Developing a Slow-release Nitrogen Fertilizer from Organic Sources: I. Using Nonviable Bacteria

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Abstract. Mineralization of N from nonviable cells of *Brevibacterium lactofermentum* (Okumura et al.) mixed into soilless substrate in elution columns occurred largely during the first 5 weeks with a peak between 2 and 3 weeks. Over a 12-week period, 73% of the total N was recovered in the eluent. To prolong the period of N release to meet the requirements of a slow-release fertilizer, the bacterium was bonded to kraft lignin, a polyphenolic substance highly resistant to degradation. To retard mineralization further, the bacterium-lignin mixture was reacted with formaldehyde to form amino cross-links within and between protein chains. Bonding to lignin was undesirable because N release occurred during the same period as from the bacteria unbound to lignin and the total amount of N recovered was reduced to only 42%. Cross-linking with formaldehyde was less desirable since N was released mainly during the first 4 weeks with a peak during the first elution (0 time) and the total amount of N released was even lower than for the bacterium-lignin mixture. Additions of urea to the latter reaction did not satisfactorily improve subsequent N mineralization. In a second set of treatments lignin was withheld and the bacterium was reacted with weights of formaldehyde (a.i.) equivalent to 0.1%, 0.5%, 1.0%, 5.0%, and 10.0% of the dry weight of bacterium. Formaldehyde quantities $\leq 1.0\%$ either had no effect or lowered the mineralization of N without altering time of release. Five percent and 10% formaldehyde successfully reduced release of N during the first 4 weeks and increased it thereafter. The best rate was 5%. In this treatment N was released from week 2 through the end of the test (12 weeks). Peak release occurred at 6 weeks. This resulting N source, while not a stand alone product, does have a slow-release property that could lend itself to use in combination with other slow-release N sources.

Many attempts have been made to use organic sources of N in greenhouses (Bunt, 1976; Handreck and Black, 1984; Post, 1956). Even though the rate of mineralization (release of nutrients in plant available forms) varies with organic source, the length of time for mineralization for most N sources is too short to sustain containerized crops through their typical growing period of 3 to 4 months (Bunt, 1976; Wilson et al., 1988). Williams and Nelson (1992) studied eight waste organic nutrient sources and indicated that release of N occurred within 6 to 7 weeks from all sources except poultry feathers. In addition, they suggested that nonviable bacteria cells have potential as a N fertilizer because the efficiency of N utilization (plant growth per unit of N released) from bacteria was higher than other organic N sources tested. Because nonviable bacterial cells are produced as an industrial by-product, their cost should be low. However, such nonsoluble N sources could not be injected through the watering system after planting thus, their application would be limited to incorporation into the substrate at the time of its preparation. This dictates the need to reduce the rate of mineralization of N from bacteria to meet typical crop schedules.

Straight chains of protein or amino acids are readily mineralized in nature, but this process is inhibited when there are branches in the chain. Milligan and Holt (1977) suggested that proteolytic

enzymes hydrolyze proteins containing quite small ranges of natural amino acid residues. Therefore, it would be expected that effectiveness of proteolytic enzymes are impaired when they encounter unnatural amino acid residues. If new cross-links were produced in nonviable bacterial cells it would reduce and retard mineralization by proteolytic enzymes of soil microorganisms.

One approach to retard mineralization of nitrogenous compounds of bacteria is to cross-link this material by a formaldehyde reaction. Wolfschoon and Vargas (1978) indicated that formaldehyde can bind two peptide chains together. Seltzer (1973) also suggested that formaldehyde and formaldehyde-yielding compounds have antimicrobial activity and formation of ammonium is inhibited with the addition of formaldehyde to nitrogenous materials. Therefore, formaldehyde can cross-link peptide and protein chains and act as an antimicrobial agent of soil microflora.

Another approach to retarding N release is bonding of nitrogenous materials to polyphenolic materials, such as lignin, which are relatively stable in the root environment. Lignin, a waste product from the paper industry, differs from natural lignin occurring in wood in that it is fragmented and modified due to the harsh chemical treatment used for extraction. The exact structure of lignin is unknown, but it has been established that it consists of polymers derived from hydroxycinnamyl alcohols and monolignols of p-coumaryl, coniferyl, and syngapyl (Freudenberg and Neish, 1968) that react with formaldehyde. Therefore, nitrogenous materials in bacterial cells when cross-linked to functional groups in kraft lignin by the formaldehyde reaction should not be readily mineralized by soil microorganisms for the afore mentioned reasons.

The objective of this research was to extend the period of N release from nitrogenous materials in nonviable bacterial cells to a period ≥ 12 weeks by developing cross-links in bacterial protein through a formaldehyde reaction and/or cross-linking nitrogenous compounds from bacteria to the relatively stable, natural, polyphenolic lignin.

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Table 1. Amounts of bacteria (B), kraft lignin (L), formaldehyde (F), and urea (U) used to make final N sources and the N content of the resulting dried N sources in Expt. 1.

Treatment ^z	B cells (g)	L (g)	F (% of B)	U (g)	N content (%) ^y
1 B	20	---	---	---	11.49
2 BL	20	20	---	---	5.75
3 BLF	20	20	16	---	5.09
4 BLF	20	20	20	---	5.53
5 BLF	20	20	24	---	5.17
6 BLUF	20	20	16	4.83	13.34
7 BLUF	20	20	20	4.83	13.31
8 BLUF	20	20	24	4.83	12.59
LSD $\alpha = 0.05$					0.71

^zAfter formaldehyde was reacted with the mixtures of bacterial cells, lignin, and urea in treatments 4 through 9, 3.75 g of H_3PO_4 was added to lower the pH followed by an addition of CaO to raise the pH to 6.

^yValues represent mean of three observations. Nitrogen concentrations reported in treatments 2 through 9 are the resulting concentrations after the background concentration was subtracted.

Materials and Methods

Cross-linking bacterial cells with Kraft lignin. In Expt. 1 (Table 1) The first treatment consisted solely of dried bacterial cells of *Brevibacterium lactofermentum*. In the second treatment (BL), 20 g dried bacterial cells was blended thoroughly with 20 g Kraft lignin and distilled water was added to this mix to make a thick slurry. This lignin is a by-product of the Kraft pulping procedure in paper manufacture. A similar mixture of bacteria and lignin was prepared in treatments 3 through 5 in which formaldehyde was mixed into these slurries in a.i. quantities of 3.2, 3.9, or 4.8 g from an aqueous 37.5% solution. These quantities of formaldehyde were equal to 16%, 19.5%, and 24% of the weight of dried bacterial cells. Treatments 6 through 8 were prepared similarly to treatments 3 through 5 except 4.83 g urea was also mixed into each slurry before adding formaldehyde. Preparation of treatments 2 through 8 were then continued as follows: 10 g concentrated H_3PO_4 was blended into each slurry to lower the pH to about 1.5 for hydrolysis to occur. Twenty to 30 min were allowed for the reaction from the addition of H_3PO_4 to come to visible completion. Signs of reaction included effervescence, evolution of heat, a change from dark to light brown color, and drying of the product. Then, the pH of each slurry was raised to 6.0 by titration with solid CaO. The final products were dried in a forced draft oven at 70 °C and ground in a Wiley mill to a particle size ≤ 1 mm.

Cross-linking bacterial cells without kraft lignin. In Expt. 2 (Table 2) the first treatment contained only dried bacterial cells. In treatments 2 through 9, 20 g dried bacterial cells were reacted with formaldehyde in quantities of 0.02 to 2.0 g, a.i. These quantities of formaldehyde corresponded to 0.005% to 10.0% of the dry weight of bacterial cells. After thorough mixing of the reactants, slurries were treated with 3.75 g concentrated H_3PO_4 and sufficient dry CaO to raise the pH to 6.0, dried, and ground to ≤ 1 mm as described above.

Columns. Clear acrylic columns with an inside diameter of 2.54 cm and a height of 25.4 cm were used. A rubber stopper with a glass tube passing through it was placed in the bottom of each column. Glass wool was placed over the stopper to insure retention of substrate in the column. A tygon tube was attached to the lower end of the glass tube. The tygon tube was secured along the side of the column until its upper end was level with the top of the column. The top of the column remained open during the tests. Tests were conducted at room temperature in a laboratory at 22 ± 2 °C. Sixty-

one cubic centimeters of a root substrate was packed in each column to a depth of 12 cm. The precise volume of 61 cm³ was determined initially by packing root substrate into a 100 ml graduated cylinder to a volume of 61 ml at a density typical of that used when potting a plant in the floral industry. After drying in an oven at 70 °C the dry weight of this volume was determined to be 9.0 g. In each subsequent test the moisture content of the root substrate used was determined and a moist weight equivalent to 9 g dry weight was packed in each column to a volume of 61 cm³ to ensure constant volume, depth, and density. Root substrate used in all column substrate tests consisted of the following volume quantities of components: 47.5% sphagnum peat (through a 6 mm screen), 47.5% vermiculite (Terralite no. 3, The Scotts-Sierra Co., Marysville, Ohio), and 5.0% peat-lite mix from an actively growing chrysanthemum crop to provide a microbial inoculum. To each liter of substrate the following amendments were added: 2.0 g dolomitic limestone, 2.7 g 20% superphosphate, 0.07 g FTE-555 micronutrient mix (Frit Industries, Ozark, Ala.), and 0.9 g AquaGroS wetting agent (Aquatrols Corp., Pennsauken, N.J.). Nitrogen products tested in all phases of this study were blended into the substrate just defined at the rate of 1 g N/L of substrate.

Table 2. Amounts of bacterial (B) cells and formaldehyde (F) used to make final N sources and the N content of the resulting dried sources in Expt. 2.

Treatment ^y	B cells (g)	F (% of B)	N content ^z (%)
1 B	20	---	11.7
2 BF	20	0.005	9.0
3 BF	20	0.01	9.0
4 BF	20	0.05	9.0
5 BF	20	0.1	9.3
6 BF	20	0.5	9.3
7 BF	20	1.0	9.7
8 BF	20	5.0	8.9
9 BF	20	10.0	9.0
LSD $\alpha = 0.05$			0.98

^zValues represent mean of three observations.

^yAfter formaldehyde was reacted with the mixture of bacterial cells in treatments 3 through 10, 3.75 g H_3PO_4 was added to lower the pH followed by addition of CaO to raise pH to 6.0.

Elution. Columns were eluted by adding distilled water to the base of the column through the tygon tube until the water level reached the top of the substrate. Water was left in contact with the substrate for 15 min, after which the tygon tube was removed and the eluent from the column was drained from the base of the column into an Erlenmeyer flask. An additional 61 mL distilled water was then passed through the column from the top. The glass tube at the base of each column was left open between elutions. Two drops of a saturated solution of phenyl mercuric acetate (solubility = 1 g/180 mL H₂O) were added to the eluent in each receiving flask as a microbicide to prevent conversion of ammonium to nitrate. Immediately upon filling columns with substrate containing the nitrogenous materials to be tested the columns were eluted once with distilled water to determine the quantities of various N forms present at time 0. In the first experiment of this study, columns were eluted three times weekly for 11 weeks and in the second experiment, two times weekly for 12 weeks. All eluents collected weekly from each column were combined and held in a refrigerator. The reduction in collection time frequency was done to save labor and was deemed appropriate from the results of the first experiment.

Chemical analyses. Column eluents were analyzed for NH₄⁺ by the Chaney and Marbach (1962) method and NO₃⁻ by the procedure of Cataldo et al. (1975) using a UV/VIS spectrophotometer (Lambda 3; Perkin-Elmer, Norwalk, Conn.). Total N in column leachate and in bacterial cell preparations listed in Tables 1 and 2 was determined by a semimicro Kjeldahl procedure (Eastin, 1978). Organic N in leachates was calculated by subtracting ammoniacal and nitrate N from Kjeldahl N. Calcium was analyzed using an atomic absorption spectrophotometer (model 373; Perkin and Elmer). Phosphate was determined by the procedure of Chapman and Pratt (1961) for P concentrations >10 µg·mL⁻¹ and by the procedure of Murphy and Riley (1962) for P concentrations <10 µg·mL⁻¹. In addition to the treatment columns in each experiment, background columns were set up with substrate but no bacterial products. These columns were eluted to determine forms and quantities of N released and these values were subtracted from all treatment values.

Experimental design and data analysis. In both experiments, the elution columns were arranged in a randomized complete block design with three replications. Each plot consisted of one column. Data were subjected to an analysis for the standard error of the com-

bined treatments within each week using the CoStat program (CoHort Software, Berkeley, Calif.). The standard error values are expressed in the form of vertical bars in the upper portion of each figure.

Results and Discussion

Experiment 1: Cross-linking bacterial cell constituents with kraft lignin. A small amount of soluble N was present in bacterial cells alone treatment in Fig. 1 A and C. This organic N probably came from hydrolysis of nitrogenous compounds in the bacterial cells during the low pH, 1.5, reaction of bacteria, kraft lignin, and phosphoric acid. Following this hydrolysis, new crosslinks likely formed at the low pH which bonded NH₄ as evidenced by the low level of NH₄ at week 0 in Fig. 1B (Robinson, 1988).

Organic N was highest at week 0 in treatments where urea was added (Fig. 1C). Hays (1987) reported that not all urea combines in the process of ureaformaldehyde production and further, that some ureaformaldehyde chains are sufficiently short to be mineralized in the first few weeks. Release of N in all treatments processed with formaldehyde or formaldehyde plus urea decreased rapidly from time 0 through week 3 and slowly thereafter with little N released after week 3. The largest component of N released in these treatments was the organic fraction followed

Fig. 1. Weekly quantities of various forms of N released into the eluent from columns containing N test materials (see Table 1) blended into a substrate (A-D), the weekly cumulative amounts of all N forms combined that were released (E), and pH of the eluents (F) in experiment 1. Each N test material was incorporated at a rate sufficient to supply 1 g N/L of substrate. Points represent mean of three replications. Vertical bars represent the standard error among treatments within each week (■ = nonviable bacteria; ● = bacteria bonded to lignin; ▲ = bacteria-lignin, 16% formaldehyde; ◆ = bacteria-lignin, 20% formaldehyde; □ = bacteria-lignin, 24% formaldehyde; ○ = bacteria-lignin, 16% formaldehyde-urea; △ = bacteria-lignin, 20% formaldehyde-urea; ◇ = bacteria-lignin, 24% formaldehyde-urea).

Fig. 2. Weekly quantities of various forms of N released into the eluent from columns containing N test materials (see Table 2) blended into a substrate (A-D), the weekly cumulative amounts of all N forms combined that were released (E), and pH of the eluents (F) in experiment 2. Each N test material was incorporated at a rate sufficient to supply 1 g N/L of substrate. Points represent means of three replications. Vertical bars represent the standard error among treatments within each week (■ = nonviable bacteria; ● = bacteria, 0.1% formaldehyde; ▲ = bacteria, 0.5% formaldehyde; ◆ = bacteria, 1.0% formaldehyde; □ = bacteria, 5.0% formaldehyde; ○ = bacteria, 10.0% formaldehyde).

and 1.0% formaldehyde resulted in progressively less mineralization of bacterial cells. The time of release was similar to bacteria alone. These products were undesirable. Reaction with 5% and 10% formaldehyde yielded desirable results. When treated with 5% formaldehyde release of N was delayed until week 3, but then continued through week 12, the final week of the experiment. Release during the first 6 weeks was reduced compared to bacteria alone. This treatment had a more desirable release pattern compared to bacterial cells alone. The total amount of N released during the 12 weeks was 48% for bacterial cells alone and 33% for bacteria treated with 5% formaldehyde (Fig. 2E). Desirable features were the delayed start of release, the higher peak of release at 6 weeks, and continued release through week 12. The undesirable features were the occurrence of a peak of release at 6 weeks and failure of the release curve to continue increasing after 6 weeks. Treatment of bacterial cells with 10% formaldehyde yielded a less desirable product than treatment with 5% formaldehyde. N release for the latter product began 1 week later and

ended in week 10. Cross linking of protein was obviously too extensive when 10% formaldehyde was used. The sequence of release of N forms was similar to that in the first experiment with organic N (Fig. 2C) and NH₄ (Fig. 2B) released first followed about one week later by NO₃ (Fig. 2D).

Substrate pH (Fig. 1F) in Expt. 1 was low, 4.7, at time 0. The substrate had only been wet for 15 min during this first extraction. The pH was low because there was insufficient time for the limestone in the substrate to dissolve. The pH rose quickly during the first 4 weeks, most likely as a consequence of limestone dissolution and the release of ammonia from the test products. Mengel and Kirkby (1987) indicated that ammonia mineralized from organic N through proteolysis picks up hydrogen to form ammonium, thereby causing a rise in substrate pH. The pH in the background treatment was lower because this treatment did not contain an organic test product. At week 2 there was a wide range in pH levels because of wide variation in N release from the test products. The level of pH related well to the level of N release. From week 4 to the end of the experiment, pH rose slowly to about 7. Substrate pH in the second experiment (Fig. 2F), in which the contact time with water was increased to one h during the initial elution of columns to allow for more dissolution of limestone, started out higher, rose rapidly to about 7.5, and then declined slowly to a little below 7 by the end of the experiment. The increased contact time plus the possibility of greater surface area

Experiment 2: Cross-linking bacterial cells without kraft lignin. Release of total N from bacterial cell treatments was similar to that in the first experiment. Reaction of bacteria with 0.1% formaldehyde had no effect on N release (Fig. 2A). Reaction with 0.5%

sequentially by NH₄ (Fig. 1B) and NO₃ (Fig. 1D). Ammonium was released from the organic fraction as it was mineralized and NO₃ was formed later from NH₄ by nitrification. The peak of N release from bacterial cells alone occurred at weeks 2 and 3 and a sizeable level of release continued through week 7 (Fig. 1A) due mainly to organic N (Fig. 1C). Bacterial cells bonded to lignin released N (Fig. 1A) in a similar time pattern to bacterial cells alone but the extent of release was much lower. The cumulative release of all forms of N combined over 11 weeks (Fig. 1E) was 73% for bacterial cells and 42% for bacterial cells bonded to lignin.

Overall, the quantity and pattern of N release from bacterial cells alone was best because a greater portion of N in these cells was available for plant use than in any other treatment and this occurred over a longer period of time. The time of release resulting from the reaction of the bacteria-lignin combination with formaldehyde or formaldehyde plus urea was undesirably shorter. The extent of mineralization of bacteria plus lignin was lower than bacteria alone while the time period of release was unaltered. Bonding of nitrogenous bacterial cell constituents to lignin with or without the aid of formaldehyde was too extensive to permit a desired level of mineralization.

Fig. 3. Weekly quantities of $\text{PO}_4\text{-P}$ (A) and Ca (B) released into the eluent from columns containing N test materials (see Table 2) blended into a substrate in experiment 2. Each N test material was incorporated at a rate sufficient to supply 1 g N/L of substrate. Points represent mean of three replications. Vertical bars represent standard error among treatments within each week (■ = nonviable bacteria; ● = bacteria, 0.1% formaldehyde; ▲ = bacteria, 0.5% formaldehyde; ◆ = bacteria, 1.0% formaldehyde; □ = bacteria, 5.0% formaldehyde; ○ = bacteria, 10.0% formaldehyde).

in the second source of dolomitic limestone could account for the higher initial pH in the second experiment. The high pH, about 7, that occurred in both experiments indicates that less liming material will be required when formulating substrate for use with a bacterial N source.

Nonviable bacteria and H_3PO_4 used in the process of reacting bacteria with formaldehyde contributed PO_4 to the eluent at week 0 and again at and after week 6 (Fig. 3A). There was a contribution of Ca to the eluent throughout the experiment from the nonviable bacteria (Fig. 3B). There were additional contributions of Ca from the CaO used in the reaction of bacteria with formaldehyde at week 0 and again after week 4. These additions of PO_4 and Ca from the bacterial products were sufficient to warrant reductions in the use of limestone and superphosphate in the substrate formulation for situations where bacteria would be used as the N source.

In general, the bonding of bacteria to lignin, with or without

formaldehyde, was too extensive to allow a desirable rate of mineralization. The N source formed by reacting bacteria with formaldehyde equal to 5% of the weight of bacteria had desirable properties. Release of N extended from weeks 2 through 12. While the occurrence of a peak of N release at week 6 was undesirable it might have a potential use. The slow-release N source developed by Choi and Nelson (1996) through the fermentation of feather keratin with *Streptomyces cn1* has a desirable N release over a 20 week period with the exception of a relatively low level of release at week 6. The combination of the bacteria source as reported in this paper with the feather source reported in a subsequent part of this report shows good potential for development of a slow-release N fertilizer.

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