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(54) Title
**THE USE OF BACTERIOLYTIC ENZYME PRODUCT AND PROTEASE AS ADDITIVE FOR IMPROVING
FEED CONVERSION IN LIVESTOCK PRODUCTION**

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(56) Prior Art Documents
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(57) Claim

1. A method of improving weight gain and feed conversion in livestock comprising feeding to livestock animal feedstuff to which has been added a bacteriolytic enzyme product from streptomyces coelicolor DSM 3030 or a mutant or variant thereof.

2. A method of improving weight gain and feed conversion in livestock comprising feeding to livestock animal feedstuff to which has been added a bacteriolytic enzyme product with N-acetylmuramidase activity and protease.

9. An animal feedstuff which contains a bacteriolytic enzyme product from Streptomyces coelicolor DSM 3030 when used as a weight gain and feed conversion additive in a method according to any one of claims 1 to 8.

10. An animal feedstuff which contains a bacteriolytic enzyme product with N-acetylmuramidase activity and protease when used as a weight gain and feed conversion additive in a method according to any one of claims 1 to 8.

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COMPLETE SPECIFICATION

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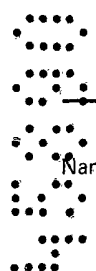
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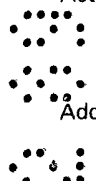
Related Art:


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 Complete Specification for the invention entitled:


 THE USE OF BACTERIOLYTIC ENZYME PRODUCT AND PROTEASE AS ADDITIVE FOR IMPROVING FEED CONVERSION IN LIVESTOCK PRODUCTION

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

US

Description

The use of bacteriolytic enzyme product and protease as additive for improving feed conversion in livestock production.

- 5 The use of additives in animal feed for improving feed conversion is known. These production promoters mainly take the form of substances with antibacterial activity, such as, for example, Flavomycin®, a mixture of various phosphoglycolipid antibiotics, the moenomycins, whose use
10 as feed additive is described in German Patent 1,203,107.

WO 8904-320 discloses the preparation of an animal lysozyme with the aid of a genetically modified micro-organism. A lysozyme of this type can be employed as feed additive for ruminants to improve the weight gain.

- 15 SU 1397-019 describes a mixture of lysozyme and citric acid or acetic acid as growth stimulator for livestock.

- It has now been found, surprisingly, that the use of bacteriolytic enzyme product from Streptomyces coelicolor DSM 3030, as well as the mutants and variants thereof
20 which likewise produce the enzyme product, is advantageous as feed additive. Moreover, a mixture of bacteriolytic enzyme product and protease can be used in feedstuffs with very good results in the weight gain and the feed conversion of livestock.

- 25 Hence the invention relates to:

1. The use of bacteriolytic enzyme product from Streptomyces coelicolor DSM 3030, as well as the mutants and variants thereof, as additive to animal feed.
2. The use of a mixture of bacteriolytic enzyme product with N-acetylmuramidase activity and protease as
30 additive to animal feed.

3. A feedstuff which contains the product specified in 1. or 2. as additive.

The invention is described in detail hereinafter, especially in its preferred embodiments. The invention, is furthermore defined by the contents of the claims.

The enzyme product with N-acetylmuramidase activity from DSM 3030 can be employed both in pure form and mixed with other substances, for example with fermentation residues or proteins.

The strain streptomyces coelicolor DSM 3030 was isolated from soil samples. The selection feature was the excretion of an enzyme product which is able to lyse bacteria at pH 3 to 7.

10 The strain grows in customary culture media, into which it releases the bacteriolytic enzyme product. After removal of the cells, the bacteriolytic enzyme product remains in the culture supernatant, from which it can be isolated by conventional methods of protein enrichment and purification, such as alcohol precipitation, ion exchange chromatography, ultrafiltration and gel filtration.

15 Streptomyces coelicolor DSM 3030 is cultured, for example, in a nutrient medium which contains sugarbeet molasses in a quantity of 5 to 50 g, preferably 10 to 20 g, per liter of culture medium. The yield can be increased further if calcium ions are added to the medium in the form of readily soluble, non-toxic calcium salts. A calcium concentration of 0.05 to 1 molar is advantageous.

20 The enzyme product is secreted into the culture medium. The enzyme with N-acetylmuramidase activity can be isolated pure from the medium by methods known per se, for example by precipitation or extraction. However, it is not absolutely necessary to work up the fermentation supernatant to isolate the bacteriolytic enzyme product, it is sufficient to add it as dry product directly to the feed. The fermentation supernatant is preferably dried by spray drying.

25 A bacteriolytic enzyme product prepared in this way is, however, associated with proteases. In order to obtain the pure enzyme product, it is possible to carry out a purification with the aid of ion exchanger chromatography on weakly acid macroporous cationic exchangers. A

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corresponding process is described in European Patent Application 280,032.

5 The resulting bacteriolytic enzyme product is stable and active in terms of its lytic activity in a pH range from below 3 to 9. After incubation at room temperature in buffer mixtures for 16 hours, the maximum activity in the range from pH 3 to 9 was still at least 80% retained.

10 The temperature optimum for the activity of the bacteriolytic enzyme product is in the range from 50 to 60°C. ^{Within} ~~Above~~ this range at least 90% of the maximum activity are reached. Starting from low temperatures, the activity increases slowly with increasing temperature up to 60°C: it is about 10% at room temperature, about 30% at 30°C, and about 60% at 40°C, of the maximum activity. There is
15 a rapid fall in activity at higher temperatures: it is still about 30% at 65°C, and 10% at 70°C, of the maximum activity.

20 It is also possible to use a bacteriolytic enzyme product mixed with proteases. In this connection, every bacteriolytic enzyme product with N-acetylmuramidase activity can in principle be used according to the invention. An enzyme product of this type is composed principally of one or more enzymes with N-acetylmuramidase activity, but it is perfectly possible to add other substances, for
25 example fermentation residues, to these enzymes. The enzyme product can be obtained from plant or animal tissue or be of microbial origin. A particularly suitable bacteriolytic enzyme product is one obtained from Streptomyces coelicolor DSM 3030, as well as one obtained from
30 the variants and mutants thereof. The proteases are, in particular, trypsin- or chymotrypsin-like enzymes. The use of the protease-containing enzyme mixture is advantageous because better results are achieved in the feed conversion with these products. The ratio of bacteriolytic enzyme product to protease ought to be in the range from 1:1 to 1,000:1, advantageously in the range from
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100:1 to 500:1, based on enzyme activity.

5 The bacteriolytic enzyme product from DSM 3030, as well as the enzyme mixture just described, can in principle be added to all feedstuffs. Suitable and preferred examples are those which comply with the provisions of the feedstuffs legislation, such as complete feed, supplementary feed and mineral feed.

10 The bacteriolytic enzyme product can be dosed in the range from 0.1 to 100,000 ppm, preferably in the range from 1 to 1,000 ppm. The lytic activity of bacteriolytic enzyme product is normally stated in units (U). The N-acetylmuramidase activity is measured as described in German Offenlegungsschrift 3,440,735. In the feed it should expediently be between 0.1 and 100 U/mg of feed,
15 preferably between 0.5 and 50 U/mg.

20 The enzyme product, alone or in a mixture, is mixed with the feed batchwise in appropriate ratios by weight. In this connection, it is important that the active substances in the feed are homogeneously distributed.

25 Feedstuffs which contain bacteriolytic enzyme product from DSM 3030 or the mixture of bacteriolytic enzyme product and protease can be employed for feeding all livestock, but particularly advantageously for feeding agricultural livestock used for the production of foodstuffs, in particular broilers, turkeys, pigs and cattle.

30 Addition of the bacteriolytic enzyme product or of the mixture to the feedstuffs brings about a considerable improvement in the utilization thereof and, connected with this, an improvement in growth of the livestock. The use thereof as additive to feedstuffs has, by comparison with additives having antibiotic activity, the great advantage that there is no risk of resistance development on use over a prolonged period.

The invention is to be explained in more detail hereinafter by means of examples.

Example 1:

Cultivation of *S. coelicolor* DSM 3030

5 Slant agar medium which contained 2% soybean meal, 2% mannitol and 1.5% agar (pH 7.5) was inoculated with *Streptomyces coelicolor* DSM 3030 and cultivated at 30°C for 10 days. 10 ml of a sterile solution of 0.9% NaCl and 0.01% of a nonionic surfactant were added to the slant culture, and the spores were rinsed out. 0.2 ml of the spore suspension was used as inoculum for shake cultures of 100 ml culture medium in culture flasks of 300 ml capacity.

15 Culture media Nos. 1-3 listed in Table 1 were made up, inoculated and shaken at a rate of 160 rpm and at 30°C. Samples were taken after 2, 4 and 6 days, the cells were removed by centrifugation, and the activity of the bacteriolytic enzyme product in the culture supernatant was determined (Table 2).

20 The highest yields of bacteriolytic enzyme product were obtained with nutrient solution 3 which, besides glucose, soybean meal and casein peptone, contained 2% sugarbeet molasses.

25 **Table 1:**
Culture media

	Medium No.		
	1	2	3
Glucose	2		1
Mannitol		2	
30 Soybean meal	2	1	0.5
Casein peptone			0.5
Molasses		1	2

Table 2:

Activity of bacteriolytic enzyme product in the culture supernatant (U/ml)

5	Fermentation time days	Medium No.		
		1	2	3
	2	21	15	363
	4	367	378	1106
	6	236	558	2088

Example 2:

10 Increasing the yield by addition of calcium

Streptomyces coelicolor DSM 3030 was cultivated in the manner described in Example 1 on a nutrient medium which contained 1% glucose, 2% molasses, 0.5% soybean meal, 0.5% casein peptone and 0.5% $\text{CaCl}_2 \times 2\text{H}_2\text{O}$. The addition of CaCl_2 resulted in a considerable increase in the yield of bacteriolytic enzyme product in the culture supernatant, 768 U/ml, 2,462 U/ml and 4,715 U/ml being obtained after 2, 4 and 6 days.

Example 3:

20 a) Determination of the activity of bacteriolytic enzyme product:

0.2 ml samples containing bacteriolytic enzyme product are pipetted into 2.8 ml of a suspension of 0.2 mg of Micrococcus luteus ATCC 4698 (Boehringer Mannheim) per ml of 0.1 M sodium acetate buffer (pH 5.0), and the decrease in turbidity is determined at 25°C by measuring the extinction at 450 nm. 1 U is defined as a decrease in extinction of 0.001 photometer scale units per minute.

b) Determination of protease activity with azocasein:

Starting material:

Buffer: 0.1 M sodium phosphate buffer (pH 7.0)

Azocasein solution: 250 mg of azocasein + 10 ml of
buffer

stir until no more azocasein
dissolves; remove undissolved
azocasein by centrifugation, and
use the supernatant for the
assay.

Procedure:

Pipette the following into 15 ml glass centrifuge
tubes

Sample

Blank

2 ml sample

2 ml sample

add 0.5 ml of buffer

preheat in a waterbath at +40°C for 5 min

add 0.5 ml of azocas. solution (40°C)

mix

incubate in a waterbath at +40°C for 30
min

add 0.2 ml of 70% strength perchloric acid
(to stop reaction)

mix

leave to stand at room temperature for 5
min

remove precipitated casein by
centrifugation

determine the supernatants by photometry
(E₄₄₀)

0 value: buffer is used in the assay in place of
the sample

the 0 value should be 0.050-0.060 (E₄₄₀).

Calculation:

$$IU_{azoc.}/ml = \frac{\Delta E \cdot 0.001}{min} \cdot \frac{(\Delta E - 0 \text{ value}) \times 1.000}{30} \times \text{dilution}$$

Example 4:

6-Month trial with fattening bulls

5 In a 6-month trial with fattening bulls, groups each of
9 animals received dosages of 100 ppm or 200 ppm enzyme
product per head and day (obtained by drying the cultiva-
tion supernatant after cells of *Streptomyces coelicolor*
DSM 3030 had been removed) per kg of supplementary feed,
which corresponds to an average dosage of enzyme mixture
of 326 mg and 454 mg, respectively, per head and day.
10 Apart from the improvement in growth and in the feed
conversion due to the product, nothing abnormal was found
in the treated stock.

Example 5:

Rat trial

15 Rats in the weight range 65-180 kg live mass received a
standardized rat feed (Altromin No. 3121, Altromin
International, Lage/Lippe, FR Germany) which was adjusted
to the needs of growing rats. 6 animals were employed for
each treatment and were distributed over the "control"
20 group (no additives), test group I (19 U of lysozyme/mg
of feed) and test group II (38 U of lysozyme/mg of feed).
The animals were housed singly in metabolism cages.

25 For each animal the feed consumption was recorded daily,
and the weight weekly. A 14-day feeding period was
followed by a 7-day nitrogen-balance period with quanti-
tative collection of feces and urine.

30 The nitrogen retention was calculated, as a measure of
the protein gain, from the difference between the
consumption of nitrogen from the feed and the excretion
of nitrogen in the feces and urine.

Result (Table 1):

The weight gains in test groups I and II were increased by 4.2 and 3.1% respectively, and the feed consumption was reduced by an average of 3.3%. The nitrogen retention was improved by an average of 28.5 and 25.2% respectively.

Example 6:

Piglet trial

Piglets in the weight range 15-25 kg live mass received a compound feed which was based on cereal, soybean and fish meals and which was supplemented with vitamins, trace elements and major elements to suit requirements (Tab. 3). The control group (n = 10) and test group (n = 7) received the feed without and with, respectively, addition of 10 U of lysozyme activity/mg of feed.

The feed consumption was recorded per meal (2/day) and the weight gain was recorded weekly. The daily gains (g/d) and the feed conversion ratio (kg of feed/kg of gain) were calculated at the end of the trial.

Result (Table 2):

The weight gain was improved by an average of 4.7% by the addition, and the feed conversion ratio was reduced by an average of 8.5%.

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Table 1

Effect of lysozyme/protease combination on rats

Group	Lysozyme U/mg of feed	Activity Protease gains g/d	Daily Feed conversion	Nitrogen retention g/d
Control		5.46 ± 0.16 100.0	3.04 ± 0.09 100.0	0.151 ± 0.010 100.0
I	19	0.04 5.69 ± 0.18 104.2	2.94 ± 0.12 96.7	0.194 ± 0.016 128.5
II	38	0.08 5.63 ± 0.13 103.1	2.94 ± 0.09 96.7	0.189 ± 0.008 125.2

p*	≤ 0.05		0.05	0.001

* probability of error

Table 2

Effect of lysozyme/protease combination on piglets

	Activity		Daily gains g/d	Feed conversion
	Lysozyme	Protease		
5	U/mg of feed			
	Control		400.7	1.89
			± 77.3	± 0.30
			100.0	100.0
10	6.7×10 ⁻⁵		419.5	1.73
			± 61.2	± 0.20
			104.7	91.5

Table 3

Feed composition in %

15	Barley	20.0
	Wheat	10.0
	Oats	6.0
	Corn	25.2
	Linseed cake	5.5
20	Alfalfa meal	4.0
	Extracted soybean meal	18.0
	Fishmeal	4.0
	Wheatbran	5.0
25	Vitamins and mineral mix	2.3

THE CLAIMS DEFINING THE INVENTION ARE AS FOLLOWS:

1. A method of improving weight gain and feed conversion in livestock comprising feeding to livestock animal feedstuff to which has been added a bacteriolytic enzyme product from streptomyces coelicolor DSM 3030 or a mutant or variant thereof.
2. A method of improving weight gain and feed conversion in livestock comprising feeding to livestock animal feedstuff to which has been added a bacteriolytic enzyme product with N-acetylmuramidase activity and protease.
3. A method as claimed in Claim 2 wherein the bacteriolytic enzyme product is from streptomyces coelicolor DSM 3030, the variants or mutants thereof.
4. The method as claimed in Claim 2, wherein the bacteriolytic enzyme product is employed in the form of the dried nutrient medium.
5. The method as claimed in any one of Claims 1 to 4, wherein the bacteriolytic enzyme product is contained in the feedstuff at an N-acetylmuramidase activity of 0.1 to 1,000 U/mg of the latter.
6. The method as claimed in Claim 5, wherein the activity is 0.5 to 5 U/mg.
7. The method as claimed in any one of Claims 2 to 6, wherein the ratio of bacteriolytic enzyme product to protease is in the range 1:1 to 1,000:1, based on enzyme activity.
8. The method as claimed in Claim 7, wherein the ratio is in the range 100:1 to 500:1.
9. An animal feedstuff which contains a bacteriolytic enzyme product from Streptomyces coelicolor DSM 3030 when used as a weight gain and feed conversion additive in a method according to any one of claims 1 to 8.



10. An animal feedstuff which contains a bacteriolytic enzyme product with N-acetylmuramidase activity and protease when used as a weight gain and feed conversion additive in a method according to any one of claims 1 to 8.

DATED this 4th day of January, 1992

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