Acetylene Reduction (Nitrogen Fixation) by Pulp and Paper Mill Effluents and by Klebsiella Isolated from Effluents and Environmental Situations

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High rates of acetylene (C₂H₂) reduction (nitrogenase activity) were observed in woodroom effluent from a neutral sulfite semi-chemical mill under aerobic (up to 644 nmol of C₂H₂ produced per ml per h) and under anaerobic (up to 135 nmol of C₂H₂ produced per ml per h) conditions. Pasteurized effluent developed C₂H₂ reduction activity when incubated under anaerobic but not under aerobic conditions. Activities were increased by addition of 0.5 to 3.0% glucose or xylose. Enrichment and enumeration studies showed that N₂-fixing Azotobacter and Klebsiella were abundant, and N₂-fixing Bacillus was present. Of 129 isolates of Klebsiella from pulp mills, lakes, rivers, and drainage and sewage systems, 32% possessed nitrogen-fixing ability.

Klebsiella strains were reported isolated from forest and vegetable produce (7), rivers (14), agricultural and other run-off (19), sugarcane (15), and pulp and paper mill effluents (18). Nitrogen-fixing strains of K. pneumoniae were isolated from animal gut contents (1), soil (12), plant material (8), decaying wood (17), plant phylloplane (2), and other sources (4, 13). Other N₂-fixing members of the Enterobacteriaceae were isolated from animals (1), soil (12), and corn rhizosphere (16). Large numbers of coliform bacteria (including Klebsiella strains) were reported in pulp and paper mill liquors and effluents (18), and Azotobacter agile was isolated from strawboard waste water (9), but nothing was known about the potential for N₂ fixation of the Klebsiella isolated or of the pulp and paper effluents themselves.

This paper describes C₂H₂ reduction activity of three types of pulp mill waste materials as well as of organisms isolated from these and other sources.

MATERIALS AND METHODS

Pulp mill effluent samples. On eight separate occasions between December 1972 and June 1974, samples of woodroom effluent (supernatant and settled sludge) were collected from a neutral sulfite, semi-chemical mill, pulping mixed species of unbarked eastern Canadian hardwoods. The temperature of the effluent at sampling time was usually in the range of 31 to 37°C. This mill recycles a considerable amount of process waters from the paper machine to the log washing and pulping areas. The log-wash effluent is passed through a 150-μm Kason screen. The retained bark and grit is land-filled, the passed fraction (woodroom effluent) is settled, and the supernatant is recycled again for log washing. In December 1972, samples were also obtained from an 8-day retention biological oxidation lagoon (pilot plant).

Cultures. Strains of Klebsiella spp. isolated from various sources were received from A. D. Tennant, Ottawa (described in 14, 18, 19) and from D. W. Duncan, Vancouver (personal communication). Others were isolated from brilliant-green bile broth most-probable-number (MPN) tubes and from positive enrichments of effluent to which 0.5% glucose was added. Azotobacter was isolated from aerobically incubated effluent by successive enrichment culture (C₂H₂ reducing) in aerobic glucose mineral salts N-free medium (5). Bacillus was isolated from pasteurized (80 to 85 C for 15 min) effluent amended with 0.5% glucose incubated under N₂, followed by successive enrichment culture (C₂H₂ reducing) under N₂ in modified Hino and Wilson N-free medium (11) supplemented with 25 μg of yeast extract per ml.

Microbial counts. Microbial counts were performed on unpasteurized and pasteurized (80 to 85 C for 15 min) samples of effluent before and after 24 h of aerobic and anaerobic incubation. Dilutions were prepared in mineral salts solution (5), and appropriate dilutions were used to inoculate the following: (i) Difco stock culture agar for an aerobic total viable plate count; (ii) glucose mineral salts N-free agar (5) for an Azotobacter plate count (colonies were checked for typical Azotobacter); (iii) Difco brilliant-green lactose bile broth tubes (five per dilution) for a coliform most-probable number estimate; (iv) 0.01-ml quantities from the highest positive dilutions of (iii) were transferred to tubes of modified Hino and Wilson

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medium, and turbid or gas-producing tubes or both were assayed for C\textsubscript{2}H\textsubscript{4} reduction (the MPN estimate was based on those dilutions which gave a positive result in this test); and (v) modified Hino and Wilson medium in aerobic tubes (five per dilution) to give an MPN estimate based on the criteria above. All plates and tubes were incubated at 35 C.

**Acetylene reduction.** Effluent samples and active cultures growing on appropriate media (10 ml in a 50-ml Erlenmeyer flask) under air or N\textsubscript{2} were incubated at 35 C on a gyratory shaker (140 rpm). At appropriate time intervals C\textsubscript{2}H\textsubscript{4} was injected to give 0.1 atmosphere pC\textsubscript{2}H\textsubscript{4}, and, after 1.0 or 1.5 h of incubation, 0.5 ml of gas phase was sampled for gas chromatographic analysis of C\textsubscript{2}H\textsubscript{4} produced. The gas chromatography system consisted of a column (300 cm by 3 mm) of 50- to 80-mesh Porapak R at 45 C with an N\textsubscript{2} carrier at 25 ml/min and an H\textsubscript{2} flame-ionization detector. In part of the study a 80- to 100-mesh Porasil C/methyl isocyanate column (200 cm by 3 mm) was used at 45 C with an N\textsubscript{2} carrier at a 14 ml/min flow rate. The experimental flasks were either discarded after assay or were aseptically evacuated and filled three times to 1 atmosphere, with the appropriate gas phase both before and after each assay to prevent carry-over of C\textsubscript{2}H\textsubscript{4} between successive assays.

**Growth measurements.** In some experiments cultures were grown in 50- or 500-ml Erlenmeyer flasks with matched side arms, and optical densities were determined at 430 nm. In these experiments, 10-ml portions were transferred by syringe into 50-ml serum-stoppered N\textsubscript{2}-filled flasks for assay as above.

**RESULTS**

**Acetylene reduction by effluents.** Figure 1 shows the C\textsubscript{2}H\textsubscript{4} reduction activity typical of liquid woodroom supernatant when incubated in air on a rotary shaker. High nitrogenase activity was recorded in unamended material after 24 h of incubation. Addition of 0.5% (wt/vol, final concentration) xylose slightly increased activity, but addition of glucose slightly decreased activity. Effluent samples which were pasteurized at 80 C for 15 min developed no nitrogenase activity during the course of the experiment.

Acetylene reduction activity typical of woodroom supernatant effluent incubated in N\textsubscript{2} on a rotary shaker is shown in Fig. 2. Unamended effluent showed low peak activity at 24 h whether previously pasteurized or not. The addition of 0.5 and 3.0% glucose to unpasteurized effluent supported somewhat greater and more prolonged activity. However, peak activity of glucose-supplemented pasteurized effluent was much greater than that of unpasteurized effluent.

The results of these and other similar studies of effluent samples are summarized in Table 1. No activity was observed in liquor from the biological oxidation lagoon pilot plant under

**FIG. 1.** Rate of production of C\textsubscript{2}H\textsubscript{4} from C\textsubscript{2}H\textsubscript{2} by woodroom effluent supernatant incubated in air unamended (O), and amened with 0.5% glucose (■), 3.0% glucose (▲), and 0.5% xylose (□). Some samples, unamended and amended with glucose and with xylose, were also pasteurized (×).

**FIG. 2.** Rate of production of C\textsubscript{2}H\textsubscript{4} from C\textsubscript{2}H\textsubscript{2} by woodroom effluent supernatant incubated in N\textsubscript{2} unamended (O), and amended with 0.5% glucose (■) and 3.0% glucose (▲). Pasteurized samples are shown with dashed lines.
Table 1. Rates of production of \( \text{C}_2\text{H}_4 \) from \( \text{C}_2\text{H}_4 \) by pulp mill effluent samples incubated under different conditions

| Sample source     | Date collected | Sugar added (% wt/vol) | Rate of production* |
|-------------------|---------------|------------------------|---------------------|
|                   |               |                        | Aerobic             | Anaerobic          |
|                   |               |                        | Not pasteurized     | Pasteurized        |
|                   |               |                        | Not pasteurized     | Pasteurized        |
| Supernatant*      | 12/21/72      | 0                      | 3 (0)               | 20 (24)            |
|                   |               | 0.5 Glucose            | 19 (24)             | 161 (24)           |
|                   |               | 3.0 Glucose            | 4 (144)             | 296 (24)           |
| Oxidation lagoon | 12/21/72      | 0                      | 0                   | 0                  |
|                   |               | 0.5 Glucose            | 0                   | 0                  |
|                   |               | 3.0 Glucose            | 0                   | 0                  |
| Supernatant       | 1/17/73       | 0                      | 350 (24)            | 0                  |
|                   |               | 0.5 Glucose            | 390 (24)            | 75 (48)            |
|                   |               | 3.0 Glucose            | 350 (24)            | 55 (48)            |
| Sludgec           | 1/17/73       | 0                      | 159 (48)            | 4 (0)              |
|                   |               | 0.5 Glucose            | 102 (72)            | 170 (24)           |
|                   |               | 3.0 Glucose            | 208 (96)            | 166 (72)           |
| Supernatant       | 2/9/73        | 0                      | 270 (24)            | 0                  |
|                   |               | 0.5 Glucose            | 197 (24)            | 19 (24)            |
|                   |               | 3.0 Glucose            | 164 (24)            | 27 (24)            |
|                   |               | 0.5 Xylose             | 303 (24)            | 21 (24)            |
|                   |               | 3.0 Xylose             | 38 (24)             | 25 (24)            |
| Sludgee           | 2/9/73        | 0                      | 5 (24)              | 0                  |
|                   |               | 0.5 Glucose            | 60 (50)             | 199 (24)           |
|                   |               | 3.0 Glucose            | 140 (146)           | 109 (24)           |
|                   |               | 0.5 Xylose             | 79 (24)             | 79 (24)            |
| Supernatant       | 2/8/74        | 0                      | 537 (24)            | 75 (24)            |
|                   | 3/28/74       | 0                      | 644 (24)            | 85 (24)            |
|                   | 5/15/74       | 0                      | 600 (24)            | 135 (24)           |
|                   | 6/6/74        | 0                      | 403 (24)            | 55 (48)            |

*Expressed as nanomoles of \( \text{C}_2\text{H}_4 \) per milliliter or per gram (wt wet) per hour. The figures in parentheses indicate the hour of incubation at which the peak \( \text{C}_2\text{H}_4 \) reduction activity noted occurred.

*Supernatant and biological oxidation lagoon liquor were incubated in 10-ml volumes per flask.

*Incubated in 20-g wet wt (9.8 g dry wt) amounts per flask.

*a incubated in 10-g wet wt (6.8 g dry wt) amounts per flask.

aerobic or anaerobic conditions, with and without a glucose supplement. All woodroom supernatant and sludge samples showed development of aerobic \( \text{C}_2\text{H}_4 \) reduction activity after about 24 h of incubation, although the sludge showed some delay in peak activity with increasing concentration of added glucose. No aerobic activity was observed in pasteurized samples. Rates of \( \text{C}_2\text{H}_4 \) reduction during anaerobic incubation in \( \text{N}_2 \) peaked at about 24 h, and higher activities were frequently obtained in samples which were previously pasteurized. The areas under activity time-course curves were consistently greater for pasteurized than for unpasteurized samples (Table 1).

**Bacteria in effluents.** Positive brilliant-green bile broth MPN tubes and anaerobic enrichment of effluents to which 0.5% glucose was added yielded \( \text{N}_2 \)-fixing *Klebsiella pneumoniae*. The isolates were MacConkey, Simmons citrate, urease, sorbitol, arabinose, and Voges-Proskauer positive, and were indole, \( \text{H}_2\text{S} \) (triple sugar iron agar), phenylalanine deaminase, ornithine decarboxylase, methyl red, and motility negative.

From aerobically incubated effluent, successive enrichment cultures were grown in aerobic shaken glucose mineral salts \( \text{N}_2 \)-free medium. These yielded an *Azotobacter* sp. which, on the basis of the absence of cysts, its inability to utilize mannitol, rhamnose, and starch, and the apparent absence of an ultraviolet-fluorescent
pigment, was probably close to *Azotobacter* (or *Azomonas*) *insignis* (9, 10).

From pasteurized effluent amended with 0.5% glucose and incubated under N₂, successive enrichment cultures were grown under N₂ in modified Hino and Wilson N-free medium supplemented with 25 μg of yeast extract per ml. These cultures yielded a facultatively anaerobic, gram-variable, C₂H₂-reducing *Bacillus* sp. having subterminal spores and swollen spore walls. Acid but not gas was produced from glucose, xylose, mannitol, and sucrose; starch was hydrolyzed, but no crystalline dextrins were apparent; litmus milk was peptonized; acetyl methyl carbinol was produced, and there was no growth at 45°C. These characteristics suggest that the organism was possibly an anaerogenic strain of *B. polymyxa* (3, 20).

Table 2 shows some microbial population estimates obtained by different methods. The total viable aerobic plate count revealed marked proliferation under both aerobic and anaerobic conditions, and counts after pasteurization (as well as direct microscope examination) showed that sporulating cells made up a small proportion of the total population. *Azotobacter* were very numerous and underwent significant proliferation during 24 h of aerobic incubation. Presumptive coliforms were also very numerous and underwent significant proliferation under anaerobic and especially under aerobic incubation. When small inocula were transferred from the highest positive dilutions of coliforms into tubes of modified Hino and Wilson medium, the numbers of such tubes showing growth and C₂H₂-reducing activity suggested that the number of N₂-[C₂H₂]-fixing coliforms were of the order of 10⁷/ml and that they proliferated under both aerobic and anaerobic conditions. A standard MPN count using Hino and Wilson medium recorded somewhat lower estimates. Pasteurization resulted in zero counts in the brilliant green lactose bile and Hino and Wilson MPN procedures.

**Klebsiella from various sources.** In view of the incidence of N₂-fixing *Klebsiella* in the effluent studied here, a survey was undertaken of the N₂-fixing ability in *Klebsiella* strains isolated from various sources. Figure 3 shows typical growth and C₂H₂-reduction data from a culture of strain 996 received from A. D. Tennant (described in 14). Similar data from 129 *Klebsiella* strains derived from various pulp and paper mill and environmental sources are sum-

![Figure 3](image_url)

**Figure 3.** Growth as optical density (O.D., circles) and rate of production of C₂H₄ from C₂H₂ (bars) of *Klebsiella* strain 996 (14) cultured under N₂. Open symbols represent cultures grown on modified Hino and Wilson medium (11) and closed symbols represent those grown on the same medium supplemented with 310 μg of NH₄⁺·N/ml.

| Table 2. Numbers of bacteria in pulp mill wood-room supernatant effluent before and after 24 h of aerobic or of anaerobic incubation and after pasteurization as indicated. |
|-----------------|-----------------|-----------------|-----------------|
| Sample          | No incubation   | Aerobic         | Anaerobic       |
|                 |                 | 0 h    | 0 h. | 24 h. | 24 h. | 24 h. | 24 h. |
| Total plate count | 6.70-8.18 | 4.10-4.70 | 8.36-12.11 | 2.70-4.78 | 7.81-11.38 | 3.22-7.28 |
| Azotobacter plate count | <5.00-6.70 | <5.00-6.70 | 7.57-7.81 | <5.00-7.00 | 7.11-7.96 | 0 |
| BGLB broth MPN  | 6.23-7.11 | 0 | 7.96-8.63 | 0 | 7.11-7.96 | 0 |
| BGLB positive to Hino and Wilson | 5.36-6.73 | 0 | 7.08-7.23 | 0 | 6.96-7.45 | 0 |
| Hino and Wilson MPN | 5.52-5.69 | 0 | 6.36-6.73 | 0 | 6.36-6.38 | 0 |

*Expressed as log₅ number per milliliter. Range of four separate effluent samples is shown.

*Samples were pasteurized.

*BGLB, Brilliant green lactose bile.*
were widely distributed, and of the 129 isolates studied 32% possessed the ability to develop nitrogenase activity.

**DISCUSSION**

Acetylene reduction was consistently demonstrated in woodroom effluent (supernatant and sludge) but not in liquor from a biological oxidation lagoon pilot plant. Lack of activity in the pilot plant liquor is consistent with the fact that NH$_4$OH was frequently added to the plant to a concentration of about 100 to 200 µg of N per ml, a concentration which would be likely to repress the synthesis of nitrogenase by microorganisms (6) or allow the proliferation of non-N$_2$-fixing species and strains. High activities in nonpasteurized samples under aerobic conditions suggested the occurrence of obligately aerobic N$_2$ fixers, such as members of the *Azotobacteraceae*. The activities which developed in both pasteurized and unpasteurized effluent under anaerobic conditions suggested the occurrence of facultative N$_2$ fixers such as bacilli or members of the *Enterobacteriaceae*, or of anaerobic N$_2$ fixers such as clostridia. The development of anaerobic activity in pasteurized effluent suggested that spore-forming N$_2$ fixers were present.

The activities observed in unamended effluent under aerobic conditions extrapolate (assuming an C$_2$H$_4$/N$_2$ conversion factor of 3) to about 60 µg N$_2$ fixed per ml of effluent. The

| TABLE 3. Occurrence of C$_2$H$_4$-reducing activity in Klebsiella sp. cultures isolated from various sources |
|--------------------------------------------------------------------------------------------------|
| **Location** | **Source of cultures** | **No. of cultures** |
| | | **Active** | **Tested** |
| Tennant, 1974 (18) |  |  |  |
| Sulfite mill 1 | Woodroom effluent | 1 | 2 |
| | Combined effluent | 1 | 2 |
| | Paper mill effluent | 0 | 2 |
| | Pilot plant (8-day retention) | 1 | 2 |
| Sulfite mill 2 | Woodroom effluent | 0 | 2 |
| | Main sewer | 2 | 2 |
| | Total effluent | 1 | 2 |
| Menon and Bedford, 1972 (14)* |  |  |  |
| Pulp and paper mill | Effluent | 2 | 3 |
| Fort Francis sewage plant |  | 2 | 4 |
| International Falls sewage plant |  | 2 | 6 |
| Fort Francis sanitary sewers |  | 1 | 4 |
| Storm sewers |  | 0 | 1 |
| Rainy Lake |  | 2 | 13 |
| Log boom |  | 1 | 10 |
| Rainy River |  | 7 | 10 |
| Creeks |  | 1 | 4 |
| Tennant et al., 1972 (19) |  |  |  |
| Land drainage water (station 3) |  | 1 | 4 |
| Land drainage water (10 other stations) |  | 0 | 18 |
| Manure |  | 0 | 1 |
| Duncan, B.C. Research Vancouver |  |  |  |
| Ground wood mill | Woodroom, bark, flume, etc. | 0 | 8 |
| Kraft mill | Slime, sewer, sea water, etc. | 5 | 11 |
| Sulfite mill | Woodroom slime, broke pit | 1 | 3 |
| Interior Kraft mill | Biobasin | 0 | 3 |
| This study |  |  |  |
| Sulfite mill | Woodroom effluent | 12 | 12 |

* Time of peak activity of cultures was between 20 and 50 h in all cases. The range in peak activities was 10 to 2,300 (mean of 708) nmol of C$_2$H$_4$ produced per ml of culture per h.

* Cultures classified as *K. ozenae* showed 10 active out of 29 tested. Those classified as *Klebsiella* sp. showed 6 active out of 26 tested.
nature of the available carbon compounds supporting this activity was not investigated.

Azotobacter insignis was isolated from fresh water in North America (10), and A. agile was reported in strawboard waste water (9). The large numbers of Klebsiella and Azotobacter in the effluent studied here appear to account for much of the activity observed. Considerable oxygen depletion occurred in aerobically incubated effluent (data not shown), and this, together with the marked proliferation of both Azotobacter and "N₂-fixing coliforms," suggests that the very high observed activities in aerobic effluent may have been caused by both Azotobacter and Klebsiella. Actively N₂-fixing Klebsiella cultures have been shown to be somewhat O₂ tolerant (11; S. Hill, personal communication). In anaerobically incubated effluent the somewhat lower proliferation of coliforms is consistent with the lower nitrogenase activities observed. To what extent N₂-fixing bacilli contributed to the observed activities is difficult to assess. Sporulating cells were a small proportion of the total population, and satisfactory counts of these organisms were not obtained.

The Klebsiella would therefore appear to be a major contributor to the nitrogenase activities observed in the effluent. The wide distribution of N₂-fixing ability observed here amongst Klebsiella strains isolated from a variety of situations is in agreement with reports elsewhere (1, 2, 4, 8, 12, 13, 17) and furthermore suggests that a potential for N₂ fixation is similarly widely distributed.

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