}_4\text{OH}$  for DMSO additions 200, 300 and 400 ppm, and ↑ for control, 50 ppm and 100 ppm.

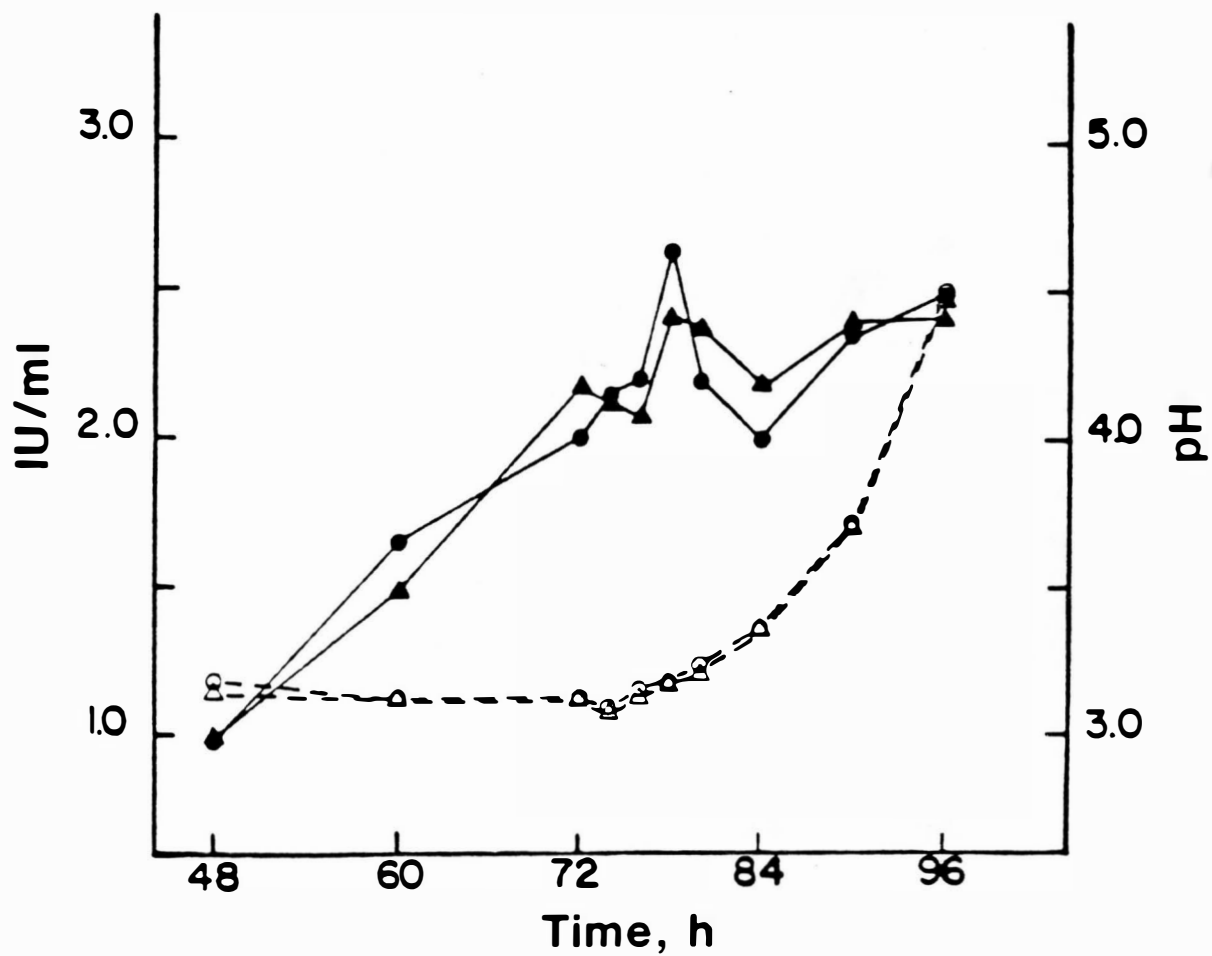


Figure 17. Run #2. Comparison of pH and IU/ml vs. time of the 50 ppm DMSO test flask to the control without DMSO. Control, pH -○-, and IU/ml -●-; 50 ppm DMSO test flask, pH -△-, and IU/ml -▲-.

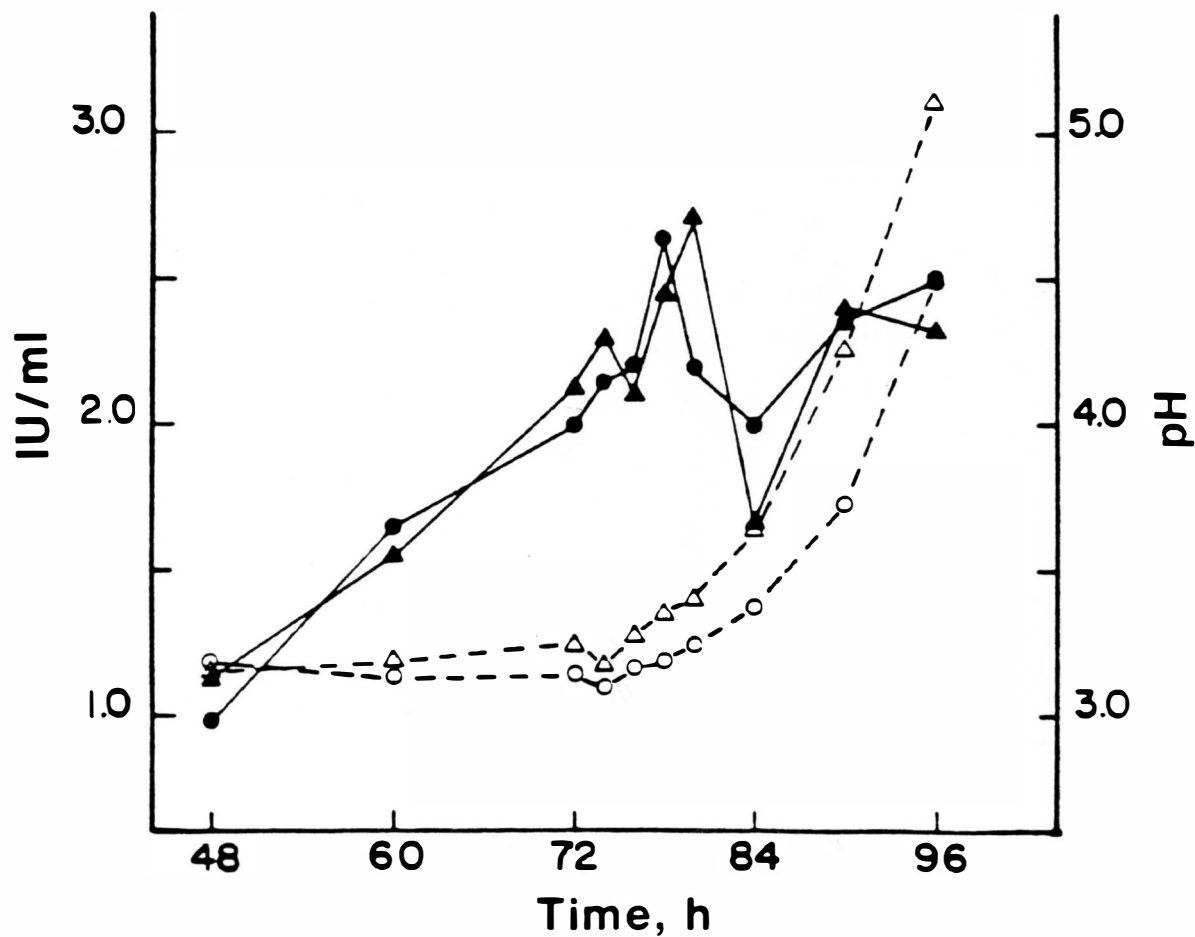


Figure 18. Run #2. Comparison of pH and IU/ml vs. time of the 100 ppm DMSO test flask to the control without DMSO. Control, pH -○-, and IU/ml -●-; 100 ppm DMSO test flask, pH -△-, and IU/ml -▲-.



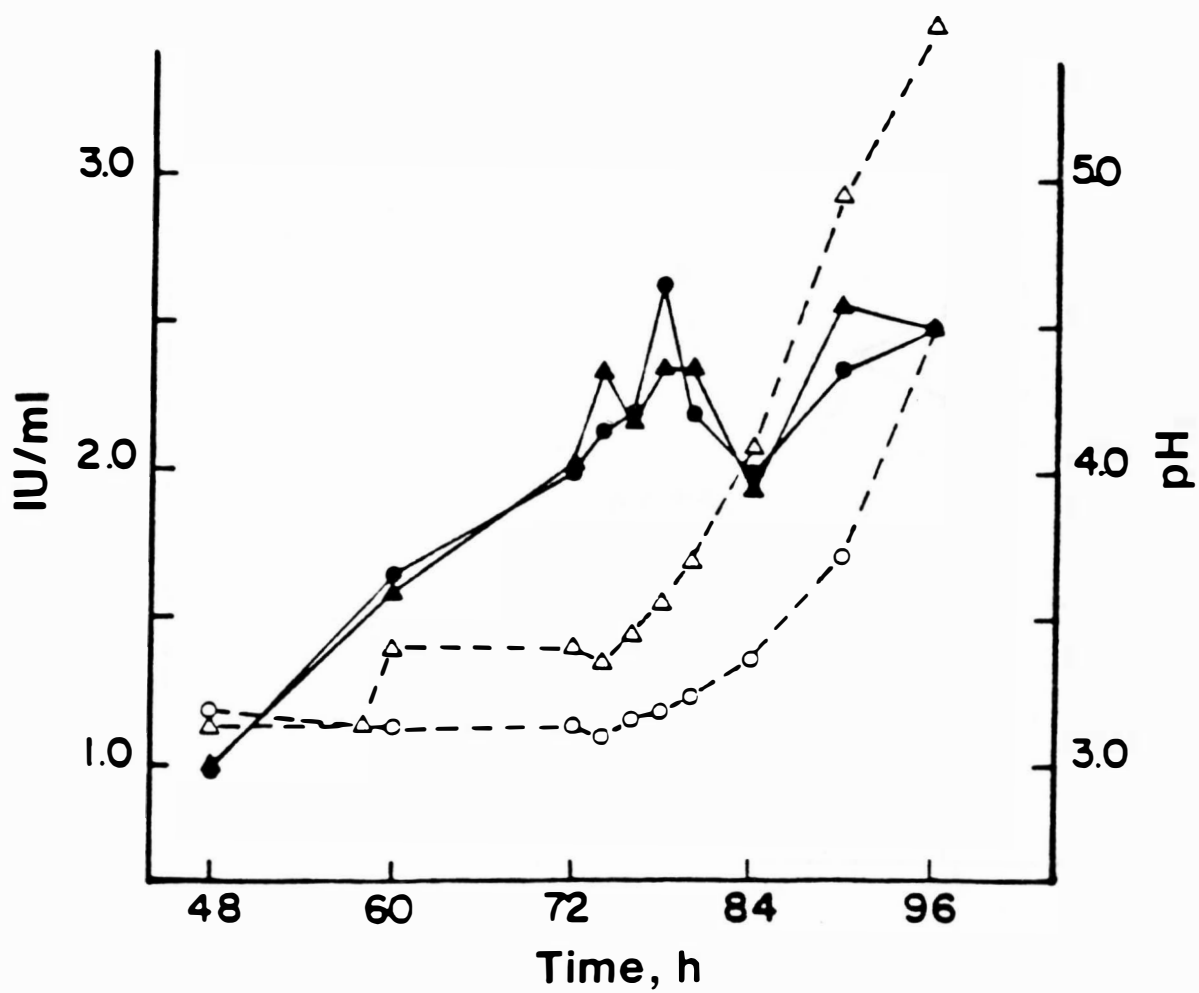


Figure 19. Run #2. Comparison of pH and IU/ml vs. time of the 200 ppm DMSO test flask to the control without DMSO. Control, pH -○-, and IU/ml -●- ; 200 ppm DMSO test flask, pH -△-, and IU/ml -▲- .

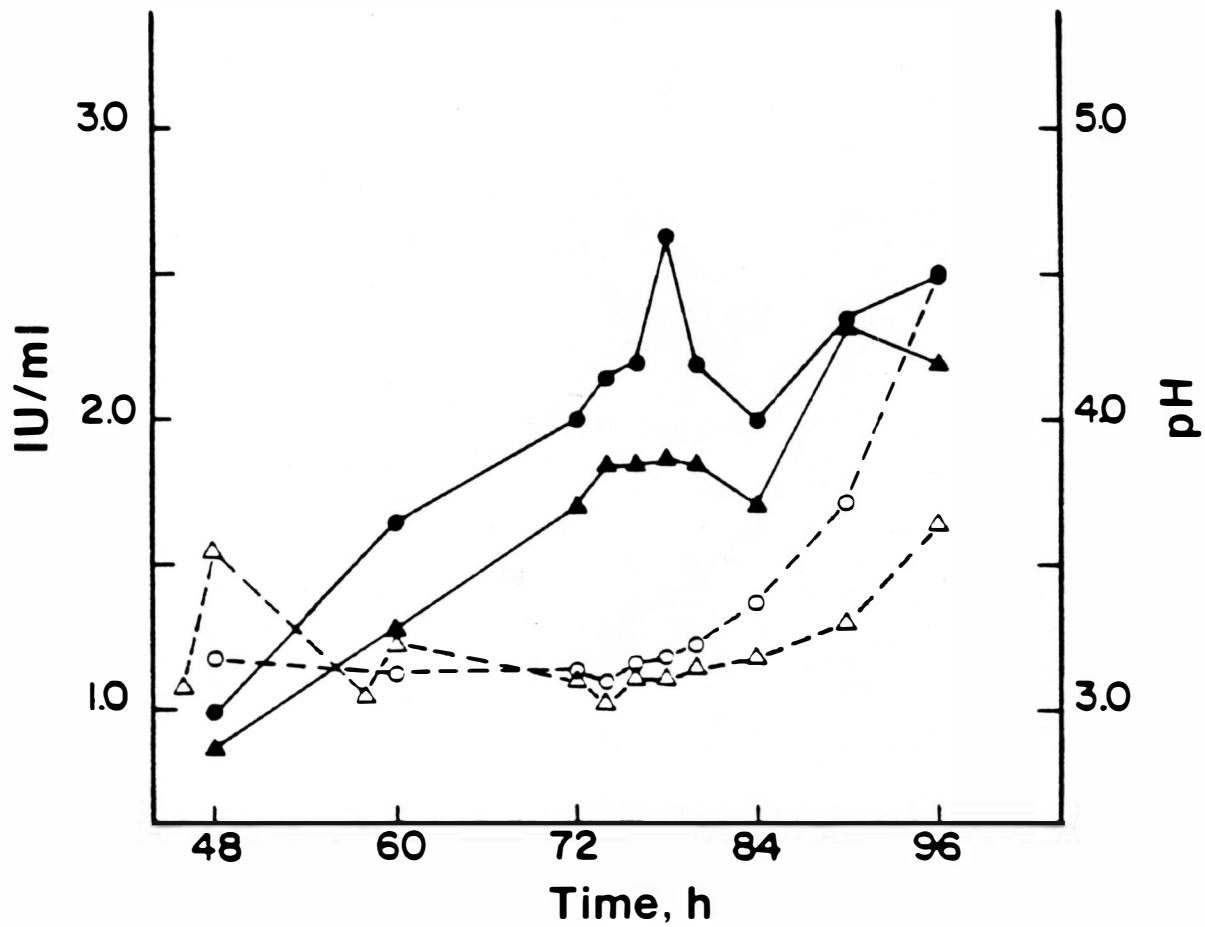


Figure 20. Run #2. Comparison of pH and IU/ml vs. time of the 300 ppm DMSO test flask to the control without DMSO. Control, pH -○-, and IU/ml -●-; 300 ppm DMSO test flask, pH -△-, and IU/ml -▲-.

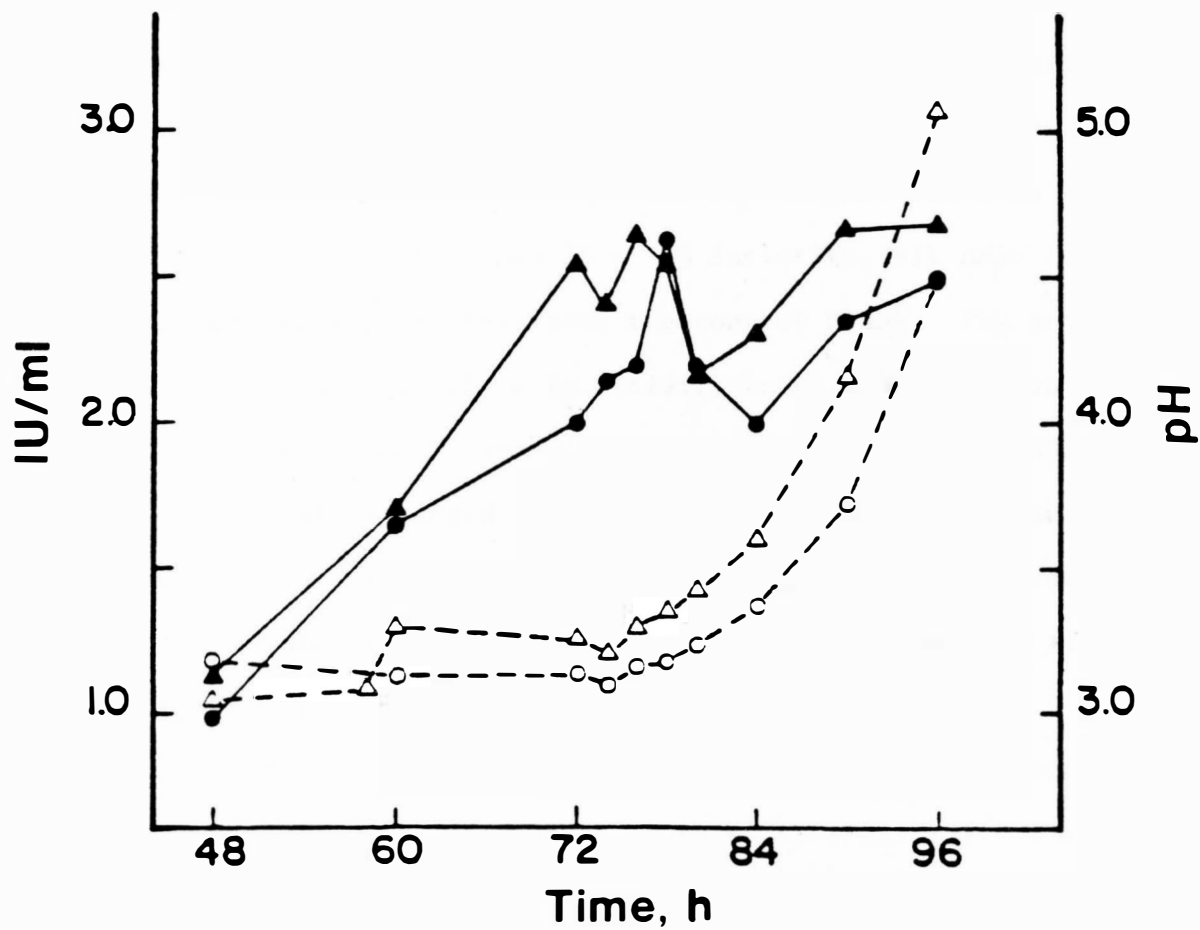


Figure 21. Run #2. Comparison of pH and IU/ml vs. time of the 400 ppm DMSO test flask to the control without DMSO. Control, pH -○-, and IU/ml -●-; 400 ppm DMSO test flask, pH -△-, and IU/ml -▲-.

deprived 300 ppm DMSO addition approximately equaled the control flask, although no advantage can be seen at the 50 and 100 ppm level. It must be noted that the activity obtained in the control flask for Run #2 was much higher than that obtained in Runs #1 and #3, or in the earlier trial runs, and the other tests (see Fig. 7--1% cellulose control). It is not known why this result occurred for the control flask in Run #2.

In Run #3, Figs. 22, and 24 to 28 inclusive, all DMSO flasks showed an improved enzyme yield over the control flask. The control flask yielded enzyme very close in activity to that obtained in the control for Run #1. The increase in enzyme activity of the DMSO additions over the control ranged from 10.9% at 200 ppm to 26.9% at 300 ppm. Unlike Runs #1 and 2 where the activity phasing is somewhat synchronous at 90-96 h, the enzyme activities of the 300 and 400 ppm DMSO flasks are out of phase with the other flasks (Fig. 22).

The fact that the pH profile is not necessarily a direct indicator of enzyme activity is apparent in this run (Fig. 23). The 200 and 400 ppm DMSO pH profiles are retarded in relation to the control but both DMSO tests yielded higher final enzyme activities. The previously mentioned dramatic increase in enzyme activity around the 72 h period is emphasized in this run at the 300 and 400 ppm DMSO additions. Figs. 22, 27 and 28 show this increase which is greater than 2.9 IU/ml for the 300 ppm DMSO level.

An overall average of the enzyme yields for all three runs shows an increase over the control without DMSO of 14.2% at 50 ppm, 14.4% at 100 ppm, 11.9% at 200 ppm, 11.0% at 300 ppm (value includes oxygen deficient yield in Run #2) and 19.4% at 400 ppm DMSO. These

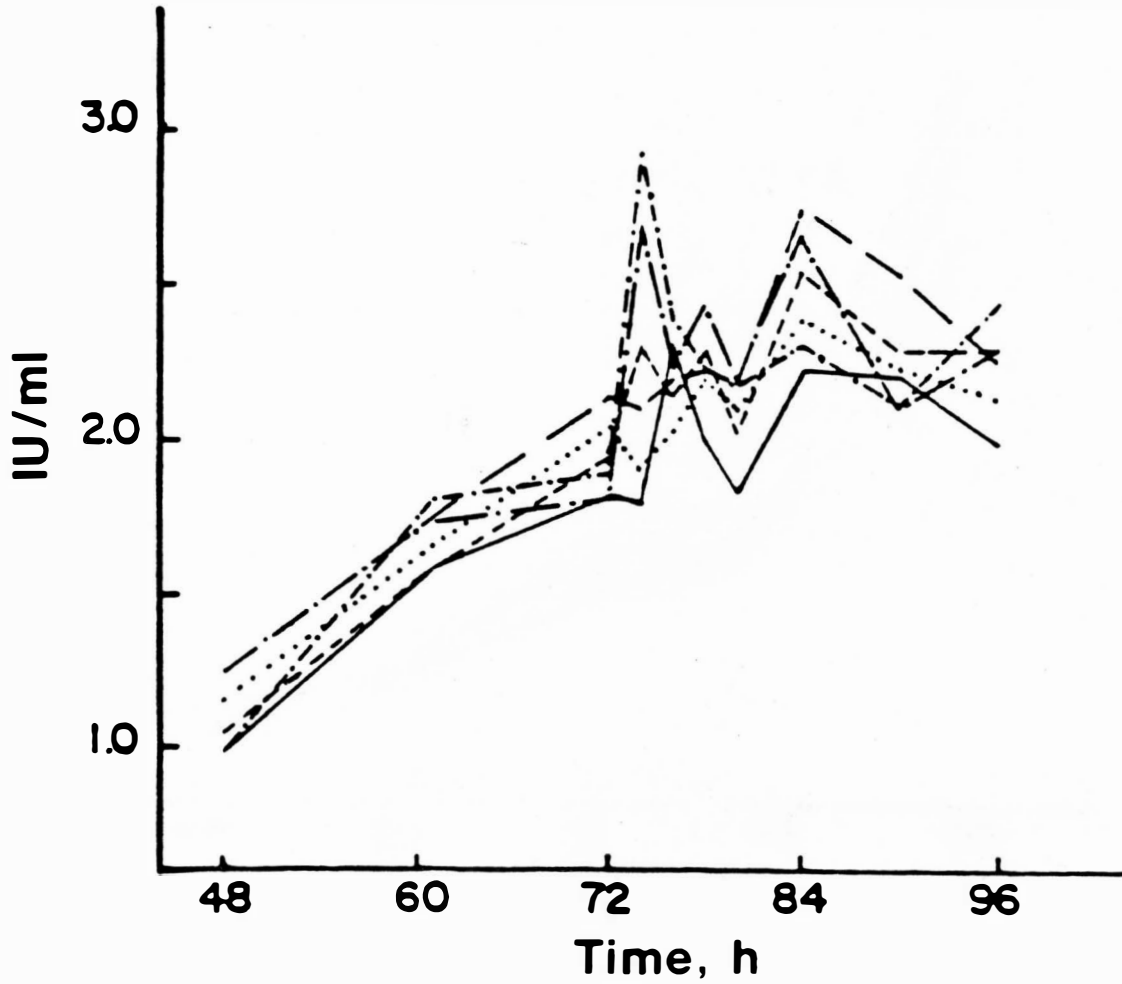


Figure 22. Composite of activity (IU/ml) vs. time for Run #3 of the DMSO test additions and the control without DMSO. Control —; 50 ppm DMSO ---; 100 ppm DMSO — —; 200 ppm DMSO .....; 300 ppm DMSO - · - ; and 400 ppm DMSO — · — .

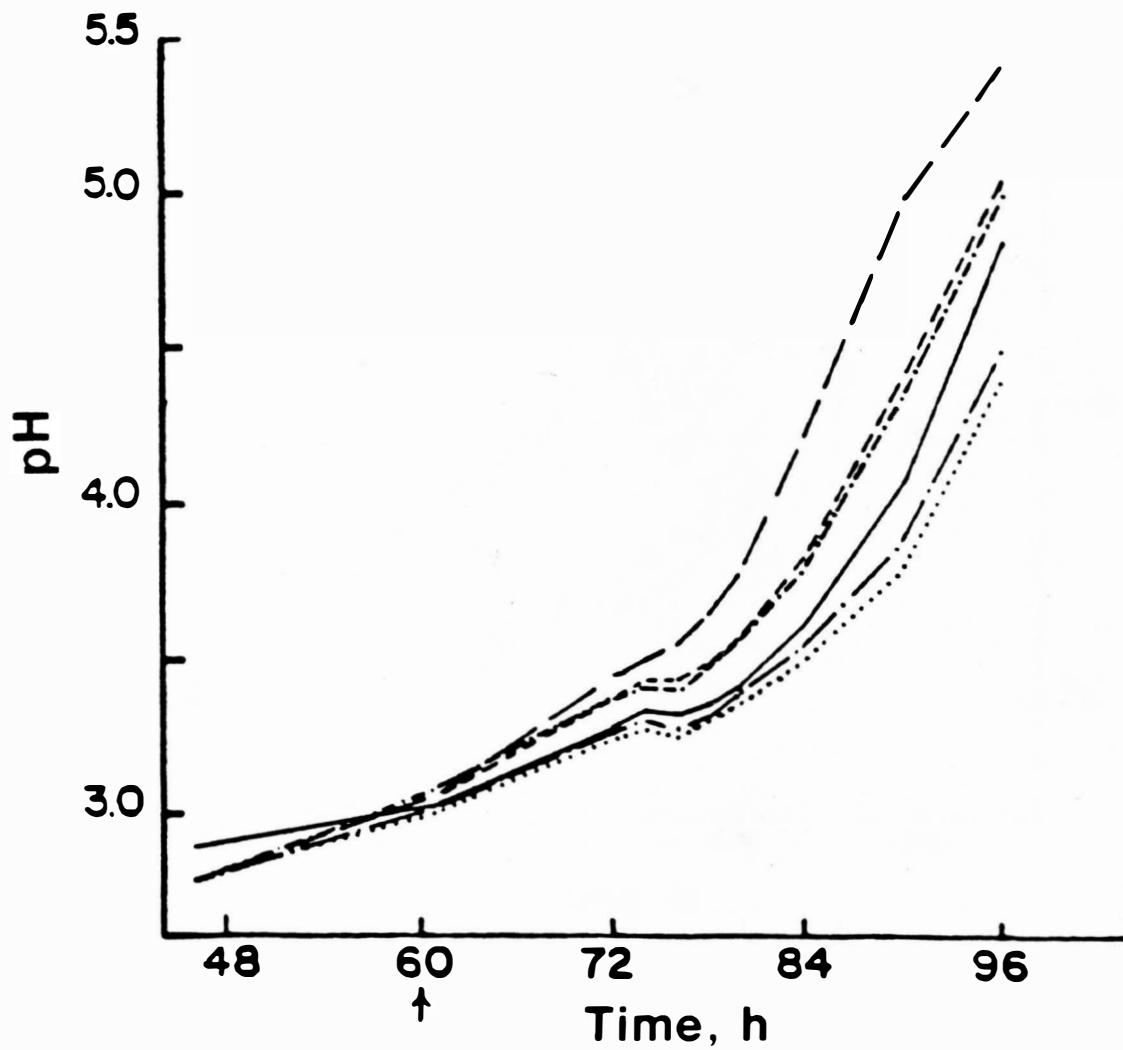


Figure 23. Composite of pH vs. time for Run #3 of the DMSO test additions and the control without DMSO. Control —; 50 ppm DMSO - - -; 100 ppm DMSO — —; 200 ppm DMSO ····; 300 ppm DMSO - · -; and 400 ppm DMSO — · —. ↑ indicates final pH adjustment with 6.5 N  $\text{NH}_4\text{OH}$ .

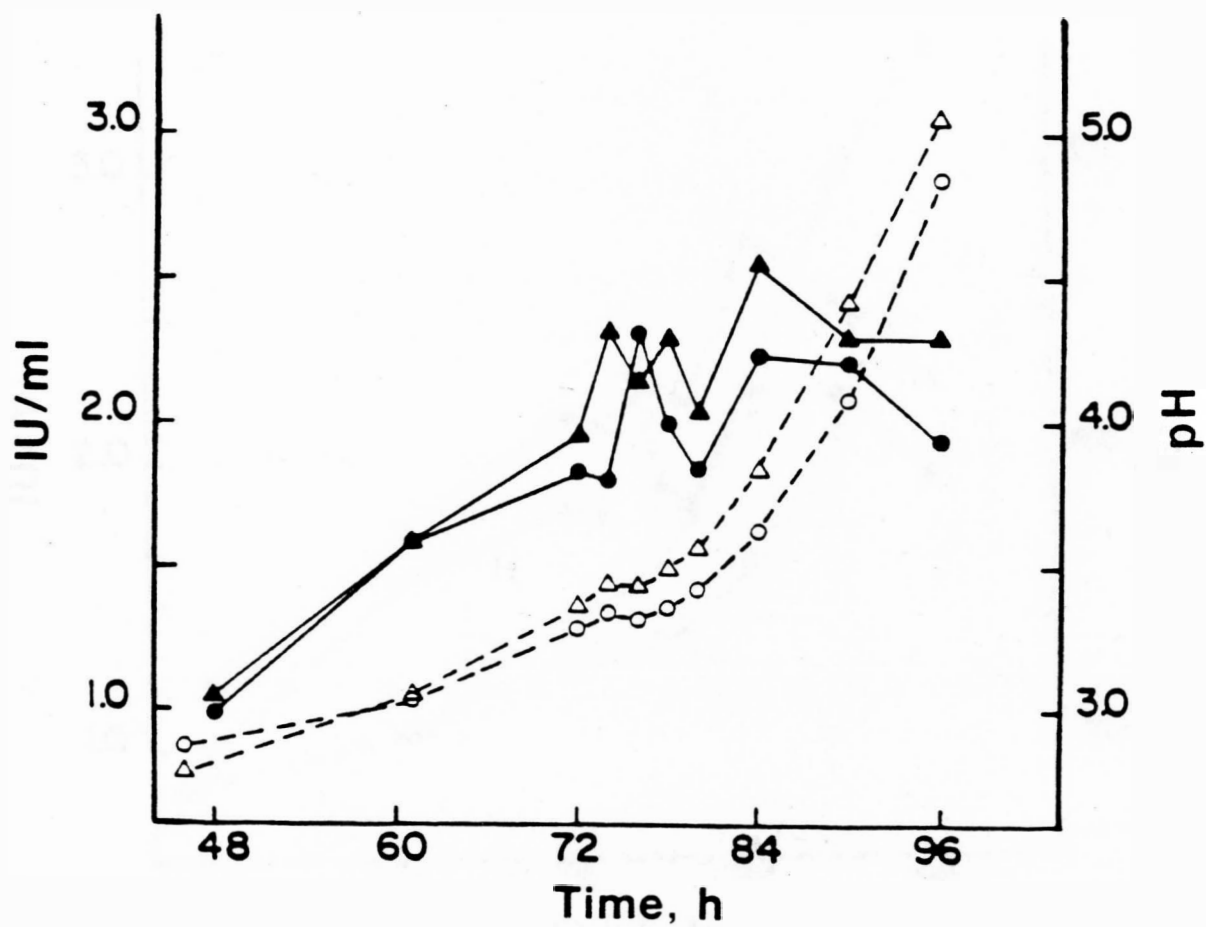


Figure 24. Run #3. Comparison of pH and IU/ml vs. time of the 50 ppm DMSO test flask to the control without DMSO. Control, pH -○-, and IU/ml -●-; 50 ppm DMSO test flask, pH -△-, and IU/ml -▲-.

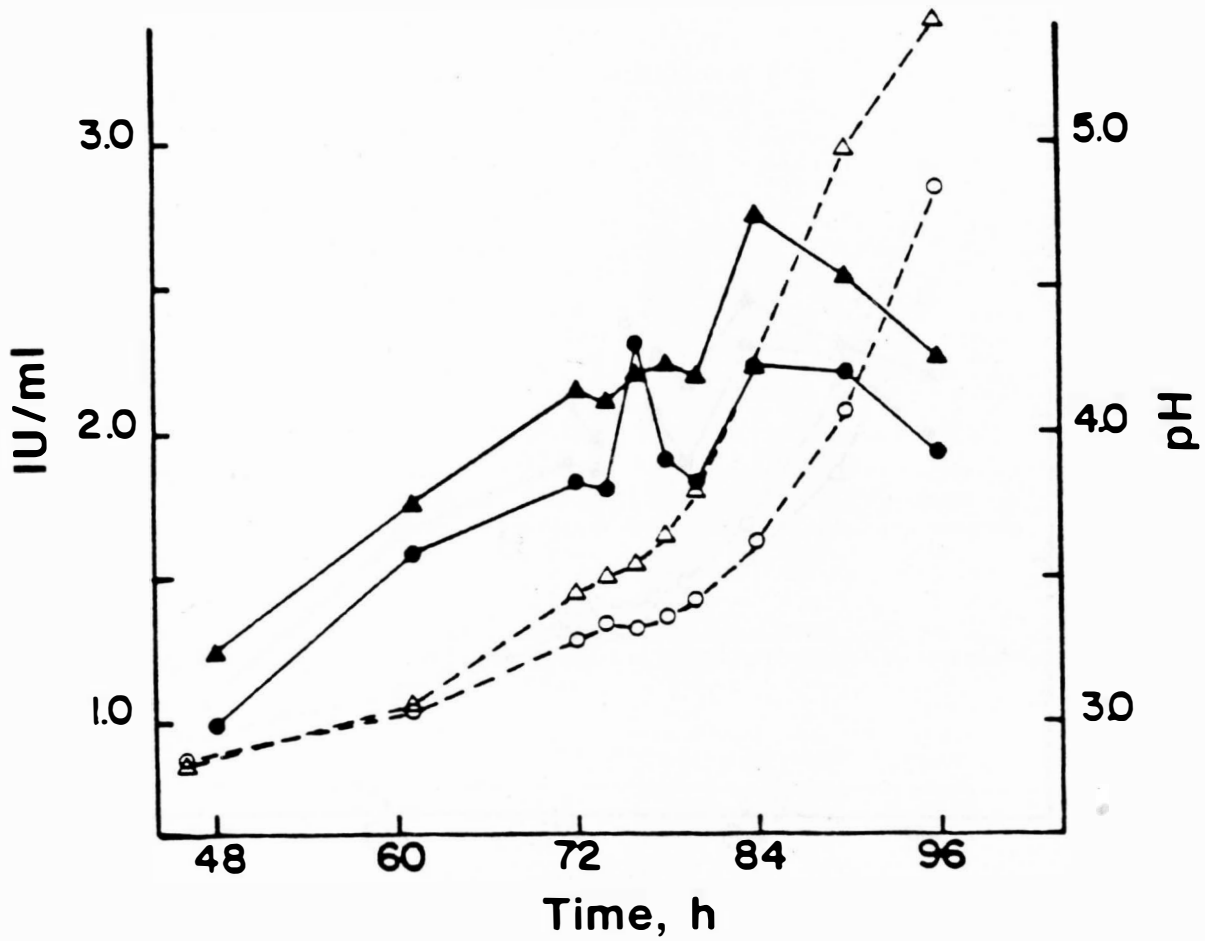


Figure 25. Run #3. Comparison of pH and IU/ml vs. time of the 100 ppm DMSO test flask to the control without DMSO. Control, pH -○-, and IU/ml -●-; 100 ppm DMSO test flask, pH -△-, and IU/ml -▲-.



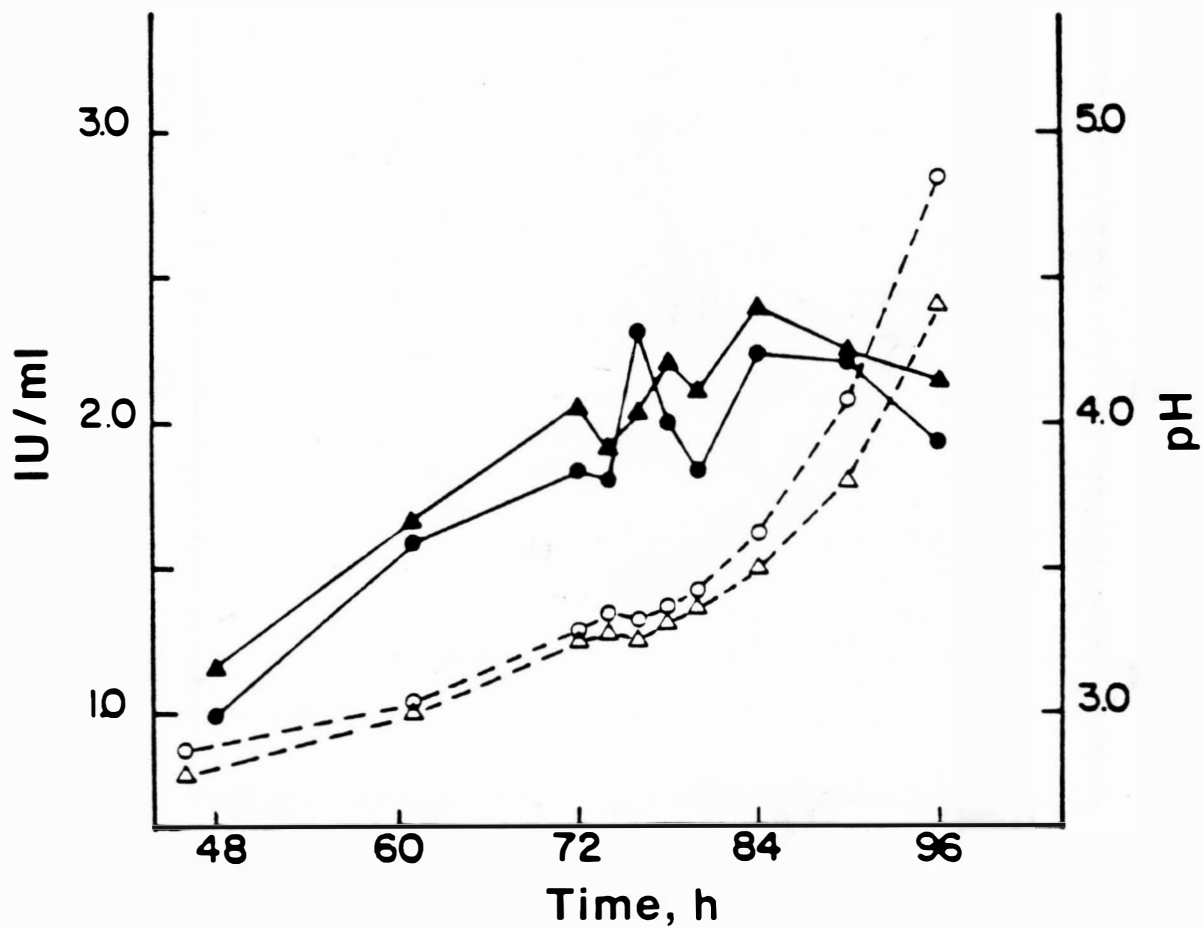


Figure 26. Run #3. Comparison of pH and IU/ml vs. time of the 200 ppm DMSO test flask to the control without DMSO. Control, pH -○-, and IU/ml -●-; 200 ppm DMSO test flask, pH -△-, and IU/ml -▲-.

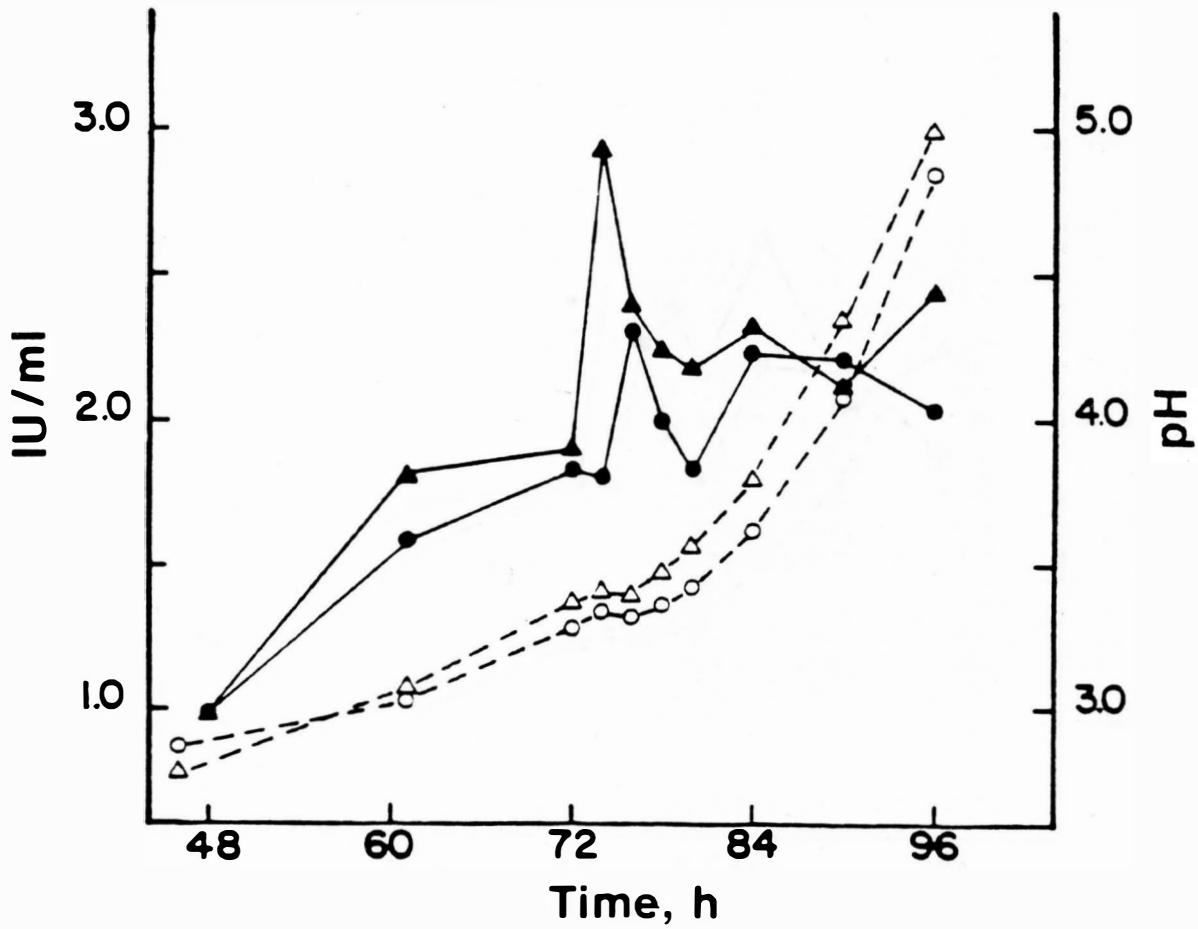


Figure 27. Run #3. Comparison of pH and IU/ml vs. time of the 300 ppm DMSO test flask to the control without DMSO. Control, pH  $\text{---}\circ\text{---}$ , and IU/ml  $\text{---}\bullet\text{---}$ ; 300 ppm DMSO test flask, pH  $\text{---}\triangle\text{---}$ , and IU/ml  $\text{---}\blacktriangle\text{---}$ .

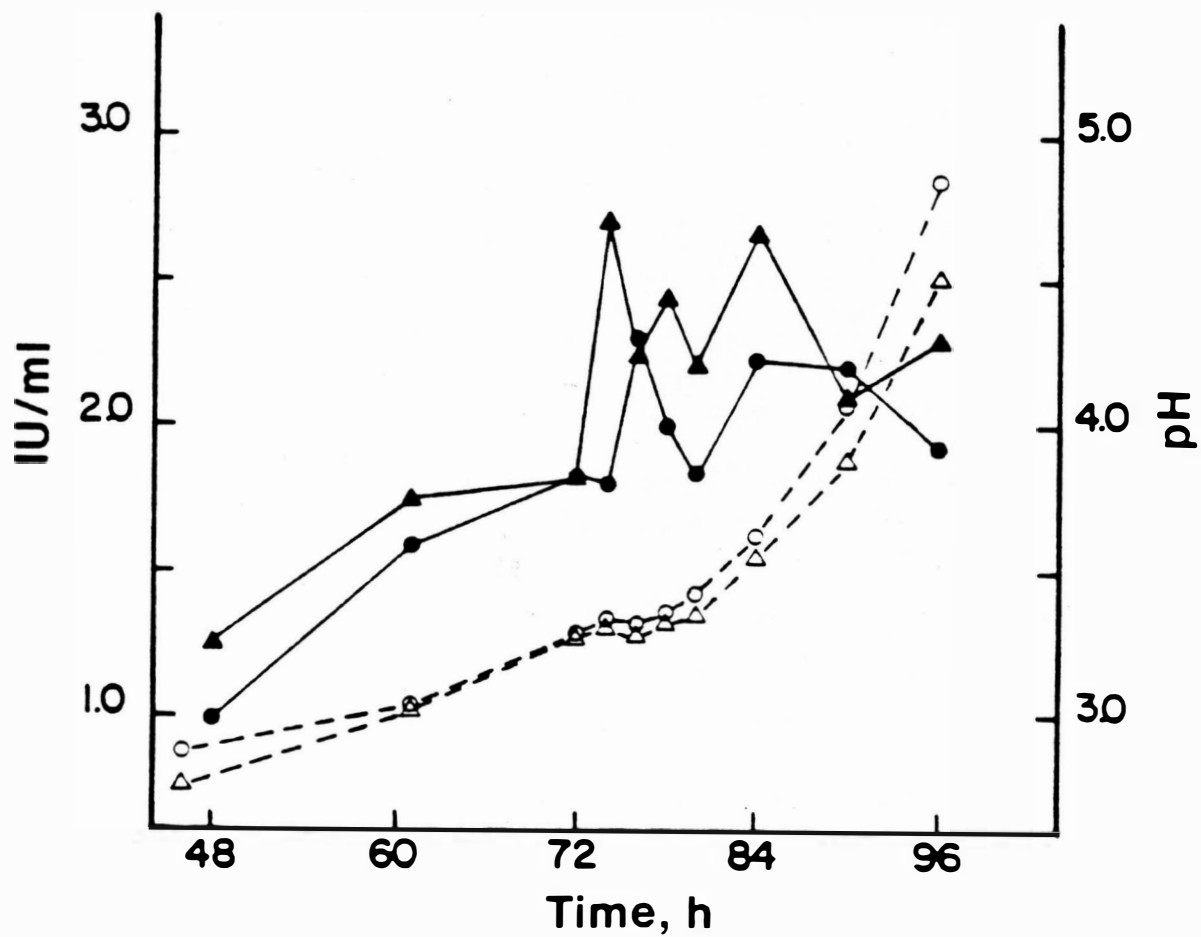


Figure 28. Run #3. Comparison of pH and IU/ml vs. time of the 400 ppm DMSO test flask to the control without DMSO. Control, pH  $\text{---}\circ\text{---}$ , and IU/ml  $\text{---}\bullet\text{---}$ ; 400 ppm DMSO test flask, pH  $\text{---}\triangle\text{---}$ , and IU/ml  $\text{---}\blacktriangle\text{---}$ .

values generally indicate that the 400 ppm level would be the best addition level. However, on the basis of the individual test comparisons to the control, a level above or equal to the 100 ppm DMSO level would be adequate.

Since this study was performed in shaker flasks, it provides a general indicator of the effect of DMSO on cellulase production. The shaker flask study is limited in the ability to maintain identical conditions in all flasks. The use of a laboratory fermenter where exact aeration rates, agitation, pH and temperature control can be made may further elucidate the effects and optimal addition of DMSO for the cellulase producer Trichoderma reesei MCG77.

## CONCLUSIONS

Pilot plant production of a crude cellulase enzyme was shown to be feasible using Trichoderma reesei QM9414 in a rudimentary batch production system. Successful production was achieved, however, the yields were dependent upon the size of the inoculum and the fermenter design. Higher levels of substrate induced higher enzyme activities but over longer periods of time. This was apparent in this study and in the study of Sternberg and Dorval (120).

Inoculum levels of not less than 10% v/v would improve enzyme production time. A better fermenter design would also favorably affect yields. A fermenter of suitable dimensions (height to diameter ratio of 1) was acquired late in this work on the basis of the data presented here but was not implemented as a part of this study.

The enzyme produced in the pilot plant runs demonstrated excellent saccharifying power. The ability to dilute the enzyme is significant in terms of costs and efficiency. Thus, the enzyme should be tested for its optimum dilution ratio before pilot plant saccharification.

The method of Montencourt and Eveleigh (74) for measuring the cellulase complex was suitable for approximating the activity of the crude enzyme. However, measuring the cellulase complex continues to be a problem. A simple method applicable to conveniently assaying a large number of samples is required. Until such a method evolves, researchers will continue to present data derived from the various methods available making comparisons difficult.

The addition of DMSO to the basic cellulose medium did not effect a clear response using T. reesei MCG77. This may be due in part to the lack of control of environmental factors, in particular oxygen supply, as a result of a shaker flask study. Overall, DMSO appears to promote an advanced pH profile. This was observed in almost all cases. However, pH can not be directly related to enzyme activity. The effect of DMSO on the pH can be regarded as favorable to the cultural conditions for enzyme production.

The enzyme activity of the DMSO test flasks reflect an overall increase compared to the control. However, the values for the individual runs fluctuated, showing no real optimal addition level. In this respect, further investigations using parallel cultures in New Brunswick Scientific MicroFermenters would be useful. This apparatus would allow strict control of the parameters in doubt here and optimize the other cultural conditions as well.

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