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Use of Fungi Imperfecti in Waste Control

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Use of Fungi Imperfecti in Waste Control

by

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for the

FEDERAL WATER QUALITY ADMINISTRATION
U.S. DEPARTMENT OF THE INTERIOR

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ABSTRACT

Forty-five species of twelve genera of the Fungi Imperfecti were screened for those fungal candidates best able to rapidly convert soluble and suspended organic material (as measured by BOD) from corn- and soy food-processing waste streams to mycelial protein. Rapidly growing fungal strains were selected which were readily removed from the digested waste effluents by coarse filtration. Trichoderma viride, Gliocladium deliquescens, and either Aspergillus oryzae or G. deliquescens gave the best results on corn, soy and SO_2-containing soy wheys, respectively. Optimal growth conditions included pH of 3.2 to 3.5, and a temperature of 30°C. Oxygen requirements were relatively low (1 lb O_2/6 to 7 lb COD removed). Nitrogen and phosphate additions were required for the corn digestion system, and additions of sulfuric acid were necessary to adjust the pH. These studies were done in 125 ml flasks containing nonsterile corn and soy wastes. The growth conditions that resulted in the highest fungal yield and greatest reduction in BOD and total solids were incorporated into 20-liter continuous culture digestions. Corn waste was reduced from an initial BOD level of 1600 mg/l to 25 mg/l in 24 hours. Soy wastes were reduced from 6200 mg of BOD/l to 125 mg of BOD/l in 36 hours of incubation. Studies of rapid fungal digestion of soy whey containing 700 mg/l of SO_2 resulted in selection of A. oryzae and G. deliquescens strains which removed SO_2 from the medium. Mycelial yields were approximately 50 to 60 g of dry mycelium per 100 g of COD utilized. The stability of the continuous fermentation with corn waste was demonstrated in a fermentation run of 140 days' length. Runs of 30 days' length have been conducted with soy whey. The protein content of mycelium recovered from the continuous culture corn digestion system was 45 percent. The recovered mycelium was light tan in color and bland in taste and smell. Feeding trials in weanling rats using T. viride grown in corn waste as the protein source gave a growth response equal to that seen with a standard casein rat diet. Digestibility was 90 percent, and no toxicity was observed in a three-week trial. Feeding trials were inconclusive with rats fed G. deliquescens fungal protein from the soy whey fermentation due to a palatability problem. Economic estimates based on the experimental results showed the fungal product to be comparable in cost to soy oil meal.

Results on both soy and corn wastes gave definite encouragement that the commercial use of selected strains of certain species of Fungi Imperfecti to remove BOD in a readily harvested form is practical.

This report was submitted in fulfillment of project 12060 EHT under the partial sponsorship of the Federal Water Quality Administration.

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SECTION I

SUMMARY

Forty-five species of twelve genera of fungi were screened to select those most capable of reducing the BOD of commercial corn and soy processing wastes by converting the soluble and suspended organic matter to mycelium. The screening led to the selection of rapidly growing fungal strains that could reduce the BOD of the corn and soy wastes from initial values of 4000 and 8000 mg/1, respectively, to <50 and 200 mg/1. The mycelium could be readily removed from the digested waste effluents by a simple, coarse filtration. Trichoderma viride, Gliocladium deliquescens, and either Aspergillus oryzae or G. deliquescens gave the best results on corn, soy, and SO_2-containing soy wheys, respectively.

The process proved adaptable to continuous fermentation and continuous runs of many weeks duration were conducted. Sterile conditions were not required and were used only in the first stage of inoculum transfer to the liquid medium.

Maintenance of the fungal strain as the dominant organism seemed to be dependent on the use of a relatively heavy inoculum, pH control in the range of three to four (by addition of sulfuric acid), and feeding at a high enough rate to prevent the culture from going into a stationary phase with extensive sporulation and lysis. If these events occurred, other organisms, including bacteria and, particularly, yeasts, appeared in the fermentation in large numbers. It was usually possible to re-establish the fungus if the period of starvation had not been too long and if refeeding was undertaken judiciously. Recovery was achieved after a feed stoppage of up to eighteen hours, but longer interruptions were likely to cause serious trouble. Loss of excessive mycelial mass through dilution and washout occurred at very high feed rates.

Corn Waste

For corn waste, the optimal retention time, once the culture had achieved heavy growth, appeared to be about twenty hours. Shorter retention times were investigated to a limited degree, but washout of fungus appeared to be occurring when the time was reduced to sixteen hours.

These data were obtained at 19-24°C. The optimal temperature was about 30°C, but the temperature response curve was relatively flat; half the maximum rate was achieved at either 10°C or 40°C.

Aeration requirements were modest, possibly because only a fraction of the BOD was totally oxidized. The rest was incorporated into the mycelium. About one pound of dissolved oxygen was required per seven pounds of COD utilized. Agitation vigorous enough to keep the mycelium

in a homogeneous suspension was required. If the mycelium clumped, it became anaerobic in the center of the mass, and lysis occurred.

Inoculation of the continuous cultures was most smoothly accomplished by adding a physiologically young culture to the fermentor containing one part medium and eighteen to twenty parts water. As soon as the culture was added, continuous feeding was begun at the desired rate. This procedure avoided large excesses of nutrients; this was desirable because excess nutrients probably would have allowed competing organisms to become established. Typically, an inoculum volume of about one-twentieth the fermentor volume was used. The use of smaller amounts was investigated only to a limited degree.

It was necessary to add nitrogen and phosphate to the corn waste because of its low content of these nutrients. Some growth occurred without added N and P, and smaller quantities than those used routinely might have supported adequate growth. Phosphate levels were regulated to control the continuous fermentation of corn waste, but reductions in nitrogen supplied were little explored because of the desirability of a high protein content in the mycelium. The two levels of nitrogen most investigated would have led to 45 and 90 percent protein in the mycelium if all had been converted to protein. Nitrogen analyses of the mycelium produced at the two levels of addition indicated 35 and 59 percent protein content, respectively. At the lower level of addition, only negligible amounts of nitrogen or phosphate escaped in the effluent. Excess phosphate was undesirable for several reasons. One is that phosphate is usually unacceptable in waste effluents; another is that a colored effluent was produced at high phosphate -to-nitrogen ratios; and a third is that the fermentation was more stable and better controlled when the growth rate was limited by phosphate supplies.

The combination of requirements for pH control and nitrogen addition contributed to the total solids content of the effluent. If pH control on the acid side had not been required, the nitrogen could have been added as ammonia, contributing no residue. Part of the sulfate and sulfuric acid additions was incorporated into the mycelium, but part appeared in the effluent stream. This might cause difficulty in meeting stream standards when highly concentrated wastes are treated. Similarly, the low pH would be unacceptable in many instances.

Harvesting the mycelium was easy. The mycelium was recovered by gravity filtration through a nylon mesh. When allowed to drip dry on the nylon mesh, the mycelium contained only 80 percent water. Filtration by vacuum was less successful because the mycelium became packed together and soon restricted the water flow. Commercial vacuum filters in which the filter cake is continuously discharged would probably work satisfactorily, but were not tested.

The utility of the mycelium as a feed seemed promising in limited studies. The amino acid composition was gratifying, particularly with regard to high lysine, threonine, and tryptophan content. The amount of sulfur-containing amino acids was lower than hoped for, but not too serious,

since methionine fortification is within the realm of economic possibility. The digestibility was excellent in weanling rats. Net nitrogen utilization was lower than ideal but was an artificially low figure because all nitrogen was assumed to be in protein. Palatability to rats was excellent. They avidly consumed even the pure fungal mycelium.

Yields of fungal mass were high, equivalent to about fifty percent of the COD utilized.

Economic estimates are presently major extrapolations. The possibility of at least breaking even (cost of processing the waste vs. return from sale of product) seems reasonable.

Soy Waste

The reduction of BOD in soy wastes by more than 97 percent was accomplished by the fungus strains used even in the presence of 700 ml/l of SO_2. Fermentation reduced the SO_2 level by 96 percent. The residual BOD seemed to be refractory to the fungal organisms. The mode of removal of SO_2 was not investigated, but the sulfur did not appear as increased sulfur-containing amino acids in the mycelial protein. The BOD was further reduced by half in one set of continuous fermentation experiments by adding a second stage fermentor containing mixed flora obtained from a soil enrichment culture. Unlike corn waste, since no nutrients needed to be added to the soy waste, no problems with added inorganic ions remaining in the stream were encountered. Phosphate and nitrogen were reduced by 70 and 90 percent, respectively, by Gliocladium deliquescens, but remained higher than ideal.

Several of the operating parameters were similar to those for treating corn waste. Sterile conditions were not required. The optimal pH was between three and four, again achieved by the addition of sulfuric acid. Optimal temperature was about 30°C, with half maximum rates at about 20° and 40°C.

Control of the fermentation to prevent appearance of BOD in the effluent or to prevent the culture from going into a stationary phase, thus allowing the invasion of competing organisms, was more difficult than for corn waste. This may have been because soy waste is a better medium for competing organisms and because there was no need for additional nutrients which can be withheld to control growth, as with phosphate for corn waste. Stability was obtained by two expedients, both aimed at maintaining an adequate balance between available nutrient and mycelial mass. One expedient was to vary the feed rate in response to variations in the COD level, which was never allowed to approach closer than 200 mg/l to the minimum attainable. The other was to remove mycelium to maintain a constant amount in the fermentation. This was 3.2 to 3.7 g/l when the COD of the feed was 10 g/l. A retention time of about thirty hours was required.

Aeration requirements were similar to those for corn waste: one pound of dissolved oxygen per 5.5 pounds of COD utilized. The yield was about fifty percent of the COD utilized.

The nutritional adequacy of the soy waste obviated the necessity of adding nitrogen or phosphate.

Experience with harvesting the mycelium was the same as with corn waste.

The feed potentialities of the mycelium remain in doubt. The amino acid composition was excellent, but the mycelium was unpalatable to rats. Washing with alcohol made it palatable; with water did not. Systematic feeding experiments were not conducted on alcohol-washed mycelium but two rats consumed a diet of washed fungus, alone, for two days without apparent deleterious effects. A brief feeding experiment was conducted on mycelium grown on HCl soy whey (no sulfur dioxide). The animals did not grow as rapidly as the controls, but several explanations are possible. One is that the experiment was too short to permit recovery of rate of gain after the first two days in which feed consumption was depressed on the experimental diet. Another is that the digestibility of the fungus was low. A third is a toxic factor. Toxic materials have been reported in some Gliocladium strains. More experimentation is called for. If the Gliocladium should prove unacceptable as a feed, Trichoderma strains which showed growth on SO_2-containing whey could be reinvestigated. The Gliocladium strain was chosen because it grew faster at high sulfur dioxide concentrations and rapidly reduced the COD.

The economics of the use of fungi on soy whey appeared more promising than those of corn waste. One reason for this was that no additions of nitrogen and phosphate were necessary. Another was the possibility of year-round operation. The year-round operation does, however, add another expense: heating will probably have to be supplied in northern climates during the winter months. The cost of such heating depends on the availability of waste heat from soy processing.

SECTION II

INTRODUCTION

The Federal Water Quality Administration awarded Grant No. 12060 EHT to North Star Research and Development Institute in August 1967 to cover 70 percent of the funding of a study of "Use of Fungi Imperfecti in Waste Control". The remaining 30 percent of the funding was provided jointly by the Central Soya Company, General Mills, Inc., the Green Giant Company, and the Ralston Purina Company. The study was programmed for two years, ending August 31, 1969.

The objective of the research was to select rapidly growing strains of fungi that would convert dissolved and suspended organic matter in waste streams from corn and soy bean processing plants into a mycelium that could be readily harvested by filtration. For the process to be practical, it was necessary that the selected fungus reduce the BOD value of the waste streams to a very low level and that the mycelium have utility as a feed product. To accomplish these objectives, it was necessary to select fungi which were capable of establishing themselves as the predominant organisms in nonsterile waste streams. Practical considerations required that the organisms used be relatively insensitive to small variations in temperature, pH, nutrients, and aeration. The mycelium would be most valuable as a feed if it was of high protein content.

Economic considerations required that the organisms be established with only minimum requirements for nutrient additions, pH adjustments, aeration requirements, operational management, etc.

This report covers the study from its initiation on September 1, 1967, to its completion on August 31, 1969.

SECTION III

BACKGROUND

For microbiological treatment of organic waste streams to be practical it was necessary for the selected fungus to reduce the BOD to a low level and for the mycelium to have utility as a feed or food product. To accomplish these ends, fungi capable of establishing themselves as the predominant organisms in nonsterile waste streams would be required. They should also be relatively insensitive to small variations in temperature, pH, nutrients, and aeration. The mycelium would be most valuable as a feed it if had a high protein content.

Several of the requirements for practical use of fungi in waste control appeared from previous work to be met by species of fungi. Lilly and Barnett, (22) , Cochrane, (23), Gray, et al., (7,8), reported that many of these fungi grew rapidly on sugar cane and sugar beet molasses as well as on crude raw plant materials. Gray, (7,8), obtained fungal mycelia containing 25 to 35 percent protein and with an amino acid composition comparable to casein. Limited tests showed some of the strains to be nontoxic when fed to mice. Another reason for interest in Fungi Imperfecti was the well known production of cellulolytic enzymes, Mandels and Reese, (12,13). Many of the vegetable processing wastes are known to contain cellulosic materials in suspension. Finally, the demonstrated capability of these organisms to grow at pH values below 5, Lilly and Barnett, (22), and Cochrane, (23), offered some hope of controlling competing organisms by conducting fermentations at low pH values.

Waste treatment at low pH values has been investigated very little. Eckenfelder, (5), reported that most of the studies in the literature concluded that biological waste treatment could not be conducted at low pH and that waste degradation at pH values below 5 was not desirable. There are, however, a few examples of low pH studies which, in the past, showed excellent decomposition of waste. The growth studies of Pipes and Jones, (17), using Geotrichum candidium and Sphaerotilus and the studies of Cooke, et al., (3), showed reduction in organic matter and BOD values as a result of increased fungal activity at pH values down to 2.9. The studies of Cooke, et al., (3), were done with nine fungal strains and showed the BOD reductions to be accompanied by significant utilization of dissolved oxygen at the low pH. Brower and Gaddis, (2), studied waste treatment by filamentous organisms at low pH values. Few bacteria appeared at the lower pH values, but occasionally large numbers of yeasts developed. The substrate used by these workers was a synthetic composition of glucose, salts, and yeast extract. In the pH range of 6.5 to 7.0, they showed the presence of a large variety of organisms including yeast, bacteria, fungi, and protozoa.

An attractive reason for using fungi in an aerated continuous culture system was that, in the conversion of carbohydrate from plant wastes to mycelial protein, nitrogen and phosphate would be required for the transformation. If plant wastes were low in these two materials, the final effluent from the controlled waste conversion could be essentially free of nitrogen and phosphate. These reductions would be added desirable features to a system which degraded organic waste to a low BOD and allowed for a harvestable protein.

Procedures of biological waste treatment are the oldest and largest application of the so-called continuous fermentation. Certain troublesome problems such as elimination of slowly decomposable substances cannot be resolved without previous theoretical considerations on the dynamics of continuous processes. Even in modern texts on waste purification no mention is made to the already extensive literature in this field. One of the few workers who recognize the duality of waste treatment and continuous processes is Herbert, (9), who included waste treatment in his discussions in continuous fermentations. According to Herbert, (9), all systems of modern biological sewage treatment works are "open continuous systems with feed-back".

One may even extend the scope of study of large continuous systems found in municipal waste treatment plants and, hopefully, in the food processing industry to even larger natural bodies of water where freely suspended microbial cells are assumed to exist under near starving conditions. Here, like sewage treatment plants, Jannasch, (10), points out that growth will be limited primarily by low concentrations of suitable carbon and energy sources. It is not inconceivable that continuous growth in natural waters occurs at extremely low rates.

Earlier studies on growth rates in continuous culture [Novick, (15), and Postgate and Hunter, (18)], showed definite minimal growth rates in continuous (chemostat) cultures. Thus, Novick, (15), grew a tryptophan-requiring mutant of Escherichia coli in well supplemented media and found the organism ceased to grow below a generation time of 15 hr at 37°C. He assumed that unbalanced and discontinuous growth occurred at shorter retention times in the chemostat, preventing establishment of a steady state. Postgate and Hunter, (18), found steady states at far longer retention times with cultures of Aerobacter aerogenes in studies of bacterial survival.

Jannasch, (10), reported indirect determinations of growth rates from washout rates of bacterial populations in continuous culture. In this manner, he hoped to estimate rates of microbial growth and transformations in natural waters. By using several organisms in the same chemostat, he could evaluate the competition for the natural substrate as well as for certain nutritional supplements.

Attempts will be made in these studies to understand the effect of retention time (dilution rate), washout rate and generation time (doubling of fungal mass) of the continuous fungal culture system growing on industrial waste. The objective will be to achieve a steady culture state where inflowing nutrient (waste) is reduced to the lowest possible organic level and the mycelium is harvested as a feed product.

SECTION IV

MATERIALS AND METHODS

Fungal Stocks

Fungal Stock cultures were maintained on Czapek-Dox, Sabouraud-dextrose, sterile corn, and soy waste agar slants at 4°C. Stock cultures were transferred every three months to freshly prepared agar slants. The organisms used in these studies and their sources are listed in Table 1.

Table 1

Name, Strain, and Source of Organism

Fungus	Strain Number	Source
Heterocephalum aurantiacum	I-9	W. Gray
Cladosporium unknown	I-75	W. Gray
Linderina pennispora	I-100	W. Gray
Dactylium dendroides	I-108	W. Gray
Paecilomyces elegans	I-134	W. Gray
Aspergillus oryzae	I-14	W. Gray
Gliocladium roseum	I-30	W. Gray
Gliocladium deliquescens	I-31	W. Gray
Morchella esculenta		W. Gray
Trichoderma viride	I-23,I-184,I-185,I-186, I-187,I-188,I-190,I-191, I-192,I-193	W. Gray
Trichoderma viride	M-114	Ralston-Purina
Trichoderma viride	QM-6a	Natick
Myrothecium verrucaria	QM-460	Natick
Streptomyces unknown	—	*Soil
Coprinus unknown	—	*Soil

*Soil from Bushton, Kansas, soy bean fields.

The cultures marked Gray in Table 1 were selected by Dr. William Gray from his stock culture collection in the Department of Botany, Southern Illinois University. His selections were based on his past experiences with these cultures in which he studied their ability to digest carbohydrates from various sources (not corn and soy wastewaters).

Before a fungal species was used for an experiment, it was transferred from the agar slant stock culture to sterile neopeptone-dextrose broth and incubated for 48 hours at room temperature. A broth culture of each organism was also stored at 4°C for two or three weeks as a working primary culture before it was either discarded or transferred to fresh broth for potential storage at 4°C in broth, the spores of the fungus were frequently used as an inoculum into fresh neopeptone broth or into corn and soy wastewaters. After one or two 24-hour transfers through corn or soy waste media, the fungus was used as an inoculum for experimental purposes.

In several special cases, a secondary continuous digestor was coupled to a primary continuous soy digestor described in a later section of these methods. The inocula for the secondary digestor were microorganisms contained in a soil sample obtained from soy bean fields in Bushton, Kansas. The culture medium was undigested residual organic effluent material resulting from continuous fungal primary digestion of raw soy whey. Thus, a continuous soil enrichment system was established to trap those organisms which were best able to grow on the primary effluent medium. This soil enrichment technique resulted in the emergence, in the secondary digestor, of a streptomycete and a basidiomycete which grew mutualistically to further degrade the primary effluent. Thus, the microorganisms were selected naturally rather than from stock culture collections in this special case. Both microorganisms were isolated in pure culture and added to our stock culture collection.

Media

The media used in these studies were corn waste, HCl (edible) soy whey, and SO_2 (industrial) soy whey. These media were collected from plant effluents in 5-gallon polyethylene containers, frozen quickly, and stored. They were thawed immediately before use in a Heinicke Instruments Co. dishwasher and, if used in part, the remainder was discarded.

Incubation

All batch-type cultures were incubated at room temperature (26° to 32°C) on a New Brunswick double tier rotary shaker. Best growth results were obtained on this shaker in Bellco flasks which had bottom indentations to improve liquid mixing and, thus, aeration. Culture volumes ranged from 100 ml to one liter per flask.

Otherwise, both batch and continuous cultures in corn and the soy wheys were incubated at room temperature in laboratory fermentor apparatus which was developed during the course of these studies.

Continuous Culture Apparatus

A design of the apparatus is shown in Figure 1. One need only adjust the inflow rate of waste material (theoretically) to maintain the operational efficiency of the system. The inflowing nutrient was adjusted to a rate which would maintain a constant fungal mass in a steady metabolic state, where any increase in mycelium was balanced by loss of mycelium at the product take-off point as shown in Figure 1. If optimal cultural conditions were maintained, one would expect to feed the continuous system with waste medium at a rate which would bring about a complete turnover of the wastewater volume. Hopefully, the time required for a complete turnover of wastewater would be shorter in a continuous culture than in a batch system because the fungal culture would always be maintained in a physiologically young state. In this state, metabolism of the waste nutrients would be at a maximum continuous rate. In addition, mycelial harvest would also be continuous.

The primary digestor was a polyethylene 25-liter carboy with the top cut off and inverted into a 300 mm glass funnel. Holes were tapped into the top (formerly bottom of the polyethylene carboy) of the digestor for insertion of various probes and tubes. The bottom of the funnel was either plugged or fitted with plastic plumbing parts for admitting air, sampling the digestion mixture, or totally draining the system. One stone sparger in a digestor of this configuration was sufficient to provide any desired dissolved oxygen concentration from 0.1 to 3.0 mg/l and with enough agitation to maintain the culture in suspension.

In the diagram (Figure 1) of the digestor apparatus, squares enclosed in dotted lines represent that part of the system which was contained within a $4^{o}C$ cold room. The raw feed was either pumped from the magnetically stirred feed tank to the digestor or elevated to allow gravity feeding at timed intervals controlled by a solenoid timer switch on the feed line. If more sensitive feeding controls were required, selected lengths of tygon tubing were inserted into the feed line to increase flow resistance. Since this tubing length control frequently plugged when very low feed rates were used, it was discarded in favor of the solenoid timer switch mechanism. These simple solenoid timers were constructed in our shop and were superior to any of the pumps or other devices that were tried at various times during the course of these studies.

Since some foaming was usually present during the early stages of waste digestion and before a low (near starvation state) COD was established, the entire digestion system was closed and air, from the sparger, served to gently push the fungal contents out the effluent tube. The air, which produced a slight positive pressure in the digestor, escaped through the nylon filter shown in Figure 1. The nylon filter served to entrap the fungal mycelium and, as the mycelial mass increased, developed efficient dewatering features. Either the effluent liquor

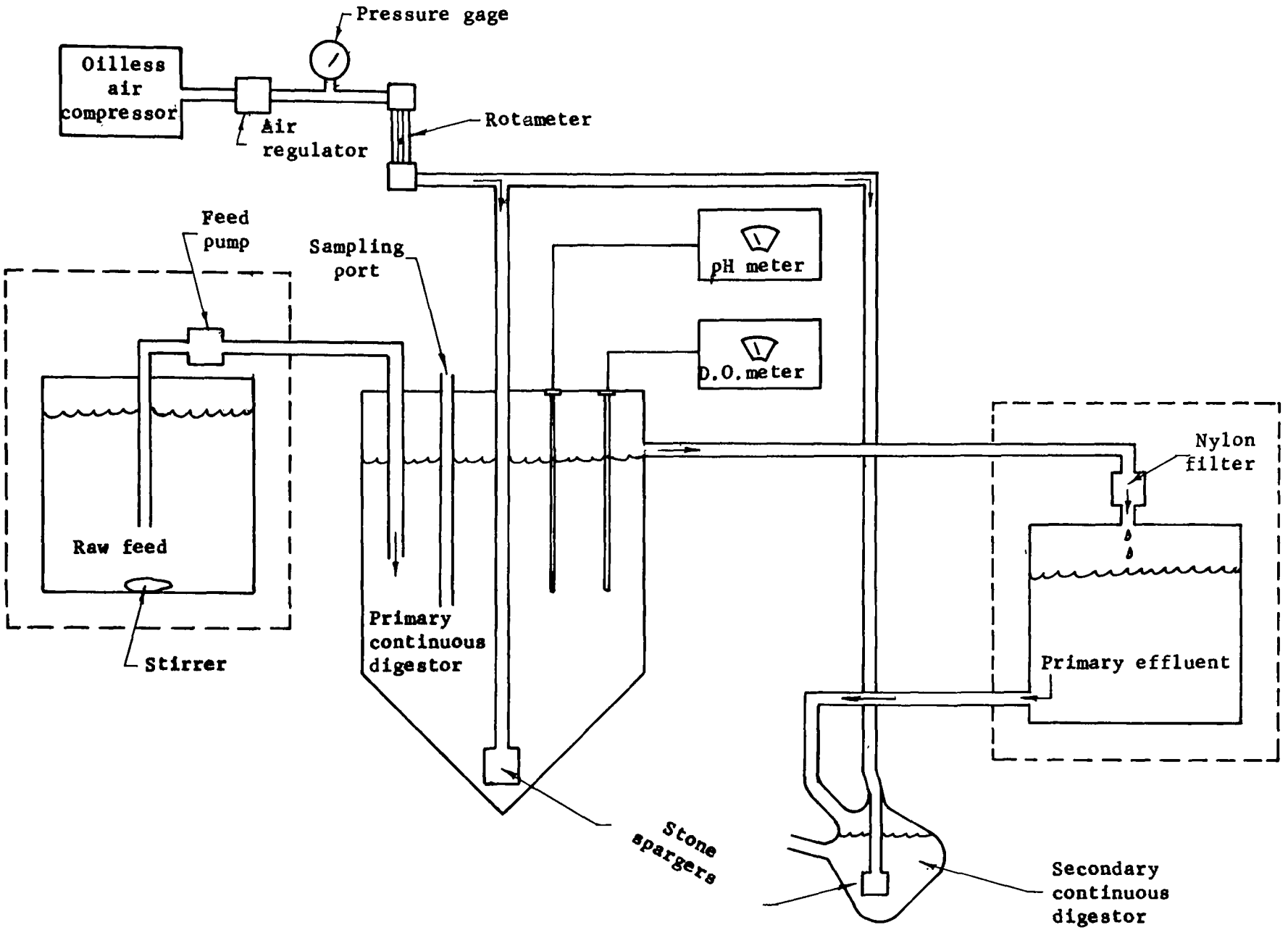

Fig. 1. Fermentor apparatus for continuous digestion of corn and soy waste by fungi.

passed through the nylon filter to the laboratory drain or the liquor was further processed (in the case of soy whey) through a secondary digestor. This secondary digestor consisted of a three-neck, 4-liter, round-bottom distilling flask fitted with rubber stoppers and the required feed, air, and effluent lines.

When changes in the nutrient composition or pH were desired during continuous digestion of the industrial wastes, such changes were made via additions to the feed reservoir contained at 4°C.

Analytical Measurements

Cultural

Measured liquid samples were removed from the continuous digestor system and vacuum filtered through tared No. 4 Whatman filter paper in a Büchner funnel. The filter papers containing the mycelium were dried to constant weight at 90°C. Fungal mycelial samples collected by filtration in this manner were recorded as mg dry weight/l of digestor liquor.

Microscopic examination of the effluent corn samples did not reveal any corn particulates which would otherwise be trapped on the filter along with the fungal mycelium. The COD of the filtrate was not increased when a small effluent sample (50 ml) from the continuous corn digestion system was filtered and washed through a nylon stocking instead of a Whatman No. 4 filter paper. We believe, therefore, that at least in the case of corn digestion the effluent COD measured after filtration was not reduced by physical trapping undigested corn particulates in the fungal matte.

The COD of soy filtered effluents may have been reduced by a small percentage due to trapping soy particulates on the Whatman No. 4 filter paper. Microscopically, occasional undigested soy particulates, large enough to be trapped with the fungal mycelium during filtration, were observed. When these effluent samples (50 ml) were filtered and washed through a nylon stocking rather than Whatman No. 4 paper, approximately 10-15 percent increase in COD was repeatedly observed.

Physical

Aliquots of filtrates from these liquid culture samples were dried at 90°C in tared aluminum pans to constant weight for determination of total solids. When ash determinations were desired, other aliquots of these filtrates were dried at 90°C to constant weight in tared nickel crucibles and then heated at 600°C for 4 hours.

Chemical

Total phosphates in corn and soy wastes were determined by the method of Fiske and Subbarow, (6).

The method of Lowry, et al., (11), was used for protein determination. Human serum albumin was used as a protein standard. Proteins were separated from other nitrogenous compounds by trichloroacetic acid precipitation.

Total carbohydrates were determined by the phenol sulfuric acid method of DuBois, et al., (4), and glucose by the Nelson, (14), modification of the Somegyi method. Standards were starch and glucose. Other carbohydrate analyses such as the anthrone and cysteine-sulfuric acid methods were found to be unsuitable in the presence of ammonium salts.

Chemical Oxygen Demand (COD) was determined by the methods described in Standard Methods for the Examination of Water and Wastewater, (19). The COD is defined as the oxygen consumed by organic constituents in a water sample in an oxidation reaction with a strong oxidizing agent, i.e., chromic acid or bichromate-sulfuric acid at boiling temperature.

Biochemical Oxygen Demand (BOD) procedure was carried out as described in Standard Methods for the Examination of Water and Wastewater, (21). BOD is defined as the biochemical oxygen demand in five days, i.e., the oxygen consumed by the respiration of the microorganisms in a water sample within five days at $20^{\circ}C$. The seed samples used in the BOD determinations were obtained from a municipal sewage source in Montgomery, Minnesota, from Mississippi mud mixed with fertile garden soil, from Minnesota river mud in an area of the river where corn wastewater was discharged, and from Ohio river mud in the area of Louisville, Kentucky, where soy wastewater was discharged.

SO_2 determinations were made according to the standard procedure described in Official Methods of Analysis of the Association of Official Agricultural Chemists, (16). Total nitrogen determinations were made by the micro-Kjeldahl procedure. Nonprotein nitrogen (NPN) was also determined after precipitating the protein.

Amino acid analyses of the dried fungal mycelium were performed with the Beckman Amino Acid Analyzer after hydrolysis of the fungal samples. These analyses were performed by the Central Soya Chemurgy Research Laboratories.

Sulfate, chloride, and nitrate analyses were conducted according to Standard Methods for the Examination of Water and Wastewater, 1956.

Feeding Studies

Weanling rat feeding studies were carried out primarily to determine whether or not toxic manifestations were inherent in the fungal proteins. Other considerations were digestibility and utilization of the fungal protein. A standard weanling rat diet was supplied by Nutritional Biochemical Corp. (NBC) and consisted of 23 percent casein, 2 percent alphacel, 59 percent starch, 10 percent vegetable oil, 4 percent salt mixture, 2 percent vitamin mixture and 0.1 percent methionine. A second NBC diet

formulated for these studies contained 46 percent dried fungi (T. viride contained 50 percent protein therefore this diet, after mixing contained 23 percent protein), 44 percent starch, 2 percent vegetable oil, 3 percent salt mixture, 2 percent vitamin mixture and 3 percent L-amino acids. The additional amino acids supplemented the fungal protein to give it the same amino acid composition as casein.

Analyses of total carbohydrate, ash, and lipid content of the fungus are shown in the test. In adjusting the composition of the diet-containing fungus to make is as nearly equivalent as possible to the control diet; the fat, lipid, and carbohydrate content of the fungus were taken into account.

The difference between the standard and test diet was probably large in the area of vitamin content. Analyses for vitamins in the fungus were not carried out, except for niacin.

The weanling rat feeding experiments were carried out in the following manner: three rats were placed on the standard diet and three were fed the test (fungal) diet. Each rat was placed in a separate metabolic cage. Fecal material was collected by means of a tube container attached to the rat's tail. Urine was collected free of fecal contamination. Feed weights and rat weights as well as total fecal and urine excretions were measured or collected daily. All six rats were started on the standard casein diet containing 1 percent chromic oxide. After one day, three rats were fed the standard casein diet without chromic oxide, and the three test rats were fed the fungal diet. Fecal collection was begun when no more chromic oxide appeared to color the fecal pellets. This loss of green stain in the feces occurred after approximately 24 hours. Thus, only fecal material from the rats eating the experimental diets were collected, and these feces were free of dietary material eaten prior to this experiment. Nitrogen analyses were carried out on selected urine and fecal samples as well as on the dietary material taken daily during the course of the feeding trial. In addition to these analyses, body weight gains and daily physical examinations for ruffled fur, scaly feet, encrusted eyes and nose, retarded incisors, etc. were taken. These measurements supplied the information required to evaluate the fungal diet as to its digestibility, protein efficiency, and toxicity.

SECTION V

RESULTS

Corn Waste

Fungal Strain Selection

Selection of the most effective organisms for use on liquid corn waste was conducted initially without adjustment of pH or addition of nutrients. Screening for rapid growth was carried out in 30 ml volumes of corn waste placed in 125 ml Erlenmeyer flasks and incubated at 27°C on a New Brunswick rotary shaker at 140 oscillations/min. Results such as those shown in Table 2 were obtained. All fungi showed an initial lag before growth, and competition with the natural bacterial and yeast biota initially proved difficult. This is illustrated in Table 2 by the differences between mycelium production in the sterile and nonsterile corn waste medium.

Table 2

Growth of Various Fungi on Corn Waste
pH 7.2

Fungus	Fungal Mass After 6 Days	
	Sterile (mg/30 ml)	Nonsterile (mg/30 ml)
Cladosporium	15.2	8.4
Linderina pennispora	17.0	5.5
Dactylium dendroides	15.1	8.0
Paecilomyces elegans	21.0	5.6
Aspergillus oryzae	23.8	4.4
Trichoderma viride	18.3	11.0
Gliocladium roseum	10.1	10.0
Gliocladium deliquescens	16.1	10.0

Adjustment of the pH to lower values permitted a more successful competition with the natural corn waste biota. The pH of the corn waste as received from Green Giant was 7.2. When this pH was reduced to 3.2 with H_2SO_4 before inoculation, rapid growth and COD digestion occurred. The reduction in COD and total carbohydrate at pH 3.2 by T. viride is shown in Table 3.

Table 3

Digestion of Corn Waste by Selected Fungi at pH 3.2

Organism	COD mg/l		Carbohydrates mg/l	
	0 hr	40 hr	0 hr	40 hr
Raw waste	2168	—	1420	—
Natural biota	—	1932	—	800
Trichoderma viride	—	482	—	320
Gliocladium deliquescens	—	546	—	440
Paecilomyces elegans	—	209	—	70

The fungal strains were next subjected to serial transfers through nonsterile corn waste to achieve even more growth and COD reductions. The effectiveness of the serial transfers in flask cultures is shown in Figure 2. A considerable increase in COD reduction was achieved during the second transfer. A fourth transfer (not shown) gave no further reduction in COD. Similarly increased rates in reduction also occurred for carbohydrate, protein and phosphate. The results obtained by growing these organisms through a series of rapid transfers on nonsterile corn wastes showed that COD reduction, carbohydrate reduction, phosphate reduction, and fungal mass were successfully increased by this transfer selection procedure.

pH Effects

It was observed during these transfer flask experiments that lowering the pH provided better fungal growth. Bacteria and yeast were depressed at the lower pH levels. A systematic study of pH effects on COD reduction by T. viride was made in shake flask cultures. Adjustments of pH were made by addition of 1 N HCl or 1 N NaOH. A heavy inoculum was used (1.5 mg/ml). The effects of pH on COD reduction after 24 hours are shown in Figure 3. The optimal pH lies between 3 and 4. This agreed well with results from other laboratories where T. viride was studied in a variety of media and with the finding that the optimal pH was between 3 and 3.5.

In view of projected pilot plant studies with continuous fermentation of nonsterile corn waste where a required initial holding period would likely occur before fungal digestion took place, we undertook an examination of pH changes that might be expected during this corn waste holding time. Several 5-gallon polyethylene containers of corn waste were allowed to remain at room temperature for 8 to 48 hours. The pH of these 5-gallon samples dropped from 6.6 to 4.5 after 12 hours, and then the pH slowly climbed to 7.0 by 48 hours. Therefore, continuous fermentation of corn waste from a holding reservoir where the pH initially dropped to a level of 4.5 to 5.0 may provide optimal pH conditions during

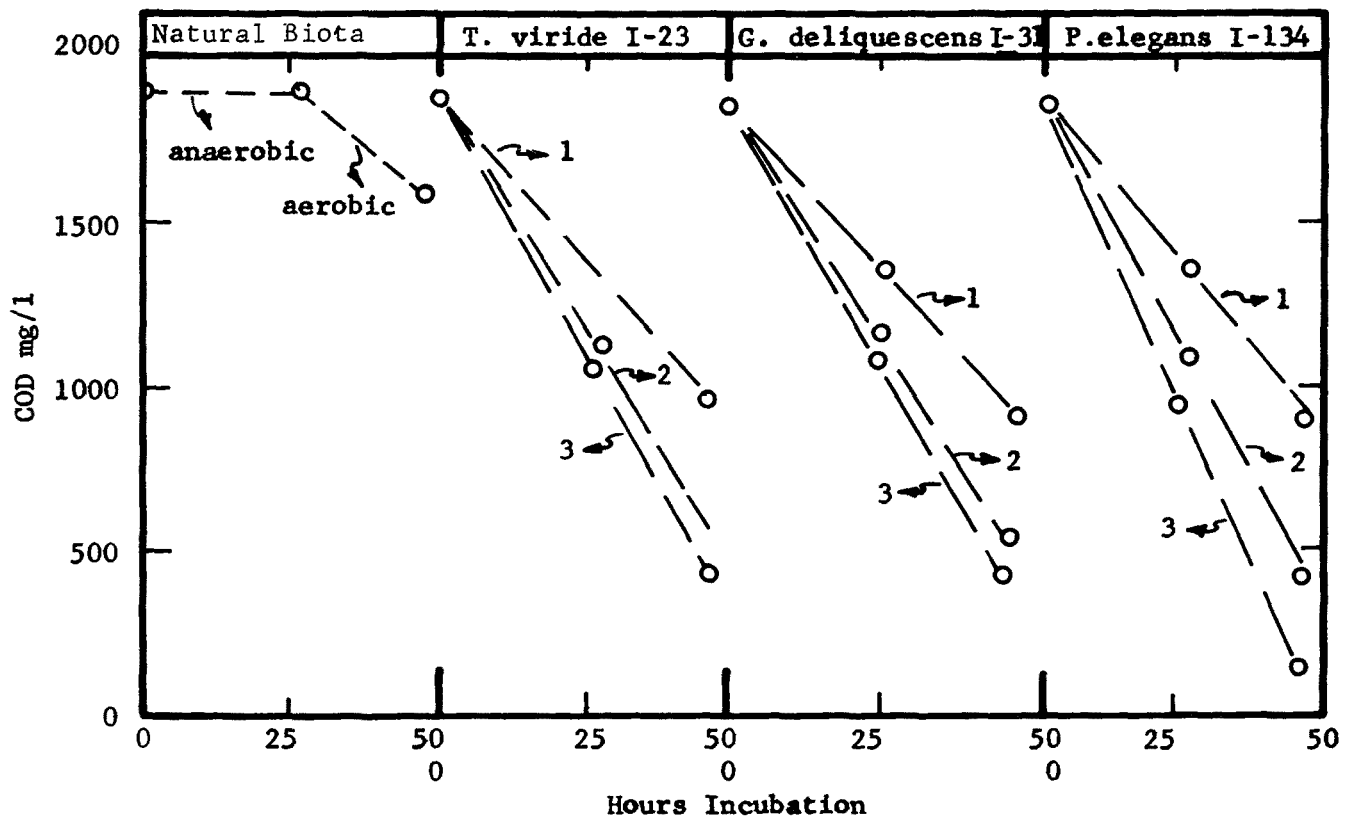

Fig. 2. Strain selection of fungi for rapid COD reduction on corn waste at pH 3.2. Numbers on lines refer to the number of fungal transfers.

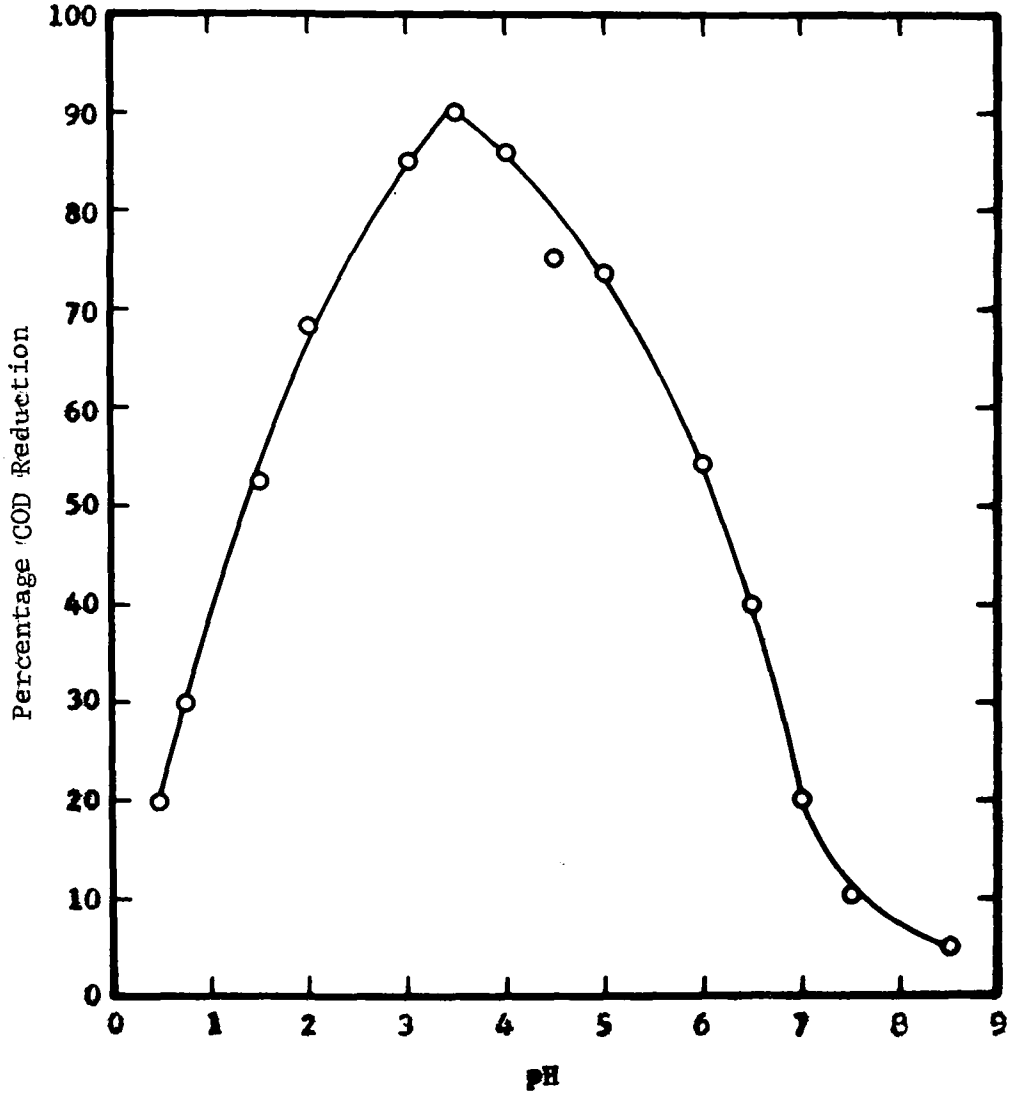

Fig. 3. Effect of pH on COD reduction by *T. viride* in corn waste after 24 hours.

fermentation. In several shake flask experiments it was shown that use of corn waste that had been allowed to stand in a static state for 12 hours before use as a culture medium, dropped to pH 3.5 during the first 24 hours of fungal fermentation. This was the period of time where fungal growth and COD digestion occurred at the most rapid rates. Adjustment of the waste to pH 5.1 with mineral acid before use had a similar effect.

Temperature Effects

The effective temperature range for active digestion of the corn waste is shown in Figure 4. Temperatures between 18° and 35°C resulted in ninety or more percent reduction of COD. This would indicate that a continuous fermentation would do well under outdoor conditions in the late spring, summer, and early fall. However, below 18° or above 35°C the percentage COD reduction decreased rapidly. The pH was held at 3.2 and other experimental conditions were the same as described for the pH experiment, except that temperature variation replaced pH variation.

Nutrient Additions

It was observed in certain shake flask experiments that the COD, carbohydrate, Kjeldahl nitrogen, and phosphate were not reduced very rapidly after 40 hours incubation. These experiments indicated that either a toxic by-product of the fermentation was produced or a deficiency in a required growth nutrient was preventing the COD and carbohydrate digestion from going to completion. The results of these studies (Figure 5) showed that near exhaustion of at least two essential nutrients (phosphate and nitrogen) occurred after 40 hours. At a time when appreciable amounts of COD (22 percent) and carbohydrate (28 percent) remained, low, perhaps metabolically limiting, levels of phosphate and nitrogen were detected. Thus, the possibility of a toxic by-product appeared to be a less satisfactory explanation for the residual COD after 40 hours of fermentation than the exhaustion of required growth nutrients.

To obtain additional information on the nutritional composition of the corn waste growth medium, certain chemical analyses were performed. It was hoped that more knowledge of the chemical composition of this waste would aid the design of future experiments to study and lead to further reduction of the residual COD. Also, knowledge of the chemical composition would act as a basis for determining what nutrient additions, other than nitrogen and phosphate, might be required to effect complete metabolism of the corn waste. The results of the chemical analyses of corn waste are shown in Table 4.

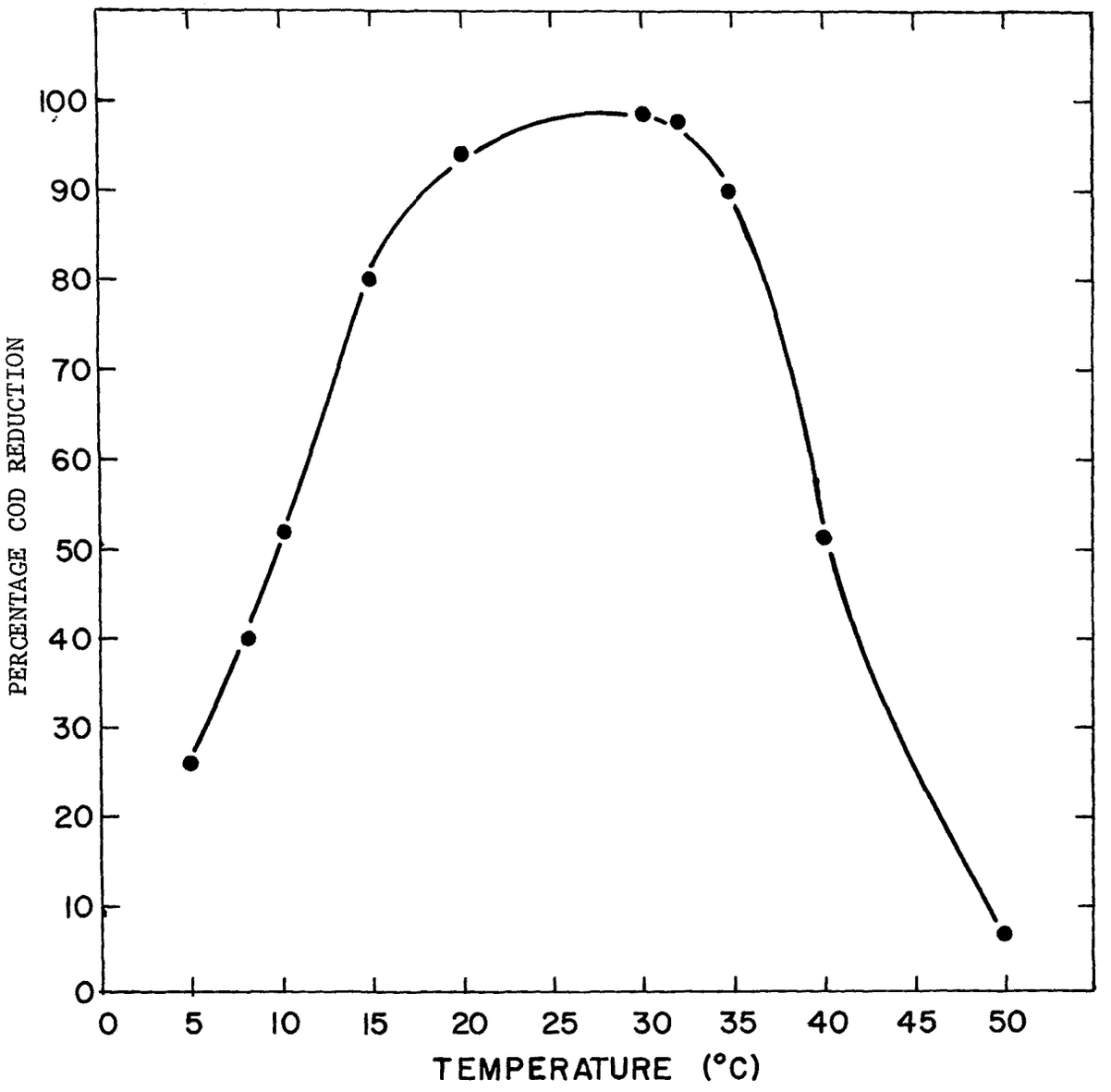

Fig. 4. Effect of temperature on COD reduction by T. viride digestion of corn waste after 24 hours.

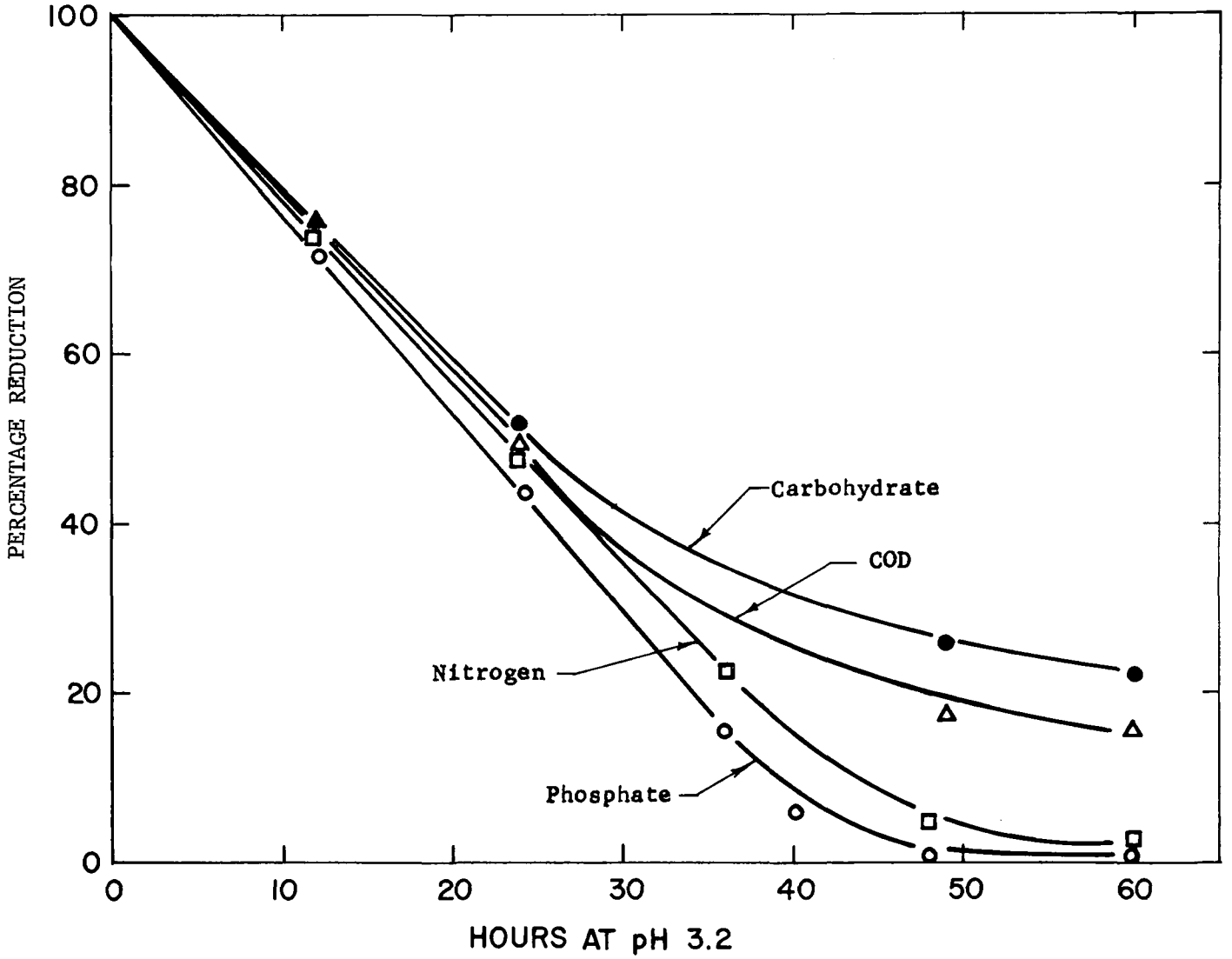

Fig. 5. Reduction of carbohydrates, COD, nitrogen and phosphate by T. viride growing on corn waste. Rates of reduction of these materials were markedly reduced after 40 hours.

Table 4

Chemical Composition of Corn Waste

Chemical Constituent	mg/l
COD	2030
BOD_5	1640
Protein	50
Carbohydrate	1360
Nitrogen (Kjeldahl)	48
Nonprotein nitrogen	38
Chlorides	784
Nitrates	0.6
Sulfates	120
Total phosphate	31
Total soluble phosphate	30
Orthophosphate	22
Total solids	3560

If one can equate BOD to chemical weight on a quantitative basis, it would appear that 83 percent of the BOD is contained in the carbohydrate faction. Both protein and total nitrogen are low. The effectiveness of nitrogen and phosphate additions in increasing fermentation rates was therefore explored. When T. viride I-23 was grown on corn waste and nitrogen was added as ammonium sulfate to cultures adjusted to pH 4.5, the results shown in Figure 6 were obtained. The COD's after 24 hours were in the 900 to 1100 mg/l range. They were further reduced by longer fermentation time to less than 200 mg/l. Thus, a three-fold increase in nitrogen concentration (as ammonium sulfate) reduced the residual COD (Figure 6) approximately 15 percent more than was obtained before its addition.

Phosphate concentrations also appeared to be low, and phosphate additions were studied. It was observed that phosphate as NaH_2PO_4, alone, did not increase the total COD reduction significantly. In combination with nitrogen, however, phosphate reduced the COD at a somewhat more rapid rate. This is also shown in Figure 6. The ratio of N to P most effective in reducing COD was 90 μg $(NH_4)_2SO_4$/ml and 5 μg NaH_2PO_4/ml or approximately a 20:1 ratio for these two salts. Further increases in nitrogen and phosphate concentrations failed to increase the digestion of the residual COD.

These experiments showed that at least two required metabolites which had limited COD digestion were deficient in the original corn waste medium.

In all these shake flask experiments, the pH had to be adjusted by the operator from time to time. This pH adjustment of 3.2 to 3.5 was required after 8 hours in the flasks containing no additions, and after 36 hours in the flasks containing additional nitrogen. One pH adjustment was made after 48 hours in flasks containing nitrogen and phosphate. After 60 hours, the COD in all the flasks began to increase, and a microscopic examination of the fungal contents revealed considerable lysis and sporulation.

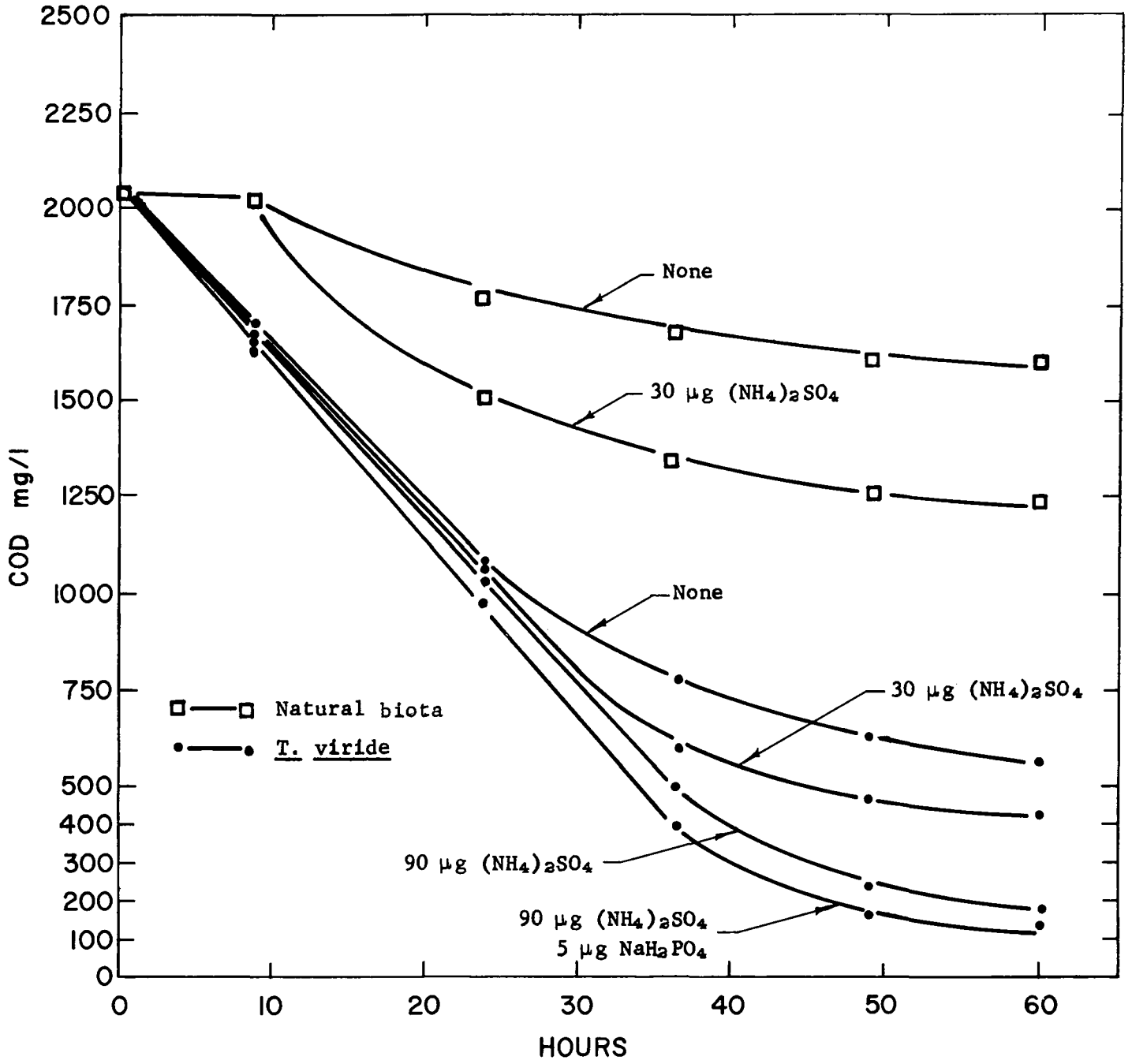

Fig. 6. Effect of added nitrogen and phosphate on COD reduction by T. viride on corn waste. Concentrations of nutrients are indicated in µg/ml of culture. Nitrogen and phosphate additions are as salts $(NH_4)_2SO_4$ and NaH_2PO_4 in the concentrations shown above.

Residual COD

Since a small residual COD remained even after prolonged digestion following the addition of nitrogen and phosphate, chemical analyses were conducted to try to determine the nature of the unutilized residue.

The chemically analyzed material came from a 48-hour batch fermentation with T. viride. The pH of the culture was maintained at 3.5, and 90 µg of $(NH_4)_2SO_4$ and 5 µg of NaH_2PO_4 were added per ml. The inoculum contained 0.5 mg/ml (wet weight) of T. viride I-23. These growth conditions were those found best for digestion of corn waste liquors in the previous experiments. Table 5 shows that the nitrogen was depleted and the phosphate reduced to low levels.

Table 5

Chemical Analyses of Corn Waste*
Before and After Growth of T. viride I-23
Batch (Shake) Culture

Analytical Procedure	Before Fungal Treatment mg/l	Additions mg/l	Fungal Treatment 48 hrs mg/l
COD	2030		210
BOD	1640		100
Nitrogen - Kjeldahl	48	19	0.8
NPN	38		0.6
Protein	50		2
Carbohydrate	1360		70
Chlorides	784		240
Sulfates	120	260	80
Nitrates	0.6		—
Nitrites	0.008		—
Total phosphate	31		5
Total soluble phosphate	29	4	3
Ortho phosphate	22		0
Total solids	3560		405
Ash	660		233

* Analyses after 48 hrs were performed on samples which were filtered through a single layer of Whatman No. 4 filter paper.

These chemical analyses suggest that further reduction in the unutilized COD would require additional nitrogen and phosphate. The remaining carbohydrate constituted 70 percent of the remaining BOD and 35 percent of the COD. In previous experiments, additional nitrogen and phosphate, beyond that shown here, did not result in further COD reduction. Therefore, either the remaining carbohydrate was refractile to digestion by this fungus or metabolites, in addition to nitrogen and phosphate, were required.

In one experiment to resolve this problem, fresh boiled yeast extract and mineral salts in the form of an ashed suspension of fungus mycelium were added to raw corn waste in flasks containing additional nitrogen and phosphate. The flasks were inoculated with 0.5 mg/l on a dry weight basis of T. viride mycelium, and the pH was adjusted to 3.5. Although the COD was reduced only slightly (180 mg/l) after 48 hours, the BOD dropped to 50 mg/l, and the carbohydrate was 30 mg/l. It appeared, from these findings, that a near complete digestion of the raw corn waste by T. viride could be achieved if an extensive study was made to find other exhausted metabolites which were growth limiting. This experiment showed that some or all of these exhausted materials were present in the yeast extract and fungal ash. What would also be required would be a quantative nutritional balance of these growth-limiting-materials before complete digestion of all the BOD was realized.

Continuous Fermentation

A continuous culture apparatus was developed in our laboratories to study the conditions required to maintain continuous corn waste digestion by T. viride I-23. A diagram of this apparatus appeared as Figure 1, page 12.

The continuous culture was operated to obtain additional mycelium for feeding trials and to obtain additional information on the performance and stability of T. viride digestion on raw corn waste under continuous conditions. Initially, the system was started with 18 l of raw corn waste and a T. viride inoculum of 0.01 g of mycelium/l at a pH of 3.2. Continuous feeding was begun after 40 hours, by which time the initial COD had been reduced from 3976 mg/l to 1260 mg/l. Feeding was set at a rate of 7.5 ml/min. in the 18-liter fermentation vessel (average retention time of 40 hours). Aeration was conducted with two stone spargers, with additional mixing by an air lift. Nitrogen was added to the feed as ammonium sulfate at a level of 1.0 gm $(NH_4)_2SO_4$/l and sodium dihydrogen phosphate was added at a level of 0.5 gm NaH_2PO_4/l. 0.1 ml of sulfuric acid/l was added to the feed for pH control. This amount of acid reduced the pH of the raw corn waste from 7.1 to 5.2. Additional reduction of the pH was accomplished by the continuous fermentation itself, and the pH remained fairly constant between 3.2 to 3.5. The results of the COD reduction in this experiment are shown in Figure 7.

This initial experiment was in operation for ten days. Competition from bacteria and other fungi was minimal. The initial number of bacteria was approximately 10^5/ml and, after eight days, was reduced to $<10^3$/ml. We believed this was due to utilization of the organic nutrients by the T. viride and to the presence of protozoa (chiefly Paramecia) which rapidly removed the bacteria. The experiment was concluded when the COD began to increase slowly from 650 mg/l at the fifth day to 800 mg/l at the eighth day and then rise to 1500 mg/l during the ninth and tenth days. Microscopically it was observed at the eighth day that the mycelium had begun to sporulate and fungal lysis was taking place. In addition, pink pigmentation appeared at the fifth day and intensified until the fermentor was reddish purple on the eighth day of operation.

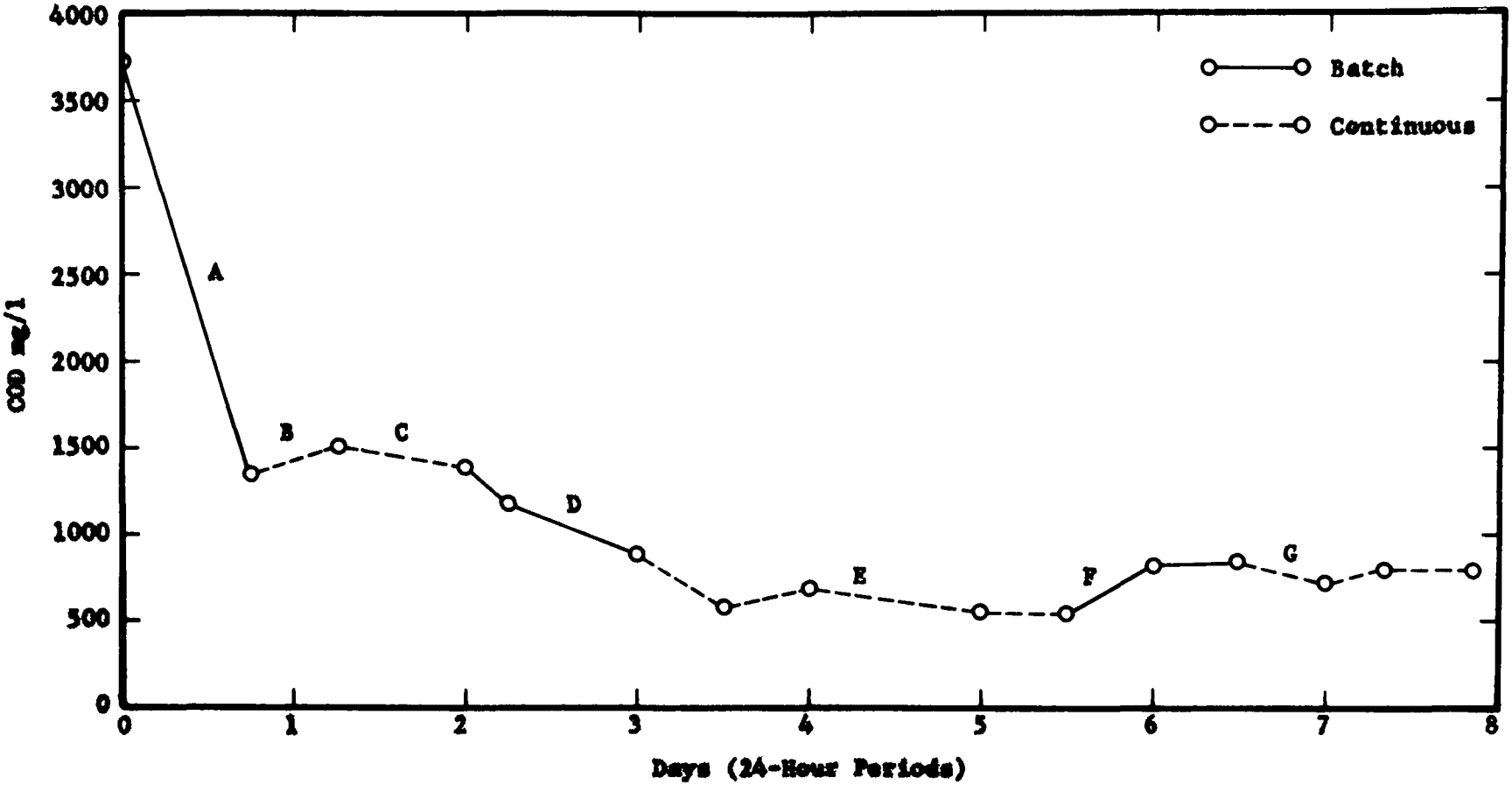

Fig. 7. COD reduction in continuous culture fermenter of corn waste by T. viride.

As seen in Figure 7, the COD was initially reduced 60 percent over the first 18-hour period. The solid line representing Area A in Figure 7 shows this initial digestion. The system operated initially as a batch fermentor and the dry weight of fungal mycelium in the 18 liters increased from 0.18 mg/ml at zero time to 0.66 mg/ml at the first sampling point.

The total volume of corn waste treated during the 8-day period was 65 liters, and 59.15 grams of fungal mycelia were recovered. Areas B and C in Figure 7 represent feed rate adjustments to 20 ml/min. at B and to 14 ml/min. at C. This latter figure represents an 18-liter volume turnover every 21 hours. Areas D and E were intermittent batch and continuous feed operations brought about by plugged feed lines and occasional stoppages in the final effluent line. During this period the mycelial dry weight increased from 0.66 mg/ml to 1.5 mg/ml. Areas F and G (Figure 7) were also troublesome operationally and various feed pumps, feed rates, collection devices, and samplers were tested. The aeration device was plugged by the fungal mass during the first 8 hours of operation. Aeration was erratic from that time on. Stone spargers were used to replace the aeration system.

Several results summarize this first attempt at a continuous culture system and bear further explanation: (1) Stability of the pH at 3.2 to 3.5 without acid addition was found during the periods when the system operated continuously. (2) The bacterial flora of the system were approximately 10^5/ml initially, $<10^4$/ml in area D of Figure 7, and $<10^3$/ml at the end of the experiment. No yeast cells were observed at any time during the 8-day run. (3) The protein composition of the fungal samples taken at areas D, E, and F ranged from 40 to 45 percent on a dry weight basis. These protein results were carried out by the Folin and micro-Kjeldahl nitrogen methods, multiplied by 6.25 after subtraction of the nonprotein nitrogen.

More extensive studies on the continuous digestion of raw corn waste were conducted over a 140-day period. The fermentor was the same used previously but with numerous mechanical modifications: pumps, aeration devices, switches, etc.. Special attention was given to the nitrogen and phosphate concentrations during this continuous experiment in view of previous problems with ecological shifts in the fungal population resulting in red pigment production. Also, the difficulty of maintaining a near steady state culture was controlled by the level of phosphate addition.

Behavior of the fermentation is illustrated in Figure 8 where points A, B, C, and D refer to unstable conditions which developed during the course of the fermentation. A and B were a result of feedline blocks resulting in fungal starvation. C and D indicated times when the feed pump stuck in the open position, causing a rapid flow of raw corn waste and thus a washing-out of the fungus. When the pump errors were corrected, a period of time was required before the fungus increased in mass and again reduced the COD. Increased COD at the peaks between D and E were a result of passage into the stationary phase, fungal lysis,

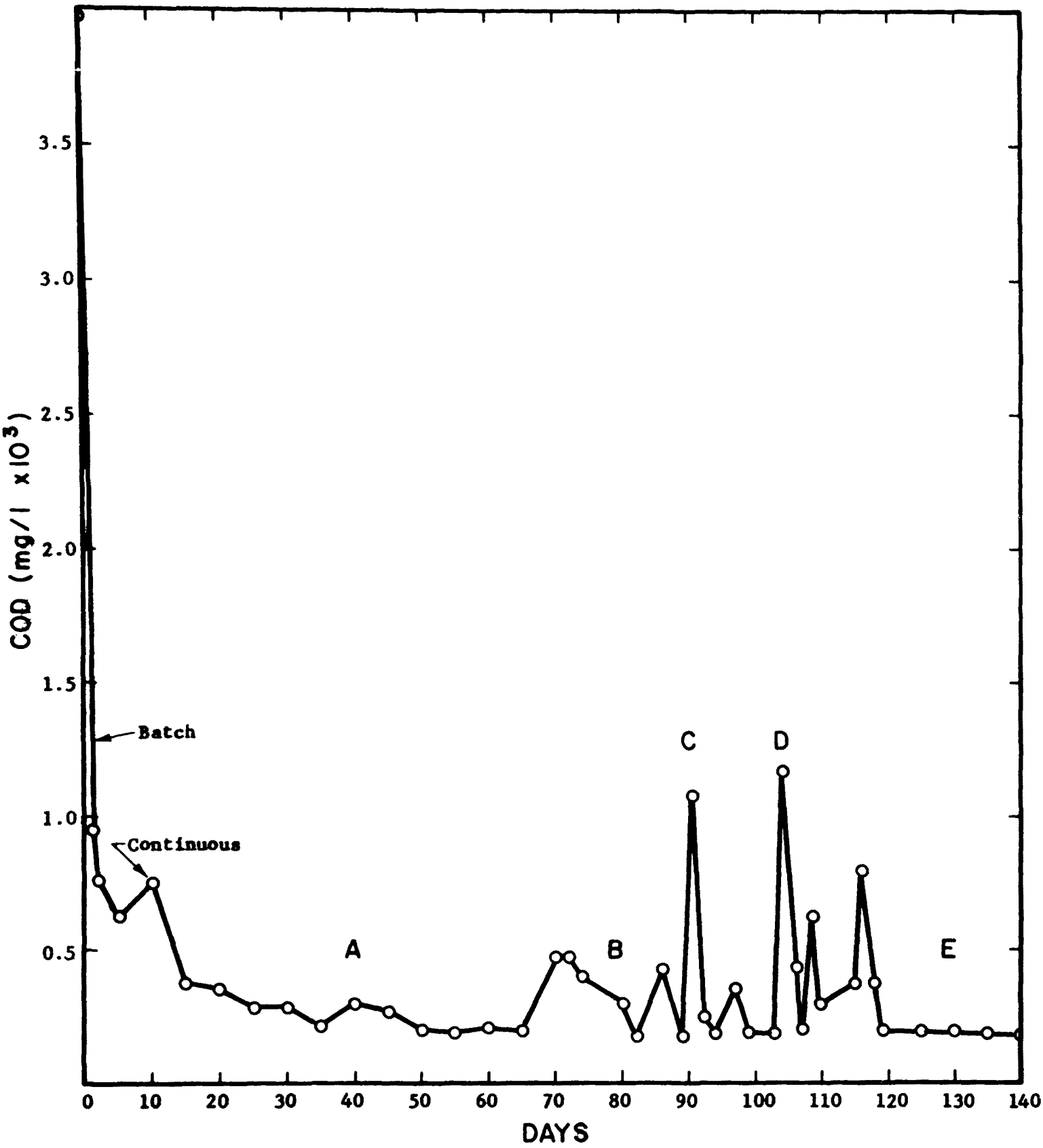

Fig. 8. Continuous digestion of corn waste by T. viride.

and related fungal cytoplasm. At point E (starting at the 120th day), the digestion was under the control of a limiting nutrient - phosphate. From the 120th to 140th day, the continuous culture was strikingly stable, as shown in Figure 8. The $(NH_4)_2SO_4$ added to the 18-liter digestion was 10 g or 0.55 g/l or approximately 0.12 g N/l. Control of the fungal digestion was accomplished by maintaining the phosphate concentration in the digestion tank close to zero. This was done by assaying the phosphate concentration in the digestor and adjusting the phosphate addition to the feed. It was found that a concentration of 25 mg/l of NaH_2PO_4 or approximately 20 mg phosphate/l added to the feed reservoir resulted in less than 1.0 mg phosphate/l in the final digester effluent. Limitation of the concentration of this required nutrient resulted in a stable fungal culture in the digestor. This stability effect produced by phosphate is shown in the continuous digestor system during the last days of the plotted data (E) of figure 8. At this time the fungal mass was 1.9 to 2.1 g/l, the residual solids 1.2 to 1.3 g/l, and the COD 150-180 mg/l. The fungus appeared mature with homogeneous cytoplasm, budding at the hyphal tips, and a white-tan color in the digestor, while maintaining a constant pH of 3.1 to 3.3. The bacterial contamination, not visible microscopically, was reduced from 8.5×10^4/ml in the feed tank to 3.1×10^3/ml in the digestor. No yeasts or protozoa were observed. The feed rate averaged 17 ml/min (ranging between 15-20 ml/min.), which corresponds to a turnover time of 18 to 20 hours.

More than five pounds of dry lyophilized T. viride fungus was collected during the course of this continuous corn digestion and was mixed, as the protein source, with a preformulated rat feed which lacked only a source of protein. The use of this fungal material as a feed for weanling rats will be discussed later.

Chemical Composition of the Effluent

During the last 3 weeks of the continuous digestion (Figure 8) a chemical analysis was performed on the effluent. The results of these analyses are shown in Table 6. Although the original raw corn waste (Figure 8) had a COD of 3750 mg/l at the beginning of the continuous operation, later feed materials from the corn processing plant were more concentrated, and the COD had increased to 5200 mg/l. Both nitrogen and phosphate were reduced to very low values, by fungal growth, as was the BOD.

Table 6

Reduction in the Chemical Components of Raw Corn During Continuous Digestion by T. viride≠

Test	Raw mg/l	Addition mg/l	Effluent from Continuous Treatment mg/l	Percentage Reduction
COD	5200		195	96.2
BOD_5	3976		35	99.2
*CHO	3500		64	98.2
Protein	200		7.5	96.0
Nitrogen (Kjeldahl)	96	116	2.4	98.8
Phosphate (Total)	32	20	<1	>98.0
Sulfate	120	280	210	48.0
Fungi	0		2200	
Solids	4000		760	81
Ash	980		510	52

*CHO = total carbohydrate as determined by the phenol-sulfuric acid method.

≠Analyses performed on samples of the effluent were made after filtering through a single layer of Whatman No. 4 filter paper.

Pigment Production

During the course of some of these studies where nitrogen and phosphate additions were made to continuous cultures of raw corn waste, a pink to red pigment occasionally developed. This usually took place after three to five days incubation. Since it would not be desirable to have red effluents present in scaled-up waste digestion systems and because the pigment may possibly contribute an undesirable factor in fungal feeding experiments, several investigations were made to learn something of the nature of this pigment production.

A series of shake flasks containing raw corn waste inoculated with T. viride were grown as in previous experiments. Specified additions of nitrogen and phosphate were made to the appropriate flasks, as shown in Table 7. Incubation was continued for 48 hours at 26°C at two selected pH's. Table 7 shows that both pH and phosphate were involved in production of the red pigment. In instances (Table 7) where pigment was formed at pH 2.5 and not at 3.5, one could drop the pH from 3.5 to 2.5 and observe the pigment. Conversely, the pigment intensity could be lessened by raising the pH from 2.5 to 3.5. In flasks where no pigment production was observed, prolonged incubation at pH 2.5 did not result in pigment. It thus appeared that less than optimal growth conditions in the presence of excess phosphate resulted in pigment formation. When optimal growth conditions for T. viride were established, as in the cases where higher levels of nitrogen were used with the phosphate - no pigment production occurred.

These results led us to examine the fungal contents of the flasks with the thought that, since the T. viride growth system was not a pure culture, there may have been other pigment producing organisms which were favored under certain growth conditions which were suboptimal for T. viride. $(NH_4)_2SO_4$ and NaH_2PO_4 were added in concentrations to supply the nitrogen and phosphate shown in Table 7.

Table 7

Pigment Production Under Conditions of Nitrogen and Phosphate Suboptimal for Growth of T. viride

$(NH_4)_2SO_4$	NaH_2PO_4	pH	Pigment	Fungus	COD
μg N/ml	μg PO_4/ml			mg/ml	mg/l
0	0	2.5	none	0.81	650
0	0	3.5	none	0.78	700
0	9	2.5	pink	0.84	670
0	9	3.5	none	0.88	690
0	18	2.5	pink	0.97	680
0	18	3.5	pink	0.90	700
40	0	2.5	none	1.02	710
40	0	3.5	none	1.00	605
40	18	2.5	red	1.11	370
40	18	3.5	red	1.13	295
80	18	2.5	pink	1.21	315
80	18	3.5	pink	1.25	285
120	18	2.5	pink	1.26	200
120	18	3.5	none	1.20	180
160	18	2.5	pink	1.35	220
160	18	3.5	none	1.46	208
300	18	2.5	none	2.28	185
300	18	3.5	none	2.65	140

Microscopic examination of the fungal organisms and streak plating on Sabouraud dextrose agar plates were done from all experimental flasks used to collect the data shown in Table 7. The flasks with pigment appeared to contain a mixed population of fungi when viewed microscopically. This was not the case with flasks which showed no pigment at either pH 2.5 or 3.5. In addition, a large proportion of T. viride mycelium was undergoing sporulation in the pigment producing flasks. The streak plates from pigmented flask cultures contained a mixture of

T. viride colonies and green colonies of another fungus. Also, much red pigment was observed on these plates. Streak plates from flasks which showed no pigment production contained only an occasional green colony among the numerous T. viride colonies. The fungi were isolated in pure culture and cross streaked on additional agar plates. The results are shown graphically in Figures 9a, 9b, 9c.

These plating experiments showed that the pigment was produced as a result of interaction between a fungus occurring naturally in the corn waste and T. viride. In cross streaking experiments such as those illustrated in Figures 9a, 9b, the pigment occurred only at the junction of the two cultures and was produced by the contaminating fungus. This fungus grew on Sabouraud dextrose agar plates as round colonies with fluffy white borders and a deep yellow zone inside the border with a dark green center. Microscopically, we observed conidiophoral branches terminated by metulae at the tips of which were clusters of phialides producing spores in parallel chains. This fungus was thus a species of Penicillium.

These studies also illustrated the ecological checks and balances which exist in such a mixed population. Understanding the factors controlling the mixed flora is necessary to controlling the fermentation.

Studies of pigment production leading to its elimination from the continuous fermentation were interesting in that optimal T. viride growth conditions of pH, nitrogen, and phosphate were conditions which apparently inhibited growth of the contaminant fungus and thus precluded pigment formation.

Dissolved Oxygen Utilization

During the early period of the continuous fermentation of raw corn waste, the dissolved oxygen supplied to the corn digester was determined. Adequate aeration was achieved by a single stone sparger in the 18-liter fermentor.

More quantitative measurements of oxygen usage were made by measuring disappearance of dissolved oxygen after interrupting aeration of a stabilized fermentation. The assumption required in this method was that the COD reduction during the period of measurement was the same as the average COD reduction per unit of time before the aeration was interrupted. A curve showing consumption of dissolved oxygen for the corn culture by this method is represented in Figure 10. A mechanical stirrer was used to provide gentle agitation and homogenization of the digestor components during interruption of aeration. The feed rate was 20 ml/min. of material containing 3650 mg/l COD. The effluent assayed 204 mg/l COD. Oxygen usage in an 18-liter fermentation was seen to be 10.2 mg/min. (0.57 mg/min./liter x 18 liters). COD reduction was 70 mg/min. This meant that one pound of oxygen was used for every 7 pounds of COD removed.

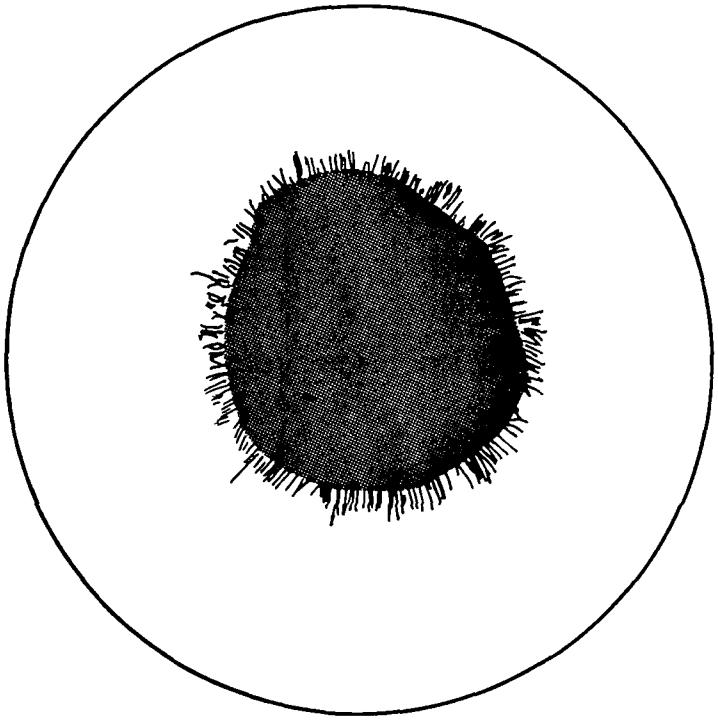

Fig. 9a. T. viride colony

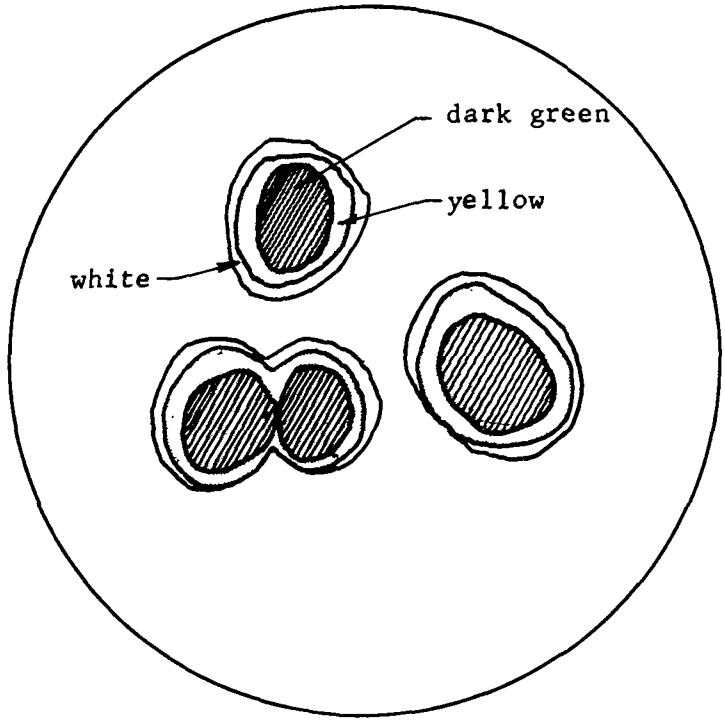

Fig. 9b. Penicillium colonies

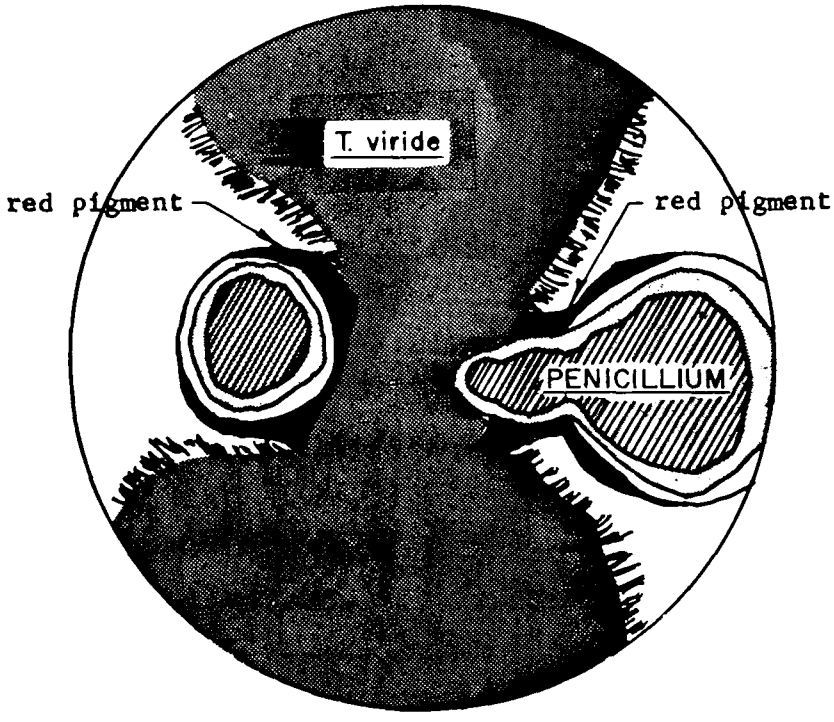

Fig. 9c. T. viride and Penicillium Note red pigment production in area where two generic colonies are in close proximity.

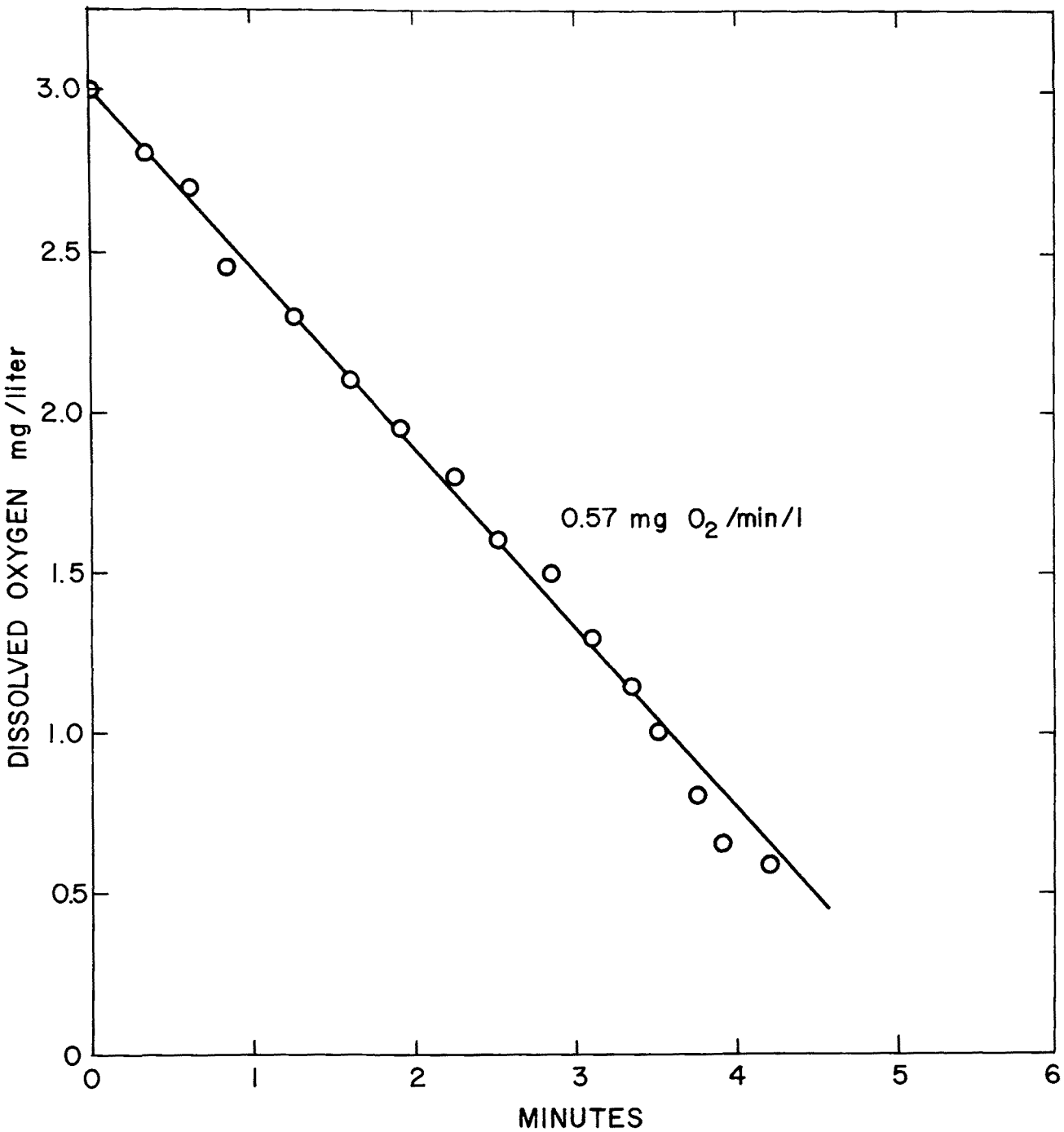

Fig. 10. Disappearance of dissolved oxygen by T. viride growing on corn waste in a continuous culture. The rate of oxygen disappearance is shown by the slope of the line and equals 0.57 mg O_2/min/l.

Amino Acid Analysis of T. viride Protein

Before undertaking feeding trials using T. viride mycelium as the protein source for weanling rats, it was necessary to determine the amino acid composition of the fungal protein. Since a protein is only as good (for feeding) as its amino acid composition and balance, any amino acid adjustments required to equate the protein to casein (standard diet protein) would necessarily be determined from the amino acid study. The amino acid composition of T. viride protein is shown in Table 8, together with the amino acid composition of several other good feed proteins.

Table 8

Amino Acid Composition of Several Proteins
grams/100 grams protein

	T. viride Fungi	Corn		Casein	Soy Bean	Skim Milk
		Normal	Opaque-2			
Lysine	3.9	2.6	4.2	8.0	6.6	8.4
Histidine	1.7	3.0	3.5	3.0	2.5	2.5
Arginine	3.0	5.1	6.8	4.0	7.0	3.2
Cystine	1.4	1.5	1.7	0.2	1.2	0.4
Methionine	1.2	1.6	1.4	3.4	1.1	2.0
Threonine	4.0	3.5	3.3	4.8	3.9	4.6
Valine	4.5	4.6	4.9	7.7	5.2	6.1
φ-Alanine	2.8	4.9	4.4	5.9	4.8	4.5
Leucine	5.4	12.1	8.4	9.8	7.6	9.9
Isoleucine	3.5	3.4	3.2	6.5	5.8	10.7
Tyrosine	2.4	4.3	3.9	6.5	3.2	6.5
Glycine	3.9	4.0	4.8	2.5	3.8	2.3
Alanine	4.8	7.9	6.5	3.0	4.5	—
Serine	3.5	3.5	4.3	6.5	5.6	4.3
Aspartic	6.5	6.7	10.0	7.0	8.3	—
Glutamic	9.0	20.8	18.7	25.0	18.5	22.0
Proline	4.3	9.7	8.6	11.0	5.0	—
Tryptophan	1.8	1.0	1.3	—	1.2	1.2

Black and Bolling, Amino Acid Handbook, 1960 was used for the amino acid reference values.
The fungal protein content = 45 percent on the basis of the amino acid analysis.

Vitamin determinations were not carried out in these studies, except for niacin. The niacin content is 34,000 μg/100 g of dried fungal mycelium. This level of niacin is approximately twice that found in beef liver. The niacin determination was performed in Dr. William Gray's laboratory at Southern Illinois University.

Table 8 shows that the lysine content was higher than normal corn protein and equal to the high lysine corn mutant, opaque-2. It was, however, lower than the other proteins shown in Table 8. The leucine-isoleucine ratio was very good and equal to the casein and soy bean proteins. The low proline value was good, since higher values are frequently associated with poor protein quality. Threonine was higher than the corn proteins, equal to soy protein, and slightly lower than casein. Especially notable was the high tryptophan level. This was higher than in all other proteins and is essential to good growth. The sulfur amino acid level was equal to soy and skim milk. Overall, the amino acid balance was excellent.

Rat Feeding

Two diets were prepared for feeding weanling rats. Both diets contained fat, starch, vitamins, and mineral salts optimal for weanling rat growth as described in the section on Methods, page 14. The protein of the standard rat diet was casein supplemented with methionine. In the test diet, the casein was replaced with T. viride fungal mycelium collected from the 140-day continuous corn digestion. The total protein of each diet (casein and fungal) was 23 percent. In several instances where there were discrepancies between the fungal protein and casein amino acid levels, the protein (fungal and casein) were balanced with small additions of specific L-amino acids. Both protein sources were supplemented with methionine to increase the S-amino acid level to that required for weanling rats.

In Figure 11 the cumulative percentage weight gains demonstrated by the standard (casein) and test (fungal) diet-fed rats are shown during the 21-day feeding experiment. This figure shows that, after a slight initial lag, the fungus-fed rats grew at the same rate as the rats fed the standard casein diet. The slopes of the weight gains are calculated for each rat, and the averages show no significant differences. These are shown in the Table insert with Figure 11. This 21-day study was a preliminary examination of fungal protein characteristics when fed to animals. It would be desirable to repeat the feeding study with more rats for a longer time period, to feed fungal protein at a low level (10 percent protein), and perhaps to use other animals such as chicks and ruminants.

The chief purposes of this study were accomplished in that the fungal test diet was proved palatable and digestible, and no toxic symptoms or gross organ changes occurred. Livers, hearts, and lungs of all the rats were examined and weighed at the conclusion of the feeding trial. No noteworthy gross changes were observed in weight or appearance in any of the organs.

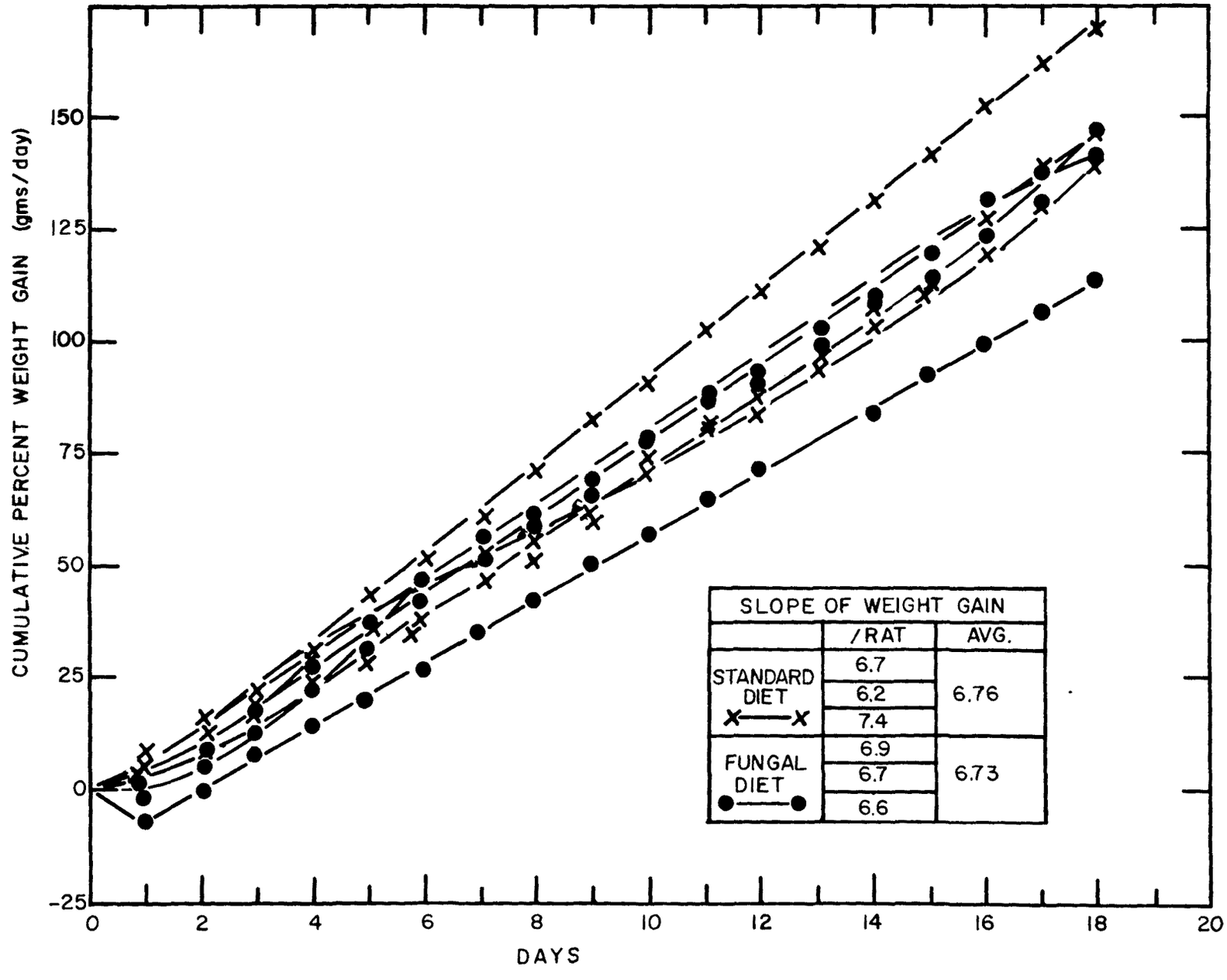

Fig. 11. Rat growth curves.

Digestibility and net protein utilization (NPU) were based on nitrogen analyses of the animal feeds, urine, and fecal samples collected separately each day during the rat feeding experiment. These are apparent digestibility and NPU values, since no endogenous nitrogen was considered. Thus, on the basis of diets containing 23 percent protein, the apparent digestibility for the standard casein diet was 97 percent and for the fungal diet, 90 percent. These values were determined from the standard nutritional formula of

$$\frac{IN - FN}{IN} \times 100$$

where IN is the intake nitrogen and FN the fecal nitrogen. The apparent net protein utilization (NPU) was also determined from the equation:

$$\frac{IN - (FN-UN)}{IN} \times 100$$

where IN and FN are as described above, and UN is the urinary nitrogen.

The results indicated an NPU for the standard casein diet of 75 percent and 50 percent for the fungal (test) diet. The 50 percent net utilization of the fungal protein is undoubtedly a low estimate, since no correction has been made for the fact that about 20 percent of the nitrogen of the mycelium is non-protein-nitrogen.

Soy Whey: HCl Soy Whey

Fungal Strain Selection

Initial selection of fungal strains for application to soy wastes was made with attention to rate and final level of COD reduction and increase in mycelial mass (fungal growth). Adaptation of the organism to the substrate was done before making judgments on the potentialities of the strain. This was done by making initial transfers through sterilized soy wastes at 24-hour intervals. In these initial strain selection experiments, no adjustments of pH or additions of nutrients were attempted. The pH of the soy whey, as received, was 4.5. Soy whey used in these studies was obtained from a process in which hydrochloric acid had been used for protein precipitation. Results are shown in Table 9.

The supernatants from these fermentations were transparent but yellow-brown in color except in the cases of *P. elegans* I-134 and *T. viride* I-185. In these two instances, the supernatant was clear and colorless. Most of the fungi produced pellet type growth, but the highly efficient *T. viride* I-185 strain (76 percent COD reduction in 24 hours) produced a heavy filamentous matted mycelium.

The rate of COD reduction was examined in greater detail using the three cultures that appeared most promising in these screening studies. These were *T. viride* I-185, *T. viride* I-187, and *P. elegans* I-134. Both I-187 and I-134 grew more slowly than I-185 and became heavily contaminated with organisms of the natural biota before maximum COD reduction was attained. Thus, it would appear from this survey that *T. viride* I-185 was the strain of choice for additional work on the HCl soy wheys.

Table 9

Digestion of Soy Waste by Various Species and Strains of Fungi Imperfecti

Fungi	COD mg/l		COD	Carbohydrate mg/l		
	0 hr	24 hr	Percentage Reduction	0 hr	24 hr	72 hr
Natural biota	7429	6315	15	3988	2970	1940
Trichoderma viride I-23	7429	3450	54	3988	2410	1095
Trichoderma viride I-23	7600	4300	43			
Trichoderma viride I-184	7600	4300	43			
Trichoderma viride I-186	7600	4300	43			
Trichoderma viride I-188	7600	4300	43			
Trichoderma viride I-192	7600	4150	46			
Trichoderma viride I-193	7600	3925	48			
Trichoderma viride I-191	7600	3900	49			
Trichoderma viride I-190	7600	3850	50			
Trichoderma viride M-114	7600	2800	64			
Trichoderma viride I-187	7600	2475	68			
Trichoderma viride I-185	7600	1825	76			
*Trichoderma viride I-185	7600	1125	86			
Gliocladium deliquescens I-31	7429	2900	61	3988	1880	1000
Gliocladium deliquescens I-31	7600	2825	63			
Paecilomyces elegans I-134	7429	3750	50	3988	1875	531
Paecilomyces elegans I-134	7600	1800	77			

*Sterilized soy waste used in this instance.

pH Effects

It is known that many fungi require acid environments for best performance, Cochrane, (23). Further, one would expect many of the competing organisms to be inhibited at low pH values. Studies of pH effects were conducted with T. viride I-185 using 30 ml samples of soy whey in shake flasks. pH adjustments were made with 1 N HCl and 1 N NaOH. Incubation was at 27°C for 24 hours. The optimal pH appeared to lie between 3 and 4 as shown in Figure 12. During the fermentation of soy whey, the pH initially dropped from 4.5 to 3.2-3.5. After 24 hours, unless acid additions were made to these flasks, the pH rose to 7.0.

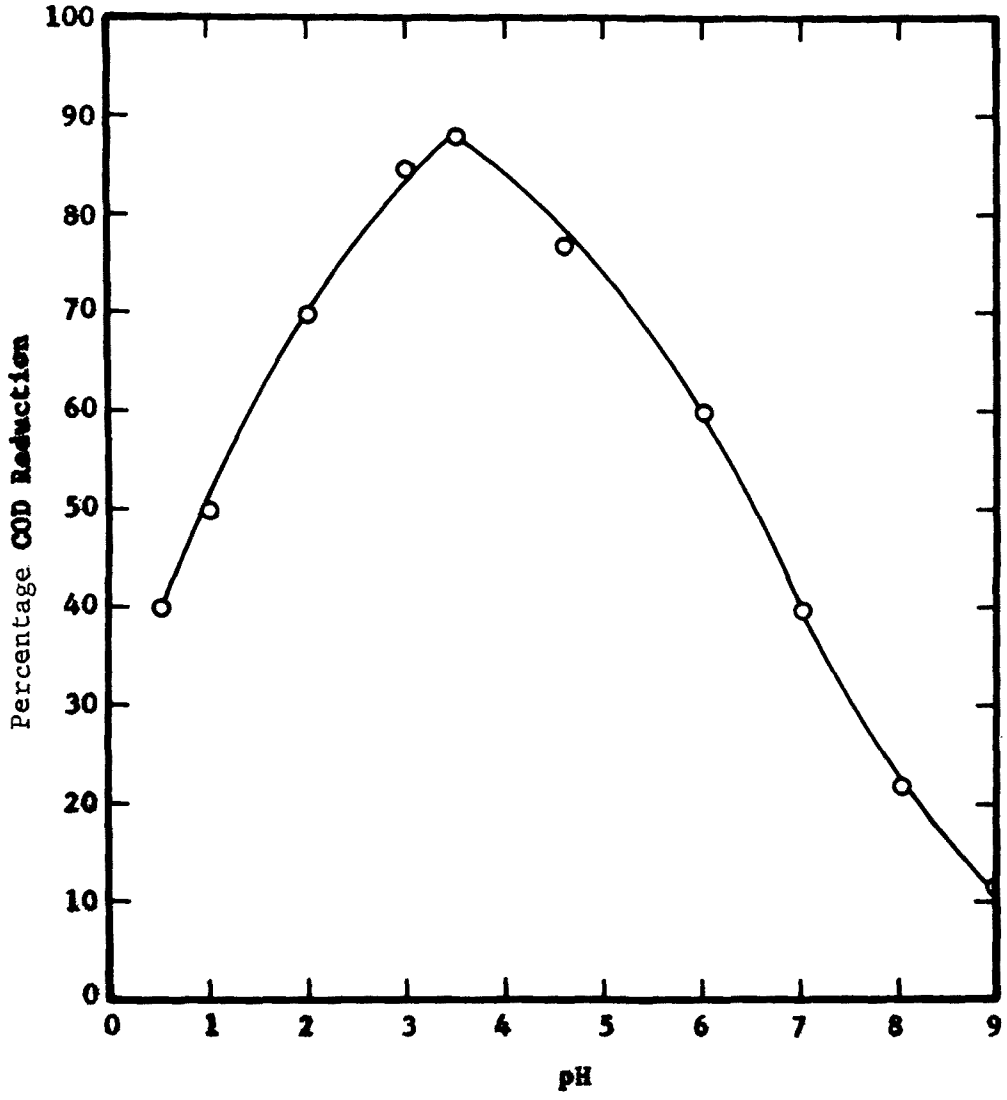

Fig. 12. Effect of pH on COD reduction by T. viride 185 in soy whey after 24 hours.

Temperature Effects

Temperature effects were also studied with T. viride I-185 in 30 ml shake flask cultures. The pH of the soy waste was maintained at 3.5 in these studies. Results are shown in Figure 13. The optimal temperature for rapid COD reduction appeared to lie between 27°C and 35°C.

Effects of Inoculum Size

Because of the high levels of carbohydrate and protein contained in soy whey it was difficult to establish T. viride as the predominating organism. It was reasoned that a large inoculum should increase the efficiency of conversion of COD to mycelial mass by overwhelming "contaminating" organisms, should increase the rate of COD reduction and should aid the fungus to establish itself in the nonsterile soy whey. These effects were examined in experiments in which various size inocula of T. viride I-185 were added to 30 ml shake flask cultures of raw soy whey. The soy whey contained 10,920 mg COD/l and was diluted by the inoculum to about 9800 COD mg/l. Results are shown in Table 10. The increased fungal mass was seen to be relatively independent of inoculum size over a ninefold range of inocula. Mycelial mass attained a constant level in every case after 24 hours. Thus, it would appear that the fungus can compete with the "contaminating" organisms in the soy whey at even the lowest inoculum level during the first 24 hours.

Table 10

Increase in Mass of T. viride I-185 Mycelium Produced as a Function of Inoculum Size

Inoculum mg/ml	Fungal mass increase in 24 hr mg/ml
0.5	2.3
1.3	2.4
2.3	2.4
4.5	2.0

The details of the soy digestion were examined in greater depth. In Figure 14 it is seen that the rate of COD reduction was initially greater when larger inocula were used. All fermentations, however, reached about the same COD level after 24 hours. The progression of initial COD digestion rates with inoculum size is plotted in Figure 15. The efficiency of conversion of COD to mycelial mass in these particular fermentations was about 40 percent on a weight basis; that is approximately 40 mg of mycelium was produced for each 100 mg of COD utilized.

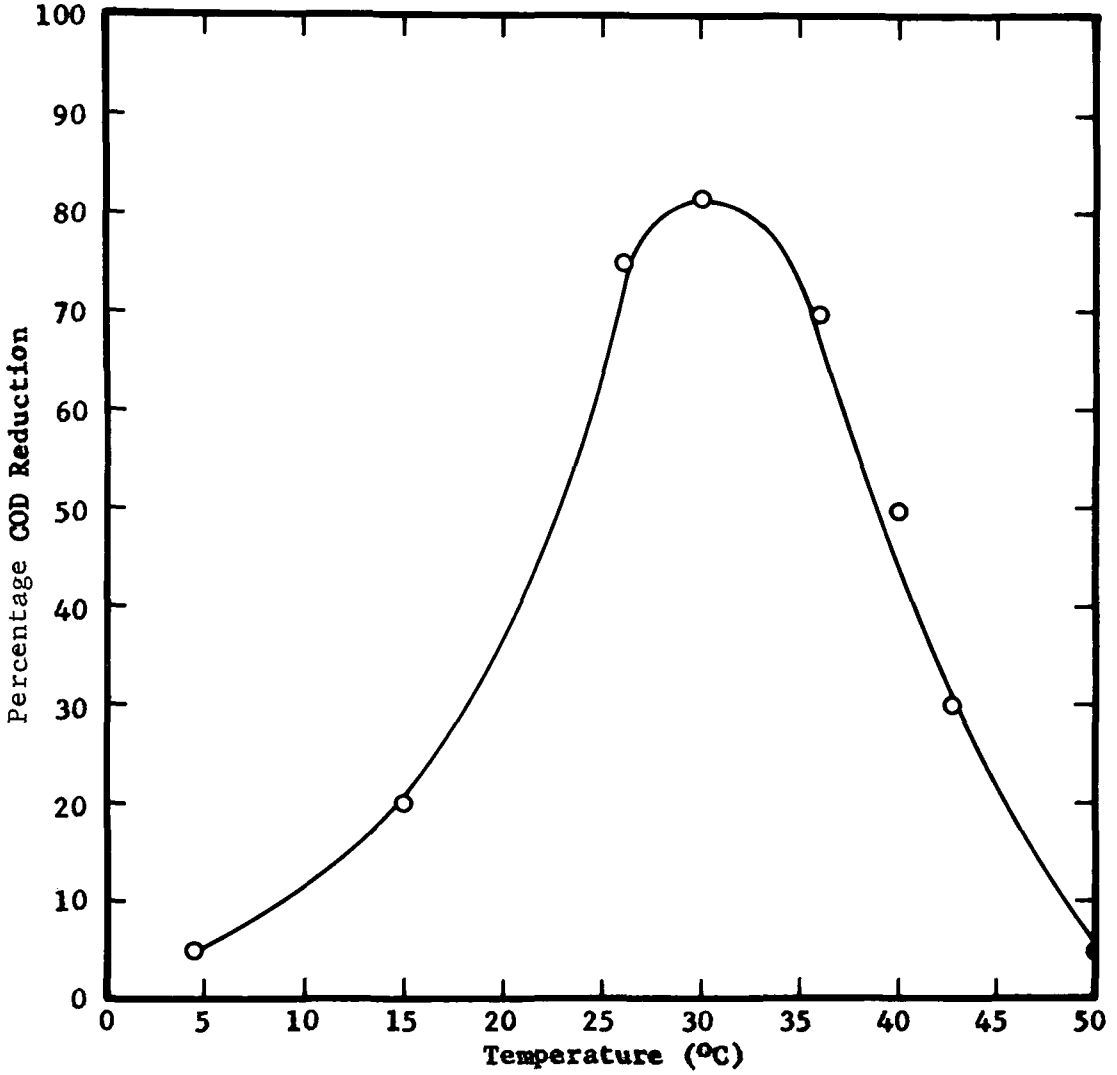

Fig. 13. Effect of temperature on COD reduction by T. viride 185 in soy whey after 24 hours.

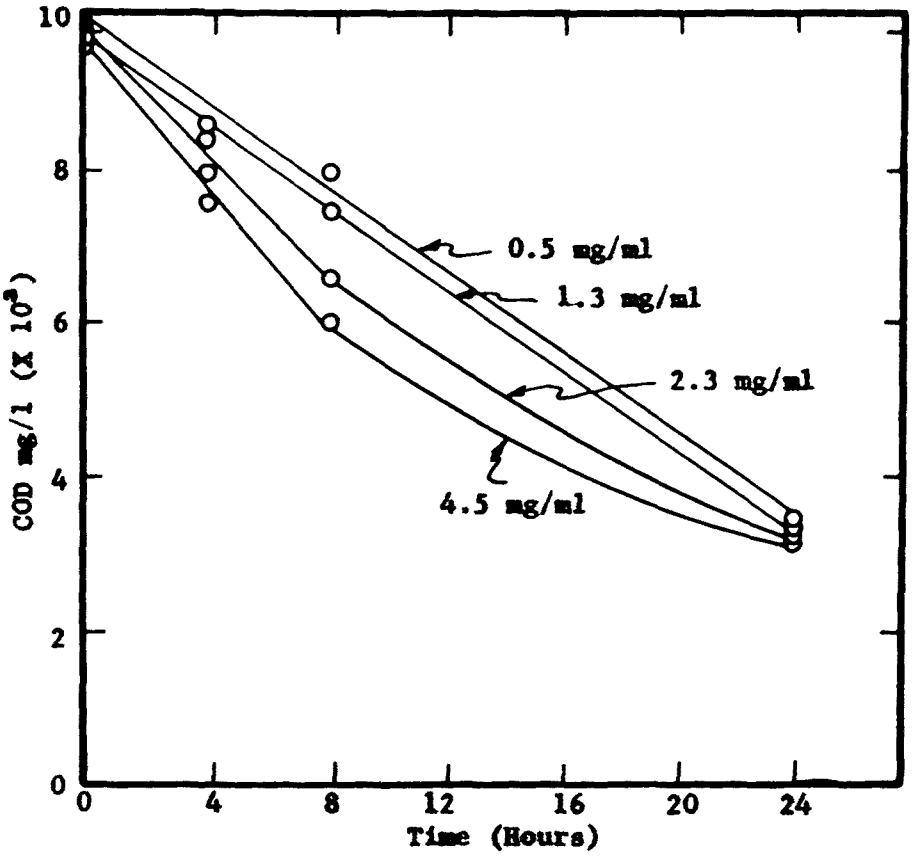

Fig. 14. COD reduction as a function of inoculum size.

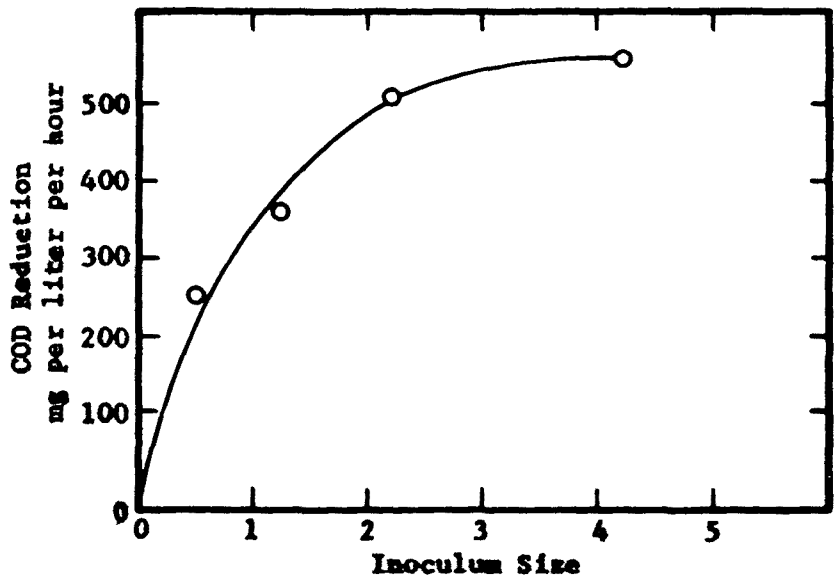

Fig. 15. Rate of COD reduction as a function of inoculum size. The early linear rates (8 hrs) in Fig. 14 above were used for the curve in Fig. 15.

Nutrient Addition

The failure of these fungal strains to bring COD levels to lower values in reasonable fermentation times prompted exploration of nutritional factors which might have been limiting fungal metabolism. As a first step, the chemical composition of the original soy whey and the filtrate after growth of T. viride I-185 on the soy waste for 48 hours were examined. Results are tabulated in Table 11.

Table 11

Chemical Analyses* of Soy Waste Before and After 48 Hours' Digestion by T. viride I-185

Test	Before Fungal Digestion mg/l	After Fungal Digestion mg/l	Percentage Reduction
COD	7800	1800	77
BOD	5420	860	84
Nitrogen (Kjeldahl)	600	96.7	84
NPN (nonprotein nitrogen)	102	71.5	30
Protein	3013	156	95
Carbohydrate	3980	800	80
Chlorides	653	557	15
Nitrates (as N)	7	5	28
Nitrites (as N)	0.01	0.13	—
Total Phosphate	87	45.6	48
Total Soluble PO_4	82	32.5	60
Ortho Phosphate	78	29.0	63
Total Solids	8300	4170	50
Ash (residual solids)	1590	1130	29
pH (units)	4.6	7.4	

*Analyses performed on samples of the effluent were made after filtering through a single layer of Whatman No. 4 filter paper.

The results indicated that the protein nitrogen had been disproportionately depleted, as compared to carbohydrates. The nonprotein nitrogen was used to a much lower degree. These findings perhaps indicated that the reduction of BOD may have been limited by the available nitrogen supply. Trials of the effect of nitrogen additions were therefore conducted in shake flask cultures of T. viride on raw soy whey. Considerably greater, but still incomplete, removal of BOD was achieved when nitrogen was added as ammonium sulfate. These results are shown in Table 12. No further reduction of COD or BOD was achieved with higher levels of nitrogen.

Table 12

Effect of Nitrogen Supplementation on Fungal Digestion of Soy Whey

Fungus	pH	COD mg/l				BOD mg/l			
		None		Nitrogen		None		Nitrogen	
		0 hr	48 hr	0 hr	48 hr	0 hr	48 hr	0 hr	48 hr
None	3.8	9,870	8,870	9,900	8,100	6,200	6,180	6,100	5,940
T. viride	3.2	10,100	2,530	9,800	1,660	5,850	940	5,620	560
G. deliquescens	3.5	10,020	2,310	9,900	1,000	5,420	869	5,860	340

Nitrogen added as $(NH_4)_2SO_4$ at a final concentration of 0.01 molar.

Residual COD

Besides nutrient depletion, other hypotheses accounting for incomplete removal of COD and BOD were formulated and tested. One hypothesis was that an inhibitor of further metabolism accumulated during soy digestion with these fungi. One test of this hypothesis was based on the assumption that a dialyzable inhibitor was formed. Spent soy whey was dialyzed for 16 hours, reconstituted by addition of ammonium sulfate and phosphate ions and reinoculated with various fungal strains. No further reductions in COD levels were achieved. The dialysis had been sufficiently extensive to remove half the residual COD. Thus, the inhibitor, if such existed, was not a small molecule.

Another test of the possible accumulation of an inhibitor substance was conducted by examining the amount of growth and COD reduction at a series of soy whey dilutions. The rationale was that the dilution of the soy whey would allow consumption of a greater proportion of the COD before an inhibitor attained critical concentrations. This would be in contrast with the findings to be expected if metabolism was limited by exhaustion of an essential nutrient. In this latter case, one would expect the same percentage removal of COD before slowing of the COD removal, regardless of the original COD concentration.

Such a study was conducted using dilutions of soy whey ranging from 10,000 to 2500 mg COD/1. Shake flasks (125 ml) were inoculated with 2 mg/ml of T. viride I-185 and sampled at 0, 8, 16, and 24 hours. At 16 hours the COD removal had reached maximum with the residual values as shown in Table 13.

The fact that a nearly constant proportion of the COD was used before the reduction of COD halted may be taken as evidence of nutrient depletion and as evidence against the accumulation of an inhibitor to critical growth-limiting levels. This line of reasoning presupposes that the amount of inhibitor produced would be a function of the amount of COD utilized and that its effectiveness would be a function of its concentration per unit volume.

Table 13

Residual COD as a Function of Dilution of Soy Whey before Inoculation

Original COD mg/l	After 16 Hours Incubation	
	COD mg/l	Percent of Original COD
9850	2900	30
8350	2410	29
5800	1700	29
4400	1000	23
2500	850	34

Still another approach to the removal of residual COD was to inoculate a spent liquor from one fermentation with other fungal strains. A liquor from a 16-hour fermentation with T. viride I-185 was filtered, adjusted to pH 4.2, and reinoculated with a variety of fungi. Of eighteen strains tested, only seven were effective in further reducing the COD (Figure 16). Four strains gave essentially identical results and are plotted on a single curve. No significant reduction of the residual COD level was observed.

A related approach was to inoculate simultaneously with two fungal strains. This was done using both the original undigested soy whey and the residual whey after an initial 24-hour digestion by T. viride. It was hoped that the metabolic capabilities of two strains might prove complementary and so permit more nearly complete digestion that could be attained by any one strain alone. Combinations used included T. viride I-185 with Gliocladium deliquescens, T. viride I-185 with Aspergillus oryzae, G. deliquescens and A. oryzae, T. viride I-192 with G. deliquescens, and T. viride I-192 with A. oryzae. None of these fungal combinations reduced the COD below approximately 2000 mg/l.

The most direct experimental evidence that residual COD was not inhibitory to fungal metabolism was shown in an experiment where cellulose was added to the soy whey supernatant. The supernatant was the filtrate from a 48 hour fungal digestion of raw soy whey. It was reasoned that any metabolic inhibition contained in the digested soy whey would block the utilization of a prime energy source (cellulose) for this fungus. The data in Table 14 show that no inhibition of cellulose was present. All added cellulose was digested and apparently stimulated further reduction of the residual soy whey. This is shown in Table 14 where the COD of 1405 (without cellulose)was reduced to 1325.

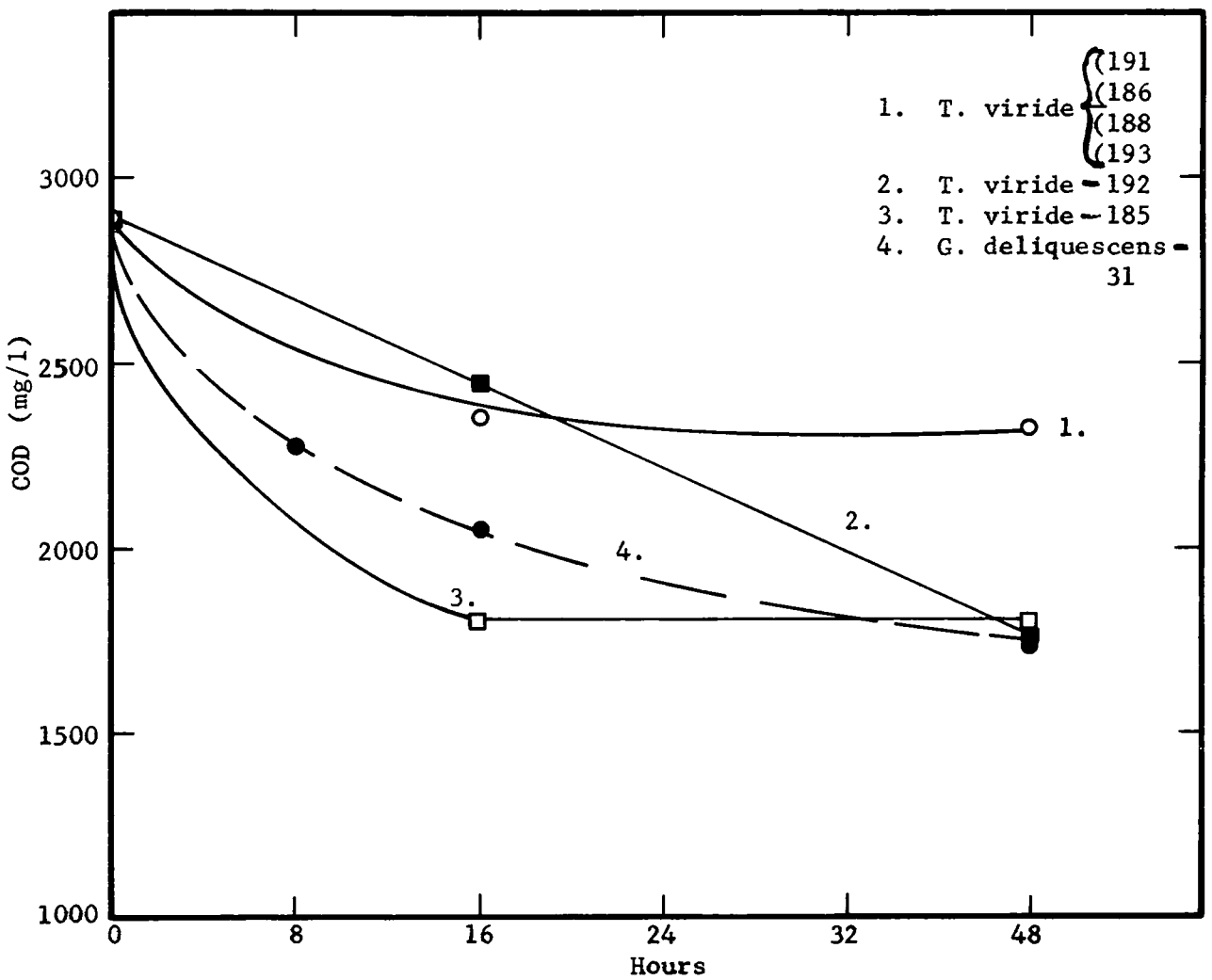

Fig. 16. Fungal growth in soy whey predigested for 16 hours with T. viride 185.

Table 14

Digestion of Spent Soy Supernatant by G. deliquescens

No.	Sample	Fungus mg	Cellulose mg	Hours	COD mg/l
1	Soy whey	0	0	0	10,800
2	Soy whey	60	0	48	2,663
3	Soy whey Supernatant	60	0	48	1,405
4	Soy whey Supernatant	0	30	0	3,683
5	Soy whey Supernatant	60	30	48	1,325

Experimental Conditions:

Soy Whey 30 ml cultures in 125 ml Erlenmeyer flasks incubated for 48 hours at 26°C in a rotary shaker at 140 rotations/min.

Soy Whey Supernatant 30 ml filtrate after 48 hr soy whey digestion by G. deliquescens. Reinoculated with fungus and incubated an additional 48 hours, as above, with and without cellulose.

It appeared from these experiments that the accumulation of inhibitors did not account for the incomplete digestion observed. Complete digestion of this remaining substrate must await additional studies designed to reveal whether or not nutritional imbalances exist, and the chemical character of the residual materials. It is entirely possible that certain carbohydrates and proteins in soy whey are refractory to further fungal metabolism. That nutritional imbalances do develop was learned earlier where at least one, the available utilizable nitrogen, was required for further fungal digestion (Table 12). There was no stimulation by additional phosphate as was observed in the corn waste studies. This was not surprising, since 45 mg phosphate/1 was found in the residual soy whey filtrates.

Continuous Fermentation

Continuous fermentation of HCl precipitated soy whey was tried in a second fermentor constructed similarly to the one used for corn waste digestion. A diagram of the apparatus was shown and described in Figure 1, page 12.

Initially, the continuous soy fermentation systems were started as "batch" type in order to establish a heavy culture of fungal mycelium before a continuous flow of soy whey was introduced. Thus, a typical experiment selected from these early attempts to establish a continuous digestion of soy whey was run as follows:

A 24-hour culture of Gliocladium deliquescens grown in sterile HCL soy whey was used for the inoculum. The raw soy whey contained 11,820 mg COD/1. 4.7 liters of this fungal culture containing 21 gm of fungus (dry weight) were added to 11.3 liters of freshly thawed soy whey. Thus, an initial digestor volume of 16 liters contained 1.3 g fungus and 8400 mg COD/liter. At zero time, the pH was 4.0 and the total suspended solids (dry weight) were 13,500 mg/1, of which 1300 mg were fungus and 12,200 mg were soy particulates. Aeration and mixing were accomplished with 3 stone spargers which supplied a dissolved oxygen concentration of never less than 0.5 mg O_2/1.

The operation of this digestion system began as a batch process as shown in Figure 17 where COD reduction is plotted vs. time in days. After 65 hours, the batch digestion was switched to continuous, and the flow rate of fresh raw soy medium was started at 2.5 ml/min. This feed rate corresponded to a soy influx of 0.15 1/hr and supplied 2150 mg COD/hr. At this feed rate there would be a complete digestor turnover every 100 hours. At zero time of continuous digestion (zero + 65 hr), the fungus appeared mature with some budding and a few lysed mycelial fragments. Large swollen budding mycelium, indicative of young, actively metabolizing organisms, was absent. Bacteria were present in very high numbers. We concluded that the culture was too aged for an efficient continuous soy digestion. However, when the feed rate was increased to 4 ml/min. (60 hr digestor turnover time), the fungus developed new growing tips and maintained a stable microscopic morphological appearance. The fungal stability was also demonstrated by the continuous decrease in the COD which leveled off at approximately 1600 mg/liter after the eighth day and continued at this level through the twelfth day.

When the COD was not reduced below 1500 mg/1 after thirteen days, the flow of soy whey was shut off and the system reverted to a "batch" fermentation for twenty hours. It was thought that by reverting to batch operation, the residual COD would be further reduced. However, the COD increased to 2500 mg/1 because of fungal starvation and lysis. Yeast and bacterial contamination increased to a high level (approximately 10^6 organisms/ml). Therefore, on the fifteenth day, the system was switched from batch back to continuous, and the feeding was set at 5 ml/min. (not shown in Figure 17). The raw feed was changed from HCl soy whey to SO_2 soy whey (the SO_2 was adjusted to 200 mg/1). This change to SO_2 soy whey was made with the hope that the SO_2 would reduce the yeast and bacterial contamination. Neither of these changes (batch of SO_2) resulted in good COD reduction. Although the mycelial dry weight increased from 2.5 g/1 at the twelfth day to 3.5 g/1 on the fifteenth day, and remained at the 3.5 g/1 level during the SO_2 soy whey feeding, the COD remained approximately 3000 mg/1. Although the COD of the incoming SO_2 soy whey was reduced during this period of SO_2 feeding from 13,000 mg/1 to 3000 mg/1, no further reduction in COD resulted through the 23rd day. The fungal appearance was that of an old, stationary phase culture, and sporulation and lysis were beginning. Since this was a sign of starvation, the feed rate was increased to 10 ml/min. The increased feed rate did not stimulate new fungal growth but increased the bacterial and yeast contamination. Thus, it was believed the "aged" fungal culture was losing out to the contaminants. The experiment was therefore discontinued.

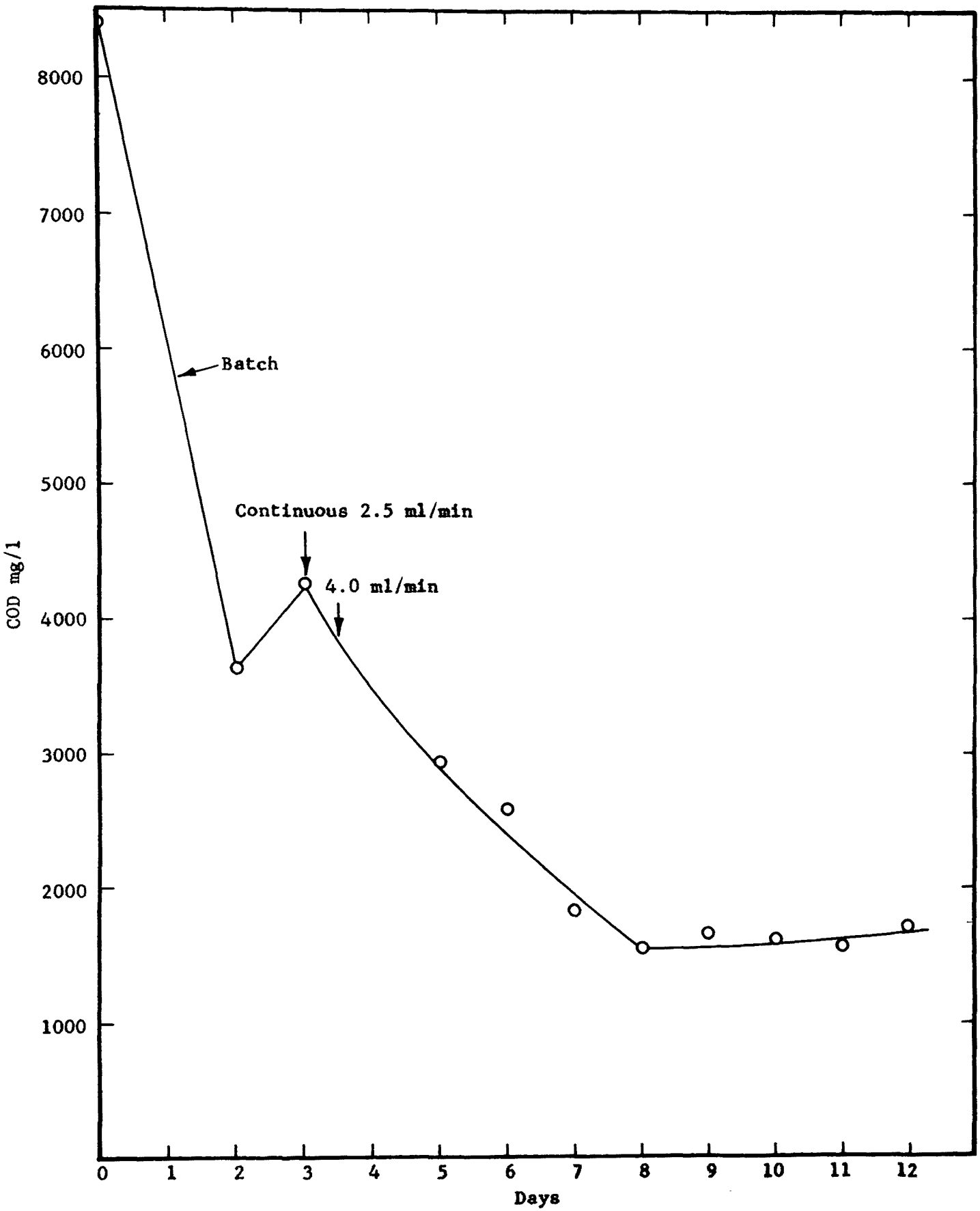

Fig. 17. Continuous COD reduction of HCl soy whey by G. deliquescens.

Inoculation-Dilution. When the previous problems with the continuous soy digestion system were described to Dr. H. Orin Halvorson, University of Minnesota, he made several suggestions. One suggestion was with regard to initiating the continuous culture system and avoiding an early build-up of other microorganisms indigenous to the soy whey (yeasts and bacteria). This technique was the inoculation-dilution procedure.

This technique consisted of introducing a small amount of soy whey into a fermentor nearly filled with water. A fungal inoculum appropriate to the amount of soy whey was introduced, and continuous feeding of the fermentation with fresh soy whey began at zero time. This technique precludes the presence of much larger amounts of nutrient than can be quickly utilized by the mycelial mass present and which might otherwise support the growth of contaminants. The mycelial mass grows as incoming soy whey gradually replaces the water initially present. The advantages of this procedure for large-scale start-up operations are obvious.

One such experiment is illustrated in Figure 18. In this experiment (Figure 18), 750 ml of HCl soy whey nutrient + 1.2 g of wet Trichoderma viride I-185 mycelium were diluted in the digestor jug with 14,250 ml of tap water. The raw soy feed (full strength) was turned on at zero time, at a feed rate of 5 ml/min. Figure 18 shows the theoretical time required for the soy concentration to reach 100 percent. The "actual" curve (COD assay) is compared to the "theoretical" COD curve. The "theoretical" curve represents the increasing concentration of raw soy COD, and the "actual" represents the impact of the fungal growth and COD reduction on the "theoretical".

Although the technique of inoculation-dilution was successful and the T. viride culture reduced COD for a few days, it soon showed microscopic evidence of deterioration and gave poor COD removal. This pattern was repeated on several trials. One difficulty appeared to be "bulking". The T. viride mycelium gathered into floating masses rather than being dispersed through the medium. Stirring was only partially successful in overcoming this difficulty. Attention was therefore turned to another organism which had given relatively good behavior in earlier trials and did not show the clumping tendency. This was Gliocladium deliquescens.

Although this organism did not give difficulties with bulking, difficulties were still encountered in maintaining a stable fermentation. Fermentations began well enough but soon deteriorated, and competing organisms began to appear. It was noted from microscopic observations that the organisms showed vacuolation and sporulation before competing organisms began to predominate. This suggested that the fungus had begun to starve and go into a stationary phase with some lysis. When this occurred, competing organisms obtained a foothold. Once in a stationary phase, the fungus responded only slowly to adequate nutrient concentrations.

Two expedients were evident as ways to prevent starvation. One was simply to regulate the feed rate by COD measurements in the fermentation so that there would always be adequate nutrient present. Of course, too great an excess of nutrient (high feed rate) would mean an outflow stream of high

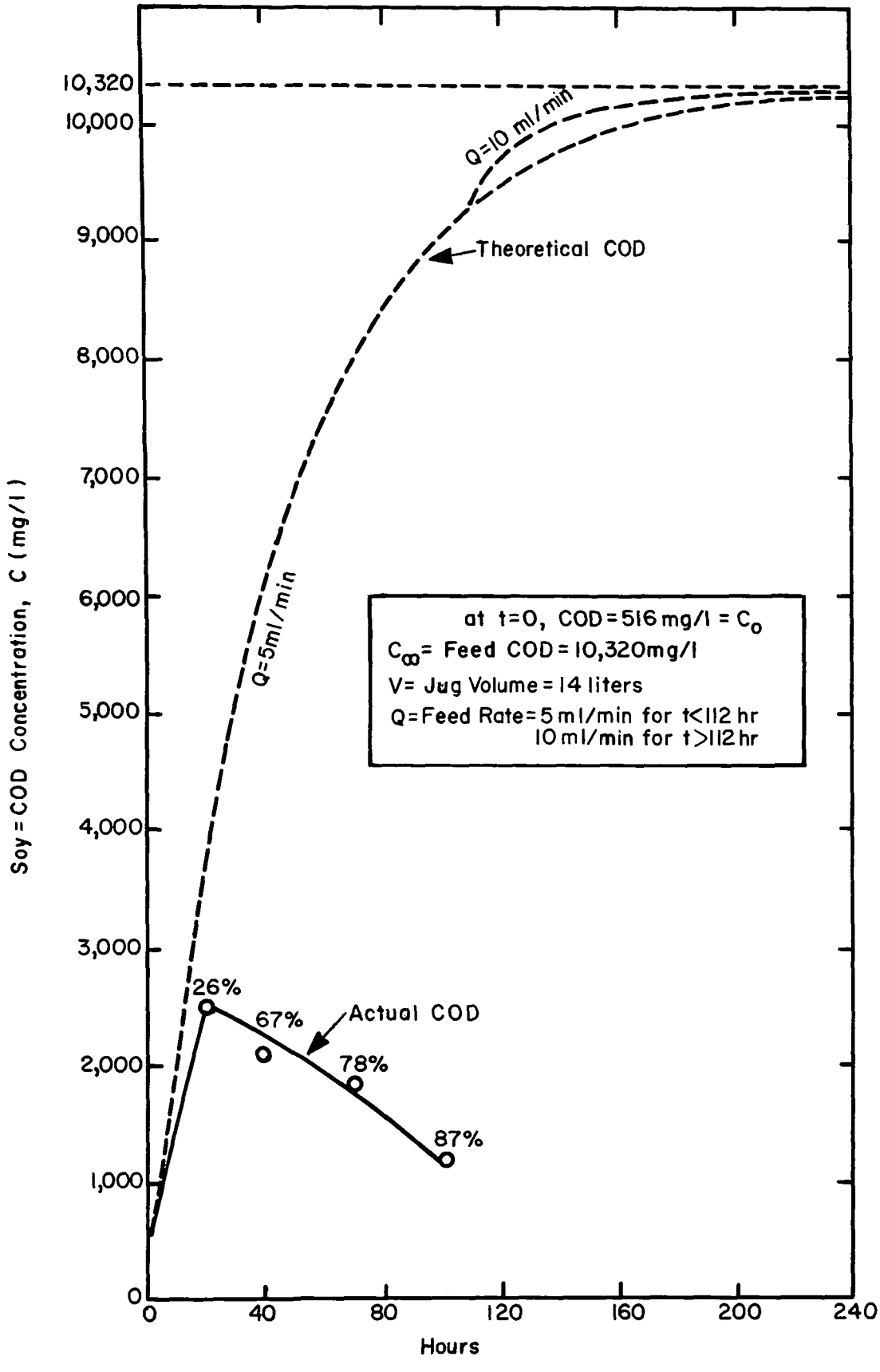

Fig. 18. Inoculation-dilution technique for starting a continuous digestion of soy whey by T. viride. Determination of the "theoretical" COD was according to the integrated formula $\frac{C-C\infty}{Co-C\infty} = e^{-\frac{Q}{V}t}$

COD and a washout of fungal mycelium. The other expedient (suggested by Dr. H. Orin Halvorson) was frequent removal of fungal mass while keeping the feed rate constant. The chief criterion in this case is the weight of fungal mass allowed to remain in the fermentation per unit volume. This is simply another way of assuring that there is not an excess of mycelium for the incoming nutrient.

Fungal Mass Control. A study of continuous soy whey digestion by employing fungal mass control is shown in Figure 19. This experiment was done using G. deliquescens. Inoculation was at the level of 3.2 g of young (24 hour) mycelium to 16 l of tap water. Feed with soy whey was at the rate of 6 ml/min. into an 18-liter fermentor.

The data shown in Figure 19 are divided for purposes of explanation into three areas (A,B,C). In Area A the fungal mass was increasing as the COD feed concentration was increasing (refer to theoretical plot). As the fungal mass increased, the actual COD concentration was maintained at a near steady state of approximately 1500 mg/l. At the five-day point, when the fungal mass was 2.8 gm/l and the COD had reached 1250 mg/l the decision was made, on the basis of microscopic examination of the fungus, to remove an aliquot of the digestion mixture, separate the fungus, and return the supernatant liquor to the fermentation jug. This removal of part of the fungal mass accounted for the decrement in mass seen in the latter part of Area A of Figure 19. Before the effectiveness of the fungal mass removal could be assessed, the feed supply system clogged. Because of feed (COD) deficiency, fungal starvation occurred over the next 24 hours (sixth to seventh day in Figure 19) and fungal cytoplasmic material was spilled into the fermentor, causing a rapid COD increase (Area B) and a heavy growth of yeast and bacteria. Between days 7 and 8 the entire fungal mass was removed from the fermentor, washed (thus removing yeast and bacteria), and the fungal mass returned to the fermentor and a fresh soy whey, adjusted to a COD of 4500 mg/l, was added. The feed flow was started again at a rate of 5 ml/min. It can be observed (Figure 19, Area B) that, as the fungal mass increased, the COD decreased until the sixteenth day, where a fungal mass of 2 g/l reduced the COD to 2000 mg/l at a flow rate of 5 ml/min. In Area C, physical removal of fungi to maintain a level of 3.3 g/l was done about every third day, and a consistent COD level was maintained between 1300 and 1500 mg/l for approximately twelve days. We believed this state could have been maintained indefinitely. Had there not been a feed block on the sixth and seventh days, the system might well have maintained the state observed over the last twelve days throughout the thirty-day period. The arrows in Area C, Figure 19, indicate the points at which the fungal removal technique was used.

The residual COD level of between 1100 and 1600 mg/l appeared to be material not preferentially metabolized in this system. In Area C we were not successful in getting the COD substantially below 1100 mg/l in this continuous culture. Although a residual COD of 1500 to 1800 mg/l was maintained at a flow rate of 7.5 ml/min. (not shown in Figure 19), feeding at 10 ml/min. resulted in a COD of 2500 mg/l and higher.

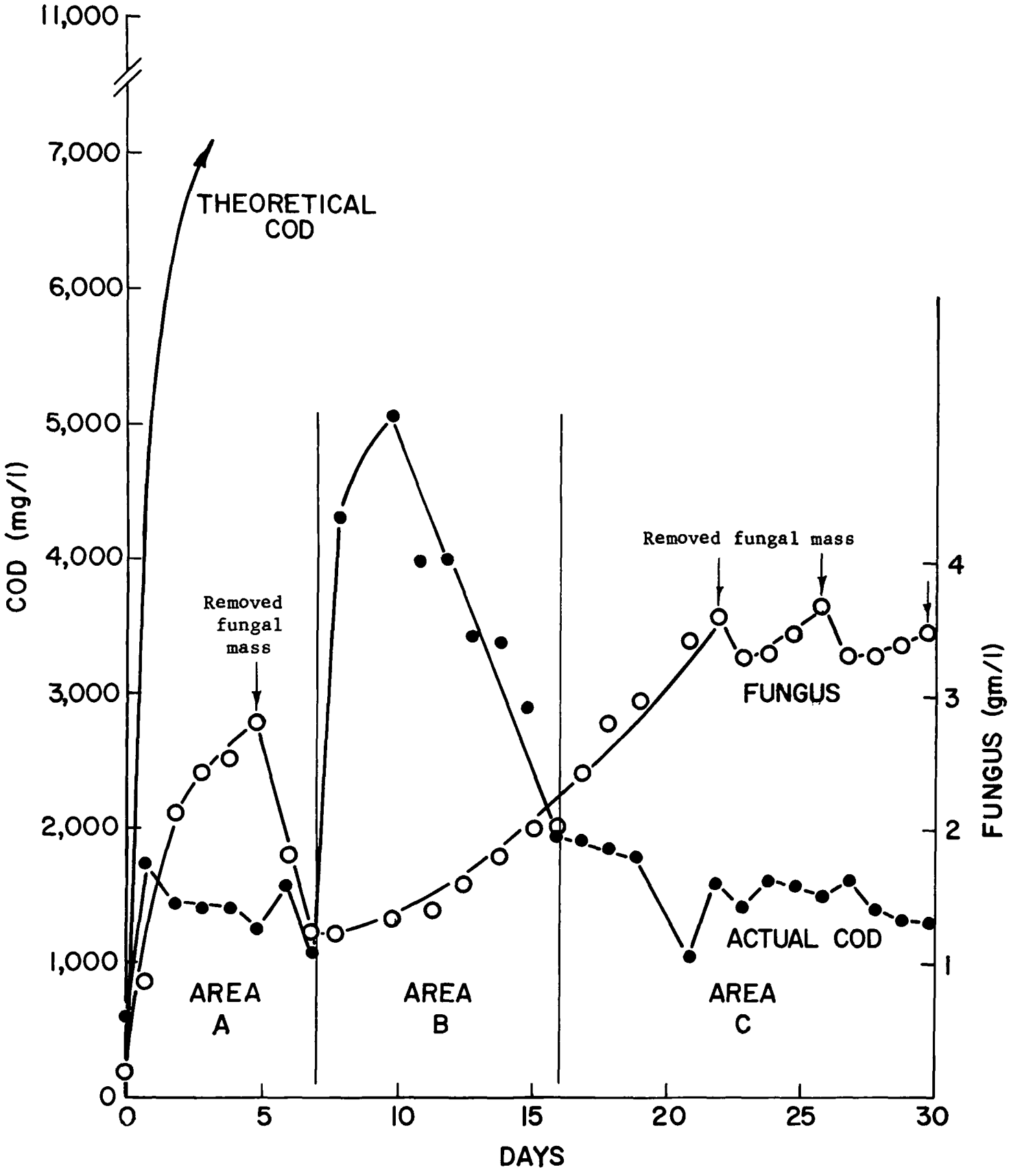

Fig. 19. Continuous digestion of soy whey by G. deliquescens. Effect of controlling fungal mass on stability of COD digestion. The theoretical COD is shown by the bent arrow, the fungal dry weight as O——O and the actual COD as ●——●.

Because further examination of these parameters could be studied with SO_2 soy whey as well as with the HCl soy whey and because the major amount of industrial soy processing is done with SO_2, it was concluded that further continuous culture studies should be done on SO_2 soy wheys.

Dissolved Oxygen Utilization

Oxygen disappearance during continuous digestion of HCl soy whey by G. deliquescens was measured immediately after interruption of aeration. The technique and assumptions for taking these data were the same as those used earlier for oxygen utilization in the corn waste digestion system. The data are plotted in Figure 20.

Making the same calculations for the soy fermentation as was done for corn in a 15-liter fermentation, we arrived at an estimate of 5.5 pounds of COD removed per pound of oxygen used. (The incoming feed contained 10,320 mg/l COD and the effluent contained 2600 mg/l. The feed rate was 4 ml/min. and the fermentor contained 15 liters total volume.)

Amino Acid Analysis

The amino acid analysis of the fungal strain used for the HCl soy whey digestion study is shown in Table 15. The analysis for G. deliquescens is compared to earlier analyses done on T. viride and to several other proteins. Especially significant are the values for lysine, threonine and tryptophan, which are 6.15, 4.86, and 2.31 g/100 g protein, respectively. This G. deliquescens amino acid analysis was considered valuable for formulation of diets for animal feeding trials.

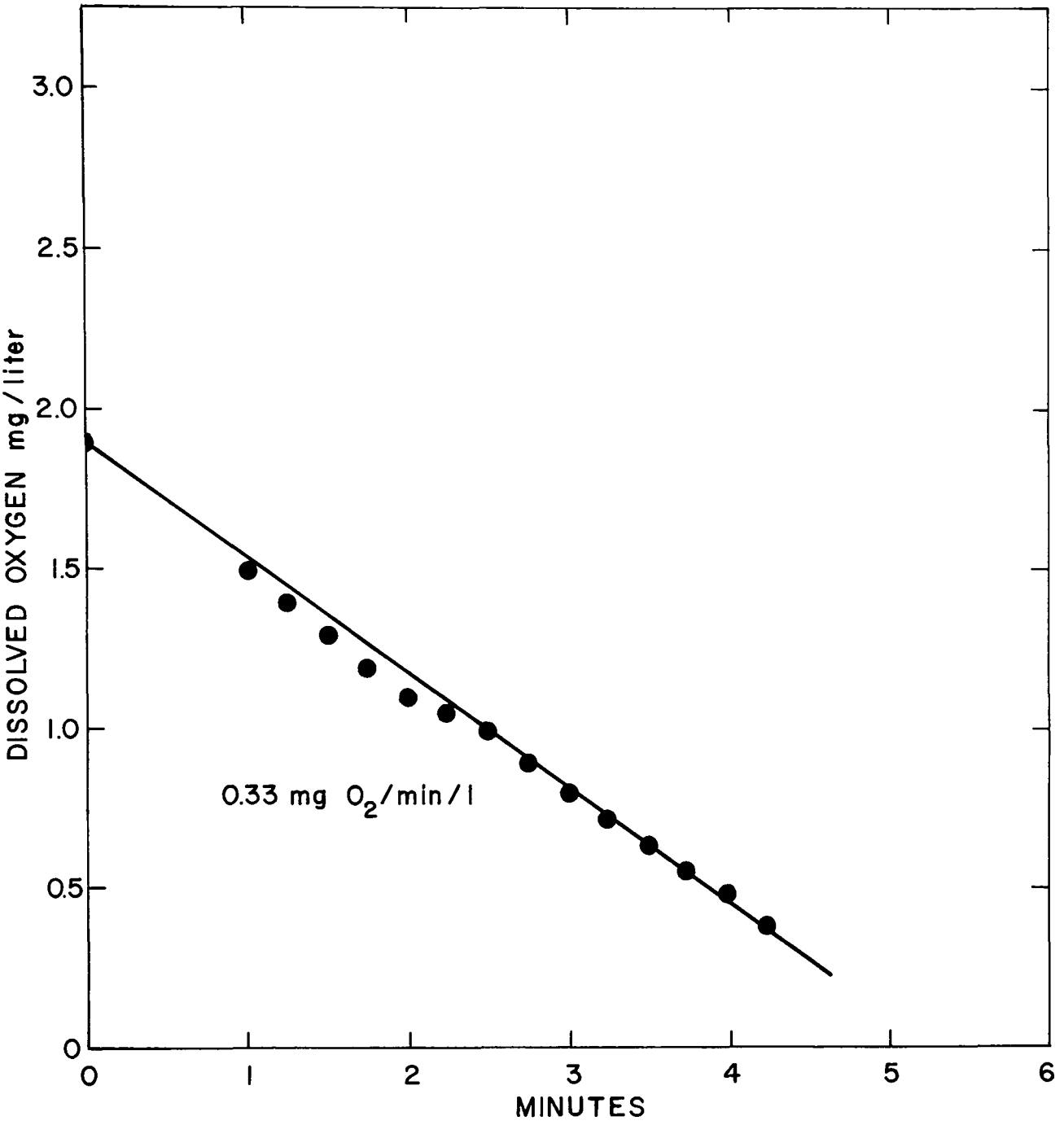

Fig. 20. Disappearance of dissolved oxygen by G. deliquescens growing on soy whey in continuous culture. The rate of oxygen disappearance is shown by the slope of the line and equals 0.33 mg O_2/min/l.

Table 15

Amino Acid Analysis of Two Fungal Strains Compared to Several Standard Proteins (grams/100 grams protein)

Amino Acid	Trichoderma viride	Gliocladium deliquescens	Casein	Soy Bean Meal	Opaque-2 Corn
Lysine	3.94	6.15	8.0	6.6	4.2
Histidine	1.67	2.33	3.0	2.5	3.5
Arginine	2.98	5.17	4.0	7.0	6.8
Aspartic Acid	6.49	8.41	7.0	8.3	10.0
Threonine	3.94	4.86	4.7	3.9	3.3
Serine	3.51	4.71	6.7	5.6	4.3
Glutamic Acid	8.98	9.47	25.0	18.5	18.7
Proline	4.34	4.25	11.0	5.0	8.6
Glycine	3.88	4.39	2.5	3.8	4.8
Alanine	4.76	5.99	3.0	4.5	6.5
Cystine	1.38	1.42	1.0	1.2	1.7
Methionine	1.20	1.19	3.5	1.1	1.4
Valine	4.48	4.85	7.7	5.2	4.9
Isoleucine	3.52	4.06	6.5	5.8	3.2
Leucine	5.35	6.18	9.7	7.6	8.4
Tyrosine	2.44	3.29	6.5	3.2	3.9
Phenylalanine	2.76	3.96	5.9	4.8	4.4
Tryptophan	1.80	2.31	1.2	1.2	1.3

The protein content of these fungal mycelia is between 42 and 45 percent, based on the amino acid analyses.

Soy Whey: SO_2 Soy Whey

Soy wheys from commercial processes in which the soy protein was precipitated by sulfur dioxide were found to present separate problems in that the sulfur dioxide markedly inhibited fungal growth.

Fungal Strain Selection

Since high concentrations of sulfur dioxide were markedly inhibitory, initial studies were conducted with a soy whey which contained 147 mg/l of sulfur dioxide. Growth of several fungus strains on this SO_2 soy whey medium are shown in Table 16.

Table 16

24-Hour Reduction of COD and SO_2 in a Soy Whey Containing 147 mg SO_2/1

Fungi	Final Values	
	COD mg/1	SO_2 mg/1
None	12,800	147.0
Natural biota	12,200	140.0
T. viride strain I-185	12,330	13.0
T. viride strain I-190	4,357	20.0
T. viride strain I-192	5,740	6.4
T. viride strain I-23	3,170	6.4
G. deliquescens strain I-31	2,110	6.4
A. oryzae strain I-14	1,750	<6.4

The more successful strains (Table 16) were seen to have reduced markedly both the COD and sulfur dioxide levels. The most promising strains were then tested in soy whey containing a COD of 12,030 mg/1 and 415 mg/1 of sulfur dioxide. This was attained by mixing 1 part of a soy whey containing 1203 mg SO_2/1 with 3 parts of a whey containing 147 mg SO_2/1. Results are plotted in Figure 21. A. oryzae I-14 was the most effective fungus of the group to reduce COD in the presence of 415 mg SO_2/1 of soy whey.

Studies with A. oryzae at a variety of SO_2 concentrations are shown in Figure 22. There was little difference in the rates of digestion at concentrations of sulfur dioxide up to 513 mg/1.

Substrain Selection for Rapid SO_2 Removal

Greater tolerance of sulfur dioxide than 513 mg SO_2/1 was required to handle several of the waste streams from commercial plants. Attempts were therefore made to select more effective fungal substrains by serial passage of A. oryzae through a soy whey containing 710 mg SO_2/1. This was a concentration which originally allowed only very slow COD utilization (Figure 22). Results of these serial transfers are shown in Figure 23. It is seen that repeated passage yielded a much more rapid growing culture at this sulfur dioxide concentration.

Extension of these techniques developed fungal cultures which grew at SO_2 concentrations up to 900 mg SO_2/1. Similar studies with G. deliquescens grown in SO_2 also showed similar results to those described for A. oryzae.

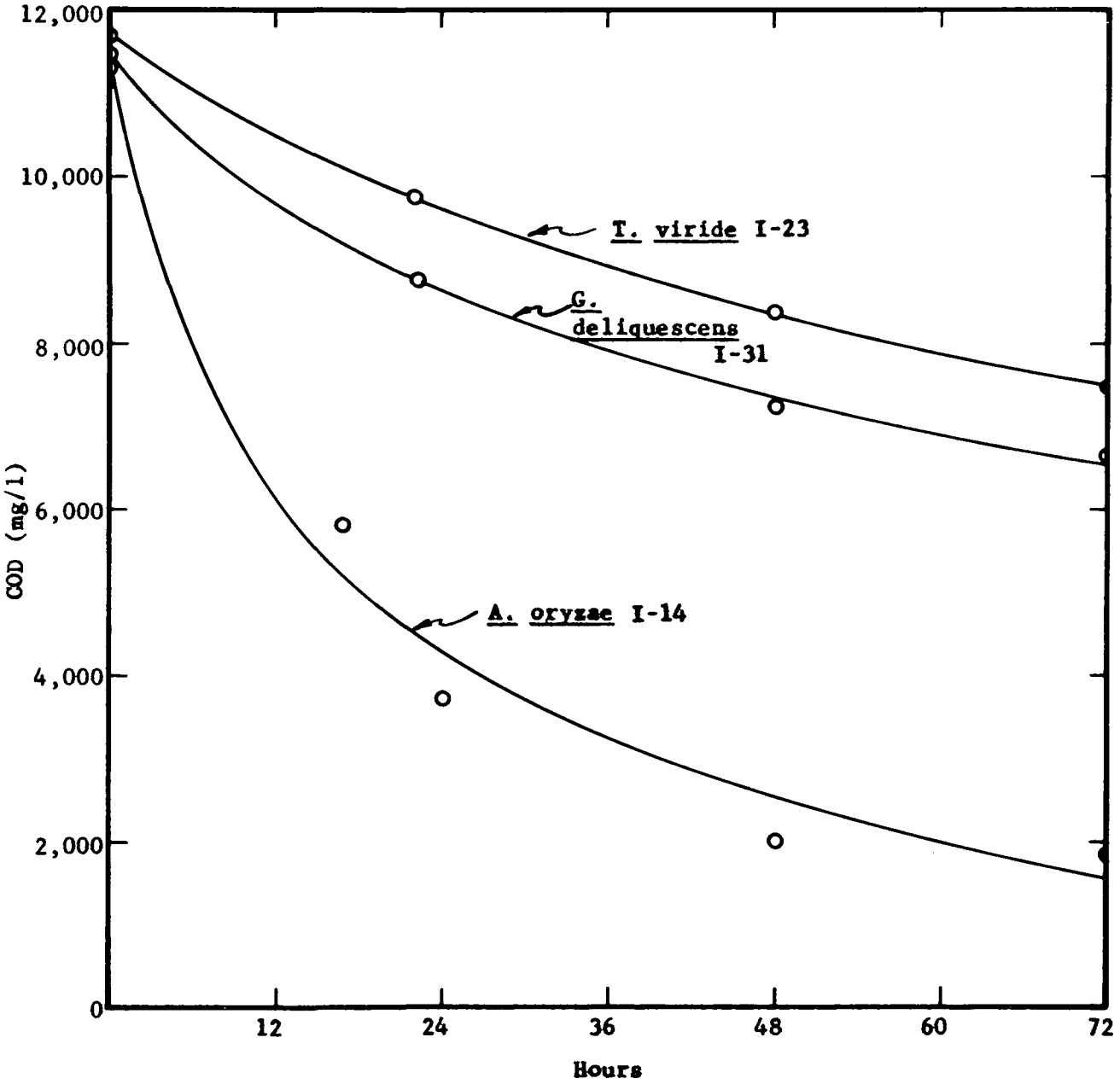

Fig. 21. Activity of fungi on COD reduction of SO_2 soy whey containing 415 mg SO_2/l.

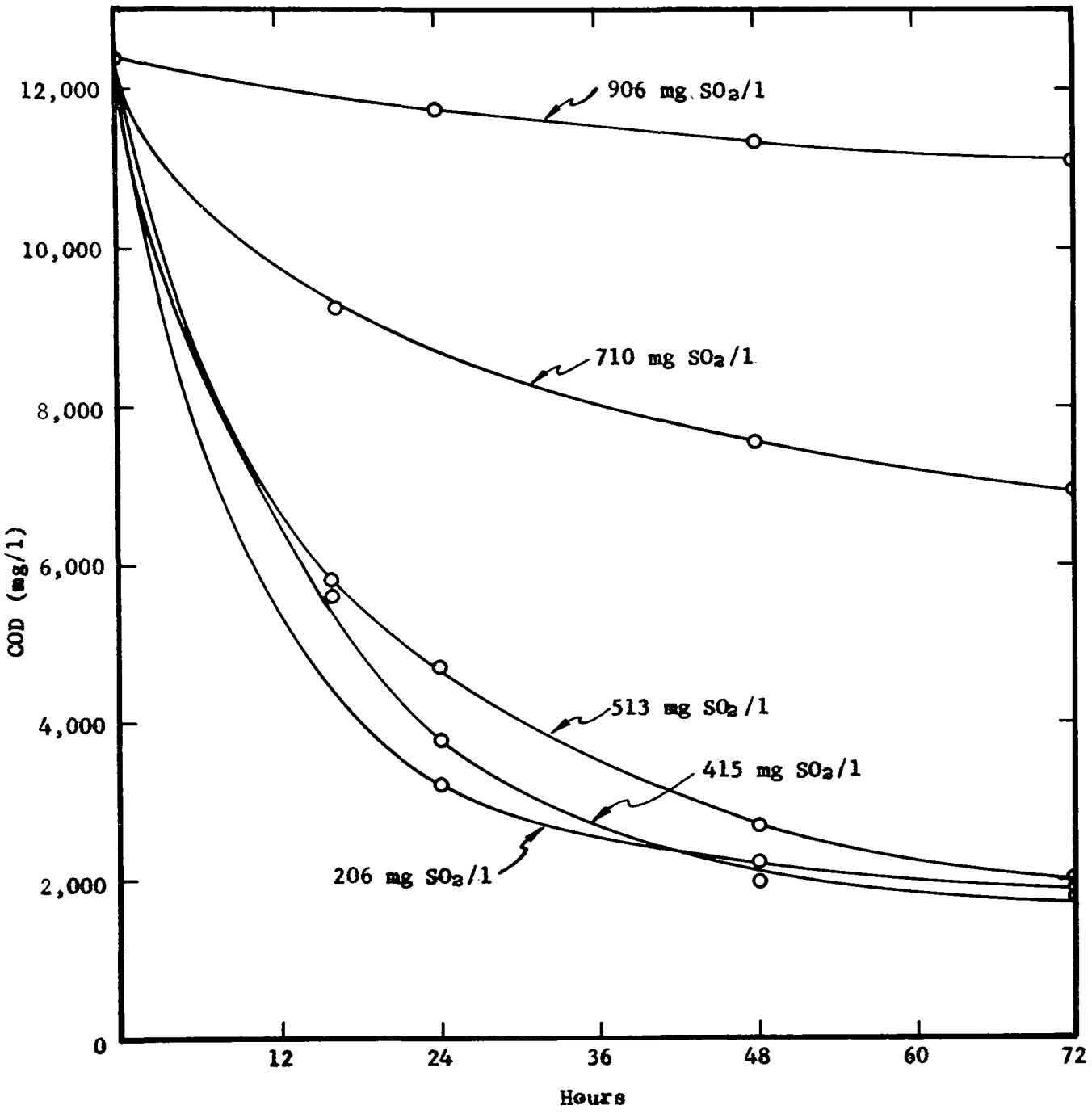

Fig. 22. COD reduction of SO_2 soy whey by A. oryzae.

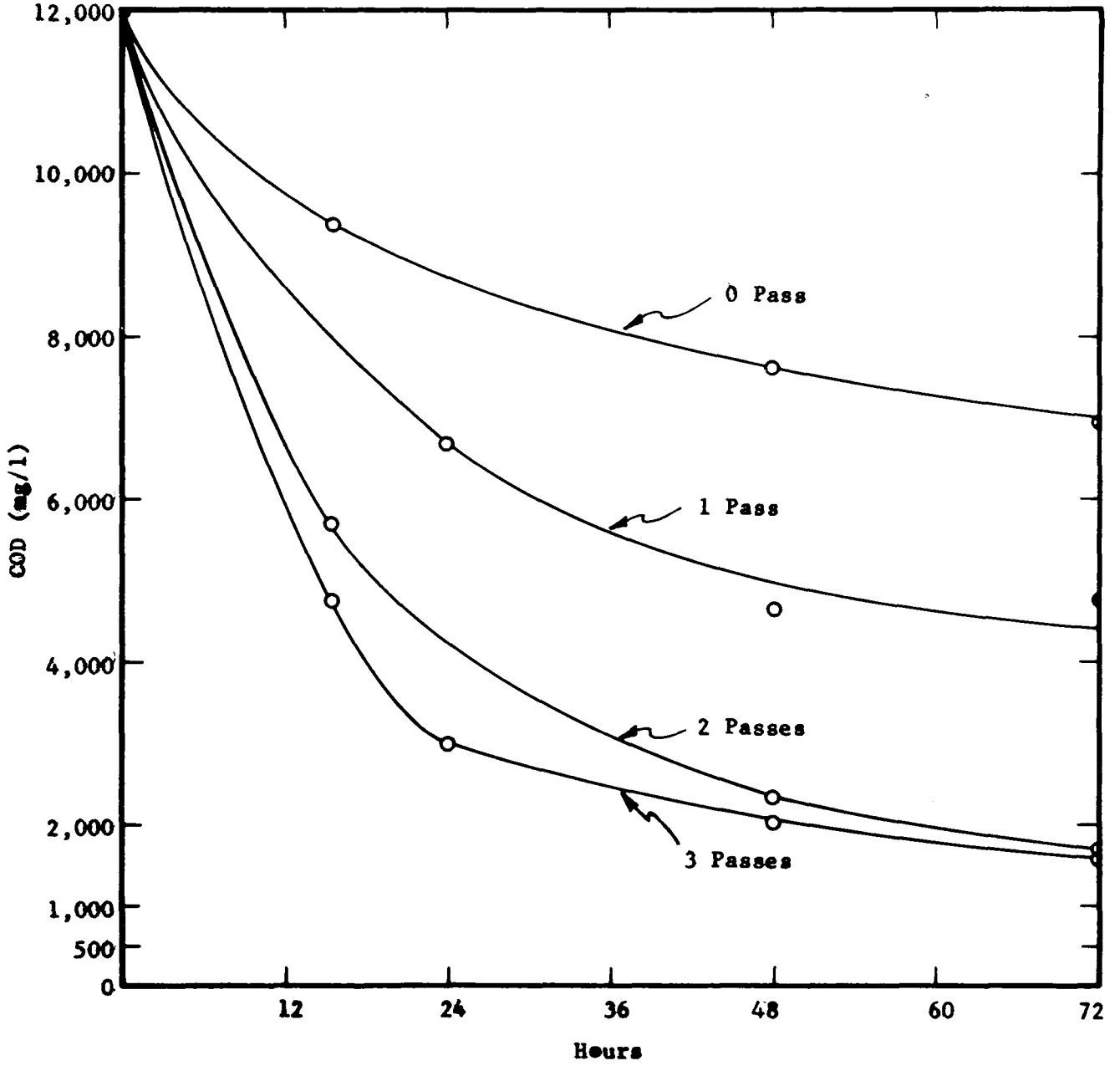

Fig. 23. Effect of rapid passage of A. oryzae on COD reduction of SO_2 containing soy whey at 710 mg SO_2/l.

The efficacy of a fungus adapted to grow at 500 mg SO_2/l was tested at a series of sulfur dioxide concentrations. Results are shown in Table 17.

Table 17

Growth of an A. oryzae Adapted to Sulfur Dioxide at Varying Sulfur Dioxide Concentrations

SO_2 mg/l	COD Reduction 24 hours mg/l	Fungal Mass mg/l	Ratio mg Fungal Mass / mg COD used
0	3160	1780	0.56
95	4690	2300	0.49
157	4710	2880	0.52
269	5870	3020	0.52
529	4690	3340	0.73
788	2900	2680	0.91

It was observed that the rate of COD utilization and the rate of accumulation of fungal mass was actually greater in the presence of SO_2 than in its absence. SO_2 inhibition was observed again at the highest levels of sulfur dioxide. It was also of interest, but must be considered as preliminary evidence, that the efficiency of conversion of COD to fungal mass was highest at the highest sulfur dioxide concentration (Table 17).

Experiments were conducted on the utilization of sulfur dioxide by rapidly transferred (adapted and nonadapted) A. oryzae at increasing levels of sulfur dioxide. "Adapted" strains were passed several times through soy medium containing sulfur dioxide. SO_2 measurements were made after 4 hours of incubation (Figure 24). The "adapted" (SO_2 pregrown) organisms showed nearly complete utilization of SO_2 at the lower levels of sulfur dioxide in the four hours incubation. The "nonadapted" (not SO_2 pregrown) showed a rate of SO_2 utilization equal to that observed for the "adapted" strain. Both curves (Figure 24) appeared to be reaching a common SO_2 level at approximately 900 mg SO_2/l. Both fungal strains appeared to have undergone SO_2 induction.

Two questions that arose as a result of these studies with the non-SO_2-pregrown fungal strain were: (1) was the fungal mycelium undergoing enzyme induction at the lower SO_2 levels which enabled it to remove (utilize) the SO_2? (2) was this the same induction process that had occurred in the fungal mycelium during pregrowth on SO_2? We rejected the hypothesis that fungal adsorption of SO_2 could explain the utilization (uptake) of SO_2. If this were true, the SO_2 utilized by both the "adapted" and "nonadapted" fungi (Figure 24) would show similar SO_2 uptake rates from zero SO_2 concentration.

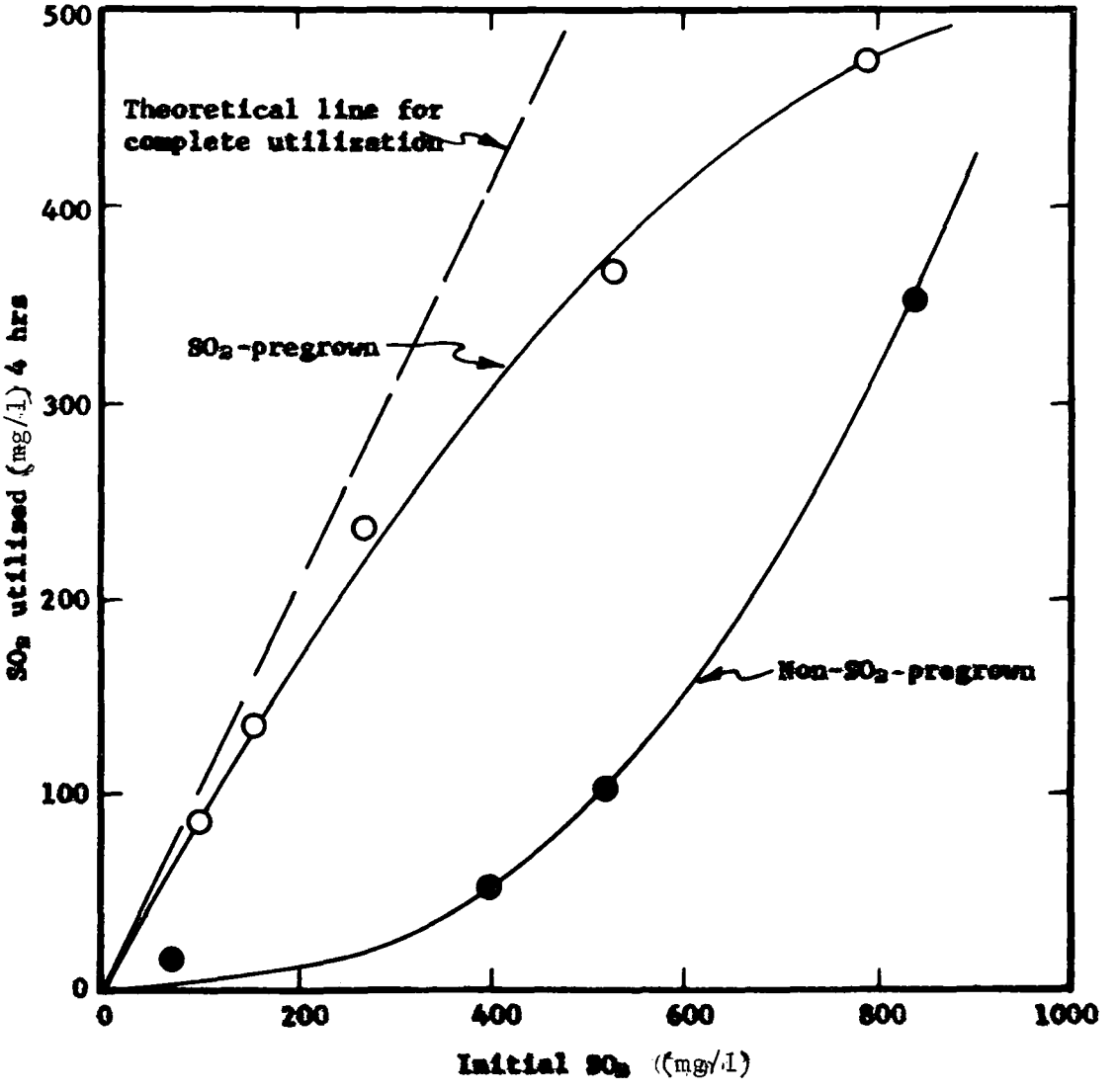

Fig. 24. Utilization of SO_2 by A. oryzae pregrown in the presence and absence of SO_2.

Continuous Fermentation

The inoculum was strain I-14 of A. oryzae obtained from Dr. William Gray. This continuous culture was also initiated by the inoculation-dilution method described for the HCl soy experiments. The A. oryzae was grown in sterile SO_2 soy whey prior to inoculating the continuous culture digestor. Transfers to fresh medium during the pre-continuous culture stage were made at gradually shorter time intervals in shake culture flasks (batch). Higher concentrations of SO_2 were employed until a rapid growing and SO_2 utilizing variant was selected for the continuous culture experiment.

Although this strain of A. oryzae was capable of rapidly removing SO_2 and had been selected for these SO_2 wheys, the COD reduction by this fungal strain in the continuous fermentation was too slow and less complete (residual COD of 3000 mg/l) than that observed in batch culture. Therefore, we proceeded to establish another continuous fermentation with G. deliquescens. This organism had also shown many desirable features in earlier batch screening investigations. In addition, if this fungal strain was satisfactory in continuous digestion, animal feeding experiments could be initiated with a fungal mycelium which had an excellent amino acid composition (Table 15) and of which we had collected a substantial amount of dried mycelium.

The G. deliquescens was removed from stock neopeptone—dextrose agar slants and used to inoculate sterile SO_2 soy whey at a level of 200 mg SO_2/l and 10,500 mg COD/l. The mycelium was transferred rapidly several times through this medium, and a 50-percent inoculum was prepared by adding the fungus from 500 ml of this medium to 1000 ml of fresh SO_2 soy whey. The one liter of inoculated soy whey was diluted by 15 liters of tap water in the continuous fermentor as previously described for our now standard inoculation-dilution procedure. The feed rate was set to deliver raw SO_2 soy whey at a rate of 5 ml/min. The results of this experiment are shown in Figure 25.

The theoretical COD was plotted to indicate the rate of COD build-up in the absence of the fungus. The data show that the critical level of actively metabolizing mycelium at a flow rate of 5 ml/min. was approximately 3.2 to 3.5 g/l. The microscopic data also confirmed the data shown here. Microscopically, at any fungal mass level above 3.5 g/l, the mycelium developed long, thin, granular strands and lysed. At the point (arrow) described as "equilibrium", optimal metabolism of the soy waste was achieved (Figure 25). Control of the fungal mass by physically removing mycelium at the 3.5 g/l level possibly would have maintained a constant COD residual level. Since this was not done, the fungus sporulated, lysed (signs of starvation), and yeasts and bacteria took over.

Fungal Mass Control. The entire procedure for preparing an inoculum, transferring rapidly through increasing SO_2 concentrations in soy whey sterile medium, and finally, inoculating the fermentor with the prepared G. deliquescens I-31 strain via the inoculation-dilution route was repeated as in previous experiments.

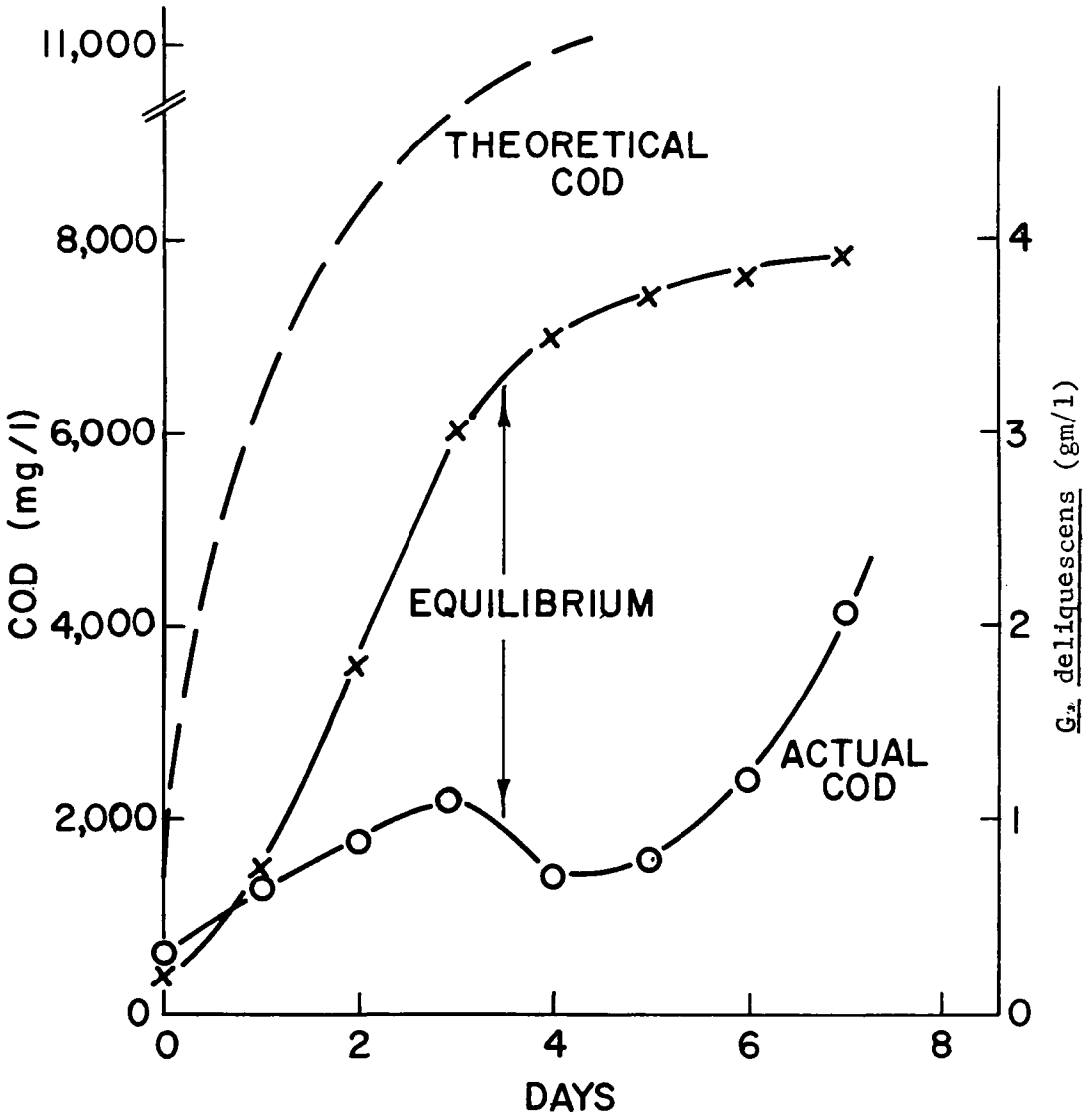

Fig. 25. Continuous digestion of SO_2 soy whey by G. deliquescens. The theoretical COD is represented by a broken line, the fungal dry weight by X——X and the COD by O——O.

In this experiment, the feed rate was held constant at 5 ml/min. in an 18-liter fermentation. Nutrient exhaustion and physiological aging of the fungi was prevented by removal of part of the fungal mass whenever it exceeded preset limits. This was in addition to the removal that constantly occurred in the effluent stream from the fermentor. Control of mycelial age could be achieved if fungal mass levels were held between 3.2 and 3.5 grams per liter. The data are shown in Figure 26.

The data for this experiment strongly indicated that COD's below 1000 mg/l could be achieved with a flow rate of 5 ml/min. in an 18-liter fermentation with control of fungal mass to levels of 3.2 to 3.5 g/l. When the fungal mass climbed above 4 g/l at this flow rate, partial fungal lysis occurred. Partial lysis occurred several times during the past thirty days and each time this occurred the yeast population rose to a high level (approximately 10^9 cells/ml). Restoration and maintenance of the fungal mass at the critical level (3.2-3.5 g/l) at this flow rate resulted in reduced COD and massive reduction of the yeast population. The minimum COD reached in this experiment was 760 mg/l which corresponded to a BOD of 235 mg/l. This was the most successful continuous fermentation achieved in a single-step digestion on soy wheys.

Although the data plotted in Figure 26 show the results obtained over a sixteen-day period, the continuous digestor was in operation for thirty days. The near steady state was achieved from the thirteenth day through the thirtieth day. The first sixteen days are expanded in Figure 26 to illustrate several features of this fermentation. Initially, the SO_2 concentration was 200 mg/l and was increased to 570 and then to 760 mg/l at the times indicated in Figure 26. There appeared to be a short growth lag at the 570 and 760 mg SO_2/l levels. It can also be seen that when the fungal mycelium exceeded 4 gm/l, the COD began to rise. The morphological appearance of the mycelium during the tenth and eleventh days was one of early sporulation and beginning lysis. Also, yeasts and bacteria began to appear in larger numbers. When the fungal mass was reduced at the thirteenth day to approximately 3 g/l, by removing mycelium, the COD rapidly decreased. The reduction in fungal mass resulted in rapid appearance of new mycelium in the form of elongated tips from the hyphal cross walls. Spores were germinating, and elongated germ tubes were emerging. After the thirteenth day, extreme care was exercised to maintain the fungal mycelium between 3.2 and 3.7 g/l.

Secondary Stage Digestor. One further attempt to remove the residual COD remaining after digestion of the SO_2 soy whey was also included in this experiment.

Two approaches were open for experimentation: (1) to isolate and characterize the residual materials and then incorporate microorganisms known to utilize these materials into a secondary stage fermentor or (2) to select microorganisms through soil enrichment on soy COD residual materials. The latter approach was tried first with rather interesting results. Three fungal isolates grew from soil enriched with the soy residual COD. The isolates were not identified although they were each isolated in pure culture on Czapek-Dox agar. Soy supernatants collected

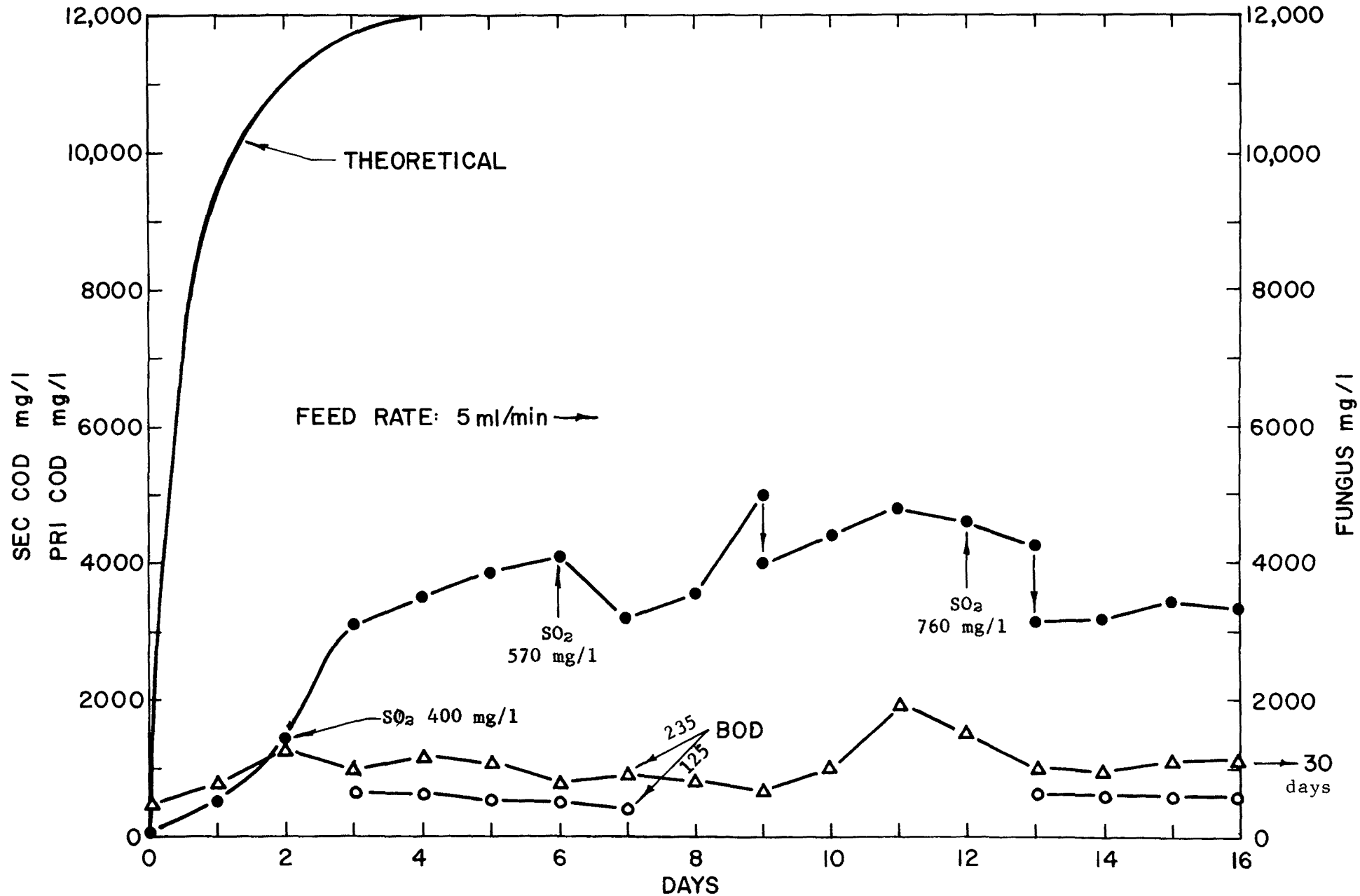

Fig. 26. Continuous digestion of SO_2 soy whey by G. deliquescens. Fungal mass control of fermentation. Solid line represents the theoretical COD if no removal was achieved. The fungal mass dry weight is represented by ●——●, the secondary stage COD reduction by O——O, and the primary (G. deliquescens)stage COD Δ——Δ. BOD's were determined as shown by arrows. The SO_2 concentrations in mg/l are also indicated by the appropriate arrows.

after removal of G. deliquescens and which contained 3000 to 6000 mg COD/l were reduced to approximately 500 mg COD/l after 60 hours. These fungi isolated from soil were tested in a secondary stage fermentor.

A secondary four-liter digestion flask was set up with an aerator, a feed line consisting of effluent from the primary SO_2 soy whey digestor, and an exhaust effluent line. This apparatus is shown in Figure 1, page 12. The fermentor was fed with effluent from the first fermentation after removing the fungal mycelium by filtration. The secondary system was inoculated with fungal isolates from soil obtained where soy beans had grown for several years. No adjustments of pH, temperature, flow rate, etc., were used for this system. The effluent COD from the primary digestor was further reduced in this system to 550 mg COD/l and a BOD_5 of 125 mg/l. These data are shown in Figure 26 during the third to seventh days and thirteenth to sixteenth days of operation.

Feed Rate Control. In this experiment, nutrient exhaustion and physiological aging were prevented by increasing the feed rate whenever the mycelium appeared slightly vacuolated or granular. Thus, the experiment started at a feed rate of 5 ml/min., and the feed rate was increased at 2 ml/min. increments when the microscopic evidence dictated a change. The data showed a COD variation of 1100 to 1800 mg/l during the time the fungus increased in mass from 1.4 to 8.0 g/l. During the course of this experiment the feed rate was increased from 5 to 13 ml/min. in a 17-liter fermentation. Although the COD was maintained at a fairly constant level between 1000 and 1800 mg/l, several features of the experiment should be noted and are plotted in Figure 27.

The step-wise increase in SO_2 concentration during the experiment produced a noticeable lag in fungal growth at each increased SO_2 increment. The fungus recovered after each SO_2 increase and grew at a rate equal to that observed prior to the SO_2 addition. These points are shown in Figure 27 as SO_2 concentrations at the second day, the fifth day, and at the eleventh day.

A second problem was the excessive foaming which prevented an accurate evaluation of the fungal mass and COD after sixteen days. The foam was found to be of higher COD than the bulk liquor of the fermentor. This meant that enrichment of the foam tended to reduce effective exposure time of influent COD. The erratic foaming also caused erratic changes in the digestor volume and a concentration of the fungal mycelium in the foam. The foaming condition was corrected with constant feeding of Antifoam B at a rate of 0.001 ml/min. Although this experiment was conducted for only sixteen days free of foaming, rather stable COD reduction was maintained during this time. At the eighth day when the feed rate was raised to 13-15 ml/min. the fungal mycelium rapidly washed out as shown by the decreased fungal mass after this time. The reduced fungal mass would have resulted in an increased COD if the feed rate had not been lowered to the 11-13 ml/min. level. Thus, control was maintained via feed rate shifts, and the COD remained fairly constant as shown. Increased COD at the eleventh day was probably due to the increased SO_2 concentration (760 mg/l) which temporarily resulted in minimal lysis of

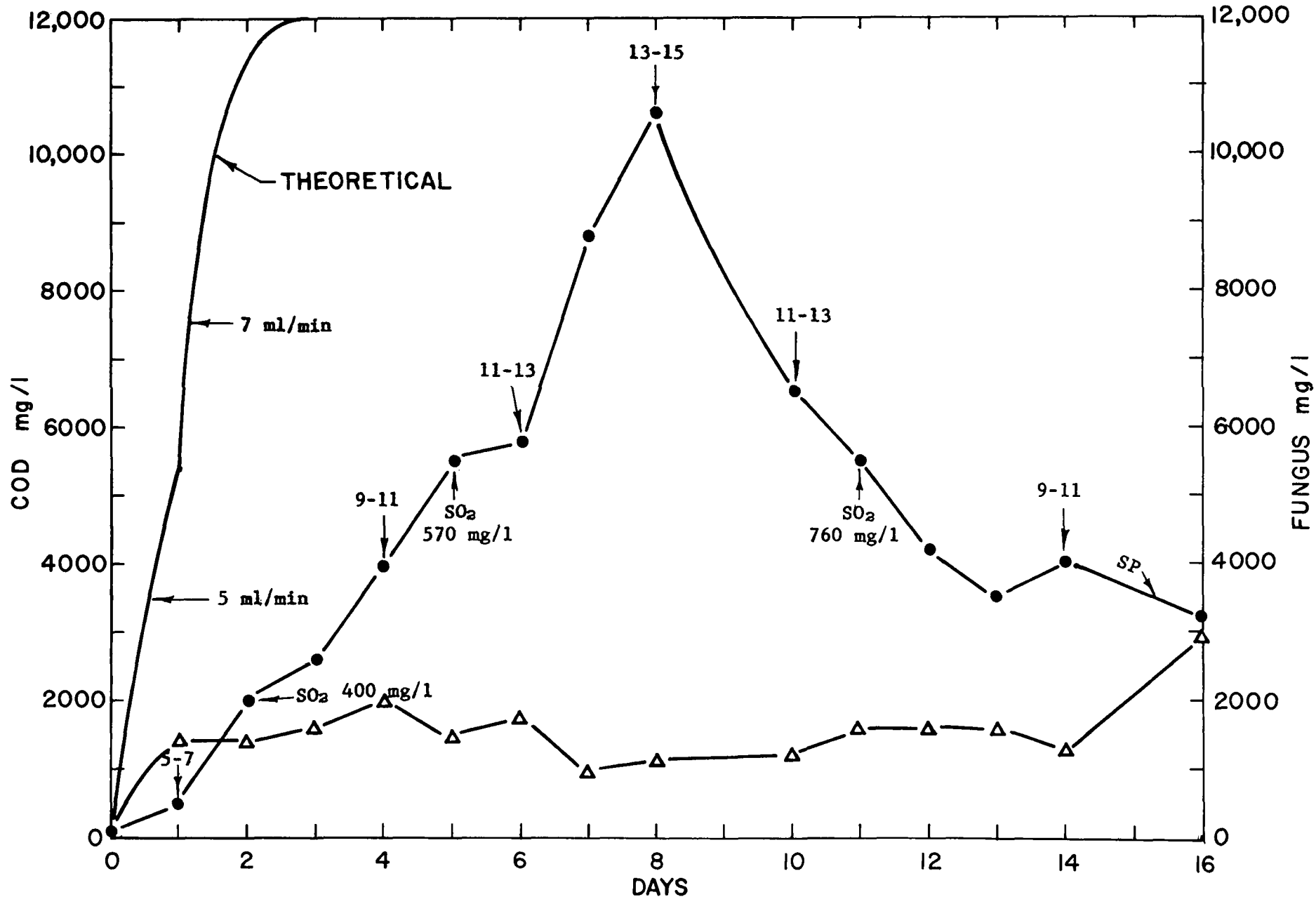

Fig. 27. Continuous digestion of SO_2 soy whey by G. deliquescens. Feed rate changes indicated by arrows pointing 5-7, 9-11, 11-13, 13-15 ml/min were used to control the fermentation. The solid line indicates the theoretical COD which shows a break where the feed rate was increased to 7 ml/min. The fungal dry weight is indicated by the ●——● line and the actual COD by the Δ——Δ line. SP, on the fungal curve, indicates the area where fungal sporulation occurred.

the mycelium. New growth appeared on the thirteenth day, and the COD decreased; however, the feed rate, unfortunately, was lowered to 9-11 ml/min. at a time when it should have been either maintained at 11 to 13 ml/min. or raised slightly. The result was a starvation condition for the fungus. Microscopically, the mycelium showed some spore formation, and the COD immediately rose to 3000 mg/l. The experiment was discontinued at this point. It appeared from this study that the optimal feed rate required to stabilize the COD between 1500-2000 mg/l was somewhere between 9 and 13 ml/min. This was equivalent to a turnover time, or retention time, of 24 to 36 hours.

Chemical Composition

The reduction in certain components of a mixed SO_2 soy whey following continuous digestion by G. deliquescens are shown in Table 18. The chemical analyses were carried out on aliquots of samples removed from the continuous fermentation on day 7 in Figure 26.

Table 18

Reduction of the Chemical Components* of SO_2 Soy Whey by G. deliquescens

1	2	3	4	5		6	
Test	Raw SO_2 Soy Whey mg/l	Raw HCl Soy Whey mg/l	Mix 75 SO_2 25 HCl Soy Whey mg/l	After Fungal Digestion mg/l		% Reductions	
				Pri. Stage	Sec. Stage	Pri. Stage	Sec. Stage
COD	11,200	14,480	12,230	808	648	93.4	94.7
BOD_5	8,130	9,750	8,537	235	125	97.3	98.6
Carbohydrate	4,700	7,000	5,450	215		97.0	
Protein	3,950	4,000	3,950	420		89.4	
Phosphate Total	122	203	144	43		70.0	
Nitrogen (Kjeldahl)	1,485	1,524	1,514	148		90.2	
SO_2	700	—	525	20		96.0	
Sulfates	196	220	208	102		50.0	
Chlorides	230	2,700	848	550		35.0	
Fungus	—	—	—	3300		—	
Solids	10,600	14,800	11,650	2800		76.0	
Ash	2,120	3,230	2,397	1620		32.0	

*Analyses performed on samples of the effluent were done after filtering through a single layer of Whatman No. 4 filter paper.

The values in Columns 2 and 3 (Table 18) are actual analyses of the two (SO_2 and HCl) raw soy wheys studied in this work. When the continuous digestor effluent was analyzed (day 7), the two raw soy wheys were mixing in the feed tank in the proportion 75:25 as shown in Column 4. Thus, the chemical levels in Column 4 were mathematical expressions of what was fed to the digestor during Day 7. The results of the G. deliquescens digestion of these chemical components are shown in Column 5 (primary digestor with G. deliquescens) and in Column 5 (secondary digestor) for the soil-enriched digestion of the primary effluent residue.

Most notable were the BOD reductions from an initial level of 8537 mg/l to 235 mg/l in the primary digestor and to 125 mg/l in the secondary-stage digestor.

Amino Acid Composition

The amino acid composition of the G. deliquescens mycelium grown on SO_2 whey was not significantly different from the same fungus grown on HCl soy whey (Table 15). However, the amino acid analysis revealed two peaks not observed in the HCl soy whey grown G. deliquescens. These were identified qualitatively as methionine sulfoxide and methionine sulfone.

Rat Feeding

G. deliquescens collected and lyophilized from the continuous soy whey digestion trials was stored in a freezer. The lyophilized fungal mycelium grown on HCl soy whey was stored separately from mycelium collected from the fermentations where SO_2 soy whey was used as the growth medium.

There was not enough dried mycelium from either growth medium type (HCl or SO_2 soy wheys) to carry out separate rat feeding trials. Therefore, the mycelium grown on both soy wheys were pooled and mixed with a special Nutritional Biochemical Corporation (NBC) diet which contained starch, fats, vitamins, and salts equivalent (except for protein) to the NBC standard weanling rat casein diet. The pooled fungal mycelium, which contained 46 percent protein, was mixed 50:50 with this special NBC formulation to provide a complete diet which contained 23 percent fungal protein. The standard diet was prepared in the same manner except that the 23 percent protein source was casein. These were the same diets as those used for the corn waste T. viride feeding experiments - except that G. deliquescens mycelium replaced the T. viride mycelium.

The whole fungal mycelium was used as the protein source and, based on the comparative amino acid analyses of the fungal protein and of casein, certain L-amino acids were added to supplement both proteins as required for weanling rats. Sulfur amino acids were low in both proteins and L-methionine was added to both standard (casein) and the test (fungal) diets. Other L-amino acid (L-methionine, L-serine, L-valine, L-leucine, L-tyrosine and L-glutamic) which totaled <5 percent of the total amino acids of the test diet, were added to the fungal protein diet to equate the amino acid composition to that provided in the casein diet. Eight rats were placed in separate metabolic cages and fed the standard (casein)

diet plus 1 percent chromic oxide. When all rats showed green fecal material (24 hours), four were placed on the test (fungal) diet. Fecal and urine samples were collected daily. Weight gain or loss, signs of toxicity, food consumed, etc., were recorded daily for each rat.

Table 19 shows the results of this feeding experiment, where the test (fungal) diet contained the combined protein of G. deliquescens grown on HCl and SO_2 soy wheys. The results show that the rats refused to eat the test diet and lost weight each day. After the third day, three rats (two shown here) were given glutamate with the fungal diet in the belief that this might improve palatability of the test diet. The rats on the test diet with glutamate did eat more of the fungal diet but continued to lose weight. All test rats died when their bodyweight reached approximately fifty grams, as shown (Final Rat Weight) in Table 19. Only two of the four standard diet fed rats and three of the test-diet-fed rats are shown here. This was done for the sake of brevity - the other data do not deviate significantly from these.

Another experiment was set up to test the palatability of this fungal mycelium. Rats were fed the fungal mycelium as their sole food source. Thus, two rats received HCl soy whey grown G. deliquescens, two received SO_2 soy whey grown G. deliquescens, two received aqueous extracted SO_2 soy grown mycelium and two received 95 percent ethanol-extracted SO_2 soy whey grown mycelium. The results of this study are shown in Table 20. The results are average values for the two rats fed each experimental fungal diet.

Table 20

Rat Feeding Trial
Fungal Mycelium as Sole Source of Diet

Day	Fungus Grown on HCl Soy Whey		Fungus Grown on SO_2 Soy Whey		Fungus Grown on SO_2 Soy Whey H_2O Extracted Mycelium		Fungus Grown on SO_2 Soy Whey EtOH Extracted Mycelium	
	Feed Consumed gm	Weight Gain gm	Feed Consumed gm	Weight Gain gm	Feed Consumed gm	Weight Gain gm	Feed Consumed gm	Weight Gain gm
1	2	-1	0	-6	0.2	-4	4	3
2	3	4	0	-9	0.25	-6	5	5
Total	5	3	0	-15	0.45	-10	9	8

These feeding trials (Tables 19 and 20) showed the mycelium harvested from SO_2-containing soy whey to be impalatable to weanling rats. The impalatability was removed by ethanol extraction but not by water extraction. The ethanol extract itself proved neither toxic nor impalatable. This was observed when rats were intubated with a fourteen-fold concentrated, ethanol

Table 19

Rat Feeding Trial Casein vs. G. deliquescens Test Diet

Day	Standard Casein Diet				Test Fungal Diet					
	Feed Consumed	Weight Gain	Feed Consumed	Weight Gain	Feed Consumed	Weight Gain	Feed Consumed	Weight Gain	Feed Consumed	Weight Gain
	gm	gm	gm	gm	gm	gm	gm	gm	gm	gm
1	9.9	6	10.0	7	1.4	-7	0.6	-7	0.6	-5
2	11.9	7	11.9	2	2.3	-8	1.3	-7	0	-9
*3	12.5	9	13.8	16	*3.1	-1	*1.9	-2	0.9	-3
4	12.0	8	12.7	8	3.7	-3	3.2	-1	1.0	-7
5	11.5	5	13.6	9	3.3	-5	4.1	-3	0.3	-4
6	12.1	6	13.8	10	2.1	-5	3.0	-4		
7	13.1	9	14.7	5	0.2	-1	2.8	-2	___	___
Total	83.0	50	90.5	57	16.1	-30	16.9	-26	2.8	-28
Initial Rat Wt.	80		80		80		82		77	
Final Rat Wt.	130		137		50		56		49	

*Day 3 - two rats on test diet received glutamate as indicated by stars.

extract. Since evaporation *in vacuo* was used in preparing the extracts for feeding, loss of aversive character in the ethanol extraction may be explained by volatility of a critical component.

A third feeding experiment was started in which HCl soy whey grown *G. deliquescens* was mixed with the NBC special preformulated diet described in the Methods section and fed to two rats along with two other rats presented the standard casein diet. This feeding experiment was run in the same manner as first described for the corn and soy grown fungi. The purpose of this experiment was to ascertain what, if any, effect the soy whey (minus SO_2) medium had on the fungal mycelium when used as a feed. Because of the limited quantity of HCl-soy-grown mycelium, only two rats could be used in the test diet. These rats were fed for only seven days before the feed was exhausted. A plot of the rat growth is presented in Figure 28. After an initial lag, the test animals began to gain weight on this diet. The growth rates, as determined by the slopes of the curves, were 7.0 for the standard rats and 5.0 for the test animals. None of the test rats died nor did they show any toxic symptoms during this one-week feeding trial. One month later the test rats were healthy and equal, in weight, to the rats fed only the standard casein diet.

Consumption of mycelium grown on HCl soy whey was initially low and may be indicative of a moderate palatability problem with this material, also. Limited supplies did not permit really meaningful experiments.

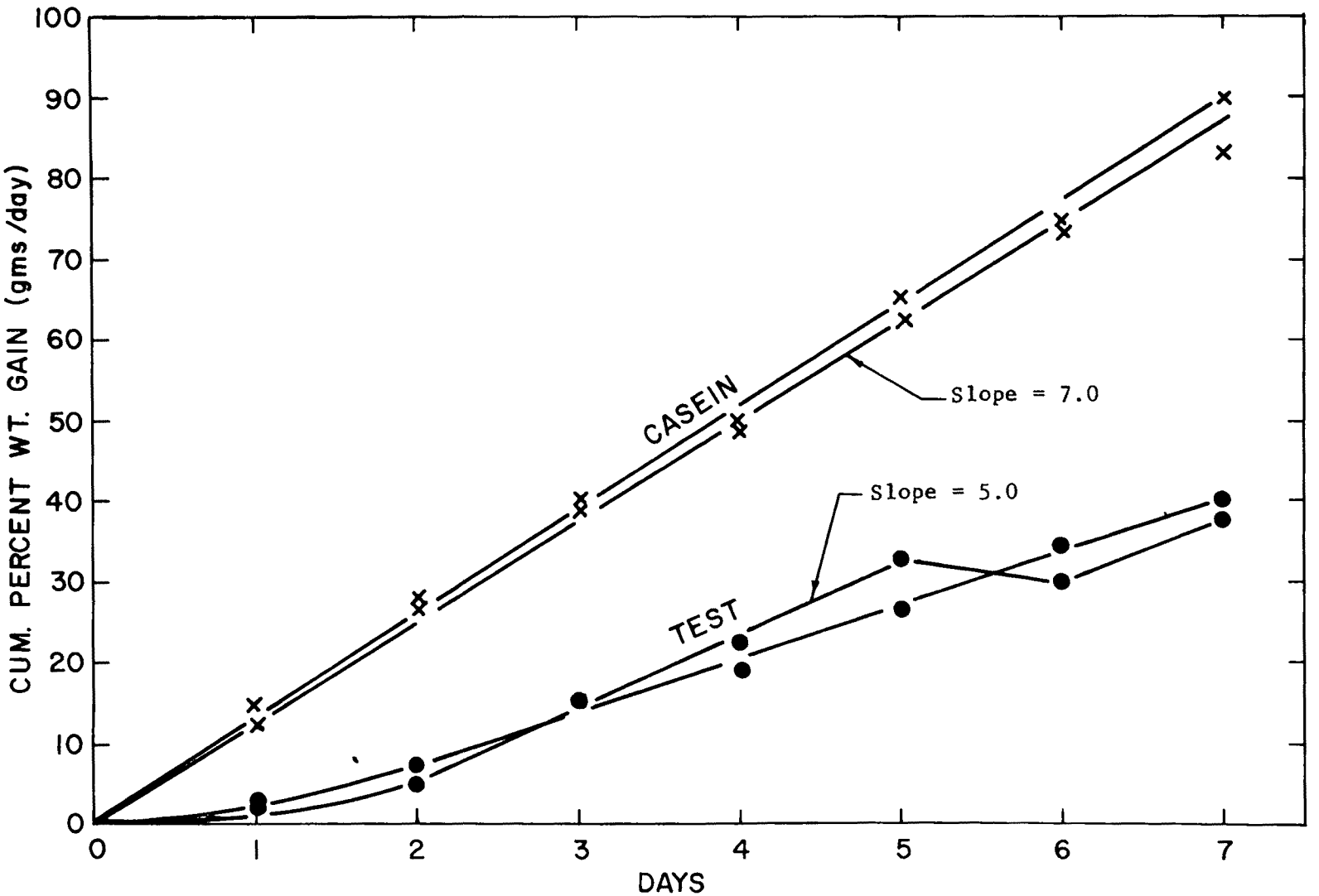

Fig. 28. Weanling rat growth rates fed a standard casein diet and a test G. deliquescens fungal diet. Slopes of weight gains are as indicated in figure.

SECTION VI

ECONOMIC ESTIMATES

Only crude estimates of the cost of waste treatment by fungi can be made from laboratory data alone. Larger-scale trials will be required as a basis for more accurate estimation. Estimates for costs of application of fungi to corn processing wastes are summarized in Table 21. The estimates were based on recovery of 0.5 pound of dried mycelium for each pound of COD utilized. This is a conservative estimate based on accumulated experience.

Table 21

Economy of Corn Waste Treatment

Item	Amount	Cents per lb fungal product
N $(NH_4)_2SO_4$	0.45 lb	0.67
$PO_4^{\equiv}$ NaH_2PO_4	0.022 lb	0.20
H_2SO_4	0.10 lb	0.14
Aeration	0.28 lb dissolved oxygen (1 hp hr = 2 lb DO) (Power cost = 1.5¢/kw hr)	0.16
	Investment at $300/hp	0.42
Labor	$100 per day	0.38
	Subtotal	1.97
Filtering and Drying		2.00
	Total	3.97
Selling Price		3.75

The amount of ammonium sulfate added is sufficient to yield a product with 60 percent protein if all the nitrogen is converted to protein. The amount of sodium dihydrogen phosphate was selected to give the phosphate-to-nitrogen ratio that has given the best control of fermentation. The amount of sulfuric acid is based on experience with the Green Giant corn waste stream. The amount actually required will depend, in some degree, on the amount and kind of materials in the water used in the plant.

Aeration cost estimates are based on the laboratory finding that 0.14 lb of dissolved oxygen was used per lb of COD destroyed. The assumption was made that 1 hp hr will provide 2 lb of dissolved oxygen. This is a reasonably conservative estimate. The $300 investment per horsepower

is meant to cover the cost of the aeration equipment, the lagoon, and costs of control equipment. This estimate also seems reasonably conservative. The investment has been amortized over 10 years at 8 percent interest. It was assumed that the COD load is 2500 mg/l and the equipment is in use 50 days per year.

Labor costs were calculated assuming eight hours of labor a day at $100 cost per day (including overhead) to operate a 2,500,000 gal/day installation.

Filtering and drying costs represent a gross estimate and are meant to cover labor, capital equipment, power, and other costs associated with this operation.

Sales returns assume the product would bring the same price as soy oil meal with which it compares in protein content and quality.

Estimates for soy waste processing have similarly been attempted (Table 22). Nitrogen and phosphate supplies are probably adequate in the incoming feed and so do not need to be added. Aeration requirements are similar to those of corn per pound of COD removed, but the costs are lower because the amortization is spread over constant operation instead of over fifty days operation per year. The constant operation does raise the need for heat in the winter in northern climates. No attempt has been made to estimate heating costs because it is not known whether waste heat would be available from processing operations.

A plant handling 3,500,000 gallons of waste per day with a COD load of 8,000 mg/l has been assumed.

It has been assumed that one pound of dry product is obtained per two pounds of COD removed.

Table 22

Economy of Soy Waste Treatment

Item	Amount	Cents per pound product
H_2SO_4	0.1 lb	0.14
Aeration	0.28 lb O_2/lb product (1 hp hr = 2 lb DO) (Power cost = 1.5¢/kw hr)	0.16
	Investment at $300/hp	0.07
Labor	$200 a day	0.17
Heat		
	Subtotal	0.54
Filtering and drying		2.00
	Total	2.54
Selling Price		3.75

SECTION VII

ACKNOWLEDGMENTS

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SECTION VIII

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23 Descriptors (Starred First)

*Fungi Imperfecti
Corn waste
Soy whey
BOD removal
Fungus feed
Economic costs

25 Identifiers (Starred First)

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Abstractor Brooks D. Church	Institution North Star Research and Development Institute

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