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**PRESERVATION OF HAY USING THE BACILLUS MICROORGANISMS**
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- (57) Claim

1. A method of preserving hay quality comprising adding to fresh hay, a quality preserving effective amount of the microorganism Bacillus pumilus.

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COMPLETE SPECIFICATION

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COMPLETE SPECIFICATION FOR THE INVENTION ENTITLED:

Bacterial treatment to preserve hay quality by addition of microorganisms of the genus bacillus

The following statement is a full description of this invention, including the best method of performing it known to me/us:-

About 150 million tons of hay is harvested annually in the United States. The loss in production is probably larger than any other crop. These losses are due to unfavorable alfalfa maturity, rain damage, field harvest loss and storage losses. The losses are typically from 20-40%, but can be as high as 70% if weather conditions are unfavorable. These losses result from the continued respiration of plants, leaf shattering from mechanical damage and leaching due to rain. Baling hay at higher than normal moistures (25-30%) will minimize the risk of rain damage and mechanical leaf losses. However, baling at moistures greater than 20% greatly increases the risk of storage losses due to oxidation and molding.

Storage losses are a major, but controllable source of nutrient loss. Hay stored inside a building at moisture contents below 20% will have a normal dry matter loss of between 5-10%. Higher moistures enhance the activity of aerobic oxidative microorganisms resulting in heat production. Excessive heat results in dry matter losses due to respiration which produces CO<sub>2</sub> and water. The heat also causes nutrient losses through nonenzymatic browning reactions. The amount of heat that is generated will depend on the moisture concentration of the hay, the ambient temperature, the existing microbial populations and the size and density of the bales.

Excessive heat damage reduces the protein digestibility and available energy. Heat damaged protein is measured by determining the nitrogen content of the fiber as acid detergent fiber nitrogen (ADIN or ADF-N). Under normal conditions, less than 7% of the total nitrogen should be present in the fiber. Excessive heat damage has occurred when this value reaches 15%.

Mold damage during storage can also cause significant losses. Only about 5% of molds produce mycotoxins,

however, they can adversely effect palatability and feed intake. Feeding moldy hay to animals will result in significantly lower dry matter intake, reduced weight gains and poorer feed conversions. These losses can be as large as 25% over nonmoldy controls.

Stored hay contains naturally-occurring opportunistic epiphytes. These epiphytes may be beneficial or detrimental to the hay quality and compete among themselves for ecological dominance in the stored hay. These epiphytes may be either bacteria or fungi. Common bacterial spoilage organisms are those which are members of the genera Sporosarcina and Bacillus. Fungal spoilage is often caused by members of the genera Aspergillus, Penicillium and Mucor.

Various approaches have been made to limit the proliferation of those epiphytes responsible for spoilage of agricultural products. One simple approach has been to spray the agricultural material at the time of storage with a mold inhibitor as, for example, propionic acid. A major disadvantage of this approach is its cost since large quantities of acid must be used to adequately treat the agricultural material. In addition, the inherently toxic and corrosive nature of acids such as propionic acid requires that special handling techniques be used by the farmer to protect personnel handling the acid. Further, all equipment coming in contact with the acid must be thoroughly cleaned to avoid corrosive damage. For further details with regard to one other approach, see my co-pending and commonly assigned application entitled HAY PRESERVATIVE, Serial No. 891,260, filed July 28, 1986, which relates to use of an inorganic salt mixture of zinc oxide, magnesium oxide and a water soluble copper ion source in combination with an organic acid such as acidic acid, propionic acid or sorbic acid.

However, in spite of extensive research into the development of various products for use as agricultural

product inoculants, spoilage of hay has remained an on-going problem.

Accordingly, it is a primary objective of the present invention to provide a hay preservative composition which adds to the hay only naturally occurring organisms at levels which hinder growth of spoilage organisms, while reducing heat in high moisture hay.

Another objective of the present invention is to provide an improved hay preservative which surprisingly acts to effectively reduce heat in high moisture hay without the necessity for adding unnatural hay additives.

A further objective of the present invention is to provide a hay preservative composition which has no environmental polluting risks.

A yet further objective of the present invention is to provide a hay preservative which will provide a quality hay product as measured by temperature, dry matter recovery, nitrogen profile, color, and micro-organism counts including yeasts and molds.

An even further objective of the present invention is to provide a hay preservative, as well as a method for preserving hay which adds to the hay, at hay preserving levels, Bacillus pumilus microorganisms.

A still further objective of the present invention is to provide hay preservative compositions which when applied to hay will reduce heat degradation risk, while simultaneously hindering growth of spoilage organisms such as Aspergillus flavus.

Another object of the present invention is to provide a method of preserving hay quality comprising adding to fresh hay, a quality preserving effective amount of the microorganism Bacillus pumilus.

The method and means of accomplishing each of these objectives as well as others will be apparent from the detailed description of the invention which will follow hereinafter.



In the present invention, hay is treated with Bacillus pumilus to preserve hay quality. Bacillus pumilus is capable of inhibiting spoilage organisms responsible for the deterioration of hay stored for long periods of time.

Thus, the present invention provides a method of treating hay to enhance its preservation which comprises administering to the hay an effective amount of Bacillus pumilus or mutants or equivalents thereof.

The invention also provides for a hay treating composition containing Bacillus pumilus. The compositions may be solid or liquid.

Spoilage of hay due to deterioration caused by the growth of spoilage organisms is a major problem in the agricultural community. Typical spoilage organisms include the genus Aspergillus and the species Aspergillus flavus, as well as the genus Penicillium and Mucor.

Hay that is baled at greater than about 25% moisture will heat and mold about 97% of the time, as indicated by field surveys. These heated bales are remarkably poorer in color, nutritional value and have higher visible mold.

It has been discovered that strains of Bacillus pumilus will preserve hay quality. While not wishing to be bound by any theory of operability, it is believed that the Bacillus pumilus may produce inhibitory compound(s) that inhibit the growth of fungi and also bacteria such as the bacterial isolate Sporosarcina. Sporosarcina are responsible for the undesirable white bloom observed on the stems of hay after only a few days of storage. These organisms are typically

observed in very hot bales. They also appear to act to encourage the growth of fungi either concurrent to their growth or at a later stage.

Understanding microbial interactions is an essential  
5 element of the use of this strain in improving the  
quality of stored hay. Initially when hay is baled a  
large bloom of one class of microorganisms occurs (the  
Gram negative group). This is observed in the first  
1-3 days. Thereafter this is replaced by a second  
10 group (the Gram positive group). Bacillus pumilus  
belong to this group, the Gram positive group. If the  
selected Bacillus pumilus are present in high numbers,  
as hereinafter defined, at this stage the hay will not  
heat or mold. Later, other groups of microflora  
15 become dominant as the populations of Bacillus pumilus  
subside. At the end of the storage period when the  
hay is fed, the Bacillus will be a part of the micro-  
flora. Again, while applicant does not wish to be  
bound by any theory, it is possible the Bacillus may  
20 effect the quality of the hay simply by competition  
for nutrients with the other organisms present.

In the method according to the invention, hay is  
treated with a hay preserving effective amount of  
Bacillus pumilus, or mutants or equivalents thereof,  
25 to enhance preservation and to maintain the quality of  
the hay, and as well to diminish the proliferation of  
spoilage organisms.

The method of the invention is especially useful  
in that the organism of the invention is non-pathogenic  
30 and occurs naturally in hay at low levels and therefore  
will not cause disease in an animal consuming the  
preserved agricultural product. Unlike many types of  
preservation which utilize the application of expensive  
often hazardous chemicals, the method of the invention  
35 relies instead upon the enhancement of colonization by  
desirable naturally occurring organisms through  
improving their ability to compete with naturally-

occurring spoilage organisms, such as Aspergillus flavus. Controlling these harmful organisms is a major consideration, since many of the organisms associated with spoilage of preserved agricultural products are fungi which are known to produce potent mycotoxins. These mycotoxins are known to cause illness and even death in animals consuming feed contaminated with these fungi.

There are certain preferred strains of Bacillus pumilus which have been isolated and found most preferable in the composition of this invention. Those strains are available upon request by the assignee and are identified as Strain Nos. 288, 289, 290, 296, 299, 302, 305 and 307. The strains ~~have~~ been deposited with the American Type Culture Collection at Rockville, Maryland, <sup>on 14th October, 1987 and were accorded</sup> ~~and are further identified as~~ ATCC accession numbers 53682, 53683, 53684, 53685, 53686, 53687, 53688, and 53689, respectively. One may use a mixture of these preferred strains which is satisfactory, or any lesser combination of them, or simply a single strain.

Bacillus pumilus was isolated from stored hay using techniques well known in the art and described generally above. Other Bacillus pumilus may be isolated and tested according to routine experimentation to assess the efficacy of the Bacillus pumilus in the preservation of hay quality.

By the term "effective mutants thereof" is intended any and all mutants of Bacillus pumilus which demonstrate the desired fungal inhibiting properties of the present strain or substantial equivalents thereof. Such mutants are considered to be functionally equivalent to the parental strain. It is well known to those of ordinary skill in the art that spontaneous mutation is a common occurrence in microorganisms and that mutations can also be intentionally produced by a





variety of known techniques. For example, mutants can be induced using chemical, radioactive, and recombinant techniques.

Regardless of the manner in which the mutations are induced, the critical issue is that the mutants function to preserve the agricultural product as described for the parent strain. In other words, the present invention includes mutations resulting in such minor changes as, for example, minor taxonomic alterations such as the fermentation of certain sugars.

While the invention is being described with particularity with regard to the preferred species Bacillus pumilus, and certain specific strains thereof, it should be understood that the invention contemplates that one may use other genetically closely related organisms of the genus Bacillus. It may therefore be possible to accomplish most if not all of the objectives of this invention with other species members of the genus Bacillus, such as Bacillus subtilus and Bacillus cereus. Bacillus pumilum is strongly preferred because it is normal flora of hay.

By the term "hay" is intended all forms of hay as the term is commonly used in agriculture. Hay is most commonly composed of alfalfa, grass, or mixtures of alfalfa and grass. It is possible that the hay preservative composition of this invention may also be used as an effective silage inoculant to reduce spoilage of silage. Another distinct possibility is that the composition may effectively function to preserve grains such as corn, wheat, rice and soybeans.

In the present invention, the inhibition of organisms responsible for hay spoilage is accomplished by treating the hay with organisms of the genus Bacillus, especially Bacillus pumilus or with compositions containing Bacillus pumilus or closely related organisms, and as well by treatment with effective mutants

or equivalents of Bacillus pumilus and compositions containing same.

5 The compositions which are used in the method of the invention may be in either liquid or dry form and may contain additional bacterial strains. In solid treatment forms, the composition may comprise the Bacillus pumilus together with a carrier. The carrier may be in the nature of an aqueous or nonaqueous liquid or a solid. In solid forms, the composition  
10 may contain solid carriers or physical extenders.

Examples of such solid carriers, solid diluents or physical extenders include malto-dextrin, starches, calcium carbonate, cellulose, whey, ground corn cobs, and silicone dioxide. In short, the carrier may be  
15 organic or an inorganic physical extender. The solid composition can be applied directly to the hay in the form of a light powder dusting, or if it is disbursed in a liquid carrier it can successfully be sprayed on the hay.

20 Typical compositions useful for treating hay according to this invention contain  $10^2$ - $10^{12}$  viable organisms/gm, preferably  $10^4$ - $10^{10}$  viable organisms/gm, and more preferably  $10^5$ - $10^7$  viable organisms/gm.

The treatment range for hay is typically  $10^5$ - $10^{15}$   
25 viable organisms/ton, preferably  $10^7$ - $10^{13}$  viable organisms/ton, and more preferably  $10^8$ - $10^{10}$  viable organisms/ton.

The composition of the present invention can include, in addition to Bacillus pumilus or mutants or  
30 equivalents of this organism, other common farm product preservation organisms as, for example, Lactobacillus, Streptococcus, and Pediococcus, and certain enzymes from fungi or bacteria.

Those of ordinary skill in the art will know of  
35 other suitable carriers and dosage forms, or will be able to ascertain such, using routine experimentation.

Further, the administration of the various compositions can be carried out using standard techniques common to those of ordinary skill in the art.

5 The above disclosure generally describes the present invention. A more complete understanding can be obtained by reference to the following specific examples which are provided herein for purposes of illustration only and are not intended to be limiting unless otherwise specified.

10

EXAMPLES

Hay was treated with inocula prepared with Bacillus pumilus strain Nos. 288, 289, 290, 296, 299, 302, 306  
15 and 307, in mixture.

Quality of hay was determined by examining the temperature, microflora and nutritional analysis. Visual scores were determined for all treatments including color, white scale and mold spores.

20 Alfalfa was cut with a mower-conditioner and allowed to dry in the field under normal field conditions. The mowed hay was placed in windrows and raked only once prior to baling. Drying rate, maturity and weather data were recorded. Hay was baled at 22 to 34  
25 percent moisture across all trials, but within a given trial hay moisture varied only 2 to 5 percent between treatments and control bales.

For small square bales experiments, the following protocol was followed. Hay was treated with water  
30 soluble treatments of the hay preservative using a sprayer. For the longer field experiment, the treatment was applied to 15-20 100-pound bales. Control uninoculated bales were prepared as well. A total of 15-20 bales was prepared for the control. Hay was transported  
35 from the field, stacked and stored on wooden pallets on a cement floor in an enclosed shed. Temperature

probes were placed in the center of each stack and temperature recorded hourly.

A smaller model system was also developed to simulate larger bale studies. One-pound of hay was packed into a styrofoam shipping container (2-inch wall thickness) with approximate dimensions of 6" x 10" x 6". The hay was compressed by placing a brick on top of the hay after a thermister had been placed into the center of the hay mass. The styrofoam lid was placed loosely on the top and the entire container placed in a 37°C incubator to initiate heating. Treatments were applied to the hay by spraying with a glass TLC sprayer.

Sampling of bales was performed by removing six core samples/sampling time with a Penn State Forage Sampler. Within each stack two lower, two middle and two top bales were sampled to minimize variability within stacks. Sample cores were placed in whirl pak sterile bags, pooled within each treatment and stored on ice for transport to the laboratory. Sampling of model bale styrofoam containers was performed by removing one sample from the center of each container. Sampling times were approximately 0, 2, 7, 14, 21 30 and 60 days.

The microorganism counts of the Bacillus pumilus as applied to the hay samples by spraying provided an organism count of  $10^6$ /gm of hay. This corresponds to an organism count of  $10^9$ /ton of hay. The organisms were a mixture on equal parts basis of strain numbers 288, 289, 290, 296, 299, 302, 305 and 307 of Bacillus pumilus. The Bacillus pumilus treatments were prepared by growing the eight strains individually in tryptic soy broth for 24 hours at 37°C. Cells were removed by centrifugation at 4°C and the cell pellet re-suspended in .04 M phosphate buffer. Cells were transported to the field on ice for immediate application or frozen

at -70° C for future use. Prior to application to the hay, cells were dispensed in five gallons water and applied (at five gallons per ton) with a sprayer attached to the baler. The colony forming units/treatment was determined at the time of application.

The samples were analyzed for dry matter, ADF, ADF-N, NDF, and N content. The temperature was monitored on an hourly basis by use of temperature thermistors accurate to  $\pm 0.2^{\circ}\text{C}$ . The data was analyzed using the covariates dry matter (DM) and day 0 temperature.

The hay was observed visually throughout the trial and at the end of the storage period for evidence of mold and white spoilage microflora. The hay was evaluated for moldiness and white spoilage on a scale of one to 10 with 10 being poorest quality. Color differences were determined at the end of the storage period using paint strips for comparisons. Colors ranged from 1 green to 23 brown.

In the smaller model system, the temperature differences between experiments represent the most informative results because thermistors are accurate to  $\pm 0.2^{\circ}\text{C}$  and hourly readings were taken. This resulted in about 500 data points/treatment/experiment. The significantly coolest treatment overall in the experimental models was the Bacillus (Tables 1 & 2). This treatment tended to have lower yeast and molds populations. The Bacillus treatment also had significantly higher nutritional values (lower ADF). NDF, ADF-N, WSC, N and available protein determinations also tended to show higher nutritional values, although these differences were not significant. The Bacillus treatments also had significantly better visual scores indicating less visual molding. Color scores were less consistent with no difference between the treatments in these small model bales.

Table 1

EFFECT OF SELECTED TREATMENT ON MEAN TEMPERATURE  
LOG10 MICROBIAL COUNT AND VISUAL QUALITY OF  
HAY IN EXPERIMENTAL MODEL BALES

5	<u>Treatment</u>	<u>Reps</u>	<u>Temp</u>	<u>Aerobes</u>	<u>YM</u> <sup>1</sup>	<u>Color</u>	<u>Mold</u> <sup>2</sup>
	Bacillus	3	36.57*	8.10	2.72	10.5	4.5*
	Control	5	37.28	8.36	3.30	9.25	7.5

<sup>1</sup> YM values are for the end of the trial

10 <sup>2</sup> Moldiness; the amount of mold observed visually in bales on a scale of 1 (not moldy) to 10 (very moldy)

\* Treatment means followed by an asterisk are significantly different from the control P<.1

15 Table 2

EFFECT OF SELECTED TREATMENTS ON FINAL CHEMICAL  
COMPOSITION OF HAY IN EXPERIMENTAL MODEL BALES

	<u>Treatment</u>	<u>Reps</u>	<u>N</u>	<u>NDF</u>	<u>ADF</u>	<u>ADF-N</u>	<u>Hemi- Cellulose</u>	<u>AP</u> <sup>1</sup>
20	Bacillus	3	3.01	55.11	39.60*	.25	15.51*	17.25
	Control	5	2.80	57.30	43.58	.32	13.72	15.50

<sup>1</sup> Available protein (N%)(%ADF-N)(6.25)=AP

25 \* Treatments followed by an asterisk are significantly different (P<.1) from the control

The same trends shown in the model system of Tables 1 and 2 were observed in the larger scale hay bales as evidenced by Tables 3, 4 and 5 below. That is, the Bacillus treated hay was cooler than the control treatment. The yeast and mold counts were similar in all bales but visual molding was reduced in the Bacillus pumilus treatments and the color was greener. Nutritional analysis also indicated similar trends to those observed in the model system shown in

Tables 1 and 2. Available protein (AP) was higher in the Bacillus treated bales and the ADF and the NDF values indicated highest nutritional quality in these treated bales.

5 Treatment of hay with Bacillus pumilus strains resulted in cooler, less moldy greener bales than uninoculated control bales. Treatment with Bacillus pumilus also gave bales that had higher available protein intake, total digestible nutrient intake and  
10 daily energy intake.

Table 3

15 THE EFFECT OF TREATMENTS ON THE MEAN TEMPERATURE, MICROBIOLOGICAL (LOG<sub>10</sub>) AND VISUAL QUALITY OF HAY IN SMALL SQUARE BALE STACKS

EXPERIMENT #590 BALES (2ND CUTTING)

	<u>Treatment</u>	<u>Temp°C</u>	<u>Total Aerobes</u>	<u>YM<sup>1</sup></u>	<u>Color</u>	<u>W</u>	<u>Mold</u>
20	Bacillus	19.92*	8.82	3.33	6.5	0.0	0.0*
	Control	24.92	9.00	3.00	10.5	3.5	2.5

<sup>1</sup> YM values are for the end of trial.

25 \* Treatment means followed by an asterisk are significantly different (P<.1) from the control.

Table 4

30 THE EFFECT OF TREATMENTS ON THE FINAL CHEMICAL COMPOSITION OF HAY IN SMALL SQUARE BALE STACKS

EXPERIMENT #590 BALES

	<u>Treatment</u>	<u>N</u>	<u>NDF</u>	<u>ADF</u>	<u>ADF-N</u>	<u>Hemi-cellulose</u>	<u>AP</u>
35	Bacillus	3.42*	52.70	38.32	.221	14.38	19.99*
	Control	3.23	56.30	40.49	.246	15.81	18.65

N initial = 3.61  
NDF initial = 51.42  
ADF initial = 38.66  
Hemicellulose = 12.76  
ADF-N = .276  
AP = available protein = 20.83  
DM = 70.15

- 5 \* Treatment followed by an asterisk are significantly different (P<.1) from the control

10 When the spray application of the examples shown is replaced with a powder dust application to achieve organism levels within the ranges specified in the specific examples shown, similar results are obtained in that the bales are cooler, that is there is a reduction in heat, there is a hinderance of growth of spoilage organisms in the microflora and there is  
15 evidence of increased nutritional value of the hay.

It therefore can be seen that the invention accomplishes at least all of the stated objectives.



THE CLAIMS DEFINING THE INVENTION ARE AS FOLLOWS:

1. A method of preserving hay quality comprising adding to fresh hay, a quality preserving effective amount of the microorganism Bacillus pumilus.

2. The method of claim 1 wherein the microorganism is Bacillus pumilus or effective mutants or equivalents thereof.

3. The method of claim 2 wherein said treating is by spraying.

4. The method of claim 3 wherein the amount of viable organisms per ton of hay treated is within the range of from about  $10^5$  to about  $10^{15}$  organisms per ton of hay.

5. The method of claim 4 wherein the amount of organisms is from  $10^7$  to  $10^{13}$  per ton of hay.

6. The method of claim 5 wherein the amount of organisms is from about  $10^8$  to about  $10^{10}$  per ton of hay.

7. The method of claim 2 wherein the Bacillus pumilus is selected from the group consisting of Bacillus pumilus, strain Nos. 288, 289, 290, 296, 299, 302, 305 and 307, accorded ATCC Accession Nos 53682, 53683, 53684, 53685, 53686, 53687, 53688 and 53689, respectively.

8. The method of claim 1 wherein the treating composition includes other farm product preservation organisms.

9. The method of claim 8 wherein said other organisms are selected from the group of Lactobacillus,

Streptococcus, Pediococcus and enzymes derived from fungi or bacteria.

10. A method of preserving hay quality, comprising: adding to fresh hay under conditions which enhance organism colonization, Bacillus pumilus, or mutants or equivalents thereof; and allowing said organisms to significantly colonize and thus forestall colony growth of naturally occurring spoilage organisms whose colonization is therefore hindered.

11. The method of claim 10 wherein said organisms are added by spraying a liquid containing them on said hay.

12. The method of claim 11 wherein said organisms are added to provide a viable organism count of from about  $10^5$  to about  $10^{15}$  viable organisms per ton of hay.

13. The method of claim 12 wherein the amount of organisms is from  $10^7$  to  $10^{13}$  per ton of hay.

14. The method of claim 13 wherein the amount of organisms is from about  $10^8$  to about  $10^{10}$  per ton of hay.

15. The method of claim 10 wherein the Bacillus pumilus is selected from the group consisting of Bacillus pumilus, strain Nos. 288, 289, 290, 296, 299, 302, 305 and 307, accorded ATCC Accession Nos 53682, 53683, 53684, 53685, 53686, 53687, 53688 and 53689, respectively.

16. The method of claim 10 wherein the treating composition includes other farm product preservation organisms.

17. The method of claim 16 wherein said other organisms are selected from the group of Lactobacillus, Streptococcus, Pedicoccus and enzymes from fungi or bacteria.

18. A composition for use as a hay preservative, comprising:

a culture of Bacillus pumilus selected from the group consisting of ATCC Accession Nos 53682, 53683, 53684, 53685, 53686, 53687, 53688 and 53689, or effective mutants thereof, dispersed in a suspension carrier.

19. The composition of claim 18 wherein the composition contains from about  $10^2$  to about  $10^{12}$  viable organisms per gram.

20. The composition of claim 19 wherein the composition contains from about  $10^4$  to about  $10^{10}$  viable organisms per gram.

21. The composition of claim 20 wherein the composition contains from about  $10^5$  to about  $10^7$  viable organisms per gram.

22. The composition of claim 18 wherein the suspension carrier is liquid.

23. The composition of claim 18 wherein the suspension carrier is solid.

24. The composition of claim 18 wherein the solid carrier is water soluble.

25. A method of claim 1 or claim 10, or a composition of claim 18, substantially as hereinbefore described with reference to the accompanying Examples.

DATED this 5th day of October, 1990

PIONEER HI-BRED INTERNATIONAL, INC.  
by DAVIES & COLLISON  
Patent Attorneys for the Applicant

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