Cellulase Production by *Trichoderma viride* on Feedlot Waste

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Feedlot waste contains essentially all the necessary nutrients for batch fermentation with the fungus *Trichoderma viride*. The organism utilizes two-thirds of the carbohydrate in feedlot waste while elaborating cellulase in quantities comparable to commercial preparations. Essentially odor-free, the fermented waste contains all of the original nitrogen but has 24% less organic matter.

Rapid development of large confinement animal rearing and feeding facilities has increased the potential for pollution of our environment by concentrating the animal wastes in localized areas (6). For example, fresh feedlot waste (FLW) has relatively high biological and chemical oxidation demand value (BOD and COD) and will readily support microbial growth. When allowed to ferment uncontrolled in these facilities, FLW produces objectionable odors and attracts insects, or when released into water it quickly depletes dissolved oxygen and kills aquatic life therein (6). Controlled fermentation with selected organisms can reduced BOD, COD, and odor and possibly convert the residual solids into economically important by-products.

Organisms that may be particularly suited for controlled fermentation of animal wastes belong to the class, Fungi Imperfecti. These organisms are capable of utilizing cellulose and hemicellulose, the major components of FLW. Sterile conditions are not necessary for fungal maintenance, only a control of pH between 3 and 4. And, finally, fungal solids are easily recovered by a coarse filtration (3). Treatment of food processing wastes with *Trichoderma viride* or *Gliocladium deliquescentes* removed 96% of the BOD₃, 88% of the COD, 93% of the total organic carbon, and 80% of the organic phosphate. Furthermore, the mycelium contains 50% crude protein and appears to be an acceptable protein supplement for animal feed (3). These findings led us to examine whole FLW as a possible substrate for *T. viride*. Reported here are the results of these studies.

**MATERIALS AND METHODS**

FLW including urine (2 to 3 days old) was collected from the concrete floor of an established operation of 5,000 to 10,000 head of feedlot cattle fed a high-energy corn ration. The sample ( > 16% solids) was suspended in distilled water (2.5% solids, final concentration), blended in a Waring Blender for 2 min, frozen in dry ice-acetone, and lyophilized to dryness at less than 0.1 mm of Hg. The lyophilized FLW was milled to pass a no. 20 screen.

Slatls of *T. viride* QM 9123 obtained from M. Mandels (8) were prepared on malt extract agar. The fungus grew normally and sporulated in approximately 6 days.

For a typical fermentation, lyophilized FLW up to 5 g was suspended in 100 ml of distilled water containing basal salts, described by Mandels and Weber (7), and 0.25 ml of phosphoric acid (85%). The pH of the suspension was adjusted to the desired value with sodium hydroxide. After this, the 500-ml flasks containing the suspension were capped with milk filters and autoclaved at 20 lb/in² steam for 30 min. After cooling, the flasks were inoculated with 2 ml of a conidia suspension made by washing one slant of *T. viride* with 10 ml of sterile distilled water. The flasks were incubated at 29 C on a rotary shaker at 400 rpm.

At appropriate intervals, the contents of the flasks were centrifuged at 12,000 relative centrifugal force (RCF). The sediment was quantitatively transferred to a lyophilization flask and lyophilized. Portions of the supernatant were immediately assayed for enzyme activity; the remainder was lyophilized. Kjeldahl microanalysis of nitrogen (1), neutral sugar by gas-liquid chromatography (GLC) (11), lignin (12), ash, moisture, and total weight determinations were obtained for each lyophilized fraction. The cellulase activity of the supernatants was measured by a modification (5) of the filter paper assay (8) and by the cotton assay (8). The filter paper assay was
modified by reducing the digestion time to 30 min and
increasing the substrate to 100 mg. In addition,
hemicellulase activity was measured by the rate of
conversion of xylan (Nutritional Biochemicals Corp.)
to apparent glucose. All assay mixtures contained the
appropriate substrate, 1 ml of 0.05 M citrate buffer,
and 1 ml of enzyme. The mixtures were digested at
50°C in unshaken tubes. After an appropriate time
interval, the reducing power of the mixture was
determined by the copper-reducing power method of
Dygert et al. (4). Filter paper and hemicellulase
activities were reported as milligrams of apparent
glucose released per hour calculated from reducing
powers measured after a 30-min digestion of the
substrate at 50°C. For the cotton activity, a 24-h
digestion interval was used, and the data were re-
ported as milligrams of apparent glucose released
after a 24-h digestion. Amino acid analyses were
performed by the method of Cavins and Friedman (2)
by using Benson and Patterson’s 3-h hydrolysis pro-
cedure (1).

RESULTS AND DISCUSSION

Starting from a spore inoculation, at least four
consecutive stages occur during batch fer-
mentation of whole FLW with T. viride. (i)
Cellulase activity develops after a 3-day lag
phase. (ii) The manure odor of the culture is
replaced by an earthy odor by the fourth day.
(iii) By 6 or 7 days, the rapid growth phase is
complete, pH has increased considerably if
uncontrolled, cellulase activity approaches its
maximal value, and changes in the quantity
and quality of the waste’s organic constituents
approach completion. (iv) Between 7 and 18
days (duration of our experiments), pH, cellu-
lose activity, and the quantity and quality of
the organic constituents remain relatively sta-
ble. Extent of these changes and level of enzyme
activity at stabilization (approximately 7 days)
depend on the initial cultural conditions which
are essentially the same as those required for
other lignocellulosic substrates (7).

Substrate concentration. Cellulase (7) and
xylanase activity were used as measures of
fungal growth; enzyme values were plotted as a
function of FLW concentration (dry basis) in
the fermentation medium (Fig. 1). Activity of
both enzymes peaks near 2.5% concentration of
FLW. This particular sample of waste contains
materials equivalent to 35% neutral sugars
which corresponds to about 0.9% concentration
of celluloses in the medium. This is in agree-
ment with the findings of Mandels and Weber
(7) who report optimal cellulase activity in
cultures containing 1.0% crystalline cellulose.

pH. Proper pH control is also necessary for
optimum enzyme yields with the FLW sub-
strate. In Fig. 2, the cellulase activity values of
several equivalent T. viride waste fermentations
are plotted versus their initial pH and their
final stable pH. For the feedlot sample used
here, maximal cellulase activity is obtained at a
final pH of 5.0. This required titrating these

![Fig. 1. Relation between concentration of FLW and cellulase production in T. viride fermentation of FLW. Spore inoculum, shake flask experiment grown 5 days at 29°C and pH 5.0. Filter paper activity (○) and xylanase activity (×) are expressed as percentage of maximal value.](image1)

![Fig. 2. Relationship between culture pH and cellulase production in T. viride fermentation of a FLW sample. Cultures were spore inoculated and grown at 29°C for 5 days in shake flask culture. Initial pH (×) was adjusted with 85% H₃PO₄ and NaOH to the indicated value. The equilibrium pH (○) was reached after 3 days' growth of T. viride and remains essentially stable thereafter.](image2)
particular waste samples initially to pH 4.5 (see Materials and Methods). The amount of acid added and the initial pH needed to spontaneously obtain a final pH of 5.0 in the fermentation liquor vary with the individual samples. For example, some waste samples had to be titrated to pH 4.0 to obtain a final broth pH of 5.0. However, our highest yields of cellulase enzymes (filter paper activities near 10 units) were produced in fermentations titrated to pH 4.0. This pH increase is probably related to the soluble nitrogenous material in the waste and its availability to the organism. According to Mandels and Weber (7), *T. viride* grown on peptone media produces a pH increase, whereas a cellulose media produces a pH decrease.

**Additives.** Addition of Tween 80 (polyoxyethylene sorbitan monoleate) or sodium oleate to cellulose cultures of *T. viride* reportedly increases the production of cellulolytic enzymes (7, 9, 10). Tween 80 and oleic acid added to FLW cultures at a 0.1% level stimulated enzyme production by 150% (Table 1). Time of enzyme synthesis in the culture remains unaffected by the additives. In both the control culture and the cultures containing the additives, enzyme synthesis did not begin until 3 days after inoculation (Fig. 3). The lag period is followed by about 5 days of rapid synthesis. During this time, the rate of synthesis is much greater for the cultures containing Tween 80 and oleate. After 5 days, enzyme synthesis decreases in both cultures to approximately the same rate and remains so until the end of the fermentation.

Action of these additives may be related to an increase in nutrient availability from FLW which stimulates enzyme production. Either Tween 80 and oleate are used directly by the organism as an energy source, or, in the case of Tween 80, emulsifying and suspending properties increase the quantity of readily available nutrients in the waste medium. Evidence for this behavior is indicated by the observation that addition of 0.1% glucose or repeated autoclaving of the waste medium before inoculation stimulates enzyme production similarly (Table 1).

**Enzyme production.** Yields of cellulase and hemicellulase complexes by *T. viride* grown on FLW substrate are equivalent to those reported for this organism grown on cellulose powder by Mandels et al. (7, 8). Direct comparison of our filter paper activity values with those reported cannot be made because the assay was modified to more accurately measure differences in cellulase preparations with filter paper activities greater than 3. However, when our best preparations are measured by the same method used by

| Additive          | Filter paper act. (mg of glucose/h) | Cotton act. (mg of glucose/day) | Xylan act. (mg of glucose/h) |
|-------------------|-------------------------------------|---------------------------------|------------------------------|
| 0.1% Tween 80     | 9.8                                 | 5.0                             | 9.8                          |
| 0.1% Glucose      | 9.8                                 | 5.5                             | 10.4                         |
| 0.1% Oleic acid   | 9.3                                 | 5.0                             | 9.3                          |
| Twice autoclaved  | 9.0                                 | 4.7                             | 9.7                          |
| Control           | 6.5                                 | 2.5                             | 4.6                          |

*The assays are described in Materials and Methods.

*To each of several Trichoderma viride fermentations of FLW prepared and treated according to Materials and Methods was added one of the additives listed.

*Autoclaving was 30 min at 20 lb/in**² steam (120°C).
fraction of FLW, forages, and grain residues (12).

Acetone powders (7) of the enzyme prepared directly from the filtered supernatants contain all of the cellulolytic and hemicellulolytic activity and are stable at least 6 months at 5 C. Typical specific activities of our powders are in the range 0.60 to 1.00 filter paper units per milligram which compared favorably with the reported range of 0.28 to 0.50 (7).

**Effect on organic constituents.** Besides producing high yields of cellulolytic enzymes, *T. viride* affects the quality, quantity, and distribution of the organic matter in FLW. Table 3 illustrates the changes in total solids, nitrogen, carbohydrate, lignin, and ash content that occur during fungal fermentation of FLW. Fermentation causes a total loss of 0.470 g or about 24% of the organic matter, primarily cellulose and hemicellulose (0.46 g or 66% of these components), which is consumed during cell respiration. No significant net change in the amount of nitrogen, lignin, and ash is observed during fermentation. Approximately 23% of the waste cannot be accounted for by nitrogen, carbohydrate, lignin, and ash determinations in either the fermented or the unfermented waste. Fermentation does shift about 16% of the unknown material into the soluble fraction. The significance of this shift is not presently understood.

Ash and lignin in the soluble and insoluble fractions remain constant during the fermentation. However, there is a net transfer of nitrogen into the soluble fraction. This nitrogen along with that already in the soluble fraction appears to be converted to enzyme protein. Quantity of the crude protein (Kjeldahl nitrogen × 6.25) fraction corresponds well with that expected (8) for our level of enzyme activity.

The insoluble residue left after fermentation weighs 31% less than that from unfermented waste. This decrease is the net effect of carbohydrate utilization by *T. viride* and the transfer of some nitrogen and other unidentified organics to the solubles. As a result, the crude protein content has increased from 18.8% in the unfermented solids to 22.6% in the fermented solids. Addition of the solubles from the fermentation liquor to the insoluble solids raises the total protein concentration by 1%.

Amino acid profiles of the unfermented and fermented waste are superior in quality to corn protein and near the quality of that from soybean meal (Table 4). Fermentation increases the quantity of lysine in waste without reduction of sulfur-containing amino acids (Table 4). Based on the amino acid data, *T. viride* mycelium contains about 40% protein. The increased lysine content in the fermented waste indicates that approximately 23% of the residual protein in the insolubles is of mycelial origin. Total mycelium content is calculated at about 13% of the residual solids.

Growth of *T. viride* on whole FLW was followed on the basis of cellulolytic enzyme synthesis in batch cultures. Three conditions were essential for maximizing enzyme production: (i) an initial substrate concentration of 2.5%, (ii) a final pH of 5, and (iii) addition of Tween 80, glucose, or oleic acid at 0.1% concentration to the fermentation broth. Under these conditions, yields of cellulolytic enzymes were equivalent to those obtained on media containing pure cellulose substrates. Primary source of energy for fungal growth was the waste carbohydrate, 66% of which was consumed in approximately 7 days.

Table 2. Comparison of cellulase produced by *T. viride* QM 9123 in FLW and cellulose cultures

| Cellulase assay | Culture substrate |
|-----------------|-------------------|
|                 | FLW*              |
|                 | Shaken flask (100 ml) | Stirred fermentor (10 liters) | Cellulose* (Mandels et al. [8]) |
| Filter paper (10%) | Act, 1 h glucose (mg/h) | 11.2 | 8.5 | 5.0 |
| Absorbent cotton (5%) | Glucose (mg/day) | 5.0 | 4.9 | 7.1 |
| Xylan (5%) | Glucose (mg/h) | 9.4 | 14.3 | |

* Cultures were grown for 14 days at 29 C, pH 5.0, in a shaken flask or stirred fermentor with 2.5% FLW and 0.1% Tween 80.

* Those of Mandels et al. (8) were grown at 28 C in a shaken flask with nutrient salts, 0.5% cellulose, 0.05% proteose-peptone, and 0.1% Tween 80.
| Fraction     | Weight | Nitrogen* | Crude Protein* | Carbohydrate* | Lignin* | Ash | Unidentified organics* (total g) |
|--------------|--------|-----------|----------------|---------------|---------|-----|---------------------------------|
|              | Total (g) | Change | % | Total (mg) | Change | % | g | % | g | % | g | % | g | % | g | % | g | % | g | % |
| Insoluble residue |         |         |   |           |         |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |
| FLW          | 2.00    | -0.62   | 31 | 0.30       | 60      | -10 | 17 | 0.690       | 34 | 9.57   | 0.19  | 20 | 0.40  | 0.35 |
| Fermented FLW | 1.38    |         |   | 3.62       | 50      | -10 | 17 | 0.235       | 65 | 14.76  | 0.20  | 29 | 0.41  | 0.22 |
| Solubles     |         |         |   |           |         |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |
| FLW          | 0.66    | +0.15   | 23 | 3.04       | 19      | +13 | 3.0 | 0.015       | 19 | 0.91   | 0.0    | 31 | 0.25  | 0.25 |
| Fermented FLW | 0.81    |         |   | 4.04       | 32      | +13 | 0  | 0           | 25 | 0.20   | 0.0    | 41 | 0.27  | 0.36 |
| Sum          |         |         |   |           |         |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |
| FLW          | 2.66    | -0.470  | 18 | 79         | 82      | -18 | 0  | 0.695       | 0.50| 0.19   | 0.67  | 0.60 |
| Fermented FLW | 2.19    |         |   | 92         | 82      | -18 | 0  | 0.235       | -0.460 | 0.20   | 0.66  | 0.58 |

*Typical values for selected insoluble and soluble constituents of FLW were isolated by centrifugation (see Materials and Methods) from cultures grown to maximize cellulase production (shake flask culture containing 2.5 g of FLW suspended in 100 ml of basal salt solution with 0.1% Tween 80 at pH 5.0 and 29 C for 15 days).

* Determinations and assays were obtained by the procedures described in Materials and Methods.

* Protein was calculated from the corresponding nitrogen determination times 6.25 the fraction of nitrogen in cereal protein.

* Unidentified organics is the total weight minus protein, carbohydrate, ash, and lignin.
lulolytic enzymes, the fungus rapidly changes the rather unpleasant odor of the waste into a pleasant earthy odor. Fermentations are achieved with total retention of the nitrogen. Further, reduction of the waste carbohydrate during fermentation increases crude protein content of the residue to a level that is feasible for refeeding as a protein supplement.

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**LITERATURE CITED**

1. Benson, J. V., and J. A. Patterson. 1965. Accelerated automatic chromatographic analysis of amino acids in spherical resins. Anal. Chem. 37:1108-1110.
2. Cavins, J. F., and M. Friedman. 1968. Automatic integration and computation of amino acid analysis. Cereal Chem. 45:172-176.
3. Church, B. D., H. A. Nash, and W. Brosz. 1972. Use of *Fungi imperfecti* in treating food processing wastes. Develop. Ind. Microbiol. 13:30-46.
4. Dygert, S., L. H. Li, D. Florida, and J. A. Thoma. 1965. Determination of reducing sugar with improved precision. Anal. Biochem. 13:387-374.
5. Griffin, H. L. 1973. Filter paper assay—effect of time and substrate concentration on cellulose activity. Anal. Biochem. 56:621-625.
6. Loehr, R. C. 1968. In Pollution implication of animal waste—a forward oriented review. U.S. Dept. of the Interior, Fed. Water Pollut. Contr. Admin., Robert S. Kerr, Water Research Center, Ada, Okla.
7. Mandels, M., and J. Weber. 1969. The production of cellulases. Advan. Chem. Ser. 95:391-414.
8. Mandels, M., J. M. Weber, and R. Parizek. 1971. Enhanced cellulase production by a mutant of *Trichoderma viride*. Appl. Microbiol. 21:152-154.
9. Reese, E. T., and A. Maguire. 1968. Surfactants as stimulants of enzyme production by microorganisms. Appl. Microbiol. 17:242-245.
10. Reese, E. T., and A. Maguire. 1971. Increase in enzyme yields by addition of surfactants to cellulose cultures of *Trichoderma viride*. Develop. Ind. Microbiol. 12:212-224.
11. Sloneker, J. H. 1971. Determination of cellulose and apparent hemicellulose in plant tissue by gas-liquid chromatography. Anal. Biochem. 43:539-546.
12. Van Soest, P. J. 1969. Composition, maturity and nutritive value for forages. Advan. Chem. Ser. 95:262-278.

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**TABLE 4. Comparison of the amino acid analysis of *T. viride* fermented FLW solids with unfermented waste solids and several feed proteins**

| Amino acid   | FLW | Corn | Soybean meal | Casein | *T. viride* mycelium |
|--------------|-----|------|--------------|--------|---------------------|
|              | Fermented | Unfermented | Mixture* |        |                     |
| Lysine       | 5.7  | 5.2  | 5.6 | 2.7 | 6.9 | 8.0 | 8.6 |
| Histidine    | 2.4  | 2.1  | 2.4 | 3.0 | 2.6 | 3.0 | 4.7 |
| Arginine     | 7.8  | 8.2  | 8.2 | 5.2 | 8.4 | 4.0 | 8.2 |
| Aspartic     | 4.7  | 4.7  | 4.6 | 6.8 | 12.6 | 7.0 | 4.2 |
| Threonine    | 3.0  | 2.8  | 2.8 | 3.6 | 4.3 | 4.7 | 2.7 |
| Glutamic     | 6.0  | 6.7  | 6.4 | 21.3 | 21.0 | 25.0 | 5.0 |
| Proline      | 2.7  | 2.7  | 2.6 | 10.0 | 6.0 | 11.0 | 2.2 |
| Glycine      | 5.8  | 5.6  | 5.4 | 4.0 | 4.5 | 2.5 | 4.3 |
| Alanine      | 5.5  | 6.4  | 6.1 | 8.1 | 4.5 | 3.0 | 4.3 |
| Cystine      | 1.3  | 0.9  | 0.9 | 1.6 | 1.6 | 1.0 | 1.0 |
| Valine       | 3.8  | 3.6  | 3.5 | 4.7 | 5.4 | 7.7 | 2.7 |
| Methionine   | 1.2  | 1.7  | 1.5 | 1.7 | 1.7 | 3.5 | 0.6 |
| Isoleucine   | 2.4  | 2.4  | 2.3 | 3.5 | 5.2 | 6.5 | 1.9 |
| Leucine      | 4.4  | 4.3  | 4.1 | 5.2 | 5.6 | 9.7 | 2.9 |
| Tyrosine     | 2.5  | 2.1  | 2.7 | 4.4 | 3.9 | 6.5 | 6.5 |
| Phenylalanine| 2.2  | 2.0  | 2.0 | 5.0 | 5.0 | 5.9 | 1.6 |

*Values calculated for a mixture of 13% *T. viride* mycelium and 87% FLW residue. The amino acid analysis is described in Materials and Methods. Data are reported as gram per 100 g of protein.*

*Grown on basal salt and glucose (2.5%) medium.