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Cellulose Decomposition and Associated Nitrogen Fixation by Mixed Cultures of *Cellulomonas gelida* and *Azospirillum* Species or *Bacillus macerans*

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Mixed cultures of *Cellulomonas gelida* plus *Azospirillum lipoferum* or *Azospirillum brasilense* and *C. gelida* plus *Bacillus macerans* were shown to degrade cellulose and straw and to utilize the energy-yielding products to fix atmospheric nitrogen. This cooperative process was followed over 30 days in sand-based cultures in which the breakdown of 20% of the cellulose and 28 to 30% of the straw resulted in the fixation of 12 to 14.6 mg of N per g of cellulose and 17 to 19 mg of N per g of straw consumed. *Cellulomonas* species have certain advantages over aerobic cellulose-degrading fungi in being able to degrade cellulose at oxygen concentrations as low as 1% O_2 (vol/vol) which would allow a close association between cellulose-degrading and microaerobic diazotrophic microorganisms. Cultures inoculated with initially different proportions of *A. brasilense* and *C. gelida* all reached a stable ratio of approximately 1 *Azospirillum*/3 *Cellulomonas* cells.

The microbial breakdown of straw and other agricultural residues which are rich in cellulose is usually limited by the low nitrogen content of these products. When straw is incorporated into the soil, the amount of plant-available nitrogen is diminished by its incorporation into the increased microbial biomass associated with straw decomposition (21), thus diminishing that available for plants. This limitation would be alleviated if an association could be formed between cellulose-decomposing and nitrogen-fixing organisms such that each supplies the requirements of the other for combined nitrogen and degradable carbon, respectively.

This concept is not new; it was investigated by Jensen and Swaby (9) and Jensen (8) with a number of cellulolytic organisms and *Azotobacter* spp. (9) or *Clostridium butyricum* (8). Mixed cultures of these organisms degraded cellulose or straw and fixed atmospheric nitrogen under conditions of limited aeration and, in the case of *Azotobacter* spp., at neutral pH. A different approach combining cellulolytic fungi and anaerobic nitrogen-fixing organisms (14, 24) has also been successful under conditions of limited aeration.

In both of these types of association, the critical factor is the degree of aeration, the cellulolytic partner usually requiring oxygen, and the nitrogen-fixing partner being inhibited by oxygen (although *Azotobacter* spp. are more tolerant to oxygen than are other diazotrophs). As a result, the two organisms must be separated spatially, the products of one partner diffusing to the other. The diffusing product is available to all other organisms in the immediate vicinity, thus reducing the efficiency of the interaction under nonsterile conditions.

Field studies (20) have shown that nitrogenase activity may be associated with the decomposition of wheat straw incorporated into moist soils. A problem associated with waterlogged or anaerobic conditions in soil is that the final breakdown products of straw tend to be phytotoxic (13). Hence, it is important that the bacteria responsible for cellulose breakdown and those fixing N_2 be able to function under conditions that do not favor the accumulation of acetic acid and other fatty acid end products.

This study examines cooperative cellulose decomposition and nitrogen fixation by mixed cultures of *Cellulomonas gelida* plus *Azospirillum* spp. and *C. gelida* plus *Bacillus macerans*. These organisms were selected as potential partners because all can function under microaerobic conditions (4, 5, 15) and *Cellulomonas* spp. tend to form a close association with a substrate (18); extracellular enzyme is only released into the medium in quantity at the end of exponential growth (1).

MATERIALS AND METHODS

Cultures. *Azospirillum brasilense* Sp7 (ATCC 29145) and *A. lipoferum* 5A (from J. Döbereiner, EMBRAPA, Séropédica, Rio de Janeiro, Brazil) and *B. macerans* 8514 (from W. J. Brill, University of Wisconsin, Madison) were provided by courtesy of F. J. Bergersen. *B. macerans* 111 was isolated from soil previously amended with straw and identified by computer-assisted procedures for the identification of dinitrogen-fixing bacteria (17). *C. gelida* UQM 2480 was obtained from L. Sly, University of Queensland, St. Lucia, Queensland, Australia.

Experimental conditions. (i) Sand cultures. Mixed cultures of *C. gelida* and *Azospirillum* spp. or *B. macerans* were established in sand (6) with cellulose or straw as the sole carbon source and with little or no combined nitrogen to follow the course of nitrogen fixation or C_2H_2 reduction. River sand (<1.4-mm mesh) was heated at 60°C in concentrated HCl for 16 h, washed in running water for 6 h, rinsed in 20 mM $CaCl_2$ for 20 min, rinsed in water for 5 min, and dried; 20-g amounts were added to Universal bottles and autoclaved twice. Cellulose (100 mg, Avicel microcrystalline; Merck) or finely ground wheat (cv. Songlen) straw (100 mg, sterilized by 10 Mrad, γ irradiation; Australian Atomic Energy Commission, Lucas Heights, New South Wales, Australia) was added aseptically to the autoclaved sand and mixed thoroughly. Washed cell suspensions (0.5 ml) of each organism were added to the sands which were adjusted to 27.5% (wt/wt) moisture content

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(almost saturated) with nutrient solution. This solution contained (per liter): $MgSO_4 \cdot 7H_2O$, 0.2 g; NaCl, 0.1 g; $CaCl_2$, 0.02 g; $Na_2MoO_4 \cdot 2H_2O$, 0.002 g; $MnSO_4 \cdot H_2O$, 0.01 g; FeNaEDTA (1.64% [wt/vol] aqueous), 4.0 ml. Sterile phosphate buffer KH_2PO_4-NaOH (pH 7.0) (final concentration of 100 mM) and filter-sterilized vitamins (biotin, 5 mg; *p*-aminobenzoate, 10 mg; thiamine, 10 mg) were added aseptically to complete the solution. When cellulose was used as the carbon source, a low level of starter nitrogen [$(NH_4)_2SO_4$, 10 mg/liter] was added to the nutrient solution. After inoculation, the bottles were sealed with autoclaved Suba seals (Wm. Freeman, Barnsley, United Kingdom) and were incubated at 30°C. The gas phase above the sand was 13 ml.

Concentrations of CO_2 in the gas phase were monitored daily before being flushed with filtered compressed air to maintain the O_2 concentration and remove accumulated CO_2. After 5 to 7 days, C_2H_2 (3.0 ml) was injected into each bottle, and 24 h later (and other intervals as indicated), the concentrations of CO_2, C_2H_4, and C_2H_2 in the gas phase (13 ml) were determined (6, 23). In the 30-day experiment, C_2H_2 and C_2H_4 were removed from the cultures by evacuation before being flushed with air and reincubated for a further 11 days. At the completion of the experiment, straw and cellulose were extracted from the sand by flotation and filtration, and dry weights were determined. Total nitrogen was determined after Kjeldahl digestion of two sets of four replicates of all treatments; these sets had not been exposed to acetylene.

All experiments were done with four replicates.

(ii) Liquid cultures. The degradation of cellulose and straw in the presence of combined nitrogen and under a range of oxygen concentrations was followed in liquid culture. A mineral salts medium (11) amended with FeNaEDTA (1.64% [wt/vol] aqueous, 4 ml/liter), dialyzed yeast extract (0.5 g/liter), and sterile phosphate buffer (KH_2PO_4-NaOH [pH 7.0], final concentration of 100 mM) was dispensed (20 ml per flask) into sterile 125-ml Erlenmeyer flasks containing 100 mg of cellulose (Avicel). The yeast extract was dialyzed to remove yeast cell walls, and the low-molecular-weight components were concentrated in a rotary evaporator before use. After inoculation, flasks were sealed with sterile Suba seals and flushed with filtered air (21% O_2) or nitrogen; O_2 was added to provide 5, 1, and <0.1% (vol/vol) O_2. The flasks were incubated at 30°C and shaken at 200 rpm. Gas phase O_2 levels were determined by using a Shimadzu GC-8A gas chromatograph fitted with a molecular sieve 5A column and a thermal conductivity detector. Carbon dioxide levels were determined daily as previously described, and each flask was then flushed with air or N_2 and the O_2 levels were adjusted; subsequent adjustments were made at 6- and 12-h intervals as required.

TABLE 1. Production of ethylene and carbon dioxide by single and mixed cultures of *C. gelida* UQM 2480 and *Azospirillum* spp. or *B. macerans* grown on cellulose or straw in sand culture[a]

Cultures	Cellulose		Straw	
	C_2H_4[b]	CO_2[c]	C_2H_4[b]	CO_2[c]
Single				
C. gelida	1	8.3	5	46.7
A. lipoferum 5A	1	3.1	1,672	47.9
A. brasilense Sp7	0	5.4	1,993	57.3
B. macerans 8514	5	3.9	13	39.5
B. macerans 111	1	4.2	115	60.8
Mixed				
C. gelida + *A. lipoferum* 5A	2,468	20.9	7,299	75.4
C. gelida + *A. brasilense* Sp7	1,311	26.4	2,533	77.7
C. gelida + *B. macerans* 8514	2,781	19.8	756	46.4
C. gelida + *B. macerans* 111	2,348	20.6	2,595	56.7

[a] Gas determinations were made over a period 72 to 96 h after inoculation. There were 4 replicates.
[b] Nanomoles of C_2H_4 produced per 24 h.
[c] Micromoles of CO_2 produced per 24 h.

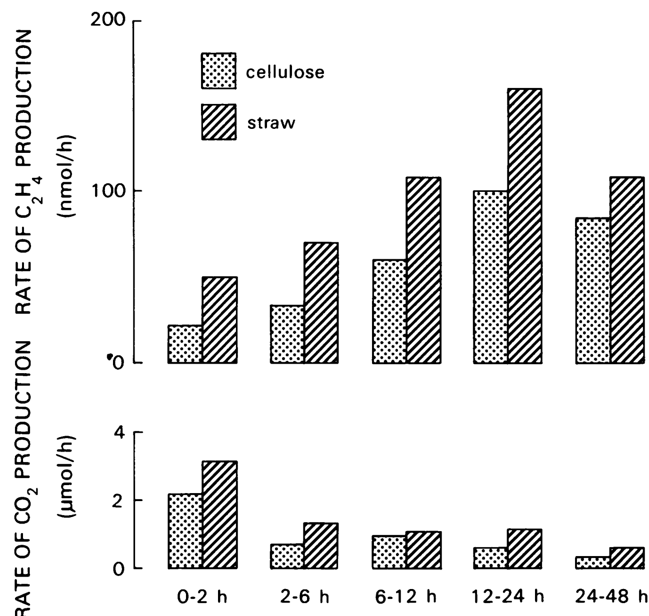

FIG. 1. Rates of ethylene and carbon dioxide production by 8-day-old mixed cultures of *A. brasilense* and *C. gelida* in sand, over 48 h after the introduction of acetylene.

After 7 days, the contents of each flask were separated into two equal portions. The first portion was filtered through a 0.45-μm-pore-size membrane filter (Millipore Corp., Bedford, Mass.), and the dry weight of the residue was determined; the filtrate was used as an enzyme preparation to determine cellulase activity. The second portion was centrifuged, and the pellet was washed with water by suspension and centrifugation. It was then suspended in distilled water to the original volume, and a portion was assayed for protein by the method of Lowry et al. (12). The centrifugal supernatant was used to determine protein, organic matter (10), and total soluble carbohydrate by the phenol-sulfuric acid method (3). All experiments were done with four replicates.

(iii) Inocula. For inoculation, *C. gelida* was grown on mineral salts medium (11) with glucose at 2 g/liter and carboxymethyl cellulose at 1 g/liter. *Azospirillum* spp. and *B. macerans* were grown on N-free malate medium (2) supplemented with $(NH_4)_2SO_4$, 5 g/liter; sucrose, 4 g/liter; KH_2PO_4-NaOH (pH 7.0), 60 mM; and vitamins (thiamine, 10 mg/liter; biotin, 5 mg/liter; and PAB, 10 mg/liter). All cultures were grown for 48 to 72 h at 30°C and shaken at 200 rpm.

Measurement of cellulase activity. Culture filtrate (5 ml) was added to the reaction mixture (5 ml) consisting of 0.1 M KH_2PO_4-NaOH buffer (pH 7.0) containing 1% sodium azide and 100 mg of Avicel in 125-ml Erlenmeyer flasks. Zero-time samples (2 ml) were taken before the flasks were plugged and

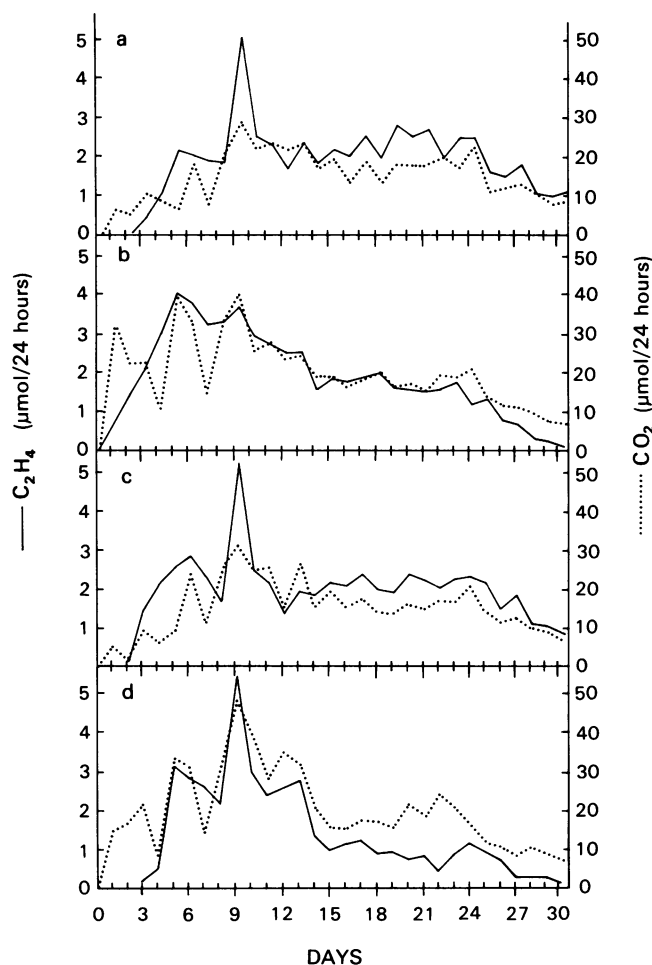

FIG. 2. Production of ethylene (—) and carbon dioxide (-----) by mixed cultures of *C. gelida*, *A. brasilense*, and *B. macerans* growing on cellulose or straw in sand culture for 30 days. (a) *A. brasilense* + *C. gelida* on cellulose, (b) *A. brasilense* + *C. gelida* on straw, (c) *B. macerans* + *C. gelida* on cellulose, (d) *B. macerans* + *C. gelida* on straw.

incubated at 30°C with a shake rate of 200 rpm for 24 h. Final samples (2 ml) were then taken, the Avicel was removed by centrifugation, and a 1-ml sample of the supernatant was assayed for reducing sugar by the Somogyi-Nelson colorimetric determination read at 500 nm (22).

Plate counts of mixed cultures of *Azospirillum* spp. and *C. gelida*. A dilution series was prepared from the mixed sand cultures by adding 4.5 ml of 1/4-strength Ringer solution with 0.01% Tween 20, to each bottle to bring the total volume of liquid to 10 ml (10^{-1} dilution). The diluted sand cultures were mixed thoroughly, and a dilution series to 10^{-8} was prepared in 1/4-strength Ringer solution with 0.01% Tween 20. Plates of potato infusion medium (2) were prepared, and 1 ml of the appropriate dilution was added to 2 ml of a soft agar overlay (1% water agar containing 0.1 mg of Congo red per ml) at 60°C and poured as a thin, even layer over the dry surface of the plates. The Congo red was taken up by the *A. brasilense* colonies, which appeared bright pink (19); it did not affect the color or growth of the *C. gelida* colonies.

RESULTS

Acetylene reduction by mixed cultures of *C. gelida* and *Azospirillum* spp. or *B. macerans* with cellulose or straw as the carbon source. When mixed cultures of *C. gelida* and either of the *Azospirillum* species or *B. macerans* were grown on cellulose mixed through sterile sand maintained at 27.5% (wt/wt) moisture content, nitrogenase activity was high, whereas in the pure cultures of the diazotrophs it was negligible (Table 1). With straw as the substrate, the increased activity due to the presence of *C. gelida* was less marked because *Azospirillum* spp. (6), and to a lesser extent *B. macerans*, are capable of using components of straw other than cellulose as an energy source for nitrogenase activity. With the cellulose treatments, CO_2 production by mixed cultures was three- to fourfold higher than from single-strain cultures. These effects were not found in the straw treatments.

The time course of acetylene reduction and carbon dioxide production followed over 48 h in replicated mixed cultures, to which the C_2H_2 was introduced 8 days after inoculation, showed that the rate of C_2H_4 production increased over the first 24 h and then decreased (Fig. 1). Suggested explanations for this delay in reaching the maximum rate of C_2H_4 production include the time required for C_2H_2 to diffuse to the site of activity and possible derepression of nitrogenase as a result of nitrogen deprivation. Carbon dioxide concentrations rose sharply in the 2 h immediately after being flushed with air, an observation probably associated with residual dissolved CO_2 equilibrating with the gas phase. As a result of this study, C_2H_4 and CO_2 determinations were made routinely 24 h after the addition of C_2H_2. If the measured rate of nitrogenase activity in the 12- to 24-h period is representative of the continuing level of activity in these cultures, the 0- to 24-h value will underestimate the equilibrium rate by 25 to 28%.

The time course of cellulose and straw degradation and associated nitrogen fixation by mixed cultures were studied over a 30-day period; 12 sets of four replicate sand cultures for each treatment were assayed for C_2H_2 reduction and CO_2 production, each set at 12-day intervals. Mixed cultures of either *A. brasilense* or *B. macerans* with *C. gelida* gave similar patterns of C_2H_4 and CO_2 production, with cellulose as the substrate (Fig. 2a and c). Production of CO_2 commenced immediately and showed sharp fluctuations in the early part of the experiment, later moderating to a more stable pattern. Production of C_2H_4 was not detected until

TABLE 2. Nitrogen fixed, ethylene and CO_2 produced, and cellulose or straw utilized over a 30-day period by mixed cultures of *C. gelida* and *A. brasilense* or *B. macerans* inoculated into sand to provide a 27.5% (wt/wt) moisture content[a]

Mixed culture	Total C_2H_4 (μmol)	Increase in N (μmol of N_2)[b]	C_2H_2/N_2	Total CO_2 (μmol)	Cellulose or straw lost (mg)	mg of N fixed/g of straw or cellulose lost
A. brasilense + *C. gelida*						
Cellulose	56	12.0	4.67	464	22.9	14.6
Straw	57	18.8	3.03	619	30.7	17.1
B. macerans + *C. gelida*						
Cellulose	58	8.8	6.59	469	20.3	12.0
Straw	41	19.5	2.10	631	28.9	18.9

[a] Results are from four replicates.
[b] Increase in N estimated by Kjeldahl digestion on a set of replicate cultures not exposed to C_2H_2.

TABLE 3. *C. gelida* UQM 2480 grown on cellulose (100 mg) or straw (100 mg) under 21 or <0.1% O_2 for 14 days in shaken liquid culture (200 rpm) in 125-ml Erlenmeyer flasks (20 ml per flask)[a]

Substrate and oxygen concn (%)	% Substrate recovered (± SE)	% Substrate utilized[b]	Total protein (mg/flask)
Cellulose			
21	39.5 ± 1.6	52.7	15.6
<0.1	86.3 ± 1.4	5.9	13.1
Sterile control	92.2 ± 0.9		11.2
Straw			
21	52.0 ± 2.0	20.7	44.4
<0.1	66.4 ± 1.1	6.3	36.3
Sterile control	72.7 ± 0.4		35.8

[a] Carbon dioxide was removed by flushing with air or N_2 at 6- or 12-h intervals as required. There were four replicates.
[b] Corrected for substrate recovered in sterile control.

day 3 and showed similar sharp fluctuations which were not necessarily in phase with those of CO_2 production. In the later part of the experiment, C_2H_4 and CO_2 production tended to fluctuate in phase.

With straw as substrate, the *A. brasilense-C. gelida* cultures commenced C_2H_4 production immediately (Fig. 2b), and the sharp fluctuations observed on cellulose were less pronounced. This is probably due to the ability of *A. brasilense* to utilize the xylan component of straw for nitrogenase activity (C_2H_2-dependent C_2H_4 production) (6). The activity of *B. macerans-C. gelida* cultures on straw (Fig. 2d) was similar to that observed on cellulose, except that the duration of nitrogenase activity was shortened. In two replicate sets for each strain mixture on each substrate, there were significant increases in total nitrogen (Table 2). The increases were greater in the straw-based cultures. Gravimetric determination of the remaining cellulose or straw indicated significant utilization of these substrates, with the greater loss occurring with the straw cultures. The apparent discrepancy between CO_2 production and cellulose consumed is explained by the increase in biomass of the cultures, based on protein determinations and the observation that cultured cells contain 43.5% protein.

Effect of oxygen concentration on the breakdown of cellulose and straw by *C. gelida*. *C. gelida* grown on cellulose with a combined nitrogen source utilized 53% of the cellulose under aerobic conditions, compared with 6% under anaerobic conditions (i.e., <0.1% O_2) over a 14-day period (Table 3). The cells were bound firmly to the cellulose so that when the cellulose was harvested by filtration (8.0-μm-pore-size membrane) the bacteria were also removed. Hence, in subsequent experiments the cells and cellulose were removed together by filtration through 0.45-μm-pore-size membrane filters. The protein content of the cellulose pellet and the supernatant was determined separately; the total protein increased over the 14 days by 4.38 mg per flask under aerobic conditions compared with 1.88 mg per flask under anaerobic conditions. Straw was not so readily attacked, only 21% being utilized over 14 days under aerobic conditions and 6% under anaerobic conditions.

The effect of oxygen concentration on the utilization of cellulose was examined in more detail using 21, 5, 1, and <0.1% oxygen. In this experiment, the production of CO_2 was followed, and the culture medium was examined in an attempt to elucidate the nature of the metabolic products under different oxygen concentrations. The percentage of cellulose utilized (Table 4) increased slightly with decreasing oxygen concentration from 48.7% at 21% O_2 to 54% at 1% O_2, but at <0.1% O_2 only 6.2% of cellulose was utilized. Hence, the ability to metabolize cellulose was sustained at oxygen concentrations in the gas phase as low as 1%.

Cell dry weight was unchanged at 5% O_2, but below this level it decreased. CO_2 production was very oxygen-dependent, showing a decrease at all concentrations below 21% O_2.

The culture supernatant contained carbohydrate material, including reducing sugars. The differential between the glucose equivalents detected by the Somogyi-Nelson method for reducing sugar and the phenol-sulfuric acid method for total carbohydrate suggests that the supernatant may have contained more di- and trisaccharides than monosaccharides; excreted bacterial polysaccharides would also contribute to the difference. Determination of total organic matter in the supernatant showed a high value for the low oxygen treatments, 1 and <0.1%, indicating the presence of noncarbohydrate compounds, but it is difficult to evaluate this assay when the compounds are unknown; different compounds give different results in terms of milliequivalents

TABLE 4. *C. gelida* UQM 2480 grown on cellulose at 30°C under various O_2 concentrations for 7 days in shaken (200 rpm) liquid culture in 125-ml Erlenmyer flasks (20 ml per flask)[a]

O_2 concn (%)	% Cellulose recovered ± SE	% Cellulose utilized[b]	Dry wt of cells (mg) ± SE	CO_2 produced in 7 days (μmol)	Organic matter in supernatant (meq of dichromate)	Carbohydrate in supernatant (μg/ml)[c]	Reducing sugar in supernatant (μg/ml)[d]	Cellulase activity in supernatant[e]
21	42.5 ± 1.2	48.70	29.0 ± 1.1	2,204	1.65	56.6	11.25	82.0
5	40.6 ± 0.3	50.57	29.3 ± 0.7	1,582	1.75	55.7	9.75	41.3
1	37.2 ± 1.1	54.00	16.0 ± 0.5	585	3.82	43.1	6.0	7.8
<0.1	84.9 ± 2.7	6.25	10.6 ± 0.9	27	2.45	21.6	6.0	1.75
Uninoculated controls	91.2 ± 1.1				2.46	24.4	7.5	

[a] Carbon dioxide was removed by flushing with air or nitrogen, and the O_2 concentration was adjusted accordingly every 6 or 12 h as necessary. Results are from four replicates.
[b] Corrected for substrate losses in uninoculated controls.
[c] Measured by the phenol-sulfuric acid method as glucose equivalents.
[d] Measured as glucose equivalents.
[e] Measured as Δglucose in micrograms per milliliter per 24 h.

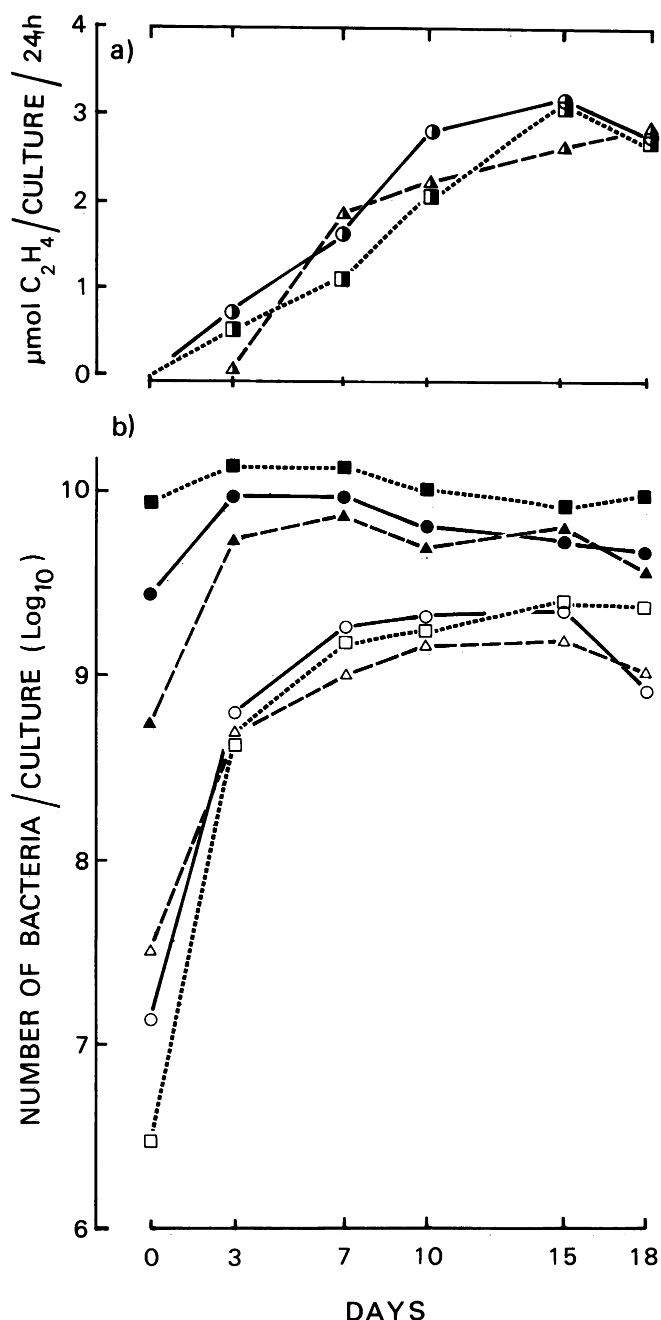

FIG. 3. Acetylene reduction and cell numbers of *A. brasilense* and *C. gelida* in sand culture when the ratio of *A. brasilense* to *C. gelida* in the inoculum was 0.0579 (△, ▲), 0.0051 (○, ●), and 0.0004 (□, ■). (a) Acetylene reduction, (b) cell numbers: *A. brasilense*, open symbols; *C. gelida*, closed symbols.

of dichromate reduced per millimole (10). The presence of cellulose-degrading enzymes in the supernatant was evidenced by the increase in reducing sugar (Table 4), when the cell-free supernatant was incubated with cellulose; the level of these enzymes was dependent strongly on the O_2 level in the gas phase during growth of the cultures.

Ratio of *A. brasilense* to *C. gelida* cells in mixed cultures. The effects of varying the ratio of cells of the two partners in a mixed culture were investigated in sand cultures, with cellulose as the sole carbon source. Three ratios of *A. brasilense* to *C. gelida* (values of 0.0578, 0.005, and 0.0004) were established with approximately 10-fold differences between them.

There was a rapid increase in the *A. brasilense* populations in the first 3 days after inoculation (Fig. 3b), especially in the treatment with the lowest *A. brasilense*/*C. gelida* ratio (3×10^6 to 4.62×10^8). The numbers of *C. gelida* doubled in this period, with the net effect that the *A. brasilense*/*C. gelida* ratios changed to 0.088, 0.067, and 0.031. Thereafter the *C. gelida* populations showed a small decline in numbers, whereas the *A. brasilense* populations showed a small increase. By day 15, the *A. brasilense*/*C. gelida* ratios were 0.25, 0.38, and 0.34. Nitrogenase activity increased to a maximum on day 15 (Fig. 3a) concomitant with the increase in the *A. brasilense* populations (Fig. 3b). The failure to detect C_2H_4 reduction on day 3 in the treatment with the highest *A. brasilense*/*C. gelida* ratio was probably due to the initial population of *C. gelida* being too small (0.5×10^9 cells per ml, cf. 2.8×10^9 and 8×10^9) to provide the necessary carbohydrate for the *A. brasilense* population.

DISCUSSION

Mixed cultures of *C. gelida* plus *A. brasilense* or *A. lipoferum*, or *C. gelida* plus *Bacillus macerans* were shown to be capable of utilizing the products of the breakdown of cellulose to fix atmospheric nitrogen. When such mixed cultures were studied over 30 days, nitrogenase activity (C_2H_2 reduction) and CO_2 production fluctuated considerably in the early stages. After 15 days the fluctuations decreased and both processes approached equilibrium rates which continued for a further 15 days. More than 20% of the cellulose was utilized (i.e., was not recovered by gravimetric determination), with 14.6 and 12.0 mg of N_2 fixed per g of cellulose lost for the *A. brasilense* and *B. macerans* cultures, respectively. These values indicate that these systems are more efficient than those involving the aerobic *Trichoderma harzianum* and the anaerobic *Clostridium butyricum* (3.3 mg of N_2 per g of cellulose [24]) and have similar or greater efficiency than mixed cultures of *Azotobacter* spp. and *Corynebacterium* sp. (6 to 14 mg of N_2 per g of cellulose [9]). Over the 30-day period, 28 to 30% of the straw was utilized, with efficiencies of 17.1 and 18.9 mg of N_2 per g of straw lost for the *A. brasilense* and *B. macerans* mixed cultures, respectively. Again, these results compare favorably with published data for other mixtures of microorganisms growing on this substrate, e.g., 11.5 mg of N_2 per g of straw with *Penicillium corylophilum* and *Clostridium butyricum* (14).

Although the mean of the four determined C_2H_2/N_2 ratios (Table 2) was 4.1:1, very close to the theoretical 4:1 ratio when H_2 evolution associated with nitrogenase function is considered, there was wide variation between the four contributory values. Furthermore, the C_2H_2 reduction rates are probably 25 to 28% lower than the true rate of nitrogenase activity. The straw values were consistently lower than those for the cellulose cultures, but no reasonable explanation can be provided for this observation. These data highlight the problem of using C_2H_2 reduction rates to estimate nitrogen fixation and support the increasing concern in the use of this technique to derive absolute values.

The oxygen concentration within the mixed cultures is critical. *Azospirillum* spp. require an oxygen concentration below 0.7% in the gas phase for nitrogen fixation to occur (16, 25). Nitrogen fixation by *Bacillus polymyxa* is also inhibited by oxygen concentrations above 1% (7). For this reason, the ability of *C. gelida* to degrade straw and cellulose at oxygen concentrations as low as 1% or less is important,

as this would enable cells of the two genera to form a close association. This would be important in a soil or compost situation where other organisms would compete for the products of cellulose decomposition. In our experimental situation, the cellulose-straw was mixed through coarse river sand maintained at a moisture content which provided many water-filled pores and therefore an abundance of microaerobic sites.

Azospirillum spp. would appear to have certain advantages over *B. macerans* as a partner for *C. gelida* on a substrate of straw in that *Azospirillum* can attack the hemicellulose component of the straw (6), thus initiating earlier nitrogen fixation and providing the *C. gelida* with starter nitrogen (Fig. 2b).

The initial ratio of cellulose-degrading organisms to nitrogen-fixing organisms does not appear to be important as the mixed cultures are capable of rapid adjustment to equilibrium ratios of 1 *Azospirillum* to 3 *Cellulomonas* cells within 7 days.

Straw residues constitute a large reservoir of "difficult-to-degrade" carbon compounds with the potential to provide sufficient energy for agriculturally significant levels of nitrogen fixation (20). Although *Azospirillum* spp. are capable of metabolizing the hemicellulose xylan and using the degradation products for nitrogen fixation (6), the full availability of these hemicelluloses could be reduced by virtue of their complex structural association with cellulose and lignin residues. Many cellulolytic bacteria and fungi appear to function aerobically, whereas bacteria fixing nitrogen are restricted largely to conditions of low oxygen tension. The current results show that cellulolytic activity associated with *C. gelida* can stimulate greatly the nitrogen-fixing activities of both *Azospirillum* spp. and *B. macerans* and that cellulase can be produced under conditions of low oxygen tension. It remains to be shown whether this is due to a close physical relationship between the various bacteria, whether diffusion of cellulose breakdown products into a suitable "nitrogen-fixing zone" is involved, or whether exocellulase diffusion is important. The high levels of efficiency observed provide optimism that these organisms can be used to promote straw breakdown under field conditions and thus enhance the nitrogen status of the soil.

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