Isolation and identification of ammonia reducing *Bacillus megaterium* from chicken cecum

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Ammonia is the most harmful ingredient in the foul odor, which causes stress response, lowered immunity, diseases, death of livestock, and poultry poisoning. To reduce ammonia emission from chicken manure, a strain of ammonia nitrogen-degrading bacteria was isolated from chicken cecum on anaerobic nitrification medium and aerobic denitrification medium. The capacity of bacteria to degrade ammonia nitrogen was also studied. The strain was firstly identified as *Bacillus megaterium* via physiological and biochemical characteristics, morphological observation, and 16S rDNA sequence analysis. The content of ammonium nitrogen in chicken manure was decreased, especially at 96 h ($P < 0.05$), which was detected by the indophenol blue method. Correspondingly, the content of nitrate nitrogen detected by phenol disulfonic acid method was increased and reached to the highest at 48 h ($P < 0.05$). In addition, the ammonia content was significantly reduced when drinking water contained 1×10^6 CFU/ml of *Bacillus megaterium*. In conclusion, a new bacterial strain derived from chicken cecum was identified and studied on its deodorization function in detail to provide the first direct evidence for improving environment of the chicken coop and surroundings.

Keywords: Chicken cecum; *Bacillus megaterium*; Isolation and identification; Ammonia reduction.

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Introduction

In recent decades, China's poultry farming has been rapid developed. The production and cultivation scale were steadily improved. However, large amount of chicken manure brought with growing problems. One of the problems is foul odor composed mostly of ammonia, which probably causes stress response, hypo-immunity and even diseases, death of livestock, and poultry poisoning [1]. Therefore, the control of ammonia emission has a positive significance on healthy farming and environmental protection.

The physical, chemical, and biological methods have been tried to remove ammonia from chicken manure [1]. Physical and chemical deamination offers high efficiency to remove ammonia, but with limitations due to the strict processing requirements, high equipment maintenance costs, complex work process, and the production of secondary pollution. Therefore, producers and researchers have focused on bio-deammonization studies recently as a potential alternative deamination method.

Because ammonium nitrogen can be transformed into nitrate nitrogen by the combination of ammonia-oxidizing bacteria and nitrite oxidizing bacteria, the bacteria are probably applied to reduce ammonia emissions of chicken manure. For this purpose, heterotrophic nitrifying bacteria have been isolated and screened for use in the control of

ammonia emissions, stimulating biological deamination applications [2].

The bacterial strains of *Candida glabrata* [3], *Bacillus subtilis* [4], plant *lactobacillus* [5], *Streptococcus thermophilus* [5], *Streptomyces microflavus* [6], and *Clostridium butyricum* [7] have been used for deodorization and are tested to be effective. In this study, a bacterium with ammonia-lowering action was isolated from chicken cecum on anaerobic nitrification medium and aerobic denitrification medium. The analytic studies of physiological, biochemical, and morphological characteristics and 16S rDNA sequence homology were performed for the isolated bacteria. The effect on ammonia reduction of the bacteria was characterized, which provide the theoretical basis for further study and application.

Material and methods

Cecal contents

The hemp cecum contents were collected aseptically, and then placed in a 100 ml sterilized saline of conical flask. After standing for 20 min, the supernatant containing the bacteria from the cecum was removed.

Preparation of medium

The following media were completely dissolved and sterilized [8].

Vicker's salt solution: K_2HPO_4 5.0 g, $MgSO_4 \cdot 7H_2O$ 2.5 g, NaCl 2.5 g, $FeSO_4 \cdot 7H_2O$ 0.05 g, $MnSO_4 \cdot 4H_2O$ 0.05 g were dissolved in distilled water to 1,000 ml.

Anaerobic nitrification medium: $(NH_4)_2SO_4$ 0.5 g, Sodium Succinate 5.62 g, Vibrelin Solution 50 ml, agar 20 g were mixed and dissolved in distilled water to 1,000 ml, with adjustment to pH 7.0.

Aerobic denitrification medium: KNO_3 1 g, KH_2PO_4 0.5 g, $FeCl_2 \cdot 6H_2O$ 0.5 g, $CaCl_2 \cdot 7H_2O$ 0.2 g, $MgSO_4 \cdot 7H_2O$ 1.0 g, Sodium Succinate 8.5 g, agar 20 g were mixed in distilled water to 1,000 ml , PH 7.0-7.3.

Screening, isolation, and purification of strains

The supernatant of the contents of the cecum was sequentially diluted into sterile water (10^{-1} - 10^{-6}). Equal volumes of the original and diluted samples were applied to the isoxime nitrification medium using sterile glass rods. Each concentration was tested in three parallel reactions. After remaining still for 30 min, the culture dishes were placed in an anaerobic incubator at 37˚C for 48 h. When a distinct single colony was formed, the colony morphology was recorded, and then stained to observe the bacterial morphology.

The isolated individual strains were next inoculated onto aerobic denitrification medium. Each sample was tested in three parallel reactions. After remaining still for 30min, the culture dishes were placed in an anaerobic incubator at 37˚C for 48 h. When a distinct single colony was formed, the colony morphology was recorded, and then stained to observe the bacterial morphology.

The colonies grown on the aerobic denitrification medium were then purified by the three-line method. After three generations of purification, the broth was expanded.

Biochemical tests

The isolated pure cultures were tested in a series of bacterial biochemical assays including Indole Test, Methyl Red Test, Voges-Proskauer Test, Citric Acid Utilization Test, Trisaccharide Iron Agar Test, Glucose Fermentation Test, Lactose Fermentation Test, Maltose Fermentation Test, Mannitol Test, and Sucrose Fermentation Test. All reactions were cultured at 37˚C for 18-24 h [9].

Bacteria morphological observation

The pure culture of bacteria was directly adhered to conductive adhesive for morphological observation. The samples were sprayed with Pt (15 mA, 100 s) and then placed

in the electron microscope (TESCAN VEGA3, Libušina tř Czechoslovakia), and observed with a lower accelerating voltage of 10 kV.

16S rDNA sequence determination

Total DNA was extracted from the strain and used as a template for PCR amplification of the 16S rDNA sequence by using the primers of 27F (5'-AGAGTTTGATCMTGGCTCAGC-3') and 1492R (5'-GGTTACCTTGTTACGACTT-3'). The reaction conditions were as follows: initial denaturation at 98°C for 5 min, followed by 35 cycles of 95°C for 35 s, 55°C for 35 s, and 72°C for 90 s, followed by extension for 8 min. After gel electrophoresis, the PCR product was purified and inserted into the pMD18-T vector and then transformed into *E. coli* DH5α competent cells. The selected clones were sequenced using the dideoxy chain-termination method on an automatic DNA sequencer (ABI Applied Biosystems Model 3730XL) at the Nanjing Jinsirui Biotechnology Co. Ltd. The 16S rDNA sequencing results were submitted to the GenBank nucleic acid sequence database for gene alignment.

Growth assays

The bacterial suspension was inoculated into nutrient broth at 1%, and then shaken at 37˚C and 200 rpm. The UV-visible spectrophotometer values were measured at 600 nm for optical density determination and sampled once every 2 h. The growth data were plotted as OD_{600} vs. culture time.

The total number of bacteria during stationary phase was calculated using the plate count method.

Determination of the amount of feeding

Experiments were performed to determine the reasonable feeding volume. Twenty chickens were housed in an animal room of 3.56 × 2.38 × 2.96 m^3. After 10 days of continuous feeding, the ammonia concentration in the room was measured using an ammonia analyzer. Three parallel tests were conducted simultaneously. The temperature of the animal room was maintained at 30-33˚C.

Determination of ammonium and nitrate nitrogen in chicken manure

The amount of 1×10^6 CFU/ml bacillus was added to drinking water for 10 days in the test group. Drinking water and diets were the same between the control group and the test group.

Data presentation and statistical analysis

SPSS software (19.0) was used for quantitative analyses of the data. All data were expressed as mean ± SEM.

Ammonia nitrogen reduction rate = (control group - test group)/control group × 100%

Nitrate nitrogen increase rate = (test group - control group)/control group × 100 %

Results

Bacterial screening

Six kinds of bacterial colonies were separated based on colony characteristics and Gram stain results using isoxymethyl nitrification medium. The bacteria were inoculated onto aerobic denitrification medium. The bacteria were named CC-1.

As shown in Figure 1, colonies were round, moist, pale yellow, opaque, with a smooth, flat surface with neat edges. The Gram stain results indicated the bacteria was Gram-positive, rod-shaped, and terminal round, with single or short chain arrangement. The size of the bacteria was 0.5 to 0.8 μm × 2.0 to 4.0 μm.

Biochemical test of strains

The biochemical characteristics of the strain were determined as shown in Table 1.

Sequencing of strains

Based on the 16S rDNA sequencing, the CC-1 strain was identified as *Bacillus megaterium*. The sequence was 100% similar to the published sequence of the strain in the NCBI gene database (KY660610.1).

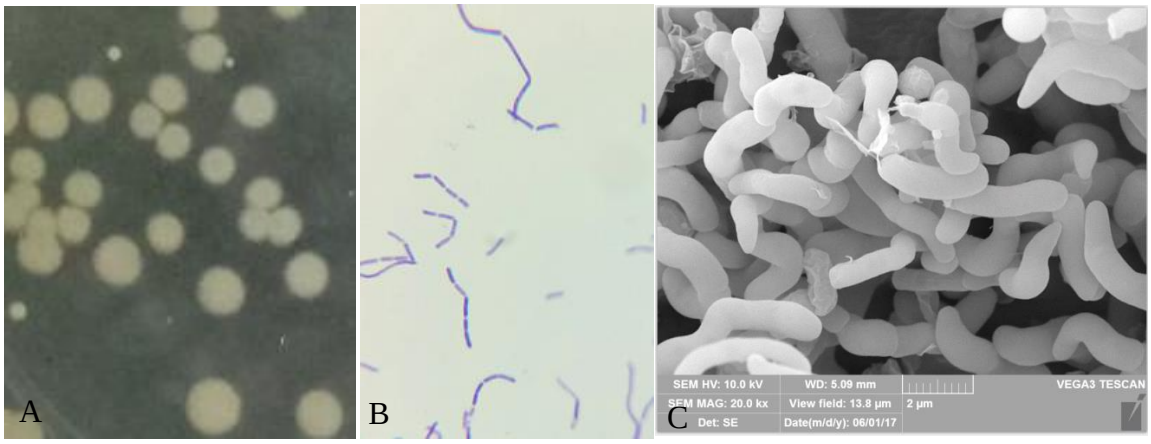

Figure 1. CC-1 colony and bacterial morphology. A: shows the colony morphology of CC-1; B: shows the image of Gram stain under the oil microscope; C: shows the image under the electron microscopy with 20,000 times magnification.

Table 1. The biochemical test results of the strain.

Biochemical Test	Results	Biochemical Test	Results
Indole Test	-	Glucose Fermentation Test	+
Methyl Red Test	-	Lactose Fermentation Test	+
Voges-Proskauer Test	-	Maltose Fermentation Test	+
Citric Acid Utilization Test	-	Mannitol Test	+
Trisaccharide Iron Agar Test	-	Sucrose Fermentation Test	+

Note: "+" indicates positive results; "-" indicates negative results.

Table 2. Changes in ammonia content of chicken coop after addition of *Bacillus megaterium*.

Group	Dose of *Bacillus megaterium* ($\times 10^6$ CFU/ml)	Ammonia content (mg/l)	Health of the flock
control group	0	$91.3^{a}\pm1.53$	no abnormality
test group Ⅰ	0.5	$88.6^{a}\pm1.15$	no abnormality
test group Ⅱ	0.8	$79.3^{b}\pm1.53$	no abnormality
test group Ⅲ	1	$72.3^{bc}\pm0.58$	no abnormality
test group Ⅳ	1.2	$71.0^{bc}\pm1.0$	no abnormality
test group Ⅴ	1.5	$70.7^{bc}\pm1.53$	no abnormality
test group Ⅵ	2.0	$81.0^{b}\pm1.0$	Unshaped faeces

Note: The different letters correspond to significant difference ($P < 0.05$), the same letter represents no significant difference.

Table 3. Effect of *Bacillus megaterium* on ammonium nitrogen and nitrate nitrogen content in chicken manure (mg/kg).

Time (h)	Ammonium nitrogen			Nitrate nitrogen		
	Control group	Test group	Decrease rate	Control group	Test group	Increase rate
24	34.40 ^{a}±1.58	18.32 ^{a}±1.63	46.74%	11.08^{a}±1.79	17.69^{a}±0.53	59.66%
48	21.83 ^{a}±2.46	10.96 ^{a}±2.54	49.79%	15.81^{a}±0.98	23.70^{b}±3.62	49.91%
72	14.56 ^{a}±1.43	6.98 ^{a}±3.12	52.06%	12.99^{a}±0.42	16.16^{b}±2.90	24.40%
96	11.85 ^{a}±1.48	2.87 ^{b}±1.37	75.78%	9.56^{a}±2.21	11.21^{b}±0.49	17.26%
120	6.06 ^{a}±1.33	3.59 ^{b}±1.14	40.76%	8.37^{a}±2.11	8.89^{b}±2.90	6.21%

Growth characteristics of strains

The growth of *Bacillus megaterium* strain entered stationary phase after 22 h of growth as shown in Figure 2. The total number of bacteria in this stage was 2.4×10^5 CFU/ml.

Determination of feeding volume

After *Bacillus megaterium* was added to the drinking water, the ammonia content of the chicken manure was determined (Table 2).

As shown in Table 2, the ammonia content in the chicken coop was significantly reduced when 0.8-2.0 × 10^6 CFU/ml *Bacillus megaterium* was added to the drinking water. The ammonia content of the test group III was significantly lower than that of experimental group I and II, and higher than that of experiment group IV and V, but the difference was not significant.

Determination of ammonium and nitrate contents in chicken manure

The contents of ammonium nitrogen and nitrate nitrogen in chicken manure were determined by the indophenol blue and phenol disulfonic acid methods, respectively (Table 3).

As shown in Table 3, the content of ammonium nitrogen in chicken manure was decreased after feeding *Bacillus megaterium*. The largest reduction was at 96 h ($P < 0.05$). The content of nitrate nitrogen was increased significantly at 48 h ($P < 0.05$).

Discussion

Nitrogen in poultry manure includes surface nitrogen, fecal nitrogen, and endogenous urinary nitrogen. Surface nitrogen is mainly the shedding of feathers, hoofs, skin, and saliva during the growth of chickens. However, this part of the nitrogen content is minimal and negligible. The nitrogen in chicken manure is mainly present as organic nitrogen, ammonium nitrogen, and nitrate nitrogen. Endogenous urinary nitrogen is mainly urea and uric acid [12].

Because the residence time of feed in chicken body is very short, nutritional ingredients in feed are not completely digested, especially protein substances, and therefore, remain in the chicken manure. The various nitrogen compounds in chicken manure were degraded into ammonia by the microbes, resulting in rapidly degradation to ammonium nitrogen. Some ammonium nitrogen was further converted to nitrate and organic nitrogen by microorganisms, most of which were volatilized with ammonia [13]. *Bacillus megaterium* was screened out through isoxigenization nitrification medium and aerobic denitrification medium. Ammonia nitrogen can be used as a nitrogen source in the anaerobic environment of the intestinal tract. The ammonium nitrogen was converted to nitrite, yet nitrite was converted to nitrate in the aerobic environment *in vitro*. Thus, the content of ammonium nitrogen was decreased, and the

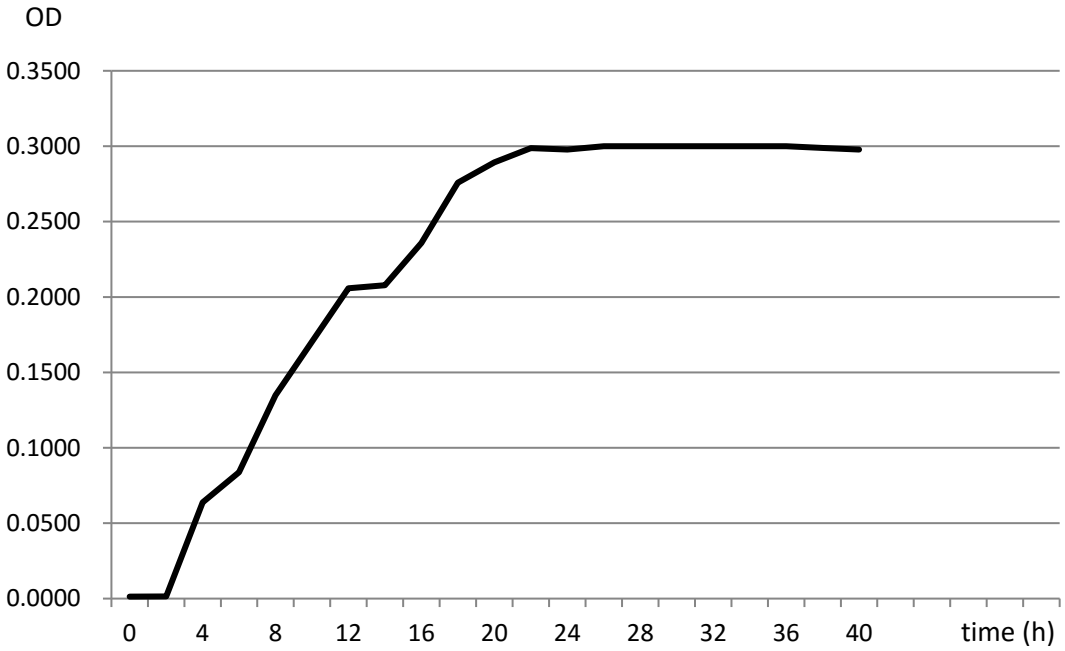

Figure 2. Growth curve of *Bacillus megaterium*.

content of nitrate nitrogen was increased in chicken manure.

In addition, extracellular enzymes are produced by *Bacillus*, including protease, lipase, amylase, polygalacturonase, and cellulose. Complex carbohydrates in feed can be degraded by polygalacturonase and cellulose [13]. After feeding *Bacillus megaterium* to the chickens, carbohydrates could be absorbed more completely by the intestines, thereby reducing chicken manure nitrogen content.

In the body, uric acid first degrades to allantoin, and then further into urea, resulting in ammonia. Uricase and urease play vital roles in this process. Chicken excrement microorganisms include *Escherichia coli*, *Staphylococcus*, *Bacteroides Fragilis* group, Lactic acid bacteria, and *Proteus* species [14], which may produce urease and uricase [15] and lead to the excretion of nitrogen-containing substances during decomposition. After feeding *Bacillus megaterium* to chickens, the growth and reproduction of microorganisms such as *Escherichia coli* were inhibited. The urease content was reduced in the digestive tract. The decomposition of urease to nitrogenous substances was also reduced. Eventually, the conversion of uric acid to ammonium nitrogen was reduced.

In addition, during growth and reproduction of *Bacillus* in digestive tract of livestock and poultry, bacterial bacteria (Bacteriocin), organic acids, or hydrogen peroxide might be secreted. The growth of intestinal microorganism could be inhibited by these metabolites, which helps to regulate the ecological balance of microbes in the animal digestive tract [16]. Therefore, after feeding *Bacillus megaterium* to chickens, the concentration of ammonium nitrogen in chicken manure was lower than that in the control group, and the concentration of ammonia in chicken coop was also decreased. These results might be explained by the following reasons. (1) the microbial flora in chicken manure may change in megacycles and the activity of bacterial urease could be inhibited to reduce decomposition of urea and, in further, decrease the yield of ammonium nitrogen in chicken manure; (2) ammonium nitrogen may be

consumed during breeding of *Bacillus megaterium*; (3) chicken intestinal digestion and absorption capacity may be increased by *Bacillus megaterium*, which may increase absorption rate of nitrogen compounds and result in decreased chicken manure nitrogen content. The content of nitrate nitrogen was increased, probably due to the presence of a large number of *Bacillus megaterium* that allowed conversion of the ammonium nitrogen in chicken manure to nitrate nitrogen. With longer time, some of the nitrate nitrogen may be converted to nitrogen and other substances by the denitrifying bacteria, which leads to a decrease in nitrate content.

In conclusion, the chicken fecal nitrogen and endogenous urinary nitrogen were reduced by the addition of 1×10^6 CFU/ml *Bacillus megaterium* into drinking water, and therefore, the overall ammonia content in the chicken coop was significantly reduced.

Acknowledgments

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