Isolation, identification, and determination of dominant desulfurizing bacterial strains from chicken manure

Gailing Wang, Mingcheng Wang, Tongbiao Li, Changli Liang, Enzhong Li*

Department of Biological and Food Engineering, Huanghuai University, Zhumadian, Henan 463000, China.

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Hydrogen sulfide is a colorless, highly toxic, water-soluble acidic gas. When the concentration in a chicken house exceeds the tolerance range of chicken, hydrogen sulfide will stimulate their mucosa and damage their sense of smell. It can also reduce the chicken's appetite, growth performance, and resistance to disease. This study was aimed to separate desulfurizing bacterial strains from fresh chicken manure to reduce the impact of hydrogen sulfide on chickens, humans, and environmental pollution more generally. With the methods of enrichment culture, plate separation, and barium chloride screening, four desulfurizing bacterial strains (L_1, L_2, L_3, and L_4) were isolated from chicken manure. They were identified as *Providencia* sp., *Arthrobacter* AMP-5, *Arthrobacter* AMP-6, and *Thiobacillus* sp. by 16S rRNA sequencing. The desulfurization effects of these bacteria were investigated. The results showed that the desulfurization rate of *Thiobacillus* sp. reached 35.3%, which is the highest among the four strains and suggested that *Thiobacillus* sp. might be an effective sulfur bacterium degrading hydrogen sulfide.

Keywords: chicken manure; hydrogen sulfide; sulfur bacteria; gas pollution.

***Corresponding author:** Enzhong Li, College of Biological and Food Engineering, Huanghuai University, Zhumadian 463000, Henan, China. E-mail: enzhongli@163.com.

Introduction

With the modernization of poultry production, large-scale breeding has become mainstream. Emissions from chicken manure have increased sharply as a result. Chicken manure cannot be disposed of quickly enough, leading to accumulations of feces odor mainly hydrogen sulfide and ammonia in the surrounding environment.

Hydrogen sulfide is a colorless, highly toxic, and soluble acidic gas at room temperature. When the concentration in a chicken house exceeds the tolerance range of chickens (generally not more than 10 mg/L), hydrogen sulfide can damage the sense of smell by stimulating the mucosa of chickens, while also reducing appetite and affecting growth. It can paralyze the central nervous system of chickens, reduce their egg-laying performance, minimize resistance to disease, and when severe, lead to substantial disease incidence. Hydrogen sulfide can also affect human health if it is released into the atmosphere. Inhalation of low-concentration hydrogen sulfide can cause dizziness, headache, fatigue, nausea, and vomiting on human being. However, at high concentrations, it can lead to palpitation, dyspnea, confusion, and even induce a coma or cause death [1, 2]. In addition, hydrogen sulfide can affect the quality of air, pollute water resources, and acidify terrestrial environments.

Physical adsorption, chemical solvent absorption, oxidation, and biological methods are often used to process hydrogen sulfide in chicken farms. Physical approaches use adsorbents to remove

hydrogen sulfide to purify the air [3]. This method is suitable for processing low-concentrations of hydrogen sulfide, but the preparation of adsorbents such as metal oxides, zeolites, ferric oxide hydrates, and zinc oxides is complicated and expensive [3-6]. Chemical absorption uses basic solvents to absorb hydrogen sulfide. This method can absorb hydrogen sulfide well, but excessive alkalinity may be resulted, and other products can cause secondary pollution [7-9]. The oxidation method results in hydrogen sulfide gas being oxidized directly to sulfur monomers such as chlorine, ozone, and $KMnO_4$ with high desulfurization efficiency. However, additional equipment is needed, and the reaction time is long. Also, oxidant consumption is high, and the waste liquid is difficult to be processed harmlessly [10, 11]. Biological methods use some particular bacteria to degrade hydrogen sulfide. The approach is simple, low cost, and does not associate to harmful gas generation. The current studies revealed that sulfur-degrading bacteria include *Thiobacillus thioparus*, *Thiobacillus thiooxidans*, *Chlorobium thiosulfatophlium*, *Rhodococcus rhodochrous*, and *Ralastonia* sp. Most of the bacterial strains that are isolated from sludge, sewage, or chicken manure are sprayed directly onto chicken manure or modified by engineering bacteria on chicken manure [12].

This study attempted to isolate new sulfur-degrading bacteria from fresh chicken manure to reduce the content of hydrogen sulfide, and further, to determine the thiosulfate-oxidizing ability of those bacteria to select an efficient strain of desulfurization. The results of this study will lay a foundation for further research on biological desulfurization.

Materials and methods

Preparation of culture media

Bacterium enrichment and screening medium was prepared by dissolving 1.0 g ammonium chloride, 0. 5 g dipotassium hydrogen phosphate, 5.0 g sodium bicarbonate, 5.0 g sodium chloride, 0.2 g sodium sulfide, 0.2 g magnesium chloride, and 10 g sodium citrate into 1 L deionized water with the pH being adjusted to 7.4, and then sterilized at 121°C for 40 min [13]. Agar screening medium was prepared by adding 1.6 percent of agar powder into the above liquid medium and sterilized at 121°C for 40 min. Tryptic Soy Broth (TSB) was used for storage medium to store bacteria. 3g TSB powder was dissolved in 100 mL purified water and autoclaved at 121°C for 20 min.

Collection and treatment of the fresh chicken manure

Ten healthy 60-day-old laying hens were randomly selected from a layer farm in Zhumadian City, Henan Province, China. 1.0 g of fresh chicken manure was weighted and put into a conical bottle containing 99 mL sterile phosphate buffered saline. The mixture tube was then shaken completely at the rotation speed of 180 rpm under sterile condition, so that the bacteria in chicken manure were fully diffused in the solution. After being placed still for 20 min, the supernatant was collected as the inoculums for the subsequent experiments.

Enrichment, isolation, and screening of sulfur bacteria

Bacterial enrichment was done by adding 1 mL of above supernatant into 50 mL enrichment and screening medium and culturing in an incubator shaker with 140 rpm at 37°C for 72 h. After that, 1 mL of the suspension containing bacteria was pipetted and inoculated into another 50 mL fresh enrichment and screening medium for 48 h with the same growth conditions. Successive enrichments of bacterial culture were performed by using 2% (V/V) bacterial culture as the inoculums for each subculture. After three successive cultures, 0.1 mL of bacterial enrichment was spread onto agar screening medium with a glass spreading rod under aseptic conditions and placed inversely in an incubator at 37°C for 36-48 h. When distinct single bacterial colonies were formed on the surface of culture medium, they were purified by using three-line method for 3 generations. Then based on the

morphological characteristics by gram staining, several strains of sulfur bacteria were initially selected. For long term storage, the bacteria were preserved at -80°C in TSB supplemented with 20% (v/v) glycerol.

16S rRNA gene sequence analysis

The nearly complete 16S rRNA sequences of preliminary screened bacterial strains were obtained as described previously [14]. In brief, the 16S rRNA gene was amplified by polymerase chain reaction (PCR) using forward primer 27F (5'-AGAGTTTGATCCTGGCTCAG-3') and reverse primer 1492R (5'-CGGTTACCTTGTTACGACTT-3'). The PCR was performed as follows: pre-denatured at 95°C for 5 min, followed by 35 cycles of 95°C for 35 s, 55°C for 35 s, 72°C for 1 min 30 s, and then, extended at 72°C for 8 min. The PCR product was purified by using E.Z.N.A. gel extraction kit (Omega Bio-Tek, Norcross, Georgia, USA), and then, linked to the pMD18-T vector (TaKaRa, Beijing, China), and finally transformed into the *Escherichia coli* competent cell (DH5α). The positive clones with inserted target fragment was confirmed by PCR, and three randomly selected positive clones of each strain were sequenced by Sangon Biotech Co., Ltd. (Shanghai, China). The comparison of 16S rRNA gene sequence of the bacterial strains to the available 16S rRNA gene sequences in GenBank was carried out by using BLASTn (https://blast.ncbi.nlm.nih.gov). Sequences and their closest matches retrieved from the database were aligned by using Clustal W software (http://www.clustal.org). Phylogenetic tree was constructed by maximum-likelihood algorithm by using Mega 6.0 software (https://www.megasoftware.net/). Bootstrap values were calculated based on 1,000 replications.

Determination of desulfurization effect

To investigate the desulfurization effect, each bacterial strain was inoculated in 2 mL liquid medium and cultivated at 37°C overnight. In the experimental group, 1 mL suspension of each bacterial strain was added to 100 mL liquid medium, while in the control group was no bacteria. 3 g sodium thiosulfate was added to all the tests. Each group was set in triplicate. All samples were shaken 140 rpm at 37°C for 48 h. After that, 1 mL culture solution was added to equal volume of 1% $BaCl_2$ solution in a tube until the precipitate was formed. The precipitate was filtered slowly through filter paper. After drying, the precipitate of experimental group (M1) and control group (M2) were weighed to calculate the desulfurization effect:

$$\text{Quality of desulfurization M (mg)} = (M_1 - M_2) \times 0.1373$$

$$\text{Desulfurization rate (\%)} = M / (30 \times 0.405) \times 100\%$$

In which 0.1373 is the content of sulfur in $BaSO_4$, 0.405 is the content of sulfur in $Na_2S_2O_3$, and 30 is the amount of $Na_2S_2O_3$ (mg).

Data analysis

The data were expressed as mean ± standard error. The quality of desulfurization rate in the experimental and control groups was analyzed by SPSS 19.0 (IBM Company, Armonk, New York, USA). $P < 0.05$ was used to assess significant differences.

Results

In the present study, four desulfurizing bacterial strains (L_1, L_2, L_3, and L_4) were isolated from the fresh chicken manure by using liquid selective medium and then purified on solid selective medium. They were identified by morphological observation and sequencing. The sulfur-oxidizing effects of the four bacterial strains were also investigated.

Morphological and genotypic characterization and phylogenetic analysis

The four bacterial strains grew well on selective medium containing agar. The colonial morphology on the solid plate and bacterial characteristics under microscope are shown in Table 1. The colonial size and morphology of strains were observed at 48 h with L_2 and L_4 bigger than the other two strains. The colonies

Table 1. Colonial morphology and microscopic properties of four sulfur bacterial strains.

Characteristics	Strain L_1	Strain L_2	Strain L_3	Strain L_4
Size	1.02 mm	2.06 mm	1.40 mm	2.08 mm
Shape	Round	Round	Round	Round or oval
Edge	Orderly	Orderly	Coarse	Orderly
Luster	Smooth	Smooth	Smooth	Smooth
Height	Flat	Bulge	Bulge	Bulge
Color	White	Milk white	Yellowish-brown	Faint yellow
Transparency	Semitransparent	Opaque	Opaque	Opaque
Degree of dry and wet	Dry	Wet	Dry	Wet
Gram staining	G^-	G^+	G^+	G^-
Microbial shape	Rod	Sphere	Rod	Rod

Table 2. Desulfurization rate of four sulfur bacterial strains (%).

	Control	Strain L_1	Strain L_2	Strain L_3	Strain L_4
Sulfur added (mg)	12.20	12.20	12.20	12.20	12.20
Sulfur desulfurized (mg)	0.20^a±0.6	1.71^b±3.4	3.43^c±2.61	1.13^d±1.8	4.54^e±1.1
Desulfurization rate (%)	1.63 %	12.30 %	26.20%	7.38 %	35.30 %

Note: Letter on the upper right of each number indicates a significant difference ($P < 0.05$).

were smooth with entire margins except L_3 strain.

The comparison of 16S rRNA gene sequences of four strains with the available 16S rRNA gene sequences in GenBank revealed that the strain L_1 belonged to *Providencia* family and shared highest sequence similarity with *Providencia vermicola* (99.93% sequence similarity). Strains L_2 and L_3 were the members of *Arthrobacter* family with the highest sequence similarity of L_2 to *Arthrobacter* sp. AMP-5 (99.86% sequence similarity) and L_3 to *Arthrobacter* sp. AMP-6 (99.93% sequence similarity), respectively. Strain L_4 was from *Thiobacillus* family and showed the highest sequence similarity to *Thiobacillus* sp. (99.23% sequence similarity). Furthermore, the maximum-likelihood phylogenetic tree analysis showed that L_1 to L_4 formed four separate lineages with the members of individual family, respectively (Figure 1).

Determination of desulfurization effect

After the identification of bacterial strains, the four isolates were tested for their thiosulfate utilization to investigate their desulfurization effect. As shown in Table 2, the precipitation in experimental and control groups were weighed and desulfurization rates were then calculated. The desulfurization rate of the control was 1.63%, suggesting the existence of some other impurities. Therefore, the rate in the control group should be subtracted from the rate in the experimental groups. The precipitation of strains L_2 and L_4 were significantly higher than that of the control group, indicating that *Arthrobacter* and *Thiobacillus* had potential capacity of desulfurization. In addition, the desulfurization rate of strain *Thiobacillus* was 35.3%, which was the highest rate among the four strains and might be used for degrading odor gas in farms in the future. However, the desulfurization rate of strain L_3 (*Arthrobacter* sp. AMP-6) was only 7.38%, which was the lowest among the four strains.

Discussion

In this study, the sulfur bacterial strains from chicken manure were primarily enriched by using liquid enrichment and screening medium, and then, were isolated by using agar screening

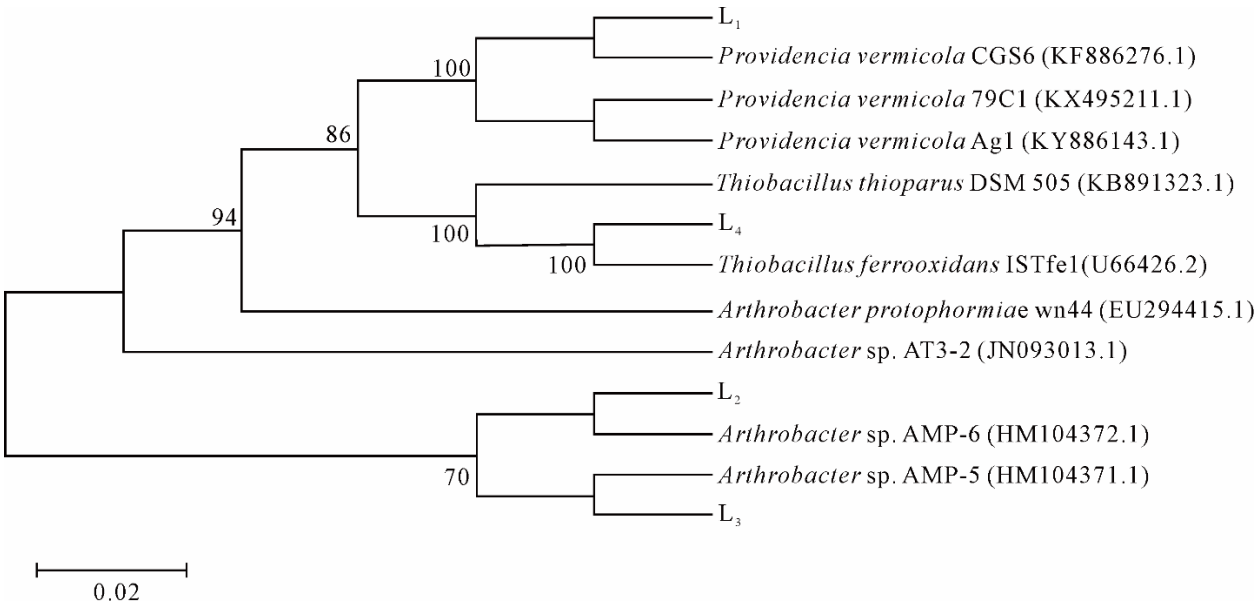

Figure 1. Maximum likelihood tree based on partial 16S rRNA gene of L_1-L_4 and selected similar sequences. The numbers at the nodes indicate the percentage of 1,000 bootstrap replicates, only values >50% are shown. GenBank accession numbers are given in parentheses. The scale bar represents 0.02.

medium. It has been proved that, by using this strategy, more sulfur bacterial strains were isolated than that using agar screening medium only. In addition, the enrichment and screening medium contained nutrients that could enable sulfur bacterial strains to grow rapidly while inhibiting the growth of other microorganisms [13]. Therefore, this study used liquid and solid screening medium to isolate sulfur bacteria and obtained four bacterial strains. After 16S rRNA sequencing and sequence alignment analysis, four bacterial strains were identified as the genus *Providencia*, *Arthrobacter*, and *Thiobacillus*. Two strains from the genus *Arthrobacter* were identified as *Arthrobacter* sp. AMP-5 and *Arthrobacter* sp. AMP-6.

Providencia is associated with denitrification and is able to oxidize deammonia of phenylalanine and tryptophan. Taylor *et al*. reported that *Providencia* could degrade indole by removing nitrogen from the indole and reduce ammonia pollution in farms [15]. In that study, *Providencia* was isolated from the chicken manure by using screening medium of sulfur bacteria and was also testified to have the thiosulfate-oxidizing ability. However, there is no report about the desulfurization of *Providencia* to date.

Arthrobacter sp. is a chemoheterotrophic bacterium, which is often V-shaped without filaments and aerobic. Many strains of this family can degrade many environmental pollutants and play important roles in catabolism. *Arthrobacter* species are thought to remove sulfur from heterocyclic sulfur. Kaufman *et al*. found that *Arthrobacter* could metabolize dibenzo-thiophene to O-Phenyl phenol, which was useful for converting sulfur in dibenzothiophene into innocuous sulfate, thus realizing the purpose of desulfurization [16]. Our results demonstrated that two strains of *Arthrobacter*, AMP-5 and AMP-6, from chicken manure had the thiosulfate-oxidizing ability.

Strains from genus *Thiobacillus* is the predominant bacterium of desulfurization microorganism, including *T*. *thiooxidans*, *T*. *thioparus*, *T*. *ferrooxidans*, and *T*. *denitrificans* [17]. The members of this bacterial family show the same characteristics as *T*. *ferrooxidans*, which include Gram negative, short rod bacteria with a

single polar flagellum under microscope [18]. However, the flagella are highly susceptible to be damaged, which result in the absence of flagella of the isolated strain in this study. Based on the sequence analysis, it was identified as *Thiobacillus* sp. in this study. The results of thiosulfate-oxidizing ability analysis showed that *Thiobacillusa* had the highest desulfurization rate among the other bacterial strains in this study. Therefore, *Thiobacillusa* sp. from chicken manure could be an excellent desulfurization microorganism with quick growth and propagation rate and high efficiency of removing sulfur compounds.

Microbial desulfurization of livestock manure is a promising technology because it can desulfurize sulfur-compounds in the manure, especially hydrogen sulfide. Sulfur bacterial strains can oxidize divalent sulfur and use elemental sulfur as sulfate for obtaining energy. Consequently, sulfur bacterial strains can change the harmful sulfur elements (H_2S) into harmless ones (sulfates) to achieve the hydrogen sulfide removal. The chemical mechanisms of sulfur bacterial desulfurization are as follows [19]:

$$2H_2S + O_2 \rightarrow 2S + 2H_2O + \text{energy}$$

$$2S + 3O_2 + 2H_2O \rightarrow 2SO_4^{2-} + 4H^+ + \text{energy}$$

$$2S_2O_3^{2-} + 3O_2 + 4H_2O \rightarrow 4SO_4^{2-} + 8H^+$$

The desulfurization quality of sulfur bacterial strains in this study was calculated by subtracting the precipitate of control group from that of the experimental group, which was the amount of precipitate formed by sulfur bacterial strains oxidizing hydrogen sulfide precursors to form SO_4^{2-} and Ba^{2+}. The desulfurization rate of *Thiobacillus* sp. was 35.3%, which was significantly higher than that of the other three strains. Therefore, the appropriate application of *Thiobacillus* sp. may reduce the content of hydrogen sulfide in chicken farms.

In conclusion, four sulfur bacterial strains were isolated from the chicken manure and were identified as *Providencia*, *Arthrobacter* AMP-5, *Arthrobacter* AMP-6, and *Thiobacillus* sp. In the desulfurization experiment, *Thiobacillus* sp. demonstrated to have the best desulfurization capacity among four bacterial strains, which suggested that *Thiobacillus* sp. played an important role in sulfur oxidation. To develop the sulfur-oxidizing ability of the exhaust gas treatment in chicken farms in the future, we would apply *Thiobacillus* sp. into chicken manure and detect its desulfurization effect.

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