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Growth of "Seeded" Cellulolytic Enrichment Cultures on Mesquite Wood

D. W. THAYER* AND CAROL ANN DAVID
Department of Biological Sciences, Texas Tech University, Lubbock, Texas 79409

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Eleven enrichment cultures were developed by a "seeded" enrichment culture technique, and one was developed by a simple enrichment technique. The seeded enrichment, the pure "seed," and the simple enrichment cultures were compared during growth on mesquite wood, cotton, carboxymethylcellulose, and cellobiose. All of the enrichment cultures were cellulolytic and exceeded the pure seed cultures in mesquite wood hydrolysis and/or viable cell count. Yeast extract improved, but was not essential for, growth of the seeded enrichment cultures on carboxymethylcellulose. Two of the seeded enrichment cultures, CAD5 and CAD11, grew best at 37°C and pH 7.0 on mesquite wood. A 1.0% (wt/vol) wood concentration was optimum for their growth.

Organisms that grew on mesquite wood were isolated from soil and decaying mesquite wood by standard enrichment culture techniques (2, 13). These pure cultures were investigated extensively in batch and semicontinuous cultures (2, 4, 11, 13, 14). Decomposition of cellulose in nature, however, results from several species acting together (1). Greater rates of cellulose hydrolysis were exhibited by mixed rather than by pure cultures (5, 6, 15). In some systems, none of the individual bacterial species was able to hydrolyze cellulose.

We now report the isolation of mixed culture systems with increased rates of wood and cellulose hydrolysis by a "seeded" enrichment culture technique. The growth characteristics of the pure "seeds" are compared with those of the seeded enrichment cultures and of a simple enrichment culture.

MATERIALS AND METHODS

Microorganisms. The following organisms were chosen on the basis of their cellulolytic activities and growth rates on mesquite wood: *Brevibacterium* JM99B, *Bacillus* JM68B, *Serratia marcescens* RW3, *Brevibacterium* JM66C, *Brevibacterium* JM98A, *Pseudomonas* JM127, *Bacillus* JM116, *Flavobacterium* JM106, *Flavobacterium* JM92, ABK1, and ABK2. These cultures were used to seed enrichment cultures as described below. The corresponding seeded enrichment cultures were designated CAD1 through CAD11, respectively. The properties of all cultures except ABK1 and ABK2 have been described in previous publications (12, 13). ABK1 and ABK2 were isolated as contaminants of *Cellulomonas* cultures and grew on wood substrates. ABK1 was a *Sarcina* species, and ABK2 was *Bacillus cereus*.

Enrichment cultures. Each seeded enrichment culture received an inoculum of 10.0 g of soil containing decaying mesquite wood and 1.0 ml of a 24-h culture in tryptic soy broth (Difco Laboratories) of one seed organism (approximately 10^9 cells). This was added to a 500-ml flask containing 100 ml of the growth medium at pH 7.0 and 1.0 g of ground mesquite wood. A 12th enrichment culture, CAD12, was inoculated only with soil. The cultures were incubated for 48 h at 37°C, and then 10 ml was transferred to sterile medium. The cultures were considered to have adapted to mesquite wood after seven transfers because the 10 g of soil inoculum was diluted to less than 0.0004 g/liter.

Culture maintenance. All cultures were maintained by weekly transfers in the mesquite medium. An inoculum of 0.1 ml was used routinely for each of the duplicate experiments. It was obtained from 50 ml of tryptic soy broth which was inoculated with 1.0 ml of culture and then incubated for 16 h at 37°C to provide more uniform inocula than could be obtained from a medium containing wood.

Media and growth conditions. The growth medium contained the following (in grams per liter of distilled water at pH 7.0): NaCl, 6.0; $(NH_4)_2SO_4$, 2.0; KH_2PO_4, 1.0; K_2HPO_4, 1.0; $MgCl_2$, 0.1; $CaCl_2$, 0.1; yeast extract, 0.5; and cellulosic substrate, 10.0. All cultures were incubated at 37°C on a rotary shaker at 250 rpm, except where indicated otherwise. All flask studies were made in 500-ml baffled Erlenmeyer flasks. Optical density measurements of culture growth were made with a Bausch & Lomb Spectronic 20 spectrophotometer, using flasks with an attached nephelometer tube (18-mm diameter).

Substrates. Dormant mesquite wood was cut in March, reduced with a hammer mill to an average size of 1.0 mm, washed with running tap water to remove fines and dirt, rinsed once with distilled water, and dried to constant weight at 100°C.

Sodium carboxymethylcellulose (CMC; Sigma Chemical Co.) and commercial absorbent cotton were used in some experiments.

Substrate hydrolysis. Substrate hydrolysis was determined gravimetrically with 1.0 ± 0.001 g of mesquite wood per culture flask. After incubation the unhydrolyzed wood was collected by vacuum filtration on tared 9.0-cm Whatman GF/A fiber glass paper and then oven dried for 24 h at 100°C before weighing. Uninoculated controls were included with each experiment. The weight loss of the control was substrated from the experimental values.

Viable cell number. A standard dilution pour plate technique (3) using Difco tryptic soy agar was used to determine viable cell numbers. Each viable cell count was the average of five replicates of the dilution yielding 30 to 300 colonies per plate.

Protein analysis. The residual mesquite wood was removed by filtration, and a portion of the filtrate was centrifuged. The cell mass was washed twice with distilled water and then suspended to 0.1 its original volume. Soluble protein was measured by the method of Lowry et al. (8), and cellular protein was measured by the biuret technique (7), with bovine serum albumin as the standard protein.

Cellulose strip hydrolysis. Strips of Whatman no. 1 filter paper were suspended in a medium consisting of 5.0 g of peptone (Difco Laboratories), 5.0 g of NaCl, and 1,000 ml of tap water (10).

Reducing sugar assay. Reducing sugar was analyzed by the dinitrosalicylic acid procedure of Miller et al. (9). Glucose was used to prepare a standard curve.

Cellulase activity. All assays for cellulase activity were made with cell-free samples, as described by Miller et al. (9). Alpha-cellulose (Sigma Chemical Co.) was substituted for CMC. The cell-free supernatant and substrate were incubated together for 1 h at 37°C in a water bath shaker.

Optimum pH. The pH of the mineral salts medium was adjusted to values ranging from 3 through 11. Culture growth was measured by viable cell counts. Because the optimum pH range was between 6.0 and 7.5, viable cell number and protein production were monitored at pH values of 6.0, 6.5, 7.0, and 7.5.

Substrate concentration. The effects of mesquite wood concentrations on viable cell numbers and protein production were evaluated at pH 7.0 in the mineral salts medium described above.

Growth parameters of pure versus mixed cultures. Growth rates, substrate hydrolyses, protein production, enzyme activities, and reducing sugars of the mixed cultures were compared with those of the pure culture seeds. The mineral salts medium was adjusted to pH 7.0, and the substrate concentration was adjusted to 1.0% (wt/vol). The effects of different concentrations of yeast extract (0, 0.01, 0.025, and 0.05% [wt/vol]) on the growth of the cultures were determined. Three control experiments were included. In the first control, alpha-cellulose was substituted as the substrate. In the second control, mesquite wood was removed from the medium after sterilization to evaluate solubilized wood as a substrate. This medium was designated wood solubles in Tables 2 and 3. In the third control, an uninoculated mesquite wood medium containing 0.05% (wt/vol) yeast extract was incubated and sampled in the same manner.

All experiments were duplicated, and the averages are reported. Variations between experimental results in the duplicate studies were small. The results shown in Fig. 2 for protein were typical.

RESULTS

The seeded enrichment cultures were compared with their respective seed cultures and with the simple enrichment culture (CAD12). All cultures grew well in a 1.0% mesquite wood medium, producing approximately 5×10^9 viable cells per ml within 24 h. All cultures hydrolyzed from 6 to 12% of the mesquite wood, with 7 of the 12 seeded enrichment cultures hydrolyzing from 1 to 3% more wood than their pure seed cultures. ABK1 and ABK2 hydrolyzed filter-paper strips in peptone-NaCl broth in 28 and 21 days, respectively. Ten enrichment cultures hydrolyzed filter paper, with CAD5 requiring only 4 days. CAD2, CAD4, CAD10, CAD11, and CAD12 required 7 days, and CAD1, CAD7, CAD8, and CAD9 required 14 days or longer for hydrolysis of filter paper. Addition of 1.0% dewaxed cotton to the mineral salts improved the growth of CAD1, CAD2, CAD5, CAD6, CAD8, CAD10, CAD11, and CAD12, compared with growth on the mineral salts alone. The seeded enrichment cultures CAD2, CAD5, CAD6, CAD10, and CAD11 were selected for further study.

The growth of all five seeded enrichment cultures on CMC was stimulated by yeast extract or Casamino Acids, but four grew in their absence (Table 1). Cellobiose supported the growth of all five cultures. A typical growth curve for the mixed cultures on CMC is shown in Fig. 1. In the presence of a 0.01% yeast extract concentration, the seeded enrichment cultures, with the exception of CAD6, had pronounced diauxic growth curves. Cultures CAD5 and CAD11 were selected for further study because they grew on CMC without yeast extract and produced the greatest viable cell counts and protein yields on CMC or alpha-cellulose plus 0.025% yeast extract.

A 37°C temperature and a pH of 7.0 produced optimum growth of the mixed cultures CAD5 and CAD11 and their pure culture seeds *Brevibacterium* JM98A and ABK2 on 1% mesquite wood. These conditions produced the highest viable cell numbers and greatest amounts of protein. The growth rates of CAD5, *Brevibacterium* JM98A, and CAD11 did not change significantly when the incubation temperature was changed from 30 to 37°C.

The seeded enrichment cultures CAD5 and CAD11 were grown with varying concentrations of mesquite wood, and the generation times, viable cell numbers, and protein yields were

TABLE 1. *Effect of yeast extract, Casamino Acids, and cellobiose on the growth of five seeded enrichment cellulolytic cultures*

Medium	Total incubation time (h)	Growth (optical density at 460 nm)[a]				
		CAD2	CAD5	CAD6	CAD10	CAD11
CMC + mineral salts	0	0.041	0.050	0.028	0.040	0.041
	24	0.13	0.12	0.069	0.12	0.12
	48	0.25	0.25	0.069	0.29	0.58
CMC + mineral salts + 0.01% YE[b]	0	0.041	0.050	0.028	0.059	0.096
	24	0.23	0.22	0.17	0.26	0.35
	48	0.73	0.740	0.17	0.75	0.80[c]
CMC + mineral salts + 0.025% YE	0	0.059	0.050	0.041	0.059	0.041
	10	0.46	0.44	0.42	0.45	0.45
	24	0.47	0.44	0.42	0.45	0.45
CMC + mineral salts + 0.05% YE	0	0.059	0.050	0.047	0.059	0.041
	10	0.72	0.62	0.62	0.62	0.62
	24	0.72	0.62	0.62	0.62	0.62
CMC + mineral salts + 0.05% Casamino Acids	0	0.05	0.05	0.05	0.05	0.05
	24	0.71	0.73	0.50	0.69	0.70
Mineral salts + 0.05% YE + 0.05% cellobiose	0	0.02	0.02	0.01	0.01	0.01
	24	1.40	1.24	1.30	1.25	1.20

[a] Samples were taken every hour for the first 12 h and then at 24, 28, 32, 44, and 48 h.
[b] YE, Yeast extract.
[c] Optical density was determined at 32 h.

determined. The following substrate concentrations were evaluated: 0.5, 1.0, 2.5, 5.0, and 10.0% (wt/vol). Both cultures produced the greatest protein yields with 1.0% mesquite. The lowest viable cell numbers were associated with a substrate concentration of 0.5%. The highest viable cell numbers for cultures CAD5 and CAD11 were with wood concentrations of 10.0, and 2.5%, respectively. The generation times were lowest with a substrate concentration of 1.0%, but the variations between the studies were minimal.

Cell protein was maximum with 0.05% (wt/vol) yeast extract in the medium (Tables 2 and 3). The CAD5 culture data with 1.0% mesquite and 0.05% yeast extract are shown in Fig. 2. The results for CAD11 were similar to those obtained with CAD5. The seed cultures produced the greatest cellulase activity when the yeast extract concentrations were low. Reducing sugar concentrations in the media decreased sharply before cellulase activities increased (Fig. 2). The reason for the sharp decrease in cellulolytic activity between 48 and 60 h is unknown. Similar decreases were not observed when 0 or 0.25% yeast extract was used in the medium.

Cultures CAD5 and CAD11 grew better on wood or alpha-cellulose than on wood solubles (Tables 2 and 3). Generation times for CAD5 and CAD11 were 2.5 and 2.2 h, respectively, on wood solubles, almost twice as long as values with either alpha-cellulose or mesquite wood. Cultures CAD5 and CAD11 produced maxima

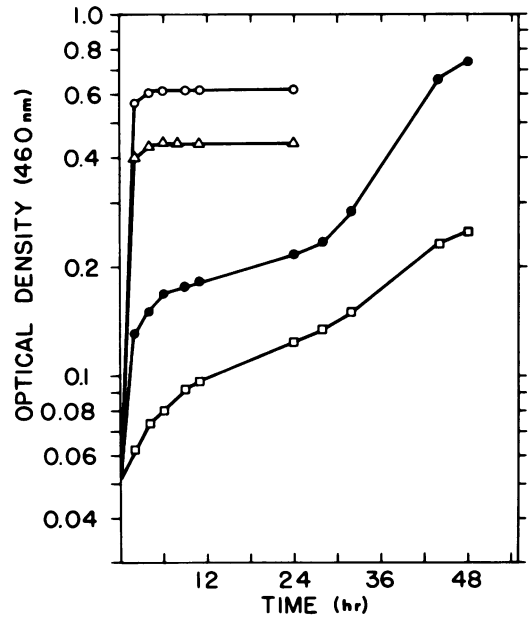

FIG. 1. *Growth of seeded enrichment culture CAD5 on mineral salts plus 1% CMC plus 0.05%* (○), *0.025%* (△), *0.01%* (●), *and no* (□) *yeast extract.*

TABLE 2. *Comparison of seeded enrichment culture CAD5 with its pure culture seed, Brevibacterium JM98A*

Substrate	Generation time (h)		Maximum protein[a] (mg/ml)		Maximum enzyme[a] (mg of reducing sugar per ml)		% Hydrolysis		Viable cells per ml[a]	
	CAD5	JM98A	CAD5	JM98A	CAD5	JM98A	CAD5	JM98A	CAD5	JM98A
Mesquite + 0.05% YE[b]	1.27	2.94	0.6	0.22	0.3	0.24	14.5	15.4	8.6×10^9	8.4×10^8
Mesquite + 0.025% YE	1.39	2.77	0.3	0.08	0.28	0.30	11.4	7.2	4.0×10^9	2.6×10^8
Mesquite + 0.01% YE	1.37	2.85	0.4	0.17	0.27	0.50	10.3	6.0	4.2×10^9	2.4×10^8
Mesquite + 0 YE	1.38	2.77	0.3	0.06	0.22	0.50	10.74	5.8	3.2×10^9	2.3×10^8
Alpha-cellulose	1.39	2.91	0.3	0.18	0.20	0.28	10.99	4.5	3.5×10^9	1.5×10^9
Wood solubles	2.5	2.5	0.11	0.12						

[a] Maximum value observed. The cultures were sampled at 12-h intervals for 72 h.
[b] YE, Yeast extract.

TABLE 3. *Comparison of seeded enrichment culture CAD11 with its pure culture seed, ABK2*

Substrate	Generation time (h)		Maximum protein[a] (mg/ml)		Maximum enzyme[a] (mg of reducing sugar per ml)		% Hydrolysis		Viable cells per ml[a]	
	CAD11	ABK2	CAD11	ABK2	CAD11	ABK2	CAD11	ABK2	CAD11	ABK2
Mesquite + 0.05% YE[b]	1.40	2.96	0.7	0.28	0.34	0.32	11.87	5.8	8.2×10^9	1.0×10^9
Mesquite + 0.025% YE	1.35	2.59	0.4	0.28	0.26	0.30	9.94	6.7	4.0×10^9	5.2×10^8
Mesquite + 0.01% YE	1.37	2.87	0.3	0.26	0.30	0.34	8.76	6.2	3.7×10^9	5.0×10^8
Mesquite + 0 YE	1.38	2.94	0.3	0.28	0.34	0.40	10.57	5.9	2.8×10^9	4.6×10^8
Alpha-cellulose	1.37	3.24	0.2	0.35	0.26	0.27	10.52	3.8	2.5×10^9	1.0×10^9
Wood solubles	2.20	2.19	0.1	0.1						

[a] Maximum value observed.
[b] YE, Yeast extract.

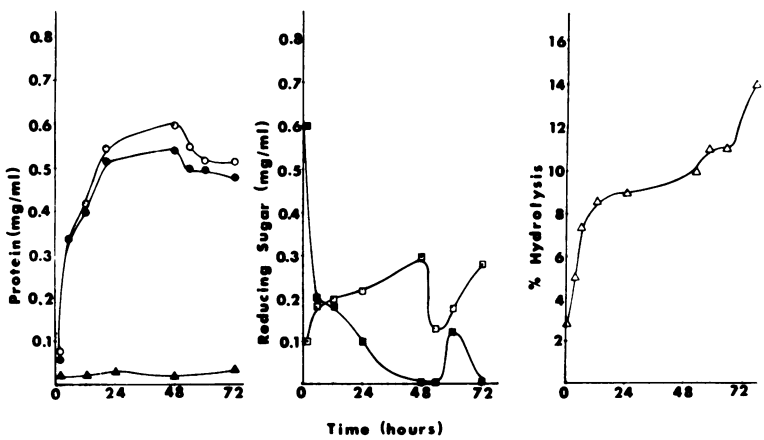

FIG. 2. *Growth of seeded enrichment culture CAD5 on 1.0% (wt/vol) mesquite wood and 0.05% yeast extract. Symbols: ○ and ●, cell protein; ▲, soluble protein; ■, reducing sugar; □, cellulase activity; △, percent mesquite wood hydrolyzed.*

of 0.11 and 0.10 mg of protein per ml, respectively, when grown on just the wood solubles.

There was no cellulase activity in uninoculated controls, and the reducing sugar concentration remained constant at 0.72 mg/ml. The biuret test indicated 0.016 mg of protein per ml. The apparent sample hydrolysis increased from 1.0 to 2.5% during 72 h of incubation, presumably due to mechanical shearing during agitation.

DISCUSSION

The purpose of this research was to develop mixed cultures having growth rates and/or protein production above that of their pure culture

counterparts and to explore the various growth parameters of the mixed cultures.

Eleven seeded enrichment cultures were obtained and compared by viable cell numbers and mesquite wood hydrolyses with their pure seed cultures and with a simple enrichment culture. All of the enrichment cultures hydrolyzed the cellulose strips. Also, the seeded enrichment cultures exceeded the pure cultures in mesquite hydrolysis and/or viable cell counts. The simple enrichment culture CAD12 was approximately equivalent in activity to culture CAD2. Therefore, seeded enrichment cultures were better than pure cultures in some respects, and it was reasonable to continue the study.

Because the growth of the enrichment cultures increased as the yeast extract concentration increased, they all used it as a substrate. This conclusion was strengthened by the appearance of a diauxie at a yeast extract concentration of 0.01% (Fig. 1 and Table 1). Therefore, exponential growth occurred at 0.025%, but at a yeast extract concentration of 0.01% there were pronounced diauxies in the growth curves. Therefore, the yeast extract was used first, followed by cellulase synthesis, and then a second growth phase occurred on the CMC. The lack of diauxic growth curves in the presence of 0.025 or 0.05% yeast extract may be due to repression of cellulase synthesis. Thus, yeast extract in high concentrations could limit substrate utilization. Greater growth occurred in the presence of 0.01% yeast extract than at higher concentrations in four of the five mixed cultures. Casamino Acids promoted growth as well as yeast extract did. Apparently yeast extract and Casamino Acids served as complex carbon and/or nitrogen sources but not as required vitamin or amino acid sources.

All five seeded enrichment cultures possessed cellobiase activity because they grew on cellobiose and produced greater cell yields with this substrate than with any other substrate tested.

Growth and protein production by the enrichment cultures were optimum at a wood concentration of 1.0% (Table 2). Because the amount of hot-water-soluble wood sugar was directly dependent on the wood concentration, an optimum substrate concentration of 1.0% is surprising. Attachment of the cells to the substrate may have resulted in erroneously low protein values at higher substrate concentrations. The viable cell number would not be as subject to this error because it was obtained from samples of the entire culture. It might be desirable to determine protein yields by analysis of a centrifuged pellet of wood and cells. Unfortunately, the wood residues contain varying amounts of protein and total nitrogen, and the wood also interferes with protein analysis by both the biuret method and the method of Lowry et al. If the data for culture CAD5 were interpreted on viable cell counts, the optimum substrate concentration would be 10%. A concentration of 7.5% (wt/vol) mesquite wood was optimum for the growth of *Pseudomonas* JM127. Inhibitory concentrations of phenolic substances may have been present when higher concentrations of substrate were used. The enrichment culture systems may unexpectedly be more sensitive to such inhibition than a pure culture of a pseudomonad.

Both the optimum tempratures and the optimum pH values for each of the enrichment cultures were representative of the conditions under which they were developed. A temperature of 37°C would be desirable industrially to reduce cooling requirements. Optimum growth at pH 7 might require rigorous control during industrial production. Our results indicated that a pH of 7 was optimum for growth initiation because the pH was not controlled during growth.

All four cultures grew on alpha-cellulose, although not as well as on mesquite wood. Alpha-cellulose hydrolysis during growth was very similar to wood hydrolysis. The error in the hydrolysis measurement probably did not exceed that for mesquite wood (1 to 2.5%). Analyses of the growth of the cultures on alpha-cellulose compared with the growth on mesquite wood provided interesting contrasts. Although both media had the same substrate and yeast extract concentrations, protein production from the mesquite wood was two- to threefold greater. Cellulase activity was greater with a wood substrate. Growth of the pure seed cultures on alpha-cellulose and wood solubles compared with growth obtained with mesquite wood culture media proved that the growth of the cultures on the wood involved more than a single substrate. Cellulose was hydrolyzed, but hot-water-soluble sugars and yeast extract also supported growth.

CAD5 and CAD11 yielded 0.6 and 0.7 mg of protein per ml, respectively. The relationship between protein yield and substrate concentration requires additional investigation. The actual yields of protein may have been greater. When the cultures were filtered to remove the unhydrolyzed substrate, some of the bacterial cells were also removed from the medium. Attachment of cells to the substrate may have lowered the viable cell numbers and protein yields.

One of the purposes of this study was to evaluate the use of enrichment cultures for production of single-cell protein from cellulosic substrates. Such cultures may have an advantage over pure cultures, but they may be more sensitive to inhibitory substances associated with

the substrates and may be unstable during industrial production. It is possible that only a single strain or two or three symbiotic strains may be responsible for the properties of these cultures. These possibilities need to be investigated before such cultures can be used industrially. With a single substrate it might be possible to design a culture by mixing strains with known properties. The pure cultures which grew on mesquite wood were not very cellulolytic (13), but they grew better than a *Cellulomonas* strain (14). The seeded enrichment technique was effective in the selection of a superior culture for growth on mesquite wood with its many substrates. Although it should be possible to obtain an equally superior culture by selection from several simple enrichment cultures, the seeded enrichment cultures were superior to the simple enrichment culture CAD12 used as a control in this study.

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