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Use of Trifluoroacetic Acid to Prepare Cellodextrins

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SUMMARY

A simple procedure for the hydrolysis of cellulose or biomass into cellodextrins using trifluoroacetic acid-water mixtures was found. Several reaction variables to get maximum conversion of the cellulose into water-soluble oligomers were determined. Fermentation of the cellodextrins produced ethanol in 75% yields.

INTRODUCTION

Recently it was reported (Freer & Detroy, 1982) that the yeast NRRL Y-2563 *Candida wickerhamii* (Capriotti) Meyer et Yarrow could ferment cellobiose and higher cellodextrins (degree of polymerization (*DP*) = 3–6) directly into ethanol. Several known methods to prepare cellodextrins have been reported in the literature. The methods use fuming hydrochloric acid (Miller *et al.*, 1960; Miller, 1963; Huebner *et al.*, 1978; Hsu *et al.*, 1980), 85% phosphoric acid (Beélik & Hamilton, 1961), acetolysis (Wolfrom & Dacons, 1952; Wolfrom *et al.*, 1956; Freudenberg, 1957; Wolfrom & Thompson, 1963) and enzymatic hydrolysis (Reese & Mandels, 1963). Since cellulose is soluble in trifluoroacetic

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acid (TFA) (Adamova & Mvasoedova, 1982) and cellulose in TFA–H_2O at high temperature will yield glucose (Albersheim *et al.*, 1967; Paice *et al.*, 1982), we evaluated a TFA–H_2O system to prepare a homologous series of cellodextrins. Several reaction variables were evaluated to produce a series of cellodextrins that were fermentable to ethanol. A comprehensive fermentation study will be forthcoming.

EXPERIMENTAL

Materials

Whatman CC-31 cellulose powder was used. Wheat straw *Triticum* sp. was obtained locally. Cellulase was purchased from Miles Labs., Research Products Div., Elkhart, Indiana, and Almond β-glucosidase was purchased from Sigma Chemical, St Louis, Missouri. All other chemicals were reagent grade.

General hydrolysis procedure

Cellulose powder (5 or 10 g) was suspended in trifluoroacetic acid (100 ml) at 25°C. The mixture was stirred magnetically for 3 days to allow complete dissolution of the cellulose. For the 10 g preparations, additional TFA (20–40 ml) was required after 24 h. Water (5–40 ml) was added and the mixture was heated for 24 h at 72°C. After heating, the mixture was placed in an evaporating dish and the TFA evaporated off in a fume cupboard. Water (400 ml) was added to the mixture and any insoluble high-molecular weight material was filtered off. The water solution was treated with anionic ion exchange resin (Dowex AG 1-X8) in the hydroxide form to neutralize the mixture to pH 6·5. The resin also adsorbed the minor amounts of colored degradation products. Resin was filtered off and the water was removed by rotary evaporation at 50°C. Analysis of several preparations is shown in Tables 1 and 2. The percent cellodextrins reported represent only the soluble portion. The insoluble portion contained $DP = 7$ and higher molecular weight cellodextrins.

TABLE 1
Trifluoroacetic Acid Hydrolysis of Cellulose into Cellodextrins[a]

	Sample (5 g)				
	A	*B*	*C*	*D*	*E*
H_2O (ml)	5	10	15	15	20
Temperature (°C)	72	72	72	78	72
Soluble weight (g)	0·8	4·0	4·5	5·1	5·0
Insoluble weight (g)	4·3	1·5	0	0	0
% Cellodextrins					
Xylose	2·9	3·2	2·9	2·9	2·9
G_1	29·6	37·6	50·1	57·9	59·1
G_2	20·2	18·1	19·1	18·3	22·1
G_3	21·5	15·5	13·0	10·4	9·5
G_4	16·5	11·9	8·6	3·8	5·0
G_5	8·0	9·4	4·0	4·2	1·4
G_6	1·3	4·3	2·3	2·5	–

[a] Follow general hydrolysis procedure

Wheat straw conversion to cellodextrins

The wheat straw was ground in a Wiley mill to pass through a 1 mm mesh sieve. Samples (5 g) were hydrolyzed using the above procedure. Other samples of wheat straw (10 g) were pretreated with NaOH-hydrogen peroxide (Gould, 1983) to partially remove hemicellulose and lignin before TFA hydrolysis.

Thin-layer chromatographic analysis

The method of Wing & BeMiller (1972) was used to obtain a rapid analysis of the cellodextrin hydrolysis products. Table 3 shows chromatographic R_f values of the mixture using *n*-butanol : HOAc : Et_2O : H_2O (9 : 6 : 3 : 1) as the developer.

TABLE 2
Trifluoroacetic Acid Hydrolysis of Cellulose into Cellodextrins[a]

	Sample (10 g)					
	F	*G*	*H*[b]	*I*	*J*	*K*
H_2O (ml)	10	10	20	20	30	40
Temperature (°C)	72	78	72	72	72	72
Soluble weight (g)	4·7	10·2	6·7	10·6	11·5	11·2
Insoluble weight (g)	5·9	0·6	5·0	1·2	0·6	0
% Cellodextrins						
Xylose	2·4	2·7	2·1	2·6	2·6	2·6
G_1	24·9	40·2	29·4	40·9	63·1	71·8
G_2	20·5	21·1	18·0	18·5	17·0	18·1
G_3	20·9	15·3	19·2	16·2	10·6	4·8
G_4	16·3	9·7	13·6	14·4	5·4	2·7
G_5	9·6	7·6	13·1	6·9	1·3	–
G_6	5·4	3·4	4·6	0·5	–	–

[a] Follow general hydrolysis procedure.
[b] 12 h hydrolysis.

TABLE 3
Chromatographic Data for Cellodextrins

Carbohydrate	R_f[a]	R_T[a] *(min)*
Xylose	0·47	2·32
Glucose	0·39	3·15
Cellobiose	0·23	3·85
Cellotriose	0·11	4·83
Cellotetrose	0·05	6·17
Cellopentose	0·02	8·04
Cellohexose	0·00	10·60

[a] See 'Experimental' section; R_f for TLC; R_T for HPLC using acetonitrile : H_2O (70 : 30) as elution solvent.

High-pressure chromatographic analysis

The method of Ladisch (Ladisch *et al.*, 1978) was used to obtain the percentages of cellodextrins in each preparation. A Waters HPLC fitted with a DuPont Zorbax-NH_2 column was used. The mobile phase consisted of acetonitrile : H_2O (70 : 30 or 72·5 : 27·5), and glucose and cellobiose were used as external standards. Table 3 lists the retention times for $DP = 1$–6.

Organism and fermentation conditions

The method of Freer & Detroy (1982) was used to ferment the TFA–H_2O produced cellodextrins. The yeast *Candida wickerhamii* NRRL Y-2563 was acquired from the ARS Culture Collection, NRRC, Peoria, Illinois.

Enzymatic degradation of cellodextrins

Cellodextrins prepared from wheat straw were suspended in 0·1 M acetic acid (pH 4·75) and either cellulase or β-glucosidase was added such that the final enzyme concentrations were 5 mg ml^{-1} or 0·5 mg ml^{-1}, respectively. The reaction mixtures were incubated at 40°C for 17 h. The carbohydrates were analyzed via HPLC using acetonitrile : H_2O (72·5 : 27·5) as eluant. The solvent system was used in this case to obtain a better separation of glucose from xylose; however, we were unable to detect cellohexose using this solvent system.

Ethanol analysis

Ethanol was determined by gas–liquid chromatography with a Packard Model 402 GLC using a Porapak Q column at 150°C.

RESULTS AND DISCUSSION

Several hydrolysis variables were evaluated using TFA to obtain maximum conversion of cellulose into soluble cellodextrins. Table 1 shows data when various amounts of water used during the hydrolysis of 5 g cellulose samples. Increasing water concentration or temperature

results in higher conversions to glucose. Similar results (Table 2) were obtained when cellulose concentrations were increased. All cellodextrin preparations showed ~3% xylose, which was formed from hemicellulose impurity in the cellulose powder. No water-soluble oligosaccharides were formed when hydrolysis was carried out at 30°C for 72 h or at 72°C when water was omitted. Heating cellulose in TFA at 72°C increased the rate of solubility; however, some cellulose decomposition occurred (solution darkened slightly). Activated carbon (Darco G-60) can be used to remove colored species in the cellodextrin preparations with less than 5% loss of the oligosaccharides. A shorter hydrolysis time (12 h) resulted in a more uniform series of cellodextrins; however, only 60% of the mixture was water soluble.

A large-scale (50 g cellulose) preparation of cellodextrins, using the conditions as for sample I of Table 2, resulted in a series of oligosaccharides with a composition similar to that reported for sample I. In this preparation, the TFA hydrolysate was divided in half. One half was evaporated in a fume cupboard; the other half was evaporated using a Büchi Rotovapor R-110 at room temperature for TFA recovery. The recovered TFA (95%) contained water (~15%) and would not redissolve cellulose powder, so TFA purification would be necessary for its reuse.

To keep the preparations of cellodextrin salt free, anion exchange resins were used to remove the final traces of TFA. Table 4 shows that most resins evaluated were effective in neutralizing and removing TFA without adsorbing the cellodextrins. Most of the resins also removed residual colored components.

TABLE 4
Evaluation of Ion Exchange Resins for Neutralizing TFA-Cellodextrin Mixtures

Resin	*Type*	*Weight remaining*[a] *(g)*	*Color removal*
Ionac A-302	Polyamine	1·1	Good
Amberlite IRA-94	Weak base	1·1	Excellent
Rexyn 201	Strong base	0·3	Excellent
Dowex AG 1-X8	Strong base	1·1	Excellent

[a] Follow general hydrolysis procedure. Theoretical amount of cellodextrin = 1·1 g.

Conversion of the cellulose in wheat straw to cellodextrins using the general hydrolysis procedure was successful. Most of the cellulose and hemicellulose dissolved and the lignin was easily removed in the insoluble portion. Pretreatment of the wheat straw with NaOH : H_2O offered no advantages in dissolving the straw or in the hydrolysis. Table 5 shows data for the wheat straw hydrolysis.

To further confirm that the oligosaccharides produced by TFA hydrolysis of wheat straw were indeed cellodextrins, the hydrolysates were tested for their sensitivity to enzymes which specifically hydrolyze β-1,4-linkages (cellulase and β-glucosidase). The results (Fig. 1) clearly indicate that 95% of the oligosaccharides which elute with retention times characteristic of cellodextrins of $DP = 2$–5 are indeed sensitive to these enzymes. In both cases glucose was the major identifiable reaction product.

Several of the previously referenced chemical treatments of residues generate either unfermentable sugar reversion products, such as isomaltose (Goldstein, 1981), or compounds toxic to microorganisms.

TABLE 5
Trifluoroacetic Acid Hydrolysis of Wheat Straw[a]

	Sample (5 g)		
	A	*B*	*C*[b]
H_2O (ml)	15	20	10
Soluble weight (g)	2·7	2·7	2·3
Insoluble weight (g)	1·7	1·6	1·8
% Cellodextrins			
Xylose	23·8	25·0	25·2
G_1	36·5	48·0	33·9
G_2	14·5	11·9	20·9
G_3	13·4	9·4	12·0
G_4	7·8	5·7	5·2
G_5	4·0	–	2·8

[a] Follow general hydrolysis procedures.
[b] Wheat straw pretreated with NaOH-H_2O_2.

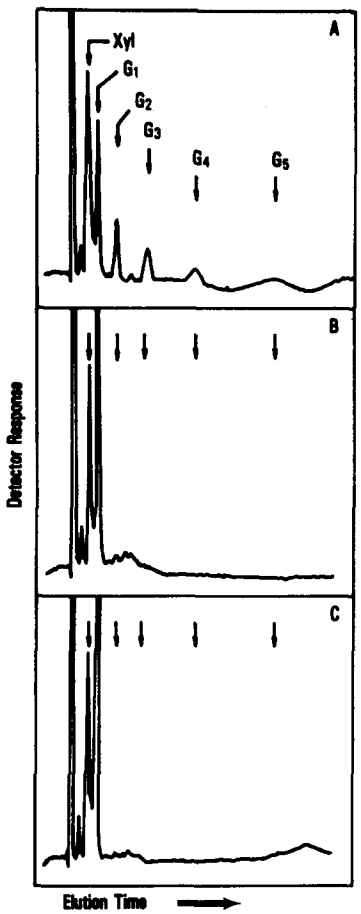

Fig. 1. Degradation of wheat straw cellodextrins by cellulase and β-glucosidase. Wheat straw cellodextrins were suspended in 0·1 M acetic acid (pH 4·75) and reacted with either cellulase (B) or Almond β-glucosidase (C) as described in the 'Experimental' section. Samples were removed from the reaction mixtures at 0 h (A) or after 17 h incubation (B and C) and the reaction products analyzed by HPLC. Symbols: Xyl = xylose; G_1 = glucose; G_2–G_5 = cellodextrins of $DP = 2$–5.

To test whether the TFA hydrolysis procedure produced compounds toxic to microbes, a portion of the cellodextrins prepared from wheat straw was mixed with medium and inoculated with *C. wickerhamii* as previously reported (Freer & Detroy, 1982). The results (Figs 2 and 3) indicated the cellodextrins prepared by this procedure were ferment-

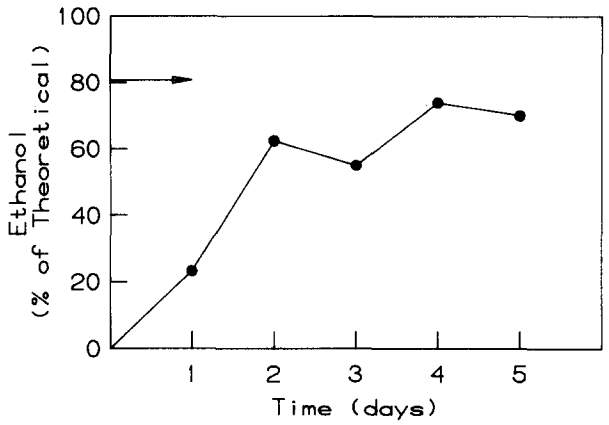

Fig. 2. Fermentation of cellodextrins prepared by TFA hydrolysis of wheat straw. Media containing 21·2 g liter^{-1} cellodextrins were inoculated with *Candida wickerhamii*. The ethanol concentrations were determined as described in the 'Experimental' section. The arrow drawn at 81% of theoretical yield represents the maximum theoretical yield of ethanol that could be formed if the residual glucose and cellobiose present in the media after 5 days' incubation are subtracted from the initial glucose/cellodextrin concentrations.

able. From an initial glucose/cellodextrin concentration of 21·2 g liter^{-1}, 8·34 g liter^{-1} ethanol was produced (approximately 75% of theoretical yield as calculated from the initial cellodextrin concentration). Furthermore, the initial lag period prior to the synthesis of ethanol was not excessive, indicating that few compounds toxic to *C. wickerhamii* were present in the cellodextrin preparation. Previous fermentations of pure cellobiose or other highly purified cellodextrin preparations yielded ethanol production curves which were very similar to that shown in Fig. 2 (Freer & Detroy, 1982, 1983). Maximum ethanol yields of approximately 80–95% of theoretical yield were obtained in 3–4 days in these previous studies.

To insure that *C. wickerhamii* was indeed utilizing cellodextrins as a substrate for ethanol production, residual media carbohydrates were analyzed via HPLC immediately after inoculation and after 5 days' fermentation. Figure 3 shows that after 5 days' fermentation, this yeast had metabolized all of the cellodextrins of $DP = 3$–5. The only detectable carbohydrates remaining in solution were relative small amounts of glucose (3·11 g liter^{-1}) and cellobiose (0·92 g liter^{-1}).

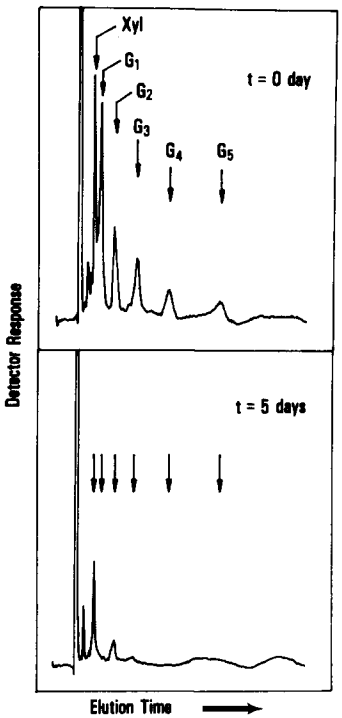

Fig. 3. Analysis of residual media carbohydrates after fermentation of cellodextrins prepared by TFA hydrolysis of wheat straw. The fermentation was performed as described in Fig. 2 and the carbohydrates were analyzed as described in Fig. 1.

If these residual sugars are subtracted from the initial glucose/cellodextrin concentration (21·2 g liter^{-1}), the ethanol produced represented 91% of the theoretical yield. *C. wickerhamii* was also capable of metabolizing xylose (Fig. 3). However, under no circumstance have we been able to demonstrate that this yeast is capable of fermenting xylose to ethanol (data not shown).

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