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Fermentation of Cellulose

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FERMENTATION OF CELLULOSE

BY

DONALD AIDALA

A THESIS SUBMITTED IN PARTIAL FULFILLMENT OF THE
REQUIREMENTS FOR THE DEGREE OF
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OF
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UNIVERSITY OF RHODE ISLAND

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Abstract

This study is concerned primarily with two aspects of submerged fermentation of solid cellulose: the effects of foaming and pure oxygen utilization (in place of air) upon growth and enzyme production of the organism T. viride QM9414. Pure oxygen flow rates are varied, and growth and enzyme production data are compared to results obtained during air fermentation runs (with flow rates five times those of pure oxygen for comparison purposes and still to meet oxygen requirements of the organism for growth).

At an initial cellulose concentration of 1.0% for all fermentation runs, aeration with pure oxygen resulted in an enhancing effect upon enzyme production rate (about 1.5-2 times as fast as with air). It appeared that better oxygen utilization was associated with the pure oxygen fermentations as indicated by slightly lower average DO levels. This was thought to be attributed to better oxygen mass transfer during the oxygen fermentations, which would favor protein synthesis. Foaming was a problem common to both air and oxygen systems, but more so to air runs, especially during the first 3-4 days. It was determined that enzyme was present in the foam, which would account in part for lower levels in solution. Cell autolysis, which favors good enzyme production, occurred at earlier times during the oxygen fermentations, which would also help to explain better enzyme production rates with pure

oxygen. Finally, the phenomenon of catabolic repression, characteristic of both glucose and cellobiose, seemed to be evident especially during the earlier stages of both air control runs, which would have inhibited enzyme production during these times.

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I. Introduction

A persistent problem of importance and concern to both the scientific community and general public is world food provision. Although much scientific research has been devoted to alleviating this problem, it is only recently that cellulose, a traditional waste material, has been recognized as a potential source of foodstuffs. Cellulose is the most abundant carbohydrate in the plant kingdom, and comprises between 40-60% of all the waste generated annually in the United States (1). In view of the plentiful existence of this compound, it would seem both fruitful and imperative that we expand our efforts toward reprocessing and developing new uses for cellulose. The development of technology to utilize waste cellulose as a fermentation or hydrolysis substrate would afford several opportunities: it could help mitigate the waste problem, provide a valuable raw material supply, and aid in providing potential food products for an ever increasing world population (2,3,4,5). Figure 1 schematically depicts some of the potential methods of cellulose utilization.

Degradation of cellulose, aside from being investigated for possible future industrial applications, is of great importance to most all living systems in nature. Microorganisms are capable of degrading cellulose into soluble oligosaccharides and monosaccharides, which are then recycled as nutrients (6). Furthermore, animals such

as horses, cows, goats, and rabbits, as well as certain insects, also possess the ability to digest cellulose to some extent (7).

Of particular concern here are methods of cellulose degradation suitable for industrial applications. Acid hydrolysis, oxidation, thermal degradation, visible or U.V. radiative destruction, pyrolysis, alcoholysis, and acetolysis have been attempted in the past, but have exhibited several drawbacks. Unfortunately, these reactions typically produce so great a diversity of products that elaborate separation techniques are required. Furthermore, many of the above reactions depend upon rather harsh reaction conditions for their success, and thus would directly increase energy requirements. Enzymatic degradation, on the other hand, is more specific in attack, would result in fewer undesired products, and at the same time, could be carried out under milder reaction conditions (8).

One problem with microbial degradation of cellulose is the lengthy time required for hydrolysis. Any improvement in enzyme production rate and yield would brighten the economic outlook of the process (9). Because of the highly crystalline complex nature and insolubility of the cellulose substrate, intimate contact between enzyme and substrate is impeded. This contact, which is required for successful hydrolysis, can only be achieved by diffusion of

the extracellular enzymes from the organisms into the highly resistant cellulose matrix (10). Such substrate characteristics have prompted investigation into possible pretreatment of the cellulosic material to produce a more reactive cellulose (11). It has been suggested that because of the foaming problem associated with cellulose fermentation, there is the possibility of the enzyme complex being swept into the foam with subsequent deactivation resulting in reduced enzyme levels for substrate degradation in the liquid phase (12).

In light of the latter information, this study sought to investigate the effect of foaming upon enzyme production, with the intention of controlling foam levels to enhance enzyme levels. To reduce foam levels pure oxygen was substituted for air. Oxygen requirements for organism growth were satisfied by maintaining pure oxygen flow rates at one fifth the value of air. Thus, the effects of pure oxygen upon enzyme productivity rates were also demonstrated. The variables of interest which affect organism growth rate and its production of the desired enzyme system were temperature, agitation, pH, dissolved oxygen level, and pure oxygen flow rate. Although many of the variables have yet to be optimized (12), some parameter operation ranges have been proposed and have resulted in better enzyme yields (13). Other approaches to the problem have been suggested by

Sternberg (13) and Mandels (14). These include generation and isolation of hyperproducing mutant strains of organisms by irradiation, and additional work in optimization of the fermentation medium. However, approaches fall more naturally into the realm of microbiology. Finally, it was also the intent of this study to determine whether fermentation data obtained here could be more properly described by one of the few existing kinetic models for enzymatic degradation such as that of Suga, Van Dedem, and MooYoung (15), Huang (16), Brown and Zainudeen (41), or Howell and Stuck (17).

Figure 1 Methods for Utilization of Cellulose (6)

II. Literature Review

Cellulose is a linear polymer comprised of D-glucose residues linked together at the one and four carbon atoms by a β -glycosidic bond. The chemical structure is depicted in figure 2. In native cellulose the average number of D-glucose residues, denoted by the letter n , is several thousand, and individual values as high as 10,000 or even 15,000 have been cited (8,18,19).

As a result of the numerous hydroxyl groups contained within cellulose molecules, there is a strong tendency for both intermolecular and intramolecular hydrogen bonding between the available hydrogen and oxygen atoms (20). The resulting configuration is typically a series of cellulose chains parallel to each other forming long slender bundles termed microfibrils which constitute the fiber (21,22). The microfibrils are in turn oriented primarily at a slight angle to the fiber axis (23). The actual diameter of the microfibril is a subject of considerable controversy because of variations in the source of cellulosic material as well as difficulties which arise in the measurement of such materials. However, recent studies indicate that the diameter is 35A (24,25), although there are other estimates as low as 16A (26,27). While many investigators prefer to consider the latter unit an "elementary fibril" (28) or "micelle string" (20),

Figure 2 Chemical Structure of Cellulose-
Haworth Formula

they commonly designate a composite unit of about 200A in diameter as the accepted microfibril.

Many suggestions have been proposed for the structure of the unit cell, but no one particular model seems to corroborate all the evidence gathered by X-ray diffraction and infrared spectroscopic measurements. The fringed micelle theory, which predicts that the microfibril includes regions of good order (crystallites) alternating with those of disorder (noncrystalline regions), is a popular model, and helps to confirm the concept of crystalline length being shorter than the cellulose molecules themselves (29). Another recent proposal suggests that the fibril is fringed so that the disorder arises essentially from the sides of the microfibril with possible interfibrillar molecular connections (30), with the inner portion of the microfibril of a comparatively more ordered nature. Finally, even systems of folded chains with the folds representing the regions of disorder have been proposed (25,31). Although there is still some question as to the exact nature of the unit cell in native cellulose, there is rather unanimous agreement as to the highly ordered configuration of the compound. It is because of this characteristic that native cellulose is highly resistant to hydrolysis by acids, and is normally susceptible to attack only at the disordered surface regions.

This limited hydrolysis feature has prompted investigation into the possible structural manipulation of cellulosic material to increase degradation rates during the hydrolysis reaction. It has been determined that the susceptibility of cellulose to attack by either acid or enzyme is significantly enhanced through destruction of its crystalline structure. This can be accomplished by several means, either chemical or physical in nature. Sodium hydroxide treatment similar to that utilized in mercerization and regeneration, as well as liquid ethylamine used in amine decrystallization, are some of the chemical techniques which effect intramolecular swelling and structural modification of the cellulose fibers. However, an important consideration here is the economic feasibility of such pretreatments. Even more successful in altering the highly crystalline cellulose structure have been physical pretreatments, primarily electron irradiation and vibratory ball milling. Both physical modes of pretreatment have enhanced hydrolysis rates of cellulosic material; however, both methods have considerable energy requirements, and their practical application will largely be determined by more favorable results in research and development (31).

During enzymatic hydrolysis, the susceptibility of the cellulose substrate to enzyme attack is largely dependent upon the accessibility of the extracellular

enzymes secreted by or bound to the microorganisms. Intimate contact is mandatory for effective hydrolysis. Because cellulose is an insoluble structurally complex substrate, it is necessary for the cellulase enzymes to diffuse through the cellulose matrix to achieve the necessary contact between enzyme and substrate. Accordingly, there are certain structural influences which will affect this diffusion process and the susceptibility of the cellulose substrate to attack. The moisture content of the fiber is significant because of the possible swelling effect which opens the pathway for enzymes, and also acts as a medium for transport. The size and diffusivity of the enzyme molecules in relation to size and surface properties of the gross capillaries (i.e. the cell lumina) and the cell-wall capillaries (i.e. those areas between microfibrils and the cellulose molecules located in the amorphous regions) would also influence the accessibility of the substrate. It has been demonstrated that many cellulolytic enzymes are capable of easily penetrating the former (2000A to 10u in diameter), but have difficulty in permeating the smaller (less than 200A in diameter) cell-wall capillaries. Other considerations such as the degree of crystallinity of the cellulose substrate, the conformation and steric rigidity of the anhydroglucose units, the degree of polymerization of the cellulose, the nature of the substances

associated with the primary cellulose substrate, such as lignin or hemi-celluloses, and finally the nature, concentration, and overall distribution of substituent groups have some regulatory effect in the diffusion process (10).

Many organisms have been reported capable of efficient degradation on various cellulosic derivatives (e.g. carboxymethyl cellulose, hydroxyethyl cellulose, and methyl cellulose), but number considerably less in their ability to utilize native cellulose. Microorganisms which are capable of the latter stem from two primary groups, bacteria and fungi. Cellulase production has been observed with the action of mesophillic and thermophillic bacteria, streptomycetes, and species of *Phizopus*, *Aspergillus*, *Penicillium*, and *Trichoderma* upon various cellulose substrates (33). It has been previously established that the fungus *Trichoderma viride* is a convenient source and an excellent producer of a system of enzymes forming the cellulase complex, which is able to totally hydrolyze native insoluble cellulose to glucose (34). Through irradiation of the conidia of *T. viride* QM6a with a linear accelerator, a mutant strain designated QM9123 was developed which is capable of producing twice as much cellulase as the parent strain (35). Additional irradiation of this strain produced a second mutant strain, QM9414, capable of even greater enzyme

levels (1,35). For this reason, this latter strain has been selected for this present study. T. viride has very simple growth requirements and will produce cellulase in a solution of nutrient salts with no special additives. Increased yields of enzyme are evident if small quantities of soluble carbon and nitrogen sources are added to such a solution. Nitrogen containing compounds, such as peptone and other protein derivatives, have proven more effective than non-nitrogenous substances with regard to enhancement of enzyme yields. Cellulose substrate concentrations between .5 and 1.0%, together with peptone concentrations in the .1 to .2% (13) range seem to yield optimum enzyme quantities, while peptone concentrations greater than .5% are known to be inhibitory to enzyme production (34). Surfactants have also been added in the .05 to .2% concentration range and have proven successful. Increased yields of enzyme of over 50% were noted with the addition of a surfactant, Tween 80 (polyoxyethylene sorbitan mono-oleate) (37). The enhancing mechanism of the Tween 80 is presently not well understood; however, it could be related to the increased permeability of the cell membrane which would allow more rapid secretion of the enzyme system necessary for the increased enzyme synthesis (13).

Because of the difficulty associated with the breakdown of native cellulose through the production

of the enzyme cellulase, it has been proposed that the cellulase enzyme system is multicomponent in nature (38). The enzyme scheme is represented in the following diagram:

Initially, the native cellulose is converted into a more reactive form by the C_1 enzyme component. Reactive cellulose undergoes hydrolysis by the C_x enzyme, the "x" indicating the multicomponent nature of the second enzyme. There are at least two β -1-4 glucanases which comprise this portion of the enzyme complex (39);

a) α -exo- β -1-4 glucanases which remove individual glucose units from the non-reducing end of the cellulose chain.

b) endo- β -1-4 glucanases which preferentially attack internal portions of the cellulose linkages. The cellobiose is subsequently hydrolyzed to glucose units by the action of the β -glucosidase enzyme component. Although the action of the β -glucosidase enzyme is highly specific for cellobiose, it is capable of hydrolyzing some of the other oligomers derived from cellulose.

A precise description as to the actual kinetics of the T. viride-cellulase system is not completely known at this time. Much ambiguity still lies in the exact mechanism of the cellulose hydrolysis. There are several theories which approach the problem with limiting assumptions and simplifications to the model (13,16,17,41,42). While important questions still remain to be answered in order to fully understand T. viride-cellulose kinetics of cellulase production, these simplified theories do help to point out and explain the existence of certain phenomena during cellulose degradation.

In the T. viride-cellulose fermentation scheme, the enzyme production phase is coincidental with the growth or trophophase (13). This can readily be seen upon examination of the reduction in dry weight versus enzyme production curves. While this trend differs from many systems where the metabolites and enzymes are produced in the idiophase which lags behind the growth phase, this phenomenon can be rationalized on the basis that,

because of the insolubility of the substrate cellulose, the activity of the cellulase enzyme system is lower than the other glucosidases which hydrolze soluble substrates. Relatively large amounts of enzyme are required to liberate metabolizable sugar from cellulose for growth. Thus, cellulase production and growth are tightly coupled. under continuous operation in stirred submerged culture, with unlimited nutrient supply, T. viride biomass production has been found to increase exponentially (43). At low dilution rates, however, systems which essentially approach batch operation, Herbert has suggested that the phenomenon of endogenous metabolism occurs with T. viride culture (40), which would consequently lower cell production rates.

Another parameter affecting metabolic activity and consequently overall growth rate is pH. Muzychenko et al attribute the adverse effect of increasing hydrogen ion concentration upon metabolic activity to a limitation in the rate of diffusional transfer of the substrate-permease complex (42). The diffusional limitation is explained by a charge build-up within the pores of the cell membrane due to the abundant supply of hydrogen ions, although it has been suggested that the cell will compensate somewhat for the diffusional resistnace by increasing either the quantity of permease or internal enzyme systems involved with glucose metabolism. Generally,

the lower the pH (or greater the hydrogen ion concentration), the slower the metabolic activity with relatively little effect upon the stoichiometry or detrimental effect upon cell structure (41).

There are several detectable phenomena which occur during the fermentation operation which undoubtedly have some effect upon cellulase production rate. Huang has observed that adsorption of the cellulase system can occur rapidly onto the cellulose substrate during the initial stages of the reaction (16). As the reaction proceeds, the enzyme is subsequently released back into the liquid phase because of lesser availability of the substrate. The entire scheme is indicated by an initial drop in protein concentration followed by a gradual increase as the reaction proceeds. In a similar manner, soluble sugars such as cellobiose and glucose are also capable of being adsorbed onto the cellulose substrate material. This would consequently reduce hydrolysis rates because of the blocking effect of the sugar, preventing the necessary intimate contact of substrate and enzyme.

It has been determined in the hydrolysis reaction that the true inducers of cellulase for a fungus growing upon cellulose are the soluble products of hydrolysis, specifically cellobiose (44). However, cellobiose can also be instrumental in repressing cellulase formation

if present in excessive amounts, usually .5-1.0%. The following diagram is useful in demonstrating the complex role of cellobiose (45):

This dual nature is also true of other rapidly metabolized carbon sources such as glucose and glycerol.

This "catabolic repression" (sometimes referred to as the "glucose effect"), occurs when the rate of carbohydrate catabolism exceeds that required for growth.

The soluble sugars become plentiful, and the organism preferentially utilizes them for growth. Cellulase synthesis then decreases or stops (13,44,45). It has also been reported that product inhibition of cellobiose and glucose combining with the enzyme system to form inactive complexes is also possible (16).

The fermentation operation has been characterized by Mandels (34) as having certain reproducible trends. The growth requirements of the organism are simple, and when grown in an agitated vessel produces threadlike mycelium whose large surface area would be desirable for growth scale-up or continuous cultivation. There is an initial lag phase which generally continues from 12 to 24 hours, after which the pH rapidly drops with the production of acid. If peptone is initially present, the pH should first rise with the production of ammonia and subsequently decrease as the organism utilizes the ammonia produced from the breakdown of ammonium ions to ammonia and hydrogen ions. The pH will normally decrease to about 2.8-3.0 and remain there until the available substrate is essentially exhausted, at which point the pH begins to rise and cellulase production ceases.

Good enzyme levels have been obtained in a 15 liter agitated fermentation vessel with 10 liters of working volume. The initial pH was nearly 5.0, the temperature from 28-30, aeration rate at .1-.3 vol/vol min of sterile air, and the agitation rate from 100-120 rpm (34,46). 5 liter reactors with 4 liters of working volume have been run at higher agitation rates (600 rpm) and aeration rates (3 vol/vol min), and have also yielded good enzyme concentrations (33). Vacuum evaporation and ultrafiltration have

been utilized to concentrate enzyme levels in culture filtrates to as much as 4 to 8-fold (46,48).

Organism

The organism utilized in this study was *Aspergillus niger* (ATCC 2641) obtained from the U.S. National Collection of Fungal Cultures, Department of Agriculture, Agricultural Research Service, Beltsville, Maryland. The organism was cultivated on malted barley agar at 25°C. It was grown in 250 ml Erlenmeyer flasks containing 50 ml of medium. The medium was prepared by autoclaving 50 ml of water, 10 g of malted barley, and 10 g of yeast extract in a 250 ml Erlenmeyer flask for 15 minutes at 121°C. The medium was then cooled to 25°C and the organism was inoculated into it.

Cellulose

The cellulose used in this study was Whatman No. 40, a high purity cellulose. It was prepared by Whatman Chemical Company (Bellingham, WA). The cellulose was dried prior to use because of its hygroscopic nature. It was stored in a desiccator over calcium chloride. The particle size was 10-20 µm. The cellulose was used as a substrate for the enzyme.

Medium

The medium used in this study was a modified Czapek-Dox medium. It contained 1% yeast extract, 0.5% malted barley, and 0.5% cellulose. The medium was prepared by autoclaving 50 ml of water, 10 g of malted barley, 10 g of yeast extract, and 10 g of cellulose in a 250 ml Erlenmeyer flask for 15 minutes at 121°C. The medium was then cooled to 25°C and the organism was inoculated into it.

III. Materials and Methods

Organism

The organism utilized during this study was *Trichoderma viride* QM9414 obtained from the U.S. Army Natick Laboratories in Natick, Massachusetts. The organism was maintained on potato dextrose agar at room temperature. As previously mentioned, this particular organism is an ample supplier of cellulolytic enzymes.

Cellulose

The cellulose substrate used for both seed flasks and larger scale fermentation runs was SW 40A Solka Floc (99.9% pure wood cellulose) purchased from the Brown Company (Berlin, N.H.). The substrate was dried prior to use because of an initial moisture content of nearly 5%. The particle size distribution was 149 -19.4%, 149 -80.5%, and 53 -11.6% (49).

Medium

The fermentation medium was that proposed by Mandels and Weber (34) with a slight modification in replacing urea with dibasic ammonium phosphate which was reported to further enhance cellulase production (50).

The medium contains in g/liter

KH_2PO_4 2.0

$(\text{NH}_4)_2\text{SO}_4$ 1.4

$(\text{NH}_4)_2\text{HPO}_4$.3

$\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$.3

CaCl_2 .3

and in mg/liter

$\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$ 5.0

$\text{MnSO}_4 \cdot \text{H}_2\text{O}$ 1.56

$\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$ 1.40

$\text{CaCl}_2 \cdot 6\text{H}_2\text{O}$ 3.66

Facto-Peptone was added to the above mineral solution at a value of 10% of the substrate level because of its reported beneficial effect upon cellulase complex yields (34). Prior to sterilization the pH of the medium was 5-5.5.

Fermentor

Submerged fermentation studies were carried out with the use of a laboratory scale 5-liter Microferm Fermentor, Model MF-102 (New Brunswick Scientific Company (NBS), New Brunswick, N.J.), equipped with agitation, aeration, temperature controller, pH indicator and controller, and dissolved oxygen probe and recorder. Figure 3 illustrates a schematic of the fermentor used in this study. The pH indicator is a Leeds and Northrup (L&N) (Leeds and Northrup Co., Philadelphia, Pennsylvania), Model #7678 pH meter, and the pH Controller-Recorder is a L&N Strip-Chart Recorder-Controller, Model Speedomax H. A NBS M1016-0201 galvanic dissolved-oxygen (DO) probe connected to a NBS D.O. Analyzer, Model #DO-40, equipped with a Hewlett-Packard Strip-Chart Recorder, Model #7100B, were used to monitor dissolved oxygen levels throughout the fermentation runs. Oxygen or air flow rates were measured continuously with a Matheson Gas Flowmeter. The sparge system used throughout all submerged fermentation runs was of an orifice (1/8-inch I.D.) type which provided an effective uniform gas stream into the medium.

Sterilization

A NBS Model #AE30-10 electric heating vertical autoclave was used to sterilize the 5-liter fermentor which con-

Fig. 3 Equipment diagram for submerged fermentation of cellulose (legend to fig. 3 is on page 24)

Legend for fig. 3

1. Spent air
2. Compressed gas (air or oxygen)
3. Oxygen-analyzer recorder
4. Heater
5. Gas filter (air or oxygen)
6. Condenser
7. pH acid and base addition lines
8. pH controller-recorder
9. Temperature control
10. pH meter
11. pH electrodes
12. Sampling line
13. Vacuum pump
14. Sampling flask, 250 ml
15. Head plate
16. Pyrex fermentor jar
17. DO probe
18. Baffle plate X 4 (3/4 inches width)
19. Orifice sparger
20. 6-blade turbine impeller (2-inch diam.)
21. Magnetically-coupled impeller shaft
22. Head space
23. Fermentation medium

Dimensions: H=12 inches

D=5.5 inches

h1=8 inches

h2=5.5 inches

h3=2.5 inches

h4=.75 inches

v1=valve #1

v2=valve #2

v3=valve #3

tained 3 liters of medium. The medium was sterilized at 121 C and 15 psig for 60 minutes and then allowed to cool to atmospheric pressure and room temperature prior to inoculation.

Incubator Shaker

A NBS Environmental Shaker, Model #G26 was used for preparation of seed cultures for the fermentation runs.

Preparation of Inoculum

250 ml Erlenmeyer flasks containing 100 ml of nutrient medium with 1% cellulose substrate were inoculated and incubated from 4-6 days at 29 C and an agitation speed of 125 rpm to serve as seed flasks for the fermentor containing 3 liters of medium.

Sampling

The high degree of heterogeneity of the culture required special sampling line construction in order to obtain representative samples. Prior to each sampling period, air or pure oxygen, depending upon what oxygen source would be used during the run, would be introduced (see figure 3) to clear the line of any previously remaining liquid and particulate matter. This flushing procedure was performed with valves 1 and 2 open and

3 shut. Valve 1 was subsequently shut while valves 2 and 3 remained open for the sample fluid to be pumped by a Peristaltic Pump, Model #AL4E (Sigmamotor, Inc., Middlepoint, N.Y.) into a receiving flask. 50 ml samples were collected at each sampling period.

Reagents

Bacto Peptone was purchased from Difco Laboratories, Detroit, Michigan. Tween 80 (polyoxyethylene sorbitan ~~mono~~-oleate), a surfactant whose effect has been to enhance cellulase production, was manufactured by BBL, Div. Bocton, Dickenson and Company, Cockeysville, Maryland. 3,5-Dinitrosalicylic Acid utilized in the enzyme and reducing sugar analysis was obtained from Eastman Kodak Co., Rochester, N.Y. Rochelle Salts (Potassium Sodium Tartrate), one of the primary reagents in the reducing sugar test, was purchased from Mallinskrodt, Inc., St. Louis, Missouri.

Dissolved Oxygen

Oxygen amount was expressed in percentage saturation of dissolved oxygen, with 100% saturation representing a solution saturated with air at 28 C. Prior to use during the fermentation, the oxygen probe was calibrated by aerating at the desired flow rate, agitation speed, and temperature for the particular run for at least 30 minutes. If pure oxygen were used during a run, the cal-

ibrating procedure would be the same with pure oxygen substituted for air. The recorder was then set at the 100% level prior to inoculation with the seed flasks.

Weights of Residual Cellulose and Fungal Cell Tissue

The method of total weight determination used in this study was that proposed by M. Mandels of the U.S. Army Natick Laboratories and further tested by Rom-anelli (51). Slight volume changes were made in this study for this particular test. All samples were analyzed immediately according to the following procedures:

1. 50 ml of bulk medium were sampled periodically from the fermentor into a 250 ml Erlenmeyer flask containing a magnetic stirrer. Four 10 ml aliquots were pipeted into test tubes (22 mm I.D. x 15 cm) using a quick transferring pipet. The remaining sample was utilized for determination of cellulase activity, reducing sugar, and glucose concentration after being filtered through a Whatman No. 2 filter pad to remove suspended particulate matter.

2. Total weight of the residual cellulose and fungal tissue was determined for the 4 portions by filtering each 10 ml solution through a preweighed 5.5 cm Whatman No. 2 filter paper in a Buchner funnel. The filter paper was subsequently washed with small

amounts of water and dried at 110 C for 20-25 hours.

3. The dried filter paper pads were weighed and the total weight of fungal tissue plus residual cellulose was determined by subtracting the filter paper weight from the filter paper plus solids weight and averaging the results. Division by the sample volume (10 ml) gave the final result expressed in mg/ml.

4. For determination of the residual cellulose, the contents of initially dried filter papers plus solids were transferred by a spatula to a 50 ml beaker. This part of the procedure involved 2 of the initially dried pads to obtain an average result of residual cellulose. 2.5% NaOH was then sprayed onto the pads to wash off any remaining solids from the filter paper.

5. A tissue homogenizer was then used to thoroughly break apart the mycelium and cellulose masses. The volume was then brought up to 30-40 ml final volume and transferred quantitatively to a 250 ml Erlenmeyer flask which was subsequently placed upon a rotary shaker from 12-18 hours at 125 rpm to digest the fungal tissue. The residue contains only cellulose and possibly a very small amount of cell wall material (primarily chitin).

6. Subsequently filtration, drying, and weighing procedures were performed in the same manner as in steps 2 and 3 to determine the residual cellulose.

7. Fungal tissue values expressed in mg/ml were then obtained by subtracting the residual cellulose values from the total solids and finally averaged, resulting in an average fungal tissue count.

Total Reducing Sugar

The glucose-dinitrosalicylic acid (DNS) method of Miller (52) was used for the determination of reducing sugars present in the reaction medium. The reagent is comprised of the following constituents:

Distilled water	1416. ml
3,5-Dinitrosalicylic acid	10.6 g
Sodium hydroxide	19.6 g
Rochelle salts (Na K Tartrate)	306.0 g
Na meta bisulfite	8.3 g

3 ml of the reagent are titrated with .1N HCl with the required amount of titrant between 5-6 ml. NaOH should be added if adjustment should be required to meet these range requirements. Samples filtered from the fermentations were analyzed by the following procedure:

1. Samples were diluted with distilled water to contain from .1-1 mg of glucose per ml. A .5 ml sample was placed into a 14 mm I.D. x 15 cm test tube containing 3 ml of DNS reagent. The mixture was then heated in a

boiling water bath for five minutes.

2. The sample mixtures were then allowed to cool to room temperature aided by tap water.

3. The percent transmittance of the cooled samples was then measured in a Bausch and Lomb Spectronic 21 Photoelectric Colorimeter at 550 nm.

4. The absorbance was calculated knowing the following relationship between transmittance (T) and absorbance (A): $A = 2 - \log T$

5. A blank consisting of .5 ml of distilled water plus 3 ml of DNS reagent and standards utilizing known concentrations of glucose made in a similar fashion, were used to formulate a standard curve from which unknown sample concentrations could be read as shown in figure 4.

6. All samples were run in duplicate, and results were reported in mg/ml of glucose equivalent.

Cellulase Activity

The assay for enzyme activity was the filter paper (FP) test proposed by Mandels et al (14,53).

1. A strip of Whatman No. 1 filter paper (1 cm x 6 cm) weighing 50 mg was placed into a test tube containing 1 ml of sodium citrate buffer and .5 ml of sample previously filtered. Test tube preparations of undiluted

Figure 4 Standard Curve for Reducing Sugar Determination

sample, .5 dilution, and .33 dilution were made up in similar fashion. Distilled water was used to make the initial enzyme dilutions. Each dilution was run in duplicate.

2. The filter paper strip was coiled into the mixture through touching each tube to a vortex mixer.

3. The mixture was then incubated in a constant temperature water bath at 50 C for 1 hour. The enzyme reaction was then stopped by the addition of 3 ml of DNS reagent. The reducing sugar content measured as glucose was then determined by the previously described DNS method. Blanks without the filter paper strip included were prepared and incubated to account for any sugar present in the original filtrate containing the undiluted enzyme.

4. Filter paper activity (FPA) is given in the units mg/ml of reducing sugar as glucose. Filter paper (FP), as it's reported in the literature, is given in International units (I.U.) per ml. If the FPA value were less than or equal to 2.0 mg/ml, the FP value would be determined by multiplying this sugar value by .185. If the FPA value were greater than 2.0 mg/ml, International units were calculated from the dilution to give 2.0 mg/ml of glucose as shown in figure 5.

Figure 5 Enzyme Test Curve

Glucose Analysis

Glucose determinations were conducted with the use of a YSI model 23A Glucose Analyzer, a quantitative device for measuring glucose levels. This instrument, originally constructed for measuring glucose levels in blood serum, blood plasma, and whole blood, has also proven useful for non-medical determinations.

The principle of operation involves the enzymatic conversion of glucose and oxygen to gluconic acid and hydrogen peroxide. In the YSI Model 23A, a constant portion of the hydrogen peroxide is then oxidized by a probe at a platinum anode. The current thus produced is directly proportional to the glucose level in the sample.

The samples for testing consisted of 25 ul portions of the undiluted filtrates obtained from the 50 ml fermentations aliquots having undergone filtration through Whatman No. 2 filter paper. The samples required no initial dilution in the 0-5 mg/ml glucose range or any special pretreatment techniques to eliminate foreign matter which might interfere in the determinations. The results were obtained from a digital read out in mg% values. These values were subsequently converted to express final glucose results in mg/ml.

IV. Submerged Fermentation Results

The variables of interest and results of six batch fermentations are given in table 1.

This study was conducted using 1% cellulose for all the runs because of reported success in obtaining good enzyme yields (34). pH was allowed to fall from initial values of nearly five to values of about 2.8-3.0 before pH control was implemented to maintain the pH between 2.8-3.4 for good cellulase yields (13). DO levels were on the average lower in the pure oxygen runs than in the air runs as shown in figure 6 and 7. Since agitation rate was constant in all runs, DO level differences were primarily dependent upon gas flow rate and rate of uptake by the organism. In all cases, the initial rapid decreases in DO level were attributed to the unrestricted growth of the microorganism. All runs were carried out at temperatures between 28.5-30 C.

Figures 8 through 13 are composite graphs with each containing five parameters measured periodically throughout each fermentation run: total solids, fungal tissue, enzyme activity, reducing sugar, and glucose concentrations.

Total solids counts seemed to follow a characteristic

Table 1 Summary of Cellulose Fermentation Parameters

Run No.	1	2	3	4	5	6
Init. Substrate (%)	1.0	1.0	1.0	1.0	1.0	1.0
Tween 80 (%)	.2	.2	.2	.2	.2	.2
Aeration (ft ³ /hr)	*.172	*.129	*.086	*.043	.430	.215
** Dissolved O ₂ (%)	71	54	57	45	63	78
Maximum Cell Dry Weight (mg/ml)	1.25	1.67	1.79	1.79	1.99	1.36
Maximum Enzyme Activity (units/ml)	1.61	1.61	1.68	1.35	.90	.31
Weighted Ave. Enz. Act. $\bar{E}$ (units/ml)	.599	.544	.949	.491	.359	.130

* Pure oxygen used during run

** Average values throughout the run

Figure 6 Dissolved Oxygen and pH readings for O_2 runs

Figure 7 Dissolved Oxygen and pH readings for Air runs

Figure 8 Composite graph for run #1, O_2 at .172 cfh

Figure 9 Composite graph for run #2, O₂ at .129 cfh

Figure 10 Composite graph for run #3, O₂ at .086 cfh

Figure 11 Composite graph for run #4, O₂ at .043 cfh

Figure 12 Composite graph for run #5, Air at .430 cfh

Figure 13 Composite graph for run #6. Air at .215 cfh

exponential pattern in all cases, although nearing the finish of the runs (i.e. after 5 days or 120 hours), the degree of cellulose conversion was lower in those runs with air. Fungal tissue growth curves were somewhat varied although certain trends were indicated. In all cases there was an initial period of growth which appeared to reach a maximum value during the early stages of the run (i.e. 40 to 60 hours). This stage was then followed by an interval of no additional growth and subsequently by a period of destruction of original growth or cell autolysis. These trends are indicated in figure 14. From the plotted data, it is difficult to determine any direct effect of pure oxygen over air upon growth except that cell autolysis did appear to arrive earlier in the pure oxygen fermentation runs.

Cellulase enzyme activity differed quite noticeably between pure oxygen and air runs. Figure 15 shows plots of enzyme activity in units/ml versus time passed for both oxygen and air runs. It was quite evident that enzyme production rates and yields were enhanced in the pure oxygen fermentations. Figures 16 through 21 are individual plots of enzyme activity from which weighted average enzyme values were obtained for each run. The area under the curves was first calculated, then divided by the run time taken as 144 hours in each case. The

resulting values represented average enzyme values for each run, and were again noticeably higher for pure oxygen runs (see table 1). These values were plotted against gas flow rates for both oxygen and air in figure 22.

Reducing sugar curves for pure oxygen runs indicated gradually increasing levels throughout the duration of the runs. During the two air control runs, this value seemed to increase rather rapidly for the first 40-60 hours and then gradually fall to a leveling value. Glucose levels were rather low in all runs with no truly significant increases or decreases throughout running times. These trends would support the idea of glucose, a more rapidly metabolized substrate, being preferentially utilized if present in sufficient quantities. Cellobiose concentrations were taken as the difference between reducing sugar and glucose values at any time during the runs. These concentrations increased as the runs proceeded (see figures 8-13) in most cases. During run 6 (fig. 13), higher concentrations of reducing sugar, primarily cellobiose, developed, which may have had some inhibitory effect upon enzyme production, while during run 3 (fig. 10), low values of reducing sugar, essentially glucose, may have had some enhancing influence.

Figure 14 Growth curves for Air and O₂ fermentation runs

Figure 15 Enzyme activity curves for O₂ and Air runs

Figure 16 Enzyme activity curve for run #1, O_2 at .172 cfh

Figure 17 Enzyme activity curve for run #2, O_2 at .129 cfh

Figure 18 Enzyme activity curve of run #3, O_2 at .086 cfh

Figure 19 Enzyme activity curve for run #4, O_2 at .043 cfh

Figure 20 Enzyme activity curve for run #5, Air at .430 cfh

Figure 21 Enzyme activity curve for run #6, Air at .215 cfh

Figure 22 Average enzyme values vs. flow rates for O_2 and Air runs

Foam levels, while not actually measured because of the lack of uniformity in foam height, were observed and qualitatively noted. Foam level comparisons between either pure oxygen or air runs alone were inconclusive. However, it appeared that during the first 3 or 4 days of the runs, the foam levels produced by air sparging were higher (touching the head plate of the fermentor, about 6 inches from the liquid level) than those produced by pure oxygen sparging (on the average, 1.5 to 2.0 inches).

V. Submerged Fermentation-Discussion

The DO data presented in figures 6 and 7 show that average DO levels for the air runs were slightly higher than those runs with oxygen. This suggests no oxygen limitation upon either air or oxygen systems. Growth patterns were similar, and fungal tissue amounts were in the same numerical range as demonstrated in figure 14. Cellulase activity however was unquestionably higher in oxygen runs (see fig. 15) which suggests an enhancing effect upon enzyme production by pure oxygen bubbles.

Although oxygen may have been available for utilization by the organism in both systems, it appears that the effective use of oxygen is in question. This implies that the oxygen transfer mechanism is more efficient for enzyme production using a pure oxygen sparging system, which is not unreasonable when separately considering the transfer possibilities of the oxygen from oxygen and air bubbles. It is quite possible that the nitrogen contained within the air bubbles acts as a resistance to the transfer of oxygen from the inner portion of the bubble to the medium. This would explain why DO levels were slightly higher in the air runs, although actual oxygen utilization by the organism w

was less efficient.

Increased enzyme levels with oxygen could further be explained in light of the previous information. In considering protein synthesis alone, we know that the energy rich compound ATP (adenosine-5'-triphosphate) is required. Furthermore, it is known that to furnish the necessary ATP for protein synthesis, oxidative reactions must occur which require molecular oxygen for their successful completion. If the oxygen were made more available for these protein synthesis reactions, increased protein levels and consequently increased enzyme levels should be observed.

Foaming was a problem associated with both air and oxygen fermentations, although it appeared more evident in the air runs during the first three to four days. This was in part due to the higher air flow rates. While it was difficult to determine whether or not the enzyme was being lost by gas fractionation and subsequently inactivated by oxygen at the gas-liquid interface (54), it was evident from several enzyme determinations on foam samples that enzyme was being swept up into the foam during the reaction. This phenomenon was particularly prevalent during the first three to four days when foam levels were highest in both systems. Higher foam levels were observed during these periods

from air runs (see results section), which would imply that a greater quantity of enzyme was being entrapped within the foam at any given sampling time. This would help to explain lower levels of enzyme present in the liquid medium during the air runs.

Another concept relating air content in foam to viscosity reported by Punton (55) assists in explaining higher enzyme yields in the oxygen runs. In figure 23 viscosity is plotted against air content of the foam created from a suspension of treated wood pulp fibers in aqueous medium containing a 1% surfactant level. This situation is similar to the fermentation system under study. Punton has observed that viscosity increased as air content in the foam increased. In observing the levels and physical appearances of the foams of both air and oxygen runs, it seemed that the air foams contained more gas than liquid, which would imply the air run medium having a greater viscosity. This was not verified because of measurement difficulties. However, figures 8 through 13 show that, on the average, higher values of total solids over five days of cultivation were associated with air runs, which would indicate less conversion of the cellulose. This in turn would mean a higher degree of polymerization which is proportional to the medium viscosity. Assuming that a medium with a

Figure 23 Relationship between foam, viscosity, and air content from suspension of treated wood pulp fibers in aqueous medium with 1% surfactant (55)

higher degree of polymerization is also more viscous, would then help support the observations of air foams containing greater quantities of gas than oxygen foams. With the above viscosity comparisons established between air and pure oxygen conditions, it is possible to employ a dimensionless relationship involving the viscosity of the medium and the degree of oxygen mass transfer to further explain the enhancement of enzyme production in pure oxygen systems (56). Assuming the gas bubbles to be nearly spherical, the equation selected to predict the mass transfer behavior was of the following form:

For Flow Past Individual Spheres

$$\text{Eq. 1} \quad \frac{kD_p \bar{M}}{\rho D_v} = 2.0 + 0.6(D_p G/u)^{1/2} (u/\rho D_v)^{1/3}$$

where

$$\text{Sherwood Number} = N_{Sh} = kD_p \bar{M} / \rho D_v$$

$$\text{Reynolds Number} = N_{Re} = D_p G / u$$

$$\text{Schmidt Number} = N_{Sc} = u / \rho D_v$$

and

u = viscosity

k = mass transfer coefficient

D_p = diameter of the bubble

$\bar{M}$ = average molecular weight of the phase

ρ = density of the medium

D_v = volumetric diffusivity

G = mass velocity

From equation 1 it is evident that the Sherwood number, which is an indicator of mass transfer, is inversely proportional to the sixth root of the viscosity, or more simply that as viscosity increases, mass transfer decreases. This relationship is important here in that it predicts that the oxygen mass transfer is less effective as viscosity of the medium increases. Consequently, lower enzyme levels might be expected from the more viscous air fermentation broths because of poorer oxygen transfer, which would impede protein synthesis.

Another possible reason for such a variance in enzyme levels in air and oxygen runs could involve the morphology of the organism in both systems. The growth of the organism T. viride has been characterized as consisting of long thread-like mycelia in typical air fermentations. It is possible that the organism's growth formation is slightly different under oxygenation, so as to affect the enzyme production scheme (57). Should the growth consist of these same thread-like mycelia, possibly not quite as lengthy, but more numerous in total number, this would afford more surface area to the organism for secretion of the enzymes forming the cellulase complex, thus increasing cellulase production.

As shown in figure 14, the growth trends for air and oxygen systems were similar in nature in that they

were characterized by the initial rapid growth usually increasing toward a maximum value in 40-60 hours, followed by a period of essentially no further growth, and finally by cell autolysis. This period of no additional cell production (net) has been predicted by Muzchenko's diffusion limitation theory which presupposes an adverse effect upon metabolic activity in the presence of high hydrogen ion concentrations (see pH curves in figures 6 and 7). The cell autolysis which followed was common to both systems with the exception that it seemed to have occurred earlier in the oxygenated systems. This may have had some beneficial effect upon enzyme production rate (54) during the oxygen runs.

In observing the sugar and enzyme curves simultaneously upon the composite graphs (see figures 8-13), it appeared that there was some evidence of catabolic repression. Figures 12 and 13 show an early build-up of reducing sugar (primarily cellobiose) which seemed to have hindered enzyme production during the first 80 hours of each run. The curves of the oxygen runs did not show reducing sugar values (specifically cellobiose) quite as high or any great build-up over a period of time. In figure 10, for instance, the sugar values were very low and consisted primarily of only glucose. This may have had some effect upon the more rapid enzyme production rate observed.

VI. Submerged Fermentation-Conclusions

The effect of pure oxygen upon growth and enzyme production for the T. viride-cellulose submerged fermentation system was studied at various gas flow rates and compared to results obtained during air control runs. It appears, from comparing enzyme production curves from both air and oxygen runs, that oxygen does have an enhancing effect upon enzyme production rate. Reasons for this phenomenon are tangible in some instances, and somewhat speculative in others.

Better utilization of oxygen by the organism in oxygen runs seems to be indicated by lower average DO levels (see figures 6 and 7). This would help to substantiate the concept of differing oxygen mass transfer mechanisms between air and oxygen fermentations, whether this was primarily due to an added gas resistance within the bubbles, or viscosity differences between the different fermentation media. With better oxygen utilization, increased protein and therefore enzyme production would be expected because of a greater number of ATP molecules made available for protein synthesis.

Although enzyme test results upon several foam samples indicated that gas fractionation of the enzyme was not occurring, it would be possible for some of the enzyme to be inactivated as it's swept up into the foam (58).

This phenomenon would be manifested by lower enzyme activity in the foam than in the liquid phase. In any event, the higher foam levels associated with air runs would mean that greater amounts of enzyme would be contained within these foams at any particular sampling time. Consequently, enzyme levels in the medium would be reduced. This effect seemed to be partially responsible for the lower enzyme activity during the first three to four days.

Differences in the morphology of the organism during growth in the two systems would provide a possible explanation, involving surface area of the organism available for secretion of the cellulase enzyme system, for better enzyme yields with oxygen. These morphological differences would first need to be ascertained. However, from the growth data accumulated in this study, it was apparent that the cell autolysis which occurred earlier during oxygen runs was in part responsible for better enzyme production in the latter stages of these runs (54).

Finally, the catabolic repression role of glucose and cellobiose seemed to be in evidence here, specifically during the initial stages of both air runs. This helps to account for lower enzyme production during these times.

VII. Suggestions for Further Study

1. Study the mass transfer of oxygen in both air and oxygen systems to determine any difference in transfer mechanisms which might account for better utilization of oxygen by the organism T. viride QM9414.
2. The effect of agitation on denaturation of the enzyme, degree of adsorption of the enzyme onto the cellulose substrate, and degree of cell autolysis should be investigated because of the influence each of these phenomena has upon enzyme production.
3. The foam resulting from the cellulose-T. viride fermentations should be further examined to determine possible deactivation of the system after being swept up into the foam. Protein analysis and possibly nitrogen analysis of both liquid medium and foam should be performed and compared. This should help to confirm or reject the concept of gas fractionation.
4. Studies should be conducted to determine any morphological differences in the organism T. viride in air and oxygen systems which might help to explain increased enzyme levels with oxygen.

5. Further experiments should be performed to verify or dismiss the idea of early catabolic repression in the air systems, a phenomenon which could have resulted in inhibited enzyme production during these times.

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Appendix

Appendix

Fermentation Raw Data

Fermentation Run #1

Volume of Inoculum: 300 ml

Date: 6/20/77

Initial Substrate Concentration: 1% (30g)

Fermentation Vol. 31

Organism: T. viride QM9414

Tween 80: .2% (6g)

Temperature: 28-29°C

Agitation: 300 rpm

Aeration: Pure O₂ at .172 cfh

Initial pH: 5.0

Note: Orifice Sparger Used

<u>Time(hrs)</u>	<u>pH</u>	<u>DO</u>	<u>C+F(mg/ml)</u>	<u>F(mg/ml)</u>	<u>FP(U/ml)</u>	<u>RS(mg/ml)</u>	<u>G(mg/ml)</u>
17.5	3.8	100	6.57	1.25	.0560	.1025	.0350
23.0	3.7	84	----	----	.0601	-----	-----
48.3	3.0	75	4.08	1.11	.2886	.1100	.0500
68.0	2.9	70	3.09	1.15	.5692	.1550	.0600
93.8	3.2	65	3.10	.90	.7872	.1925	.0400
119.5	3.2	55	1.33	.13	1.0570	.2075	.0500
137.5	3.2	60	.44	----	1.3214	.1850	.0300
166.2	3.2	60	.22	----	1.6087	.2125	.0250

Note:

C+F Cellulose plus Fungal Tissue

F Fungal Tissue

FP Filter Paper Enzyme Activity (Units/ml)

RS Reducing Sugar

G Glucose

DO Dissolved Oxygen, Percent Saturation

Fermentation Run #2

Date: 7/31/77

Volume of Inoculum: 300 ml

Fermentation Vol. 31

Initial Substrate Concentration: 1% (30g)

Organism: T. viride QM9414

Tween 80: .2% (6g)

Temperature: 28-29°C

Agitation: 300 rpm

Aeration: O₂ at .127 cfh

Initial pH: 5.0

Note: Orifice Sparger Used

<u>Time(hrs)</u>	<u>pH</u>	<u>DO</u>	<u>C+F(mg/ml)</u>	<u>F(mg/ml)</u>	<u>FP(U/ml)</u>	<u>RS(mg/ml)</u>	<u>G(mg/ml)</u>
22.0	4.1	56	7.16	1.19	.0814	.1150	.0750
44.5	3.0	60	7.25	1.67	.1166	.1325	.0600
75.2	2.9	60	4.50	1.57	.3441	.1600	.0650
92.6	3.1	35	4.60	1.66	.6727	.1550	.0350
120.7	2.9	55	3.24	1.49	1.2542	.2425	.0750
148.6	3.0	53	2.78	----	1.5745	-----	-----
151.1	3.0	58	1.65	.73	1.6087	.2425	.0950

Note:

C+F Cellulose plus Fungal Tissue

F Fungal Tissue

FP Filter Paper Enzyme Activity (Units/ml)

RS Reducing Sugar

G Glucose

DO Dissolved Oxygen, Percent Saturation

Fermentation Run #3

Volume of Inoculum: 300 ml

Date: 6/2/77

Initial Substrate Concentration: 1% (30g)

Fermentation Vol. 31

Organism: T. viride QM9414

Tween 80: .2% (6g)

Temperature: 28-29°C

Agitation: 300 rpm

Aeration: Pure O₂ at .086 cfh

Initial pH: 5.0

Note: Orifice Sparger Used

<u>Time(hrs)</u>	<u>pH</u>	<u>DO</u>	<u>C+F(mg/ml)</u>	<u>F(mg/ml)</u>	<u>FP(U/ml)</u>	<u>RS(mg/ml)</u>	<u>G(mg/ml)</u>
15.7	4.7	66	7.19	1.26	.0592	.0342	.0250
37.2	2.8	48	5.29	1.79	.3441	.0775	.0700
65.7	3.1	50	4.61	1.61	.7400	.0650	.0600
88.9	3.2	40	3.96	1.69	1.5102	.0996	.0900
111.7	3.1	50	2.57	1.05	1.4800	.0983	.0700
139.4	3.4	80	.94	..22	1.6818	.0904	.0350
165.9	3.4	68	.61	----	-----	.0929	.0300

Note:

C+F Cellulose plus Fungal Tissue

F Fungal Tissue

FP Filter Paper Enzyme Activity (Units/ml)

RS Reducing Sugar

G Glucose

DO Dissolved Oxygen, Percent Saturation

Fermentation Run #4

Volume of Inoculum: 300 ml

Date: 1/14/77

Fermentation Vol. 3l

Initial Substrate Concentration: 1% (30g)

Organism: T. viride QM 9414

Tween 80: .2% (6g)

Temperature: 28-29°C

Agitation: 300 rpm

Aeration: Pure O₂ at .043 cfh

Initial pH: 5.0

Note: Orifice Sparger Used

<u>Time(hrs)</u>	<u>pH</u>	<u>DO</u>	<u>C+F(mg/ml)</u>	<u>F(mg/ml)</u>	<u>FP(U/ml)</u>	<u>RS(mg/ml)</u>	<u>G(mg/ml)</u>
18.0	5.1	32	7.22	1.31	.0879	.0800	.0090
36.2	3.4	26	6.6080	1.21	.2368	.1100	.0080
61.0	3.1	48	5.34	1.21	.3737	.1850	-----
85.9	3.3	60	4.16	.93	.3854	.2250	.0085
111.4	3.7	46	3.98	1.79	.8132	.2450	.0800
131.2	3.6	46	3.01	1.59	1.3455	.2750	.0115
161.4	3.5	60	2.61	1.50	.7629	.2650	.0135

Note:

C+F Cellulose plus Fungal Tissue

F Fungal Tissue

FP Filter Paper Enzyme Activity (Units/ml)

RS Reducing Sugar

G Glucose

DO Dissolved Oxygen, Percent Saturation

Fermentation Run #5

Volume of Inoculum: 300 ml

Initial Substrate Concentration: 1% (30g)

Organism: T. viride QM9414

Tween 80: .2% (6g)

Temperature: 28-29°C

Agitation: 300 rpm

Aeration: Air at .430 cfh

Initial pH: 5.0

Note: Orifice Sparger Used

Date: 8/22/77

Fermentation Vol. 31

<u>Time(hrs)</u>	<u>pH</u>	<u>DO</u>	<u>C+F(mg/ml)</u>	<u>F(mg/ml)</u>	<u>FP(U/ml)</u>	<u>RS(mg/ml)</u>	<u>G(mg/ml)</u>
22.0	3.8	80	7.80	1.72	.0648	.1300	.0250
47.5	3.2	40	6.63	1.81	.0944	.2800	.0950
74.0	3.1	50	5.28	1.99	.2257	.1300	.0300
96.8	3.1	60	4.36	1.65	.5441	.1475	.0050
124.8	3.2	70	3.90	1.64	.7957	.1450	.0200
143.3	3.3	70	3.62	1.32	.8916	.1300	.0200
169.0	3.2	70	3.13	----	-----	.1550	.0150

Note:

C+F Cellulose plus Fungal Tissue

F Fungal Tissue

FP Filter Paper Enzyme Activity

RS Reducing Sugar

G Glucose

DO Dissolved Oxygen, Percent Saturation

Fermentation Run #6

Volume of Inoculum: 300 ml

Initial Substrate Concentration: 1% (30g)

Organism: T. viride QM9414

Tween 80: .2% (6g)

Temperature: 28-29°C

Agitation: 300 rpm

Aeration: Air at .215 cfh

Initial pH: 5.0

Note: Orifice Sparger Used

Date: 6/18/77

Fermentation Vol. 31

<u>Time(hrs)</u>	<u>pH</u>	<u>DO</u>	<u>C+F(mg/ml)</u>	<u>F(mg/ml)</u>	<u>FP(U/ml)</u>	<u>RS(mg/ml)</u>	<u>G(mg/ml)</u>
18.8	5.1	90	6.34	.95	.0476	.1025	.0700
40.0	4.5	90	5.60	1.36	.0782	.2325	.0600
67.0	3.4	--	3.92	1.31	.1087	.4350	.0600
88.0	3.1	56	3.58	1.05	.1758	.3675	.0750
118.0	2.9	80	3.33	1.22	.2146	.2025	.0700
141.7	2.9	70	2.65	1.19	.2294	.1700	.0450
158.8	2.9	80	1.97	.69	.3094	.1575	.0350

Note:

C+F Cellulose plus Fungal Tissue

F Fungal Tissue

FP Filter Paper Enzyme Activity

RS Reducing Sugar

G Glucose

DO Dissolved Oxygen, Percent Saturation

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