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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 deliquescens* removed 96% of the BOD_5, 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 Blendor 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.

Slants 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/in^2 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 reported 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 procedure (1).

RESULTS AND DISCUSSION

Starting from a spore inoculation, at least four consecutive stages occur during batch fermentation 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, cellulase activity, and the quantity and quality of the organic constituents remain relatively stable. 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 cellulosics in the medium. This is in agreement 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 substrate. 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

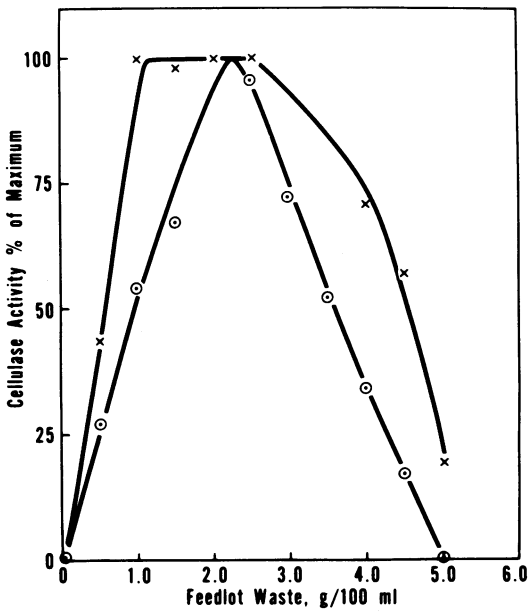

FIG. 1. *Relation between concentration of FLW and cellulase production in T. viride fermentation of FLW. Spore inoculum, shake flask experiment grown 5 days at 29 C and pH 5.0. Filter paper activity (⊙) and xylanase activity (×) are expressed as percentage of maximal value.*

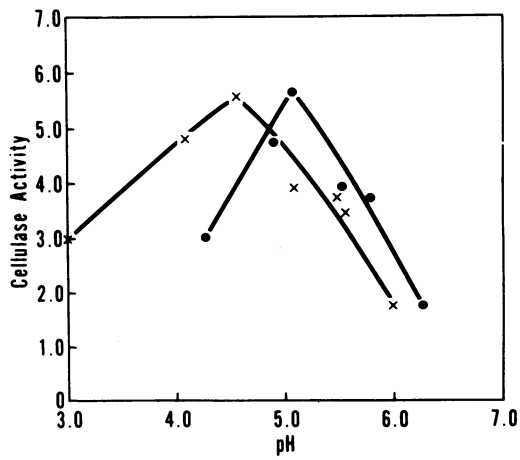

FIG. 2. *Relationship between culture pH and cellulase production in T. viride fermentation of a FLW sample. Cultures were spore inoculated and grown at 29 C for 5 days in shake flask culture. Initial pH (×) was adjusted with 85% H_3PO_4 and NaOH to the indicated value. The equilibrium pH (●) was reached after 3 days' growth of T. viride and remains essentially stable thereafter.*

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 monooleate) 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 Mandels and Weber (7), we also observe values of 5 or above (Table 2).

The xylanase activity is included to demonstrate the action of our enzyme on hemicellulose, a substantial portion of the carbohydrate

TABLE 1. *Effect of additives on production of cellulase*

Additive[b]	Cellulase assay[a]		
	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[c]	9.0	4.7	9.7
Control	6.5	2.5	4.6

[a] The assays are described in Materials and Methods.

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

[c] Autoclaving was 30 min at 20 lb/in² steam (120 C).

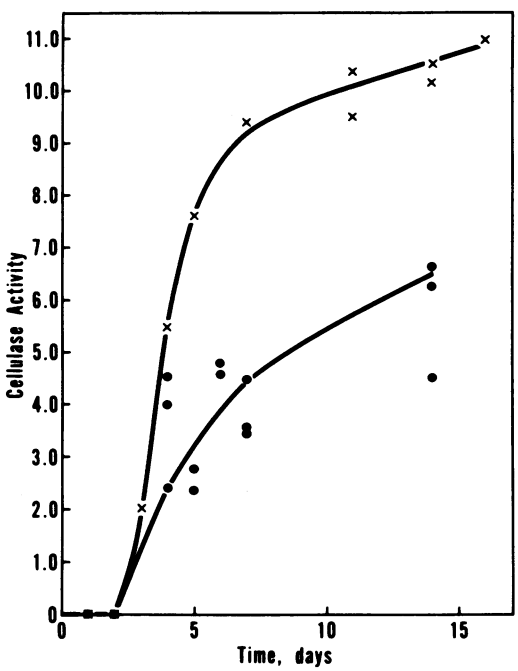

FIG. 3. *Effect of Tween 80 on production of cellulase in T. viride ferementations of FLW. The fermentations were run in shake flasks at 29 C and pH 5.0. Crude filtrates from cultures containing Tween 80 (×) and cultures without Tween 80 (●) were analyzed for filter paper activity at the indicated intervals.*

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 $\times$ 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%. However, addition of only the acetone-precipitated crude protein of the supernatant to the insoluble fraction would increase its protein content to 32.3%.

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.

Besides producing copious amounts of cel-

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

Cellulase assay		Culture substrate		
		FLW[a]		
Enzyme substrate	Measurement of act	Shaken flask (100 ml)	Stirred fermentor (10 liters)	Cellulose[b] (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	

[a] 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.

[b] 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.

TABLE 3. *Organic constituents of T. viride-fermented FLW*[a]

Fraction	Weight			Nitrogen[b]				Crude Protein[c]		Carbohydrate[b] neutral				Lignin[b]		Ash		Unidentified organics[d] (total g)
					Weight													
	Total (g)	Change		%	Total (mg)	Change		%	g	%	g	Change		%	Weight (g)	%	Weight (g)	
		g	%			mg	%					g	%					
Insoluble residue																		
FLW	2.00			3.00	60			18.8	0.38	34	0.680			9.57	0.19	20	0.40	0.35
Fermented FLW	1.38	−0.62	31	3.62	50	−10	−17	22.6	0.31	17	0.235	0.445	65	14.76	0.20	29	0.41	0.22
Solubles																		
FLW	0.66			3.04	19			19.0	0.12	3.0	0.015					41	0.27	0.25
Fermented FLW	0.81	+0.15	23	4.04	32	+13		25.2	0.20	0	0					31	0.25	0.36
Sum																		
FLW	2.66				79				0.50		0.695				0.19		0.67	0.60
Fermented FLW	2.19	−0.470	18		82				0.51		0.235	−0.460			0.20		0.66	0.58

[a] 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).

[b] Determinations and assays were obtained by the procedures described in Materials and Methods.

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

[d] Unidentified organics is the total weight minus protein, carbohydrate, ash, and lignin.

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*[b] mycelium
	Fermented	Unfermented	Mixture[a]				
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

[a] 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.

[b] Grown on basal salt and glucose (2.5%) medium.

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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