Development of an Environmentally Friendly Biofertilizer with Keratin Degrading and Antibiotic Producing Actinomycetes

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(Received Sep. 1, 2004 / Accepted Oct. 12, 2004)

Currently used chemical treatment of the keratinous material is the preferred method of poultry waste disposal in Australia, however it is costly, and environmental pollution problems from the chemical leachates as well as the poor nutritional quality of the end-product render this method undesirable. As the microbial community of the composting cycles have been reported to reduce the odours and pathogens of the domestic animal waste, local industries look for improved technologies of biological origin to treat poultry manure, carcasses and feather.

This study reports on an alternative method utilizing the degradative abilities of the actinomycetes in a composting system developed specifically to break down recalcitrant material and to produce a cleaner and pathogen-free endproduct with particular reference to waste usage and disposal on the Sunshine Coast Region in Queensland, Australia. Keratinolytic actinomycetes from a local poultry farm environment were isolated and used to augment the compost microflora. The inclusion of the organisms into poultry waste compost was successful in producing an odourless, pathogen free product with complete biological degradation of the feather waste in 17 days. The application of this system may improve the rapid disposal of poultry waste as well as the public and environmental health in the Region.

INTRODUCTION

Biodegradation of intensively farmed animal waste is now viewed as an alternative avenue for creating a viable end product with visible benefits to the primary producers in environmental and economic management strategies¹. A combination of specific waste products and processes produced via biological methods would lead not only to an improved consumable product but also to consumer confidence in waste management practices.

At present, the poultry industry manages waste (carcass, feather and manure) through several disposal methods. Carcass waste is generally rendered into bone and meat meal². Feather waste is also rendered by either steam or chemical treatments to produce feather meal¹. The manure is stockpiled to be sold in either untreated or treated forms to consumers as fertilizer or disposed as landfill. Disposal pits and trench burial incineration are also common methods used for disposal of diseased mortality². In each of these processes, however, the outcome has limitations with respect to quality, cost efficiency^{1,3} as well as environmental management.

Furthermore, human pathogenic organisms are known residents of poultry farms. For example, Craven *et al.*⁴ concluded that the populations of *Clostridium perfringens* followed throughout the production and processing of the waste were at levels of 94% in the bird faecal and caecal samples and 53% on wall swabs and 29% on the workers' boots. Mallinson and Snoeyenbos⁵ also showed that the untreated poultry waste provided an ideal environment for pathogenic

species such as *Vibrio* and *Salmonella* to thrive. In addition, as a result of the continuous use of the glycopeptide antibiotics as a feed supplement, resistant enterococci and salmonella organisms compete with normal microflora constituting a significant human health risk⁶.

Investigations into feather degradation began in 1950s on alpha keratin using keratinolytic *Bacillus* sp. and *Microsporum canis*^{7,8}. More recently, ranges of microorganisms were tested *in vitro* including *Bacilli*, *Thermoactinomyces*, and *Vibrio* species. They were found to be keratinolytic as well as utilizing collagen, elastin and casein^{9,10,11,12}. Actinomycetes were also reported to break down feathers such as *Streptomyces pactum* and *Streptomyces albidoflavus* isolated from soil showed a broad range of enzymatic activity^{10,13}.

Therefore, in the search of environmentally friendly processes to replace currently used environmentally hazardous ones, composting the poultry manure with indigenous microorganisms displaying keratin degrading and antibiotic producing properties might represent an alternative route.

Accordingly, the study presented here exploits the keratin degrading and antibiotic production abilities of the actinomycetes to produce a pathogen free and environmentally friendly poultry manure compost.

MATERIALS AND METHODS

Actinomycete isolations and identifications

A total of 20 poultry manure samples (10g) were collect-

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ed randomly from a local poultry farm and a composite sample was produced. Actinomycetes were isolated using serial dilution technique and starch-casein agar¹⁴). The selected isolates were then screened for their ability to hydrolyse keratin on feather meal agar plates¹²). Colonies producing clearing zones in this medium were selected for further work.

A range of morphological, biochemical studies were conducted to characterize the actinomycete isolates^{14,15,16,17}). Molecular sequencing of the isolates was done at the Genetic Analysis Facility, Advanced Analytical Centre, James Cook University, Townsville, Australia.

Testing for enzymatic activity and production of antimicrobial compounds

Keratin degrading actinomycete isolates were also tested to detect a range of enzymatic activities additionally following the previously described protocols. These enzymes were casease¹⁸), hemicellulase¹⁴), endoglucanase¹⁹) and amylase¹⁴).

The production of inhibitory metabolites by the actinomycete isolates was tested against the laboratory strains of *Escherichia coli*, *Bacillus subtilis*, *Candida albicans*, *Pseudomonas aeruginosa*, *Staphylococcus aureus* and *Mycobacterium smegmatis* as well as using the antibiotic resistant strains of the *E. coli* (methycillin resistant), two different strains of *Enterococcus faecalis* (vancomycin resistant) and *S. aureus* (vancomycin resistant) using disk diffusion assay.

In addition, to detect the survival abilities of the actinomycetes under antagonistic pressures of other indigenous microorganisms in a competitive compost environment, a basal medium adapted from Sangali and Brandelli¹²) was augmented with samples of sterilized and unsterilized manure placed onto the middle of the solidifying agar plate. After solidification the plates were inoculated with the each actinomycete isolate and were then incubated at 37°C. Growth of actinomycete isolates was monitored under competitive and uncompetitive conditions using light microscopy.

Compost trials

Actinomycete isolates, according to their ability to breakdown feathers, were placed into different groups and these groups were later used as actinomycete inoculum during composting (Tables 1 and 2).

Sterilized ryegrass seeds infested with actinomycete isolates were used to deliver the actinomycete inoculum into the manure as described by Kurtböke *et al.*,²¹) and the quantity of inoculum was 10% (w/v)²²).

Black polypropylene bins (45 kg) were used with a final compost weight of 40 kg. The ratio of the additives applied to the manure was maintained at a 1:1:2 ratio (2.5 kg feather, 2.5 kg straw, and 5 kg manure)²³). A control containing sterile ryegrass seeds (10% w/v) was also prepared. The timescale of the composting was 15 days with 3 days pasteurisation at the end of the treatment. Actinomycete inocu-

Table 1. Details of the actinomycete isolates used as compost inocula

Compost Type 1
USC-902, USC-906, USC-907 [<i>Streptomyces thermosacchari</i> (AF306657*)],
USC-901, USC-904, USC-905 [<i>Streptomyces</i> sp. VA26256_03 (AY436366*)],
USC-909, USC-911 [<i>Streptomyces ambofaciens</i> (M27245*)],
USC-912 [<i>Streptomyces</i> sp. 060386 (AY529645*)],
USC-901, USC-904, USC-905 [<i>Nocardioideus thermolilacinus</i> (AJ399486*)],
USC-900, USC-903 [<i>Actinomadura</i> sp. S2 (AJ293715*)]
Compost Type 2
USCMP-11, USCMP-12, USCMP-13 [<i>Saccharomonospora azurea</i> (AFO 12792*)]
Compost Type 3
USCMP-H1 [<i>Saccharomonospora azurea</i> (AFO 12792*)],
USC-146 [<i>Nocardiosis</i> sp. 20057 (AY336518*)],
USC-4 [<i>Nonomuraea rubescens</i> (AY039255.1*)]
Compost Type 4
All actinomycetes together

*: Accession number

Table 2. Feather degradation in different compost types used to treat poultry manure

Details of the Compost Inoculum groups	Number of feathers degraded	Observations on feather degradations
Compost Type 1	5/14	Shaft snapable in 3-4 places, barbs intact
Compost Type 2	4/14	Shafts flexible, only large feathers break slightly, tips showing slight degradation
Compost Type 3	14/14	Breaks easily-dry inside, barbs pull out easily, fine feathers turned into pulp
Compost Type 4	7/14	Shaft snaps and peeled but thick sections don't break, barbs come away with peeled sections

lum was introduced at day 2 and the moisture content was set to the recommended level of 60%²⁴) before the inoculation event. Compost was turned on alternate days and all sampling and testing was performed at the same time of day (1.00 pm) to avoid bias in results, particularly in relation to external temperature drops that may impact on the outer compost edge readings.

For analytical tests to be performed in the laboratory, composite samples were produced from five 100 g samples collected randomly from the piles. All analysis was conducted within 2 hours of sampling for temperature²⁵), pH²⁶) and moisture content²³).

Malodour changes were monitored using gas chromatography (Varian Saturn 2100T GC/MS). The percentage of

volatile solids was then calculated using standard methods²³. Analysis was performed prior to composting and after pasteurisation.

Changes in the numbers of coliform bacteria population were assessed using colony counts technique and reported as the geometric mean total coliform units per gram²⁷.

For pasteurisation of the end product after 15 days of composting, the individual composts were placed on separate plastic bags exposed to solar radiation for 3 days²³. Compost core temperature during this period was monitored at mid-day to ensure achieving the temperature ranges above 55°C. Each compost sample was turned regularly to ensure a homogenous mixture during pasteurisation. At the end of pasteurisation composite samples were collected for maturation and stability analysis following guidelines from Australian and International Standards^{23,29,30,31}.

Bulk density was analysed from composts on day one and after pasteurisation and reported as kg/m³³¹. Particle size-grading test was performed to determine which category it would belong (e.g. soil conditioner, mulch or fine mulch)³¹.

A Dewar self-heating test was performed using the method recommended by Brinton *et al.*³¹. Recordings of the highest temperature achieved over the 5-day period were compared with classifications based on the European System³¹.

To detect the phytotoxic effect of the compost end product, the germination index (GI) was determined on tomato seeds (cv Roma) and cress seeds^{28,30}, and later compared to a retail potting mix approved by Standards Australia.

Electrical conductivity and salinity of the compost end product were determined as described by Wu *et al.*²⁶. Sampling for total carbon and nitrogen was performed after the pasteurisation period with the randomly collected 10 × 10g samples from each compost and the 3 × 1g samples were drawn to form a composite from these samples. The samples were dried for 48 hours at 65°C in a vacuum oven. They were then ground using a ball mill (Spex 8000) and encapsulated for isotopic analysis.

Results were analysed using one-way ANOVA. The maturity and stability calculations were made on formulations as described in the literature cited.

RESULTS

Identification of actinomycetes

Following the morphological, biochemical and 16s rDNA sequencing studies, actinomycetes isolates were identified as the members of the actinomycete genera *Streptomyces*, *Saccharomonospora*, *Nocardioides*, *Nocardiopsis* and *Nonomuraea* (Table 1). Characteristics and the antimicrobial compound production and enzymatic abilities of the isolates are listed in Tables 3a and b.

Although actinomycete some actinomycete isolates were identified as the same species due to the differences in their antimicrobial properties they were included in the compost inocula preparation as different strains (Tables 1 and 3b).

Compost trials

Physical changes

All composts maintained moisture content levels above control (Fig. 1a). The levelling of the moisture content between days 13 and 17 indicates stability of water holding capacity of the composts. Higher levels of moisture content in the composts inoculated with actinomycetes at 36% (Compost Type 1), 31% (Compost Type 2), and 34% (Compost Type 4) were within acceptable guidelines for compost end product²³. Compost Type 3 at moisture content of 22% was at the lower end of acceptability. The control displayed poor water holding capacity with the final moisture content of 11% ±4.5.

The outer temperatures of all compost piles remained over the composting period on an average of 39°C ±0.3, the average outside temperature of 34°C ±1.4 (Fig. 1b) over the same period shows that biological processes within the piles were extending the thermal production to the outer areas. The highest recorded average temperature was 50°C ±2.3 in the Compost Type 3 and the Compost Type 1 on day 9. The final pH values of the composts were between 8.6±0.05 and 9.6±0.29.

Feather degradation and growth in competitive environment

The feather degradation was evident in all treatments. The shafts peeled apart with evidence that the inner core had been removed leaving a soft outer keratin skin (Table 2) (Fig 2a). The control of the final compost showed no degradation at all and the feathers were tough and resisted any crushing effect applied. At the end of composting, the shafts were still intact and quills remained on the barb end. Microscopic examination showed barbules intact and not bent. Whereas, in the inoculated composts, the barbules were bent at right angles every 0.5 mm along. These may be the sites where disulphide bonds breakage was occurring, subjected to enzymatic attack. Downy feathers in the composts disintegrated into a fine powder when crushed in the hand (Fig 2b).

All of the actinomycetes were found to grow on the plates inoculated with unsterilized manure, indicating their competitive survival ability.

Malodor reduction

The primary odorants present in the poultry manure were identified as dimethyl-disulfide (DMDS), propanol, n-pentane, n-butane, dimethyl-2,2- propane, 3-methyl pentane and methyl-cyclohexane (Figure 3).

The Compost Type 1 and Compost Type 4 had the highest reduction in volatile compounds (Table 4). The reduction of volatile peaks in the composts showing the highest decrease of volatile solids was seen on day 12 of the cycle.

End-product characteristics

According to the Dewar test all composts were classed in category IV that refers to moderately stable compost (Table 5).

The bulk densities increased the highest in Compost Type 1 and Compost Type 2 when comparing initial density to final density. Compost Type 1 recorded 166 kg/m³ (initial)

Table 3a. Characteristics of the actinomycete isolates

Isolate ID	USC-900	USC-901	USC-902	USC-903	USC-904	USC-905	USC-906	USC-907	USC-908	USC-909	USC-910	USC-911	USC-912	USC-4	USC-MP-11	USC-MP-12	USC-MP-13	USC-MP-H1	USC-146
Optimal growth range at:																			
pH	5-9	5-7	4-9	5-7	5-7	5-7	4-9	4-9	6-9	5-8	6-9	5-8	7-9	5-8	6-8	7-9	5-7	7-8	7-9
Temperature (°C)	28-45	28-45	28-45	28-45	28-45	28-45	28-45	28-45	28-45	28-45	28-45	28-45	28-37	28-45	28-45	28-45	28-45	28-45	28-37
NaCl tolerance (%) up to	7	13	4	7	13	13	4	4	7	4	7	7	7	7	4	<1	<1	<1	7
Hydrolysis of:																			
Feather meal	+	+	+	+	+	+	+	+	+	+	+	+	+	+	-	+	-	+	+
Casein	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Xylan	+	+	-	-	+	+	-	-	+	+	+	+	+	-	-	-	-	-	-
Starch	+	+	+	+	+	+	+	+	+	+	+	+	-	+	+	+	+	+	+
Cellulose	-	-	-	-	-	-	-	-	+	+	+	+	+	+	-	-	-	-	+
Indole production	-	-	-	-	-	-	-	-	-	+	-	+	+	-	-	-	-	-	-
Nitrate reduction	-	+	-	-	+	+	-	-	-	-	-	+	+	+	-	-	+	+	+
Gelatin liquefaction	+	+	-	-	+	+	-	-	-	-	-	-	-	-	-	-	-	-	-

+: positive;

-: negative

and 224 kg/m³ (final) bulk densities whereas the Compost Type 2 recorded 170 kg/m³ (initial) and 226 kg/m³ (final). The initial bulk density of Compost Type 3 and Type 4 was 194 kg/m³ and only slightly increased by the end of the cycle. Carbon/Nitrogen Ratio after pasteurisation was highest in Compost 2 at 5.75, followed by Compost Type 4 at 5.27 and Compost Type 3 at 4.65. Compost Type 1 produced the lowest carbon/nitrogen ratio of 3.76 (Table 5).

Electrical conductivity in the control increased from 6.54 dSm⁻¹ to 6.78 dSm⁻¹ during the composting cycle. Equally, the control salinity levels increased from 3.56 to 3.75. Salinity levels were relatively equal in the composts with Compost Type 1 recording the highest at 3.11 and Compost Type 4 recording the lowest level at 2.87. Compost Type 4 also returned the lowest Electrical Conductivity at 5.29 dSm⁻¹, which corresponds with the highest recorded germination in the phytotoxicity test (Table 5).

Germination indices of the composts after pasteurisation of 3 days at 55°C are illustrated in Table 6. Mature compost is considered to be one where the germination percentage is over 80%²³. Only the Compost Type 4 containing all the actinomycete isolates achieved this rating. The Compost

Type 2 showed maturity towards cress but not tomato. The same result was also obtained in the leading retailed potting mix currently used in the Sunshine Coast Region, which was used as a comparison for this test.

The particle size-grading test performed over 2 replicates categorizes the Compost Types 2, 3 and 4 as a soil conditioner with less than 20% retention of mature compost particles in the sieve. Whereas the Compost Type 1 yielded a final product with a category classed as fine mulch with over 20% mature compost particles retention during sieving. The control when sieved also fell into this category at 40% retention of the mature compost particles.

The reduction in the numbers of pathogenic bacteria at the end of the compost cycle was equivalent across all composts containing different actinomycete inocula at the end of the cycle. Initial population's geometric average was 5.3 log¹⁰ cfu/g. This decreased at a rate of between 0.26 and 1.13 log log¹⁰ cfu/g over the composting cycle whereas the control treatments had 1.85 log¹⁰ cfu/g at day 11 and remained there until pasteurisation.

Following pasteurisation coliform reduction was significant in the Compost Type 1 whereas the Compost Type 3

Table 3b. Antibacterial activities of the Actinomycete isolates

Isolate ID	USC-900	USC-901	USC-902	USC-903	USC-904	USC-905	USC-906	USC-907	USC-908	USC-909	USC-910	USC-911	USC-912	USC-4	USC-MP-11	USC-MP-12	USC-MP-13	USC-MP-H1	USC-146
Test organism																			
<i>E. coli</i>	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
<i>Bacillus subtilis</i>	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	+	+	-	-
<i>Candida albicans</i>	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	+	+	-	-
<i>Pseudomonas aeruginosa</i>	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	+	+	-	-
<i>Staphylococcus aureus</i>	+	+	-	-	+	+	+	-	+	+	+	+	+	+	+	+	+	-	+
<i>Mycobacterium smegmatis</i>	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	+	+	-	-
<i>E. coli</i> (Methicillin resistant)	+	+	-	+	-	+	-	-	+	+	+	-	-	+	+	-	-	-	+
<i>Enterococcus faecalis</i> -1 (vancomycin resistant)	+	+	-	+	+	-	-	-	+	+	-	+	-	+	-	-	+	-	+
<i>Enterococcus faecalis</i> -2 (vancomycin resistant)	+	+	-	-	+	+	-	-	+	+	-	+	-	+	-	-	+	-	+
<i>S. aureus</i> (vancomycin resistant)	+	+	-	+	+	+	-	+	+	+	+	-	-	+	-	-	+	-	+

+: inhibitory activity is observed in the agar disk diffusion assay;

-: no inhibitory activity is observed in the agar disk diffusion assay

showing coliform presence below detection limits. The Compost Type 2 and the Compost Type 4 had minimal coliform presence ranging between $0.69 \log^{10}$ cfu/g (Compost Type 2), $0.80 \log^{10}$ cfu/g in (Compost Type 4) and $0.92 \log^{10}$ cfu/g in the control.

DISCUSSION

Augmentation of the poultry manure with indigenous actinomycetes resulted in the production of environmentally friendly biofertilizer as well as biodegradation of feathers within 17 days. Use of indigenous actinomycetes belonging to taxa *Streptomyces*, *Nocardioopsis*, *Nocardioidea*, *Saccharomonospora* and *Nonomuraea* species as an inoculum increased their chances of survival in a competitive environment.

In an earlier study, Tiquia and Michel³²⁾ found that the inoculation of nonindigenous feather degrading *Bacillus licheniformis* and *Streptomyces* sp. did not enhance the composting process. Based on CO₂ evolution and temperature patterns they found that the uninoculated compost community structure and degradation rate was the same as the inoculated one. Within their study there was no consideration for competitive exclusion of the two inoculated species by the existing microbial community with antagonistic properties within the compost. The inoculation of indigenous actinomycetes can be valued as a contributing factor in the success of a decreased composting time frame when compared to usually used compost cycles of 3 to 6 months²⁴⁾. Furthermore, degradation of the feathers did not occur in the control compost without the actinomycete inoculum. Accordingly the rapid composting process and feather degradation

Fig. 1. Final compost moisture content (a) and core temperature values (b)

Fig. 2. Degradation of indicator feathers by actinomycete inoculum

observed in this study clearly indicates that the bioaugmentation of the compost microflora with indigenous actinomycetes speeds up the process.

The actinomycetes used in this study showed predominant growth activity between pH levels of 7.0 and 9.0. Findings agreed with Guerra-Rodriguez *et al.*²⁸⁾ who composted chestnut burr/leaf litter and poultry manure and also with Ignatova *et al.*¹⁰⁾ who conducted studies on the digestion of keratinaceous substances.

Structural differences in the barley straw after composting indicates that the actinomycete isolates used in this study might have ligninolytic activity. This finding agrees with Horwarth *et al.*³³⁾ and Leifeld *et al.*³⁴⁾.

The gas chromatography analysis showed that malodour causing compounds traced during the composting depleted well. These observations agree with the findings of Reichert *et al.*³⁵⁾ who discovered that *Pseudonocardia asaccharolytica* sp. and *Pseudonocardia sulfidoxydans* sp. as dimethyl

Fig. 3. Gas chromatography mass spectrometry identifying commonly detected volatile compounds in fresh poultry manure

Table 4. Detection of changes in the volatile solids before and after the composting

Compost Type		1	2	3	4	Control
VS%	Initial	51	55	54	52	49
	Final	37	55	42	40	55

VS% change is pre and post composting cycle (including pasteurization period).

Table 5. Maturity assessment of the final compost end-product

Compost Type	Dewar temperature rise above ambient °C	Particle size grading	Bulk Density kg/m ³	C/N Ratio	Electrical conductivity (dS m ⁻¹)	Salinity
1	12	21	224	3.76	5.73	3.11
2	13	16	226	5.75	5.67	3.21
3	12	19	196	4.65	5.86	3.09
4	13	9.25	218	5.27	5.29	2.87

disulfide degrading actinomycetes and as well as with the findings of Ohta and Ikeda³⁶⁾ who described *Streptomyces griseus* and *Streptomyces antibioticus* as malodour deodorizers of pig faeces.

All composts types used in this study resulted in faecal coliform reduction under the required Environmental Protection Agency level of 1000 cfu/g²³⁾ for end use. Elimination or reduction of pathogenic species and odour nuisances reduces the risks associated with disease dispersal, air quality problems, flies, vermin and scavenging animals, protection of surface and ground water.

The final level of moisture content of the composts was within guidelines³⁷⁾. The dehydration rates were similar to those recorded by Hayashida *et al.*²²⁾ in the treatment of pig faeces and were at the required levels for palletisation as set by Naylor and Girenes³⁷⁾ for marketing purposes.

Composting using actinomycetes resulted in rapid removal of the recalcitrant feather content and the resulting composts were friable high value product that had accept-

able land use applications. Reduction of the numbers of pathogenic bacteria and malodours was another significant achievement of the composting procedure used in this study and the phytotoxicity levels were lower than an established potting mix used as control.

Alternative poultry manure composting strategies such as the one illustrated in this study would result in the reduction of retention time in the compost cycle performed and would decrease operational costs, which would in turn significantly increase the success of on farm composting. The method used in this study might present an alternative to on farm composting to produce a community orientated and environmentally friendly facility. It would also relieve nursery facilities of environmental liability, farmers of liability and promote recycling while ensuring human and environmental health. In addition, further enzymatic studies on the keratinolytic performance of the actinomycetes in this study might lead to the biological production of feather meal.

ACKNOWLEDGEMENTS

Authors thank Dr Peter Brooks and Dr Mark Holmes of the Faculty of Science, University of the Sunshine Coast for providing expert advice on the chemical and biochemical aspects of the study and to Dr Lynn Woodward, Genetic Analysis Facility, James Cook University, Townsville for sequence analysis of the isolates.

Lyndall Pettett gratefully acknowledges the Zonta Noosa "Women in Science" Encouragement Award.

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