

Full Length Research Paper

Evaluation of *Bacillus amyloliquefaciens* as manure additive for control of odorous gas emissions from pig slurry

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Two *in-vitro* experiments were conducted to evaluate the efficacy of *Bacillus amyloliquefaciens* culture broth (BA) on reduction of odorous gas emission from pig slurry. In experiment 1, the treatments included control (no spray), water spray, and spray with 1% (BA1), 5% (BA2), 10% (BA3) and 100% (BA4) BA. Each treatment was replicated three times. The only significant difference in NH₃ emission was observed at 48 h, when BA1, BA3 and BA4 showed significant reduction compared to the control. The H₂S emission was significantly reduced only at 3 h in response to treatments with BA compared to control. The SO₂ emissions from slurry were not affected by the treatments. The treatments of experiment 2 were: control (no spray), water spray, 10% BA spray one time/day and 10% BA spray one time/two day. The NH₃ emissions were significantly reduced in response to treatments with BA at day 4, 6 and 7 compared to the control. Significant reduction in H₂S emissions were observed from day 3 to 7 from the BA one time/day treated slurry compared to the control slurry. The SO₂ emissions did not differ among treatments, with the exception of a tendency to decline in response to treatment with BA one time/day at day 4 and 6. Overall, treatment with 10% BA one time/day was effective in reducing NH₃, H₂S, and SO₂ from pig slurry and can be used as manure additive.

Key words: *Bacillus amyloliquefaciens*, pig slurry, *in-vitro* fermentation, ammonia, sulfur dioxide, hydrogen sulfide.

INTRODUCTION

The term manure was used in the past to describe excreta that was predominantly used as fertilizer and soil conditioner. However, increasing use of chemical fertilizer in crop, animal manure is no longer in demand for its fertilizer value, but with the development of intensive, confined housing and feeding practices, it is now

considered a pollutant and odor nuisance. Odors from animal feeding operations are produced via an incomplete fermentation of manure by anaerobic bacteria. Schiffman et al. (2001) identified a total of 331 different compounds in the air and lagoon water from pig production facilities. Odor emissions from swine facility

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are complex mixture of ammonia (NH₃), volatile sulfur (H₂S, SO₂) and a large number of volatile organic compounds (VOCs). Exposure to high levels of odorous gases not only adversely affect the health and performance of animals but also affect the health of workers and cause environmental problems such as the nitrification and acidification of rain (Ushida et al., 2003). Therefore, reduction in odor nuisance plays an important role for strategies concerning where to permit pig production facilities to be located and determines the maximum size of the facilities. So far, strategies to reduce odor mainly focused on technical approaches such as bio-filter (Sheridan et al., 2002), bio-scrubbers (Hahne et al., 2003), manure storage covers (VanderZaag et al., 2008), mechanical aeration (Al-Kanani et al., 1992), diet manipulation (Sutton et al., 1999) and segregation of feces and urine. Some of these techniques are effective, but tend to be expensive (VanderZaag et al., 2008) and their effectiveness period is short. To date, limited information is available on whether direct application of microbial additives is effective in reducing odor and noxious gas emissions from pig slurry (Kim et al., 2008; Rahman et al., 2011).

Pig manure is primarily a mixture of urine and feces, and it contains undigested dietary components, endogenous end products, and indigenous bacteria from the lower gastrointestinal tract (Sutton et al., 1999). Generation of odors from stored swine slurry is a complex process that involves many bacterial species, producing an extensive array of volatile compounds. Hence, microorganisms play a major role in both production and reduction of malodors (Zhu, 2000). A number of studies have been conducted to investigate the effects of direct fed microbials (DFM) on reduction of noxious gas from pig slurry. *Bacillus*-based DFM were effective at breaking up manure solids, and demonstrates its effectiveness in odor control (Davis et al., 2008; Wang et al., 2009). Direct application of microorganisms have been extensively practiced in municipal wastewater to degrade organic matter (Low and Chase, 1999) since degradation of organic matter in wastewater relies on microorganisms (Sund et al., 2001). A previous study (Rahman and Mukhtar, 2008) suggested that direct application of microbial additive is also effective in reducing solids and nutrient contents in manure from anaerobic dairy lagoons. This study was designed to evaluate the effectiveness of a microbial treatment technology; spraying of *Bacillus amyloliquefaciens* culture broth (BA) in reducing noxious gas (NH₃, H₂S, and SO₂) emissions from pig slurry under *in vitro* fermentation condition.

MATERIALS AND METHODS

This *in-vitro* study was conducted at Animal Nutrition and Feed Science Laboratory, Suncheon National University, Republic of Korea. The experimental protocols were approved by the Animal Care and Use Committee of Suncheon National University, Republic of Korea.

Source of bacterial stock culture

The bacterial stock culture used in this experiment was *B. amyloliquefaciens* KB3 culture broth provided by the Jeonnam Biodiversity Foundation, Jeonnam, Republic of Korea. It was isolated from pig feces and there were 1×10⁹ cfu bacteria per ml.

Sample collection

A total of 12 crossbred (Landrace × Yorkshire × Duroc) growing pigs (average body weight 40 ± 0.12 kg) were housed for a period of 8 days in individual elevated solid-sided stainless steel metabolism cages (1.6 × 0.8 m²) equipped with plastic slatted floors. Pigs were allowed to consume feed and water *ad libitum* and feces and urine were collected on day 6, 7 and 8 of the period. The slurry samples were collected from the tray placed below the cage, 3 times in the morning (half an hour apart) and 3 times in the afternoon (half an hour apart). Each time, about 200 g of fresh feces was collected from each pig and was put into plastic sample bag. The sample of day 6 and 7 were stored at 4°C to avoid pre-fermentation and loss of water. After completion of day 8 collection, all samples were homogenized, mixed well and brought to room temperature (24 to 28°C) before the commencement of experiment.

In vitro fermentation and measurement of noxious gas concentration

Two experiments were conducted to investigate the effects of BA on odorous gas emissions from pig manure under anaerobic condition. The *in-vitro* trials were carried out in glass reaction chamber to facilitate anaerobic fermentation with air circulation and stirring device.

Experiment 1

There were six treatments including: control (no spray), water (water spray), BA1 (spray with 1% BA), BA2 (spray with 5% BA), BA3 (spray with 10% BA), and BA4 (spraying with 100% BA). The stock culture was diluted with distilled water (DW) to prepare 1, 5, 10 and 100% culture solution. Approximately 2 kg of the stock slurries was stored in each glass fermentation chamber in triplicate for each treatment. The glass chambers had a small hole at one side of the top cover to facilitate gas measurement which was equipped with a small tube with cover. A circulating fan run by electricity was used for uniform distribution of heavy and light gas in each fermentation chamber. Following one pretreatment sampling at 0 h, gas samples were again recorded at 0 h following sprayed with 100 ml of bacterial culture. The gas was sampled using a Gastec gas sampling pump (model AP-20; Gastec Corp., Japan) and Gastec detector tube (No. 3M for NH₃; No. 4HM and 4LT for H₂S; No. 5LA for SO₂). Prior to measurement, the slurry samples were shaken manually for approximately 30 s in order to disrupt any crust formation on the surface of the slurry sample and to homogenize the samples. The cap of the adjacent tube was open and headspace air was sampled within 10 s, approximately 2.0 cm above the slurry surface at a rate of 100 ml/min. After sampling, the tube was closed using the cover and allowed to ferment at 32°C, with additional samples being collected at 3, 6, 12, 24 and 48 h following sprayed with 100 ml of each bacterial culture.

Experiment 2

In Experiment 2, a 10% dilution of the stock culture was prepared by mixing with 90 ml of DW. There were four treatments including:

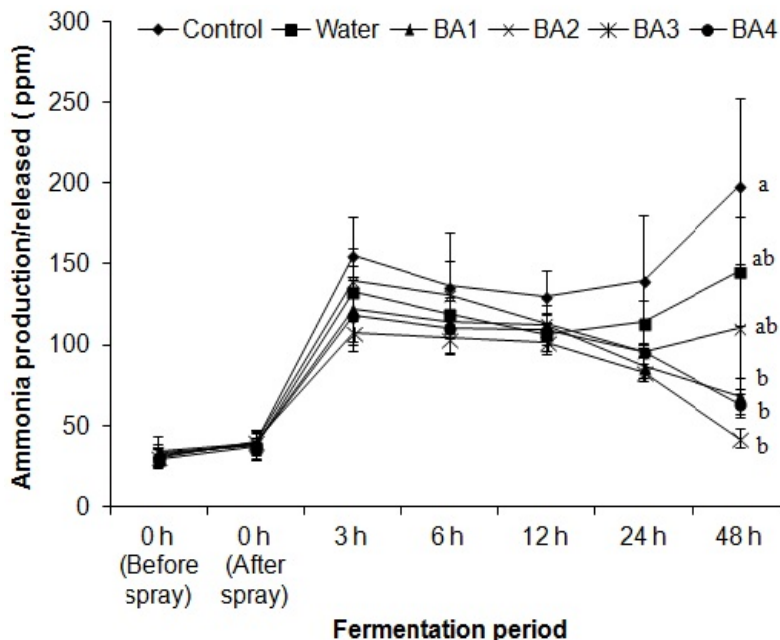


Figure 1. Effects of spraying different concentration of *Bacillus amyloliquifaciens* culture broth (BA) on ammonia emission from pig slurry for 48 h. Control, no spray; Water, water spray; BA1, BA 1%; BA2, BA 2%; BA3, BA 10%; BA4, BA 100%. Different letters at a particular time points indicates significant difference ($P < 0.05$).

control (no spray), water (water spray), 10% BA spray one time/day and 10% BA spray one time/two day. Approximately, 2 kg of stock slurry was stored in each fermentation chamber and allowed to ferment for 7 days at 32°C. The odorous gases were sampled on day 1 to 7, following spray with 100 ml of each treatment bacterial solution. The gas was sampled using a Gastec gas sampling pump (model AP-20; Gastec Corp., Japan) and Gastec detector tube (No. 3M for NH₃; No. 4HM and 4LL for H₂S; No. 5LA for SO₂). The gas measurement technique was same as experiment 1.

Statistical analysis

All experimental data was analyzed in accordance with the General Linear Model Procedure established by the Statistics Analysis Systems Institute (SAS, 2003). The variability of all of the data was expressed as the standard error (SE) and a probability level of $P < 0.05$ was considered to be statistically significant, whereas a $P < 0.10$ was considered to constitute a tendency. Treatment means were computed with the LSMEANS option of the SAS program.

RESULTS

Experiment 1

The effects of spraying with different levels of BA on the emission of NH₃ from slurry are shown in Figure 1. The NH₃ emission from the slurry in the control treatment was higher than that of slurry in the water, BA1, BA2, BA3, and BA4 treated groups throughout the entire experimental period. However, the only significant

difference was observed at 48 h, when NH₃ emission was reduced in response to treatment with BA1, BA3 and BA4 compared to the control, with BA3 showing the highest efficacy ($P < 0.05$).

The effects of BA on slurry H₂S emission are shown in Figure 2. The only significant difference observed in H₂S emission was between that of slurry from the control group and the BA treated groups at 3 h ($P < 0.05$). As shown in Figure 3, SO₂ emissions from slurry were not affected by treatment with water or BA.

Experiment 2

The effects of direct application of 10% BA on NH₃ emission from pig slurry over 7 day are documented in Table 1. There were no significant differences among treatments in NH₃ emission at day 1 to 3 and day 5. However, it was significantly reduced in response to treatment with 10% BA one time/day and one time/two day at day 4, 6 and 7 compared to the control group ($P < 0.05$), with the lowest emissions being observed in the BA one time/day treated group.

As shown in Table 2, treatment with water and BA did not affect the H₂S emissions from pig slurry at day 1 and 2. On day 3, the H₂S emissions were significantly lower from both of the BA treated slurry compared to the control slurry ($P < 0.05$). From day 4 to 7 significant differences were observed in H₂S emissions between that of slurry from the control and 10% BA one time/day treatment

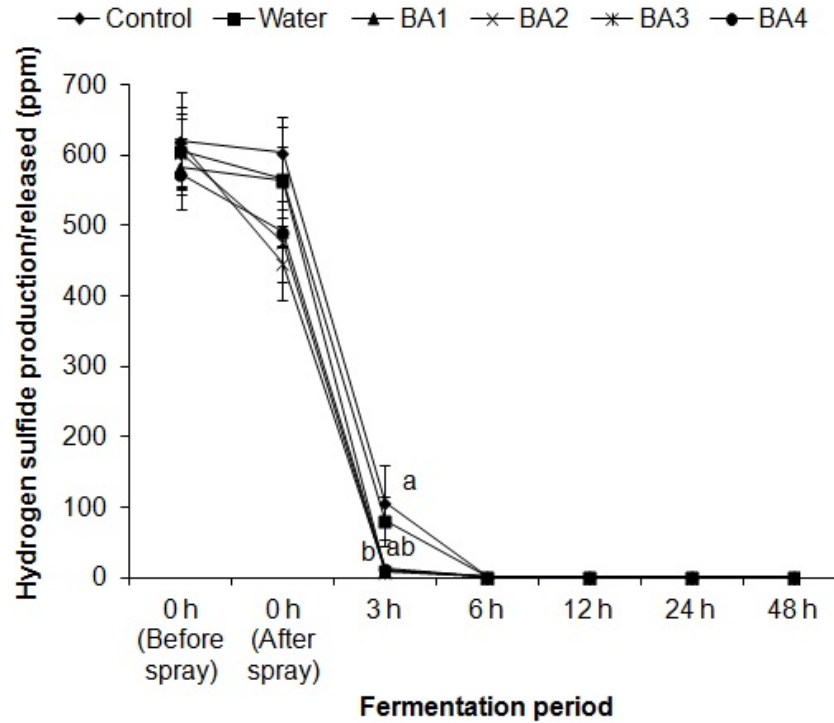


Figure 2. Effects of spraying different concentration of *Bacillus amyloliquifaciens* culture broth (BA) on hydrogen sulfide emission from pig slurry for 48 h. Control, no spray; Water, Water spray; BA1, BA 1%; BA2, BA 2%; BA3, BA 10%; BA4, BA 100%. Different letters at a particular time points indicates significant difference (P < 0.05).

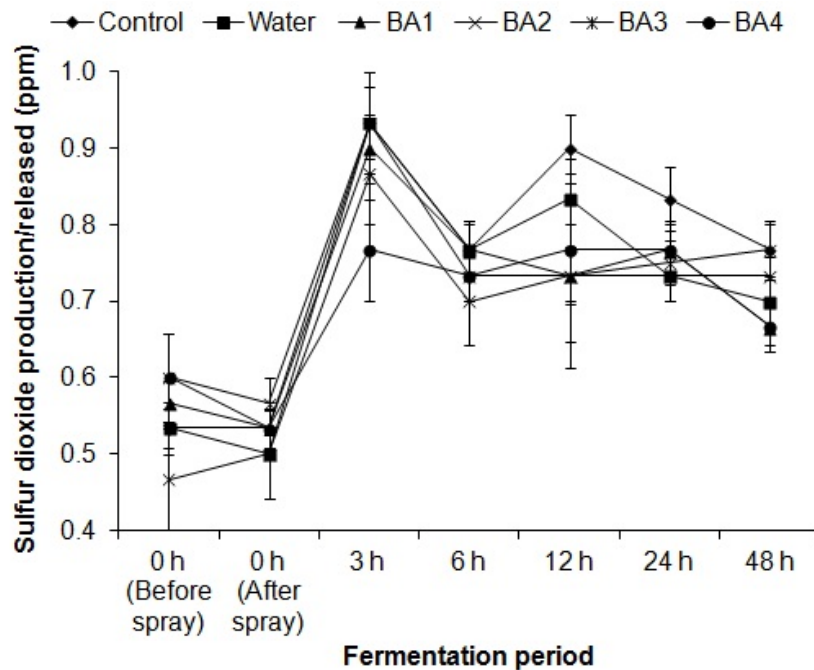


Figure 3. Effects of spraying different concentration of *Bacillus amyloliquifaciens* culture broth (BA) on sulfur dioxide emission from pig slurry for 48 h. Control, no spray; Water, Water spray; BA1, BA 1%; BA2, BA 2%; BA3, BA 10%; BA4, BA 100%.

Table 1. Effects of spraying 10% of *Bacillus amyloliquifaciens* culture broth (BA) on ammonia emission from pig slurry^A.

| Incubation period | Treatments | | | | SEM ^B | P-value |
|-------------------|---------------------|----------------------|--------------------|----------------------|------------------|---------|
| | Control | Water | BA one time/day | BA one time/ two day | | |
| Day 1 | 41.67 | 50.33 | 74.67 | 65.33 | 10.31 | 0.24 |
| Day 2 | 143.33 | 95.33 | 108.00 | 11.67 | 18.32 | 0.42 |
| Day 3 | 118.00 | 126.67 | 66.67 | 81.33 | 16.30 | 0.11 |
| Day 4 | 153.33 ^a | 103.33 ^{ab} | 51.67 ^b | 76.00 ^b | 18.89 | 0.03 |
| Day 5 | 163.33 | 98.00 | 48.33 | 63.33 | 28.49 | 0.11 |
| Day 6 | 236.67 ^a | 121.67 ^{ab} | 43.33 ^b | 59.33 ^b | 29.08 | 0.02 |
| Day 7 | 213.33 ^a | 164.33 ^a | 39.33 ^b | 45.33 ^b | 23.45 | 0.01 |

^{a,b}Means in a row with no common superscripts significantly differ ($P < 0.05$). ^AEach value represents the mean of 3 replicates. ^BStandard error of the means.

Table 2. Effects of spraying 10% of *Bacillus amyloliquifaciens* culture media (BA) on hydrogen sulfide (H₂S) emission from pig slurry^A.

| Incubation period | Treatments | | | | SEM ^B | P value |
|-------------------|---------------------|---------------------|-------------------|----------------------|------------------|---------|
| | Control | Water | BA one time/day | BA one time/ two day | | |
| Day 1 | 339.33 | 430.00 | 346.67 | 403.33 | 75.32 | 0.83 |
| Day 2 | 400.00 | 406.67 | 325.67 | 350.00 | 52.23 | 0.69 |
| Day 3 | 25.00 ^a | 14.33 ^{ab} | 0.33 ^b | 1.17 ^b | 2.89 | 0.01 |
| Day 4 | 550.00 ^a | 360.35 ^a | 9.67 ^b | 400.07 ^{ab} | 55.36 | 0.001 |
| Day 5 | 533.33 ^a | 320.00 ^a | 9.33 ^b | 373.33 ^a | 78.39 | 0.02 |
| Day 6 | 333.33 ^a | 183.33 ^a | 4.83 ^b | 223.40 ^a | 42.16 | 0.02 |
| Day 7 | 216.67 ^a | 133.33 ^a | 2.50 ^b | 173.33 ^a | 23.15 | 0.01 |

^{a,b}Means in a row with no common superscripts significantly differ ($P < 0.05$). ^AEach value represents the mean of 3 replicates. ^BStandard error of the means.

Table 3. Effects of spraying 10% of *Bacillus amyloliquifaciens* culture media (BA) on sulfur dioxide (SO₂) emission from pig slurry^A.

| Incubation period | Treatments | | | | SEM ^B | P value |
|-------------------|-------------------|--------------------|-------------------|----------------------|------------------|---------|
| | Control | Water | BA one time/day | BA one time/ two day | | |
| Day 1 | 0.53 | 0.50 | 0.70 | 0.60 | 0.10 | 0.58 |
| Day 2 | 0.83 | 0.70 | 0.77 | 0.73 | 0.09 | 0.79 |
| Day 3 | 0.87 | 0.90 | 0.67 | 0.60 | 0.09 | 0.14 |
| Day 4 | 0.80 ^a | 0.63 ^{ab} | 0.57 ^b | 0.63 ^{ab} | 0.05 | 0.10 |
| Day 5 | 0.77 | 0.60 | 0.50 | 0.67 | 0.07 | 0.16 |
| Day 6 | 0.90 ^a | 0.60 ^{ab} | 0.53 ^b | 0.63 ^{ab} | 0.09 | 0.10 |
| Day 7 | 0.80 | 0.67 | 0.43 | 0.47 | 0.11 | 0.14 |

^{a,b}Means in a row with no common superscripts tended to differ ($P < 0.10$). ^AEach value represents the mean of 3 replicates. ^BStandard error of the means.

group ($P < 0.05$).

The SO₂ emissions from pig slurry in response to spraying with 10% BA are shown in Table 3. During the 7 day measurement period, the SO₂ emissions from pig

slurry did not differ significantly among treatments, with the exception of a decreasing tendency in response to treatment with 10% BA one time/day when compared with control group at day 4 and day 6 ($P < 0.10$).

DISCUSSION

Odor production and accumulation of manure solids are characteristics that manifest as a result of inadequate microbial decomposition of manure (Davis et al., 2008). Undigested carbohydrates and protein (nitrogen) that have passed through the gastro-intestinal and urinary tract undergo microbial anaerobic decomposition to produce odorous compounds. This is further compounded by swine diet formulations, which commonly contain high concentrations of trace minerals and antibiotics that have deleterious effects on the bacteria needed for effective manure decomposition (Gilley et al., 2000). Therefore, direct application of microbial culture to improve manure digestion would provide a convenient mean to reduce odor emission from pig slurry. *B. amyloliquefaciens* (BA) is a potent spore-forming *Bacillus*, produces a number of extracellular enzymes including α -amylase, cellulose, metalloproteases and proteases (Gould et al., 1975; Gracia et al., 2003) to promote manure digestion and thereby may attenuate odor generation (Schreier, 1993). Ohta and Ikeda (1978) identified *Bacillus* spp. as effective microorganisms for reducing malodors. This study demonstrates that direct application of BA is an effective means of reducing odorous gas emission from pig slurry.

Feed nitrogen which is not utilized as body protein is excreted with feces and urine. According to Muck and Steenhuis (1981), the main part of ammonia (NH_3) originated from the decomposition of urea nitrogen in the urine by urease producing bacteria such as *Bacteriodes*, *Bifidobacteria*, *Proteus* spp. and others. Others, notably *E. coli*, do not have urease activity, so that release ammonia by deamination of organic nitrogen other than urea (Vince et al., 1973). As soon as the urine comes in contact with feces, the urea is converted into NH_3 and carbon dioxide by the microbial urease enzyme present in feces in the presence of high pH (Stevens et al., 1989; Aarnink, 1997). Therefore, reduction in the concentration of ammonia-producing bacteria is a key aspect to reduce emission of ammonia. It has been reported that, BA generates antimicrobial bacteriocin (barnase) (Lisboa et al., 2006), which may reduce ammonia producing *Clostridium perfringens*, *E. coli* and *Yersinia* in the feces, thereby attenuating the release of NH_3 in the present experiment. By contrast to our result Rahman et al. (2011) reported no effect of microbial additives on odor and ammonia reduction from farrowing-gestation swine operation. Lim et al. (2011) reported that bacteriocin produced by BA has antimicrobial activity against a wide range of microorganisms. Another possibility is that, BA produces several extracellular enzymes including metalloproteases and proteases, which may improve the digestion of fecal organic nitrogen and thereby reducing the ammonia production. The pH of the slurry is another important factor influencing the ammonia emission (Freney et al., 1983). *Bacillus* has been reported to reduce the pH of

slurry via the production of organic acid (Wang et al., 2009), which may cause a reduction in hydrolysis of urea and deamination of other forms of nitrogen, thereby reducing NH_3 emissions in the present experiment.

Hydrogen sulfide (H_2S) and sulfur dioxide (SO_2) have been identified as the most dangerous volatile sulfur gases (VS) among the by-products of manure decomposition generated under simulated anaerobic fermentation conditions (Banwart and Brenner, 1975). Production of VS by anaerobic bacteria involves dissimilatory sulfate reduction and metabolism of sulfur-containing amino acids (Ushida et al., 2003). VS could be removed from the air with the use of chemoautotrophic or heterotrophic bacteria (Kanagawa and Mikami, 1989). Sato et al. (1999) demonstrated that a range of heterotrophic bacteria could decompose H_2S *in vitro*. They also demonstrated that soil isolates belonging to the genera *Bacillus*, *Pseudomonas* effectively decomposed H_2S . Ushida et al. (2003) isolated a VS degrading *Bacillus* spp. (Strain KPU 0013) and reported their ability to reduce H_2S emission from pig slurry. The possible explanation of reduction in H_2S emission in this experiment is that, BA may decompose the H_2S *in vitro*. Nakada and Ohta (1997) also reported removal of H_2S by applying a deodorant bacterium *Bacillus* sp. BN53-1. Another possibility is that, BA reduced the pH of the feces, which prevent sulfate reduction by the sulfate reducing bacteria (Tuttle et al., 1969) and metabolism of sulfur-containing amino acids by anaerobic bacteria (Arakawa et al., 2000; Ushida et al., 2001).

Sulfur dioxide (SO_2) is one of the six criteria pollutants defined in the US Clean Air Act. However, information of livestock related SO_2 can hardly be found. To the best of our knowledge, no other studies have been carried out to evaluate the effects of microorganisms on the emissions of SO_2 from pig slurry. In experiment 1, we found no significant effect of BA on SO_2 emissions from pig slurry. However, in experiment 2, slightly reduced SO_2 emissions were found on days 4 and 6 in response to treatment with 10% BA one time/day, which may be due to degradation of VS by BA or reduced growth and activity of sulfur-reducing bacteria in the slurry.

Conclusion

The results of this study indicate that direct application of 10% BA one time/day is more proficient in reduction of fecal NH_3 , H_2S , and SO_2 emissions. Therefore, this level can be used as manure additives for odor reduction in pig facility. However, the underlying mechanisms by which reduction occurred should be further assessed by evaluating community structure of fecal bacteria and fecal pH which may better explain the relationship between *B. amyloliquefaciens* and native bacterial populations in the slurry.

Conflict of Interests

The author(s) have not declared any conflict of interests.

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