Developing a Slow-release Nitrogen Fertilizer from Organic Sources: III. Isolation and Action of a Feather-degrading Actinomycete

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Abstract. An actinomycete designated *Streptomyces cn1* with a high proteolytic activity and capacity to degrade feather keratin was isolated and its effectiveness for altering feathers to yield a slow-release N fertilizer was evaluated. The pattern of N release in column elution tests from feathers ground to a particle size ≤ 1 mm, but otherwise unaltered, was characterized by a first period of release from weeks 2 through 5 with a high peak at week 3 and a second period of release from 14 to 20 weeks. The release of N during the first period was 10.5% and during the second period it was 7.3% for a total of only 17.8% of the N contained in these feathers. Grinding feathers to a finer particle size ≤ 0.5 mm caused increases in N release during the two periods to 14.7% and 15.8% N, respectively, for a total of 30.5% and second period N release began 5 weeks earlier at week 9. Microbial hydrolysis with *Streptomyces cn1* for 1 through 5 days resulted in an adverse reduction in total N released, due in part to drying of feathers after hydrolysis. Hydrolysis of feathers for 7 days resulted in 42.6% of total N released over 20 weeks with 77.0% of this released during weeks 6 through 20. The second period of release began at week 8. Hydrolysis of feathers for 9 days was best for purposes of a slow-release fertilizer. Forty five percent of total N was released over 20 weeks with 89.3% of this released during the second period that began in week 7. Root substrate pH was increased in all treatments where feathers were applied. This would require a reduction in the rate of limestone incorporated into a commercial substrate when feather N is used. Pepsin digestibility and ninhydrin tests provided some insight into the N release mechanism but did not effectively predict N release from the feather products.

Feathers contain about 15% N on a dry weight basis and huge quantities are produced as an industrial by-product (U.S. Dept. of Agriculture, 1991). However, they have not been used effectively as a plant fertilizer since N mineralization is too slow to meet plant requirements (Williams and Nelson, 1992). This is due mainly to the feather keratin structure in which N is held.

Keratin structure can be altered by cleaving disulfide bonds by steam hydrolysis and peptide bonds by enzymatic hydrolysis with *Bacillus licheniformis* (Weigmann) (Choi and Nelson, 1996b). Steam hydrolysis resulted in an excessively rapid increase in mineralization during the first 5 weeks that was not desirable for a slow-release fertilizer. While enzymatic hydrolysis reduced mineralization during the first 5 weeks and increased it during the later weeks, which was desirable for a slow-release fertilizer, the extent of these shifts were not sufficient to yield a commercial product. The earlier study suggested that feathers be ground before enzymatic hydrolysis, a microorganism with higher keratinase activity be sought, and the length of the hydrolysis period be investigated further.

When whole feathers are subjected to microbial hydrolysis, the peptide bonds formed by hydrophobic amino acids usually located inside the feather keratin (Wong, 1991) are not cleaved by microbial enzymes. These uncleaved peptide bonds probably account for the slow and variable mineralization rates when N release is

determined in column experiments. Extension of microbial hydrolysis time and grinding of feathers to provide more surface area for microbial attack could possibly result in increased enzymatic cleavage of peptide bonds, and consequently in faster mineralization by soil microorganisms.

Several attempts have been made to isolate microorganisms from soil which have keratinase activity (De and Chandra, 1982; Elmayergi and Smith, 1971; Williams et al., 1990). However, most of this effort has been directed toward keratinase enzymes used for increasing the digestibility of feathers as an animal feed. Therefore, the objectives of this research were to isolate a strain of actinomycete with a high proteolytic activity from soils and to study the effectiveness of this organism with respect to developing a slow releasing N fertilizer of four months or longer duration from feather keratin.

Materials and Methods

Isolation. Soil samples were collected from several poultry waste dumping sites in Raleigh, N.C., and were plated using dilution plate techniques. The medium used for isolation and maintenance contained the following gram quantities of materials per liter distilled water; 5 feather, 0.5 K_2HPO_4 , 0.2 $MgSO_4 \cdot 7H_2O$, 0.05 $CaCl_2$, 0.015 $FeCl_3 \cdot 6H_2O$, 0.05 $NaNO_3$, and 20 agar. The final pH was 7.4. Feathers used as the C plus N source in the medium were ground to a particle size ≤ 1 mm in a Wiley mill, defatted with acetone, washed with distilled water, and dried overnight at 70 °C. About 100 actinomycete cultures were obtained and screened for proteolytic activity by liquefaction of gelatin (Gordon and Mihm, 1957). The culture that hydrolysed (liquefied) gelatin most extensively in 24 h was selected for further study.

Preparation of actinomycete culture. The organism was grown for 7 d at 22 ± 2 °C in nutrient solution with continuous shaking (200 rpm) to provide a spore suspension. This was done in 200-mL Fisher Brand Qorpak Prescription narrow-mouth, square, glass bottles with rounded corners (Fisher Scientific, Pittsburgh, Penn.).

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The nutrient solution consisted of the following gram quantities of materials in 1 L of distilled water; 0.5 feathers, 1.5 K₂HPO₄, 0.05 MgSO₄·7H₂O, 0.05 CaCl₂, 0.015 FeSO₄·7H₂O, and 0.05 ZnSO₄·7H₂O. Feathers chopped with scissors, defatted with acetone, and dried overnight at 70 °C were used as the C and N sources in the medium.

Portions (100 mL) of this spore suspension containing 10⁵ spores/mL were used to inoculate 1 L liquid medium contained in 2-L Erlenmeyer flasks. They were then incubated at 28 ± 1 °C on a gyrating shaker (200 rpm) for 10 d. The basal salts medium used for this culture was the same as that used for preparation of the spore suspension except that it contained 1 g feathers/L of medium. In both procedures, liquid medium and feather substrate were sterilized separately for 15 min at 121 °C and a pressure of 138 kPa.

Preparation of actinomycete-hydrolysed feather. Thirty-gram aliquots of dry feathers ground in a Wiley mill to a particle size ≤1 mm were placed in 1-L Erlenmeyer flasks. Each flask was sealed with a rubber stopper fitted with cotton plugged inlet and outlet tubes to permit continuous flushing with sterile air during subsequent microbial hydrolysis. Before use, the flask assemblies were sterilized in an autoclave at 121 °C and 138 kPa for 15 min.

The actinomycete culture described earlier was passed through two layers of cheesecloth to remove large particles of nondegraded feathers. Aliquots of actinomycete culture (150 mL) were added to 30 g ground and sterilized feathers in flasks. These were incubated for various treatment times at 28 °C with continuous aeration. After incubation, the contents of flasks were dried at 70 °C for 2 d in a forced-air oven and ground once again to a particle size ≤1 mm in a Wiley mill.

Experimental setup. Eight treatments were used to test the pattern of N release from raw feathers ground to particle sizes of 1.0 mm versus ≤0.5 mm (treatments 1 and 2) and from feathers ground to ≤1.0 mm and then hydrolyzed with *Streptomyces cn1* for 0, 1, 3, 5, 7, or 9 h (treatments 3 through 8) (Table 1). Feathers hydrolyzed for 0 h in treatment 3 were treated by placing dry ground feathers in a forced-air oven at 70 °C for 2 d. Feathers hydrolyzed for 1 to 9 d differed from treatment 3 feathers in that

they were first exposed to the microorganism and then while in a moist state they were placed in the oven for 2 d of drying.

Column elution procedures. The column elution procedure described in the previous paper of this series (Choi and Nelson, 1996a) was used to determine the profile of N release from hydrolysed feathers. In addition to accommodating the eight treatments described above, additional columns were set up with no feathers to establish the background levels of N contributed to the eluent from the substrate that was common to all columns. The columns were eluted twice weekly with distilled water. The two elutions were combined within each week and stored in a refrigerator until analysis.

Chemical analysis procedures. Hydrolyzed feathers were subjected to the following tests: pepsin-HCl (Elmslie, 1958), ninhydrin-NH₃ (Moore and Stein, 1954), and semimicro Kjeldahl N (Eastin, 1978). Column leachates were analyzed by the procedures outlined in the first paper of this series (Choi and Nelson, 1996a).

Experimental design and data analysis. Data from feather analysis were subjected to a randomized complete block analysis of variance and means were tested by LSD. There were three observations within each mean. The elution columns were arranged in a randomized complete block design with three replications. One column was used in each plot. The standard error for all treatments was determined weekly for N release data from column leachate analysis using the CoStat program (CoHort Software, Berkeley, Calif.). The standard error for all treatments over the whole experiment was determined for the pH data. Standard error values are expressed as vertical bars in each figure.

Results and Discussion

Descriptive testing of the unknown actinomycete strain was performed by the American Type Culture Collection (ATCC) (Rockville, Md.) where it was compared to other strains of actinomycetes. Identification of the organism was mainly based on criteria suggested by the International Streptomyces Project (Shirling and Gottlieb, 1966), but additional criteria found in

Table 1. Effects of particle size and microbial hydrolysis time with *Streptomyces cn1* on feather N concentration, pepsin digestibility and ninhydrin reaction of feather keratin, percentage of N released in various time periods from feathers incorporated in root substrate in elution columns, and the percentage of the 20 week N release that is released in the week 6 through 20 period.

No.	Treatment			N concn (%) ^{sv}	Pepsin digestibility (%) ^{xv}	Ninhydrin (mM leucine/ kg feather) ^{wv}	N release (weeks)			
	Particle size (mm)	Hydrolysis time (d)	Dried at 70 °C ^z				0–5 (%) ^{vu}	6–20 (%) ^{vu}	0–20 (%) ^{vu}	(6–20/0–20) ×100
1	1	0	No	14.7	22.7	53.5	10.5	7.3	17.8	41.0
2	0.5	0	No	14.1	26.2	44.0	14.7	15.8	30.5	51.8
3	1	0	Yes	15.1	18.1	42.5	5.3	10.1	15.4	65.6
4	1	1	Yes	15.3	18.9	22.0	7.2	0.4	7.3	5.5
5	1	3	Yes	14.7	19.7	26.4	5.6	0.6	6.7	9.0
6	1	5	Yes	14.2	20.1	23.5	10.7	0.4	11.0	3.6
7	1	7	Yes	14.6	21.2	35.2	9.8	32.8	42.6	77.0
8	1	9	Yes	15.0	23.6	46.2	4.8	40.1	44.9	89.3
LSD _{0.05}				1.24	2.82	8.62	3.2	8.3	9.9	

^zAfter microbial hydrolysis, feathers were dried two days at 70 °C.

^vN contents were determined by a Kjeldahl method (Eastin, 1978).

^xPepsin digestibility was determined by the method of Elmsie (1958). The equation used to calculate digestibility was (N content before digestion – N content after digestion)/(N content before digestion) × 100.

^wNinhydrin reactive N was determined by a modified ninhydrin method and expressed as leucine equivalent (Moore and Stein, 1954). This indicated the degree of peptide bond cleavage.

^uValues are means of three replications.

^vPercentage of total feather N in elution columns released. The amount of N released from background columns that contained root substrate but no feathers, was deducted before reporting the above values.

literature were also used (Lechevalier and Lechevalier, 1980; Locci, 1989). These criteria follow.

The number of conidia per aerial hypha was 5 to 50 and no conidia were detected per substrate hypha on yeast extract-malt extract agar, oatmeal agar, inorganic starch agar, and glycerol asparagine agar. The color of aerial hyphae were light gray on yeast extract-malt extract agar, very light beige on oatmeal agar, light brownish gray on starch agar, and light grayish brown on glycerol asparagine agar. However, the color of substrate hyphae was light yellowish brown on yeast extract-malt extract agar, very light beige on oatmeal agar, and very light olive brownish on glycerol asparagine agar and inorganic starch agar. Melanin production was positive on those agar plates but negative on tyrosine agar. In carbon utilization, the unknown microorganism showed good growth on arabinose, D-fructose, inositol, mannitol, sucrose, and galactose but no growth on raffinose, rhamnose, and salicin. In other physiological tests, the organism responded positively to nitrate reduction, xanthin degradation, hypoxanthine degradation, adenine degradation but negatively to hippurate hydrolysis, tyrosine decomposition, and guanine degradation. The organism showed antimicrobial activity to *Micrococcus luteus* (Schroeter) Cohn but no activity to other organisms tested. In resistance to antibiotics, the organism responded negatively to neomycin and rifampin but positively to penicillin.

Based on the above results, the strain was assigned to the genus *Streptomyces*. This assignment was based primarily on cell wall composition and typical morphology in which L-diam-inopimelic acid was detected by descending chromatography. Tests were insufficient for unequivocally associating this organism with a species. The two species closest to this organism were the major clusters *Streptomyces chromofuscus* (Preobrachenskaya, Blinov, and Ryabova) Pridham, Hesseltine, and Benedict and *Streptomyces exfoliatus* (Waksman and Curtis) Waksman and Henrici. Until further taxonomic tests are conducted we designated the strain as *cn1*.

The N concentration in feathers was not affected by the particle size to which the feathers were ground (1 mm vs. 0.5 mm, treatment

1 and 2), 2 d drying at 70 °C (0 d of microbial hydrolysis, treatment 3), or microbial hydrolysis with *Streptomyces cn1* for 3 through 9 d (treatments 5 through 8) (Table 1). The average for these treatments was 14.7% N. The only difference appeared as a higher N concentration in feathers hydrolysed for 1 day versus feathers ground to a particle size ≤ 0.5 mm (treatments 4 versus 2). No explanation is obvious for this difference.

On the basis of pepsin digestibility level, there was more cleavage of disulfide bonds in feathers ground to ≤ 0.5 mm than feathers ground to ≤ 1 mm (Table 1). Papadopoulos (1985) indicated that pepsin digestibility correlated positively with the degree of disulfide bond cleavage in keratin protein. During drying, the degree of disulfide cross-linking of feather keratin increased over the level in control feathers ground to ≤ 1 mm (treatment 1 versus 3). This is supported by Milligan and Holt (1977) who indicated large numbers of isopeptide crosslinks of the disulfide and/or amino types are introduced into wool keratin on prolonged heating and that many of these unnatural bonds are resistant to microbial

cleavage. Cherry et al. (1977) also indicated that the solubility of protein declined when keratin was heated above 20 °C. The process of microbial hydrolysis with *Streptomyces cn1* caused cleavage of disulfide bonds as indicated by the rise in pepsin digestibility with increased hydrolysis time. Microbial hydrolysis for 5, 7, and 9 d counteracted the disulfide cross-linking caused by drying and resulted in a level of cross-linking equivalent to that in the control feathers of treatment 1.

The ninhydrin test measures free ammonium and primary amine groups. Less ninhydrin-reactive N was present with

feathers ≤ 0.5 mm compared to feathers ≤ 1 mm (Table 1). This may be due to greater contact of reactive sites during the longer grinding time and to heat generated during grinding. Further drying of the relatively dry feathers in treatment three caused a reduction in ninhydrin reactive N. By contrast, the feathers in treatments 4 to 7 were very wet

from the microbial hydrolysis mixture when drying began. Ninhydrin-N was very low in these latter treatments apparently due to moist heat where more extensive recombination of amino groups

Fig. 1. Effects of feather particle size and various microbial hydrolysis times of *Streptomyces cn1* on the release of N from feather keratin in a column test where 1 g keratin N was incorporated into each liter of substrate. (A and B) Total N ($\text{NH}_4 + \text{NO}_3 + \text{organic}$) released, (C) $\text{NH}_4\text{-N}$, and (D) $\text{NO}_3\text{-N}$. Columns were eluted twice weekly with distilled water and the amounts of N released weekly are expressed. Points represent the mean of three replications. Vertical bars represent the standard error among treatments within weeks (■ = raw feathers ≤ 1 mm, treatment 1, ● = raw feathers ≤ 0.5 mm, treatment 2, ▲ = feathers hydrolyzed with *Streptomyces cn1* for 0 d, treatment 3, ◆ = feathers hydrolyzed with *Streptomyces cn1* for 1 d, treatment 4, □ = feathers hydrolyzed with *Streptomyces cn1* for 3 d, treatment 5, ○ = feathers hydrolyzed with *Streptomyces cn1* for 5 d, treatment 6, △ = feathers hydrolyzed with *Streptomyces cn1* for 7 d, treatment 7, ◇ = feathers hydrolyzed with *Streptomyces cn1* for 9 d, treatment 8).

was possible than in the drier heat of treatment three. Asquith and Otterburn (1977) and Cherry et al. (1977) indicated that unnatural amino acids such as lysinoalanine, lanthionine, and β -aminoalanine are formed during heating. Lanthionine formed from protein bound cysteine, lysinoalanine from protein bound lysine, and β -aminoalanine from protein amides account in great part for the resistance of heat treated keratin to microbial degradation. These bonding effects of autoclaving and drying in feathers were gradually counteracted as increasing microbial hydrolysis time with *Streptomyces cn1* resulted in increased cleavage of peptide bonds seen in the increase in ninhydrin N (treatments 4 through 8).

Nitrogen was released from feathers in the column elution test in two periods (Fig. 1 A and B). The first occurred during weeks 2 through 5 with a peak at weeks 3 and 4. Nitrogen released during this period probably came from small feather particles produced during grinding. Noval and Nickerson (1959) indicated single passage of wool keratin through a Wiley mill (≤ 0.5 mm) resulted in a 10% decrease in cystine content, a measure of disulfide bonds, and keratin that was more susceptible to tryptic enzyme attack, also an indicator of less disulfide bonds. The second peak of release from feathers began between weeks 7 and 14, depending on the treatment, and continued through the end of the experiment at week 20. A slow-release fertilizer should release nutrients in proportion to plant uptake demand. Crop biomass is low at the beginning and increases with time, thus, availability of N should increase over time. Therefore, the first period of release should have been lower than the second and there should not have been a decrease between periods.

The percentage of total feather N released over 20 weeks from 1-mm particles was 17.8% while from 0.5-mm particles it was 30.3% (Table 1). The release pattern from 0.5-mm feather particles was better than from 1-mm particles for a slow-release fertilizer because 51.8% of the 20 week N release occurred during weeks 6 through 20 for the 0.5-mm feathers compared to 41.0% for the 1-mm feathers. Also, the second period of N release began at week 9 for 0.5-mm feathers, 5 weeks earlier than from 1-mm feathers, thus filling in more of the valley between the two release periods. The greater overall release of N from 0.5-mm feathers compared to 1-mm feathers might be due to more surface area in a unit weight of small particle feathers for hydrolyzing enzymes to act upon or fewer disulfide bonds in the 0.5-mm feathers (Gordon and Mihm, 1957) to reduce activity of proteolytic enzymes (Table 1).

Treatment 3 feathers, ground to 1 mm and dried at 70 °C for 2 d, released a higher percentage of N during weeks 6 through 20 than treatment 1 feathers, ground to 1 mm and not dried, but a similar total amount of N over the whole 20-week period (Table 1). Feathers microbially hydrolyzed with *Streptomyces cn1* for 1, 3, and 5 d (treatments 4 to 6) were very undesirable since only 6.7% to 11.0% of feather N was released over 20 weeks and the percentage of this released during weeks 6 through 20 was smaller than for any other treatment (Table 1). Feathers microbially hydrolysed for 7 d (treatment 7) were more desirable than the previous six treatments because 42.6% of feather N was released in 20 weeks and 77.0% of this was released during weeks 6 through 20 (Table 1). Treatment 7 feathers began the second period of N release at week 8, 6 weeks earlier than the second period release from feathers hydrolyzed for 5 d (Fig. 1B). The release pattern from feathers microbially hydrolysed for 9 d (treatment 8) was the most desirable from the standpoint of slow-release fertilizer requirements. This feather product released 44.9% of its N in 20 weeks with 89.3% released during weeks 6 through 20 (Table 1). Also, the second period of release began at week 7, earlier than in any other treatment (Fig 1B).

The first N released from feathers in the column test was detected as NH_4 (Fig. 1C). Ammonium was detected primarily from week 2 through 4 with only traces thereafter. Nitrate derived from feathers was detected during two time periods, from week 3 through 5 and from week 7 until the end of the experiment (Fig. 1D). Nitrogen is in reduced form in feathers, therefore the NH_4 form would be expected to appear first in the substrate during mineralization. A nitrifying bacteria population, that originated from the old chrysanthemum substrate in the column, developed in response to the appearance of NH_4 resulting in NO_3 appearing 1 week later than NH_4 . After week 5 the large population of nitrifying bacteria converted NH_4 rapidly to NO_3 such that NO_3 rather than NH_4 was detected. Only a trace of organic N, <0.2% of feather N, was detected and only during the first 3 weeks (data not shown).

The pH level of substrate in all treatments was similar (data not shown). As dolomitic limestone in the column substrate dissolved, the pH increased from 4.5 at the start to 7.1 at week 8 and then remained at that level. Organic N sources typically cause a rise in substrate pH. A reduction in limestone application in the root substrate would be warranted during use of a feather keratin N source in commercial production.

In summary, neither the pepsin digestibility test nor the ninhydrin test fully assessed the slow-release properties of the feather products. Each offered some explanation for the N mineralization pattern of the feather products. However, it was necessary to use a test such as column elution to obtain a satisfactory evaluation of N mineralization. Microbial hydrolysis of feathers for 9 d with *Streptomyces cn1* yielded a slow-release N source with large improvements over ground, but otherwise unaltered, feathers. This product shows promise as a slow-release fertilizer and should be tested on crops under commercial plant production conditions. This N source released 44.9% of its N in 20 weeks compared to 17.8% for ground feathers. It undergoes 4.8% mineralization from weeks 2 through 5 and 40.1% from weeks 6 through 20 as opposed to 10.5% and 7.3%, respectively, in untreated feathers. The lower initial and higher final release of N brings the total time course of release into better synchrony with typical crop demand. Also, it would be advisable in future studies to microbially hydrolyze feathers previously ground to the smaller particle size (≤ 0.5 mm) since N release is greater and the proportion released in the later weeks is greater than particles ground to ≤ 1 mm.

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