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Biological control of H_2S-producing bacteria in raw poultry materials destined for rendering process

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Isolation and Characterization of Bacteriophages Specific to Hydrogen Sulfide Producing-Bacteria

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Abstract: The objectives of this study were to isolate and characterize bacteriophages specific to hydrogen sulfide producing-bacteria (SPB) from raw animal materials, and to develop a SPB-specific bacteriophage cocktail for rendering application. Meat, chicken offal and feather samples collected from local supermarkets and rendering processing plants were used to isolate SPB (n=142). Bacteriophages (n=52) specific to SPB were isolated and purified from above samples using 18 of those isolated SPB strains as hosts. The host ranges of bacteriophages against five selected strains (*Escherichia coli*, *Citrobacter freundii* and *Hafnia alvei*) of SPB were determined. Electron microscopy observation of nine phages selected for phage cocktail revealed that six phages belonged to the family of *Siphoviridae,* whereas three phages belonged to *Myoviridae*. Restriction enzyme digestion analysis with endonuclease DraI detected six distinguished patterns among nine phages. Phage treatment prevented the growth of SPB up to 10 h with the multiplicity of infection (MOI) ratio of 1, 10, 100 and 1,000 in the Tryptic Soy Broth (TSB) at 30°C, and extended the lag phase of SPB growth for 2 h at 22°C with MOI of 10, 100 and 1,000. These results suggested that the selected bacteriophage cocktail has high potential for phage application to control SPB in raw animal materials destined for rendering process.
Key Words: Hydrogen sulfide producing-bacteria; Hydrogen sulfide; Bacteriophages; Raw animal materials; Rendering process

Introduction

In the United States, approximately 200 rendering plants process about 54 billion pounds of inedible animal by-products every year (Meeker and Hamilton, 2006). These raw animal materials contain many species of microorganisms that can cause the rapid spoilage of raw animal materials under ambient temperature. Among them, hydrogen sulfide producing-bacteria (SPB) utilize the sulfur and sulfur-containing compounds as the terminal electron acceptor under anaerobic conditions to produce hydrogen sulfide (H_2S) gas. Production of H_2S can occur during transportation and storage prior to the rendering process when temperature abuse occurs. The genera of *Pseudomonas, Citrobacter*, and *Aeromonas* are reported as the predominant SPB (McMeekin et al., 1975; Hinton et al., 2004), and some other bacteria such as *Salmonella* and *E. coli* are also producer of H_2S in an anaerobic environment (Layne et al., 1971; Barrett and Clark et al., 1987). Hydrogen sulfide is extremely toxic for humans and animals. The production of H_2S not only decreases the quality of finished animal meal products, but also harms the workers' health by creating an unsafe working environment in rendering facilities. During a typical transportation and storage time of 12 h, the amount of H_2S in animal raw materials can easily increase to a lethal level of 700 ppm that can cause the immediate death of humans (Beauchamp et al., 1984).

To control the H_2S production caused by SPB, many studies were conducted using different antibacterial agents. Antibiotics such as ciprofloxacin and metronidazole were used to control the SPB growth in rat (Ohge et al., 2003). For clinical application, chlorhexidine and triclosan are reported to inhibit SPB in the oral cavity and saliva (Sreenivasan, 2003; Sreenivasan and Gittins, 2004). In oil fields, nitrate salts were applied to suppress the SPB growth and diminish sulfate reduction, consequently controlling the H_2S production (Bodtker et al., 2008). However, these methods may have limited use in foods. For example, in the application of

nitrate, toxic nitrite, a carcinogen-producing agent, is produced in the nitrate reducing reaction as a result of H_2S reduction (Londry and Suflita, 1999). In recent years, bacteriophages have been explored as a biological control method for pathogen control in the food industry. Although some other chemical agents were also applied to eliminate pathogens in food industry, such as organic acids and chlorine, but there is no report on controlling SPB and H_2S production using these sanitizers.

Bacteriophages (phages) are viruses that target and lyse specific bacterial cells during their rapid replication without harming humans, animals, plants and other species of microorganisms (Garcia et al., 2008). These viral particles can be found in the natural environment such as water, soil, and air. In 2006, the FDA approved the use of bacteriophages for controlling *Listeria monocytogenes* in ready-to-eat foods (Intralytix, 2006). Bacteriophage therapies have been reported to reduce *Salmonella*, *E. coli* and *Campylobacter jejuni* in broiler chickens, beef, sprouting seeds and other food products (Huff et al., 2003; Pao et al., 2004; Wagenaar et al., 2005; Atterbury et al., 2007). However, there is no known study applying bacteriophages to control SPB in raw animal materials destined for rendering process.

The objectives of this study were to isolate and characterize bacteriophages specific to SPB from raw animal materials, and to develop a SPB-specific bacteriophage cocktail for rendering application.

Materials and Methods

Isolation of hydrogen sulfide producing-bacteria (SPB)

Raw meat samples, either expired or near expiration, of poultry (n=6), pork (n=5), fish (n=4) and beef (n=4) were obtained from local retail stores for isolation of SPB. Chicken offal (n=1), feathers (n=1) and feather processing water (n=1) samples from a rendering facility (Carolina By-Products, Ward, SC, USA) were also used for SPB isolation. Meat and chicken offal samples were homogenized in a blender (Model: 51BL30, Waring, Torrington, CT, USA), and serial dilutions of meat homogenate, feather and feather processing water (10^{-1}~10^{-6}) were spread-plated onto tryptic soy agar-hydrogen sulfide (TSA-H_2S) plates containing 40 g/L of tryptic soy agar (TSA, Becton Dickinson, Sparks, MD, USA), 0.3 g/L of sodium thiosulfate (Fisher, Rochester, NY, USA), 0.3 g/L of ferric citrate (MP Biomedicals, Solon, OH, USA), and 0.6 g/L of L-cysteine (MP Biomedicals, Solon, OH, USA). L-cysteine was filter-sterilized through a 0.2 μm filter (VWR, West Chester, PA, USA) prior to addition to sterile TSA-H_2S medium. For fish samples, 5 g/L of NaCl was added to TSA-H_2S medium. Plates were incubated for 3 days at room temperature (22~25°C) in anaerobic jars (Becton Dickinson, Cockeysville, MD, USA). Black colonies growing on TSA-H_2S plates were picked and purified by restreaking on TSA-H_2S plates several times. Pure cultures growing on TSA plates were preserved in Tryptic Soy Broth (TSB; Becton Dickinson, Sparks, MD, USA) containing 20% glycerol and stored at -80°C.

Characterization of SPB isolates

Gram staining was performed on each SPB isolate. A 3% KOH (Fisher Scientific, Pittsburgh, PA, USA) test was also done to confirm the results from Gram staining (Gregersen, 1978). The morphology of each SPB isolate was observed under a microscope (Leica Microsystems, Bannockburn, IL, USA). To determine the species of each SPB isolate, 16S

rRNA gene sequencing was performed. Briefly, the genomic DNA from bacterial cells was isolated using a Microbial DNA Isolation Kit (MoBio, Carlsbad, CA, USA). Then the polymerase chain reaction (PCR) was employed to amplify 16S rRNA gene according to the procedure described by Washio et al. (2005). PCR products were further purified using a DNA Extraction Kit (MoBio, Carlsbad, CA, USA) by following the manufacturer's directions. The purified PCR products were analyzed by Clemson University Genome Institute (CUGI, Clemson, SC, USA) for DNA sequencing. The 16S rRNA gene sequencing data were analyzed by Basic Local Alignment Search Tool (BLAST) and Ribosomal Database Project (RDP) in the nucleotide collection database for species identification.

Bacterial strains and culture conditions

To detect the host range of bacteriophage isolates, five selected SPB strains from raw meat samples were used. A preliminary study revealed that all five SPB strains had higher growth rate at 37°C than at room temperature. Therefore, these strains were grown overnight in TSB with shaking (New Brunswick C25 incubator shaker; Edison, NJ, USA) at 37°C. After centrifugation at 5,000 × *g* for 5 min, the bacterial pellets were washed with 0.85% saline, and the optical density was adjusted to approximately 0.5 at 600 nm wavelength as determined by a spectrometer (μQuant; Bio Tek, Winooski, VT, USA).

Bacteriophage enrichment, isolation and propagation

Bacteriophages were isolated from raw meat and feather processing water samples by conventional enrichment method (Van Twest and Kropinski, 2008). Initially, all 142 SPB isolates were used as host strains to enrich bacteriophages from above samples. To 5 mL of the double strength Tryptic Soy Broth (TSB, 60 g/L) inoculated with 100 μL of each of SPB host strains, blended raw meat sample or feather processing water sample was separately added to the

total volume of 10 mL. The tubes were incubated at 37°C overnight. The enrichment culture was then centrifuged at 2,500 × *g* for 20 min and the supernatant was filtered through a 0.2 μm filter (VWR, West Chester, PA, USA).

Since completely clear plaques were formed on the lawn of 18 SPB isolates based on above experiments, indicating strong lytic activity of phages on these isolates as compared to others, these SPB isolates were used to screen, isolate and purify different phages from enrichment broth. Bacteriophages were isolated using soft agar overlay method (Heringa et al., 2010). Three milliliter of 0.6% agar (Becton Dickinson, Sparks, MD, USA) containing a single SPB strain at a density of 10^7 CFU/mL was overlaid onto a TSA plate. After the overlaid agar plate solidified, a 10 μL drop of phage suspension was spotted onto the surface. Bacterial strains that had a clear zone around phage drop were then tested against serial dilutions of phage suspension by the soft agar overlay method. Briefly, bacterial suspension at density of 10^7 CFU/mL was mixed with bacteriophage suspensions for 10 minutes in a 96-well microplate (96-Well Cell Culture Cluster, Corning, NY, USA). The mixture was then transferred into 3 mL of 0.6% agar and overlaid onto a TSA plate. After incubation at 37°C overnight, plaques were isolated by cutting from the agar with a 1 mL sterile pipette tip. Isolated plaques were subsequently suspended in 1 mL of SM buffer [100 mmol/L NaCl, 8 mmol/L $MgSO_4 \bullet 7H_2O$, 50 mmol/L Tris-HCl (pH 7.5)]. Following overnight incubation at 4°C, plaque purification was repeated at least two times.

Phage stock suspension was prepared by using soft agar overlays of serial dilutions of phage as described above. To lowest density plate on which all bacterial growth was cleared by phage, a 10 mL of SM buffer was added and held at 4°C for 2 days. The surface mixture of SM buffer and soft agar was collected and centrifuged at 11,000 × *g* for 15 min. The supernatant was

transferred into a sterile test tube and filtered through a 0.2 μm filter. The titer of phage stock suspension was determined by pipetting drops of 10 μL serially diluted stock suspension onto the plate overlaid with host bacteria, and plaques were counted after overnight incubation at 37°C.

Host range test of bacteriophages

A total of 52 bacteriophage isolates specific to SPB were collected for host range study based on the different sizes of plaque formation. TSA plates overlaid with soft agar were seeded with each of five selected SPB strains as host at a density of 10^6 CFU/plate. A 10 μL drop of phage suspension in the titre of 10^8 PFU/mL was pipetted onto the agar. Plates were incubated at 37°C overnight, and the characteristics of plaques were recorded.

Transmission electron microscopy

A 5 μL drop of phage suspension (>10^{10} PFU/mL) was pipetted onto the surface of a copper grid (400 mesh, EMS, Hatfield, PA, USA) and incubated for 1 min at room temperature, and was then drawn off with filter paper. The copper grids were stained for 60 seconds with 5 μL of 2% uranyl acetate (UA; EMS, PA, USA) and air-dried for 2 h. A Hitachi H-7600 electron microscope (Hitachi, Tokyo, Japan) was used at 120-keV accelerating voltage to observe the bacteriophages on the grids. Phage size was calculated by scale bar in each image. Size of each phage is the average value measured on 10 random phage particles.

DNA isolation and restriction enzyme analysis of bacteriophage isolates

Based on host range and bacterial lytic activity, nine phages were selected for phage cocktail. The DNA of fresh phage stock suspensions were used extracted using MoBio Ultra Clean Microbial DNA Isolation Kit (MoBio, Carlsbad, CA, USA). Before the restriction enzyme analysis, DNA samples were subject to ethanol precipitation according to standard protocols (Sambrook et al., 2001). The concentrated DNA samples from nine selected SPB phages were

digested with restriction endonucleases DraI at 37°C in a water bath according to the supplier's recommendations. DNA fragments were separated electrophoretically in 1.5% agarose gel (Bio-Rad, Hercules, CA, USA) at 50 V for 3.5 h, stained with ethidium bromide and de-stained with distilled water.

Inhibiting SPB mixture by bacteriophage cocktail in nutrient broth

A cocktail of nine selected strains of phage was tested for inhibition of a five strain mixture of SPB. The SPB strains were grown in TSB separately and washed by 0.85% saline as described above, and then diluted to 10^4 CFU/mL. Equal volume of each of the five selected SPB strains was mixed as the bacterial mixture which was added into a 96-well plate followed by adding the phage cocktail at multiplicity of infection (MOI) of 0.001, 0.01, 0.1, 1, 10, 100 and 1000. The wells containing 270 µL of bacterial mixture and 30 µL of SM buffer served as controls. The inoculated micro-well plates were incubated at 22 and 30°C and optical densities were measured at 0 h, every hour from 4 to 16 h, and 24 h using a spectrometer (µQuant, Bio Tek, Winooski, VT, USA).

Statistical analysis

Bacterial count data were converted to $\log_{10}$ CFU/g for statistical analysis. An analysis of variance (ANOVA) for a completely randomized design was conducted to determine if general differences existed between treatment means using the general linear model (GLM) procedure. Specific comparisons among different phage treatments were accomplished with Tukey's test. All statistical analyses were performed using Statistical Analysis System 9.1 (SAS; SAS Institute, Cary, NC, USA).

Results

Isolation and characterization of SPB

Raw meat (fish, poultry, pork and beef) purchased from local grocery stores contained ca. 4 log CFU/g of SPB. Raw animal by-products from a rendering plant contained SPB counts ranging from 4 to 6 log CFU/g or CFU/mL in chicken offal, chicken feather and feather processing water. Hydrogen sulfide producing-bacteria isolates (n=142) from different raw meat products and raw animal by-products were successfully collected using the TSA-H_2S medium (Table 1). All of SPB isolates, cultivated as Gram negative, formed either white or light yellow colonies on TSA media and black or dark gray colonies on TSA-H_2S plates.

Five strains of SPB were selected from 142 SPB strains based on their growth rate in nutrient media, rapid production of H_2S, and diverse sources of isolation. All of these five selected SPB strains were able to grow at a broad range of temperatures such as 20 and 37°C. Isolates S12, S201, and S203 were isolated from poultry meat samples whereas isolates S183 and S211were isolated from beef and fish, respectively. The isolates were further identified as *C. freundii* (S12), *E. coli* (S201, S203), and *H. alvei* (S183, S211) using 16S rRNA gene sequencing technique. Additionally, all these five host strains of SPB were tested for the presence of prophage using mitomycin C method as described in our previous publication (Heringa et al., 2010), and none of them is lysogen.

The growth rates of five selected SPB strains were lower at 25°C than at 30°C (data not shown). There was a 9 h lag phase for the five SPB strains at 25°C as compared with approximately a 4 h lag phase at 30°C. Overall, there was no significant (p>0.05) difference of the growth rate among these five strains at each temperature.

Isolation, restriction enzyme analysis, and host range of SPB-specific bacteriophages

Bacteriophages (n=52) were isolated using the 142 SPB isolates obtained from meat samples and feather processing water as hosts initially (Table 1), and then screened against a subset of 18 SPB isolates using soft agar overlay method.

Restriction enzyme DraI successfully digested all genomic DNA samples extracted from above nine selected phages, however, this analysis could not differentiate all nine isolates. The restriction analysis result revealed phage isolates 211a, 214a and 214c have the same DNA pattern, while isolates 214b and 213a shared another same pattern (Figure 1). The other phages 217a, 218a, 12a and 201a appeared to have unique restriction patterns.

The host range analysis revealed differences among 211a, 214a and 214c (Table 2). The phages 214b and 213a could be differentiated by plaque size (Table 3). The host range for most bacteriophage isolates were relatively narrow, and most of the phages from raw meat sources had no lytic activity toward SPB isolates from chicken feather and feather processing water samples (data not shown). To develop a phage cocktail for controlling SPB, nine phages were selected based on plaque size and host range.

Bacteriophages characterized by electron microscopy

Transmission electron microscopy images of the nine SPB-specific phages showed that phages 211a, 214a, 214c, 217a, 218a and 12a had flexible non-contractile tails and were of the family *Siphoviridae*. The presence of a contractile tail for 213a, 214b and 201a suggested that they belong to the family of *Myoviridae*. Figure 2 presented the TEM images of representative phage of each phage family. Each phage had a unique morphology according to the dimensions of the head and tail (Table 3).

Broth test of bacterial lytic activities of phage cocktail with different multiplicity of infection (MOI) values

Based on the host ranges of 52 bacteriophage isolates described above, nine phages were selected to make a phage cocktail covering a broad host range including against the five selected SPB strains. A total of seven MOIs (0.001, 0.01, 0.1, 1, 10, 100 and 1000) were evaluated on inhibition of SPB growth in TSB.

At 30°C, the bacteriophage cocktail was able to prevent the growth of SPB for at least 10 h at a MOI of 1 or higher as compared with 4 h of the lag phase in control samples without phage treatment (Figure 3A). Phages applied at MOIs from 10 to 1000 had a similar inhibitory effect on SPB growth, and reduced the total bacterial cell yields by 61% as compared with control after 24 h of incubation. As the MOIs decreased to 0.1, 0.01 and 0.001, the inhibitory effect decreased, and there was no extension of lag phase, but slight reduction of growth rates and total bacterial cell yields was observed (Figure 3A).

At 22°C, bacteriophage treatment with MOI of 10 to 1,000 extended the lag phase by 2 h longer than the control. Although phage treatment with MOI of 0.001 to 1 did not significantly reduce the SPB growth rate in early phase of treatment ($p>0.05$), there is a significant reduction in total bacterial cell yields ($p<0.05$) at the end of test as compared with control (Figure 3B).

Discussion

The purpose of this study was to isolate bacteriophages specific to SPB and then apply those phages to control H_2S production from raw rendering by-products by SPB. To isolate SPB strains from raw animal materials, TSA-H_2S plates were used in this study. Similar media such as lead acetate agar, peptone-iron agar, plate count agar-H_2S, and *Pseudomonas* agar medium were used in previous studies to isolate SPB from raw meat (Levin et al., 1968; Nicol et al., 1970; McMeekin and Patterson, 1975; Malle et al., 1998; Hinton et al., 2004;). Using this

selective medium, SPB populations in different raw animal materials were enumerated in the range of 4~6 log CFU/g. However, the SPB population may vary on a large scale according to the freshness of raw materials. Hinton et al. (2004) reported the level of SPB, which were grown on iron agar plate, ranged from 1 to 4 log CFU/mL on chicken broilers or rinse before scalding and increased to 9 log CFU/mL in refrigerated storage of 14 days in rinsate of poultry carcasses. Malle et al. (1998) suggested that fish should be considered as spoiled when the count of SPB exceeds 5 log CFU/g.

In this study, five selected SPB strains were identified as *C. freundii* (S12), *E. coli* (S201, S203), and *H. alvei* (S183, S211). Although several studies reported *Aeromonas* spp., *Pseudomonas* spp. and *Shewanella putrefaciens* were predominant SPB species in poultry carcasses, fish flesh, pork loins, etc. (Levin et al., 1968; Kadota et al., 1972; Borch et al., 1996; Gram and Huss, 1996; Malle et al., 1998; Hinton et al., 2004; Holley et al., 2004; Katikou et al, 2007), *C. freundii* was found as one of the major SPB from poultry products and processing plants in addition to *S. putrefaciens*, *Proteus* spp. and *Corynebaterium* spp. (McMeekin and Patterson, 1975). *C. freundii* also was isolated in this study from raw poultry meat. Both *E. coli* and *H. alvei* belong to the family of *Enterobacteriaceae* that are known to produce H_2S (Borch et al., 1996). *E. coli* was reported to have the ability to produce H_2S from cysteine (Kadota et al., 1972).

In this study, the host range for most bacteriophage isolates were relatively narrow (Table 2), and most of the phages from raw meat sources had no lytic activity toward SPB isolates from chicken feather and feather processing water samples (data not shown). For example, the phage 214c can only lyse strains of *H. alvei* (S183 and S211) strongly, but does not inhibit the growth of strains of *C. freundii* (S12) and *E. coli* (S201, S203). This result was not surprising in

considering the specificity of bacteriophages. For example, bacteriophages specific for *E. coli* O157:H7 have no effect on *L. monocytogenes* or *C. jejuni* (Atterbury et al., 2003; Leverentz et al., 2003; Callaway et al., 2008). In this study, not all SPB isolates were identified into species level yet. It is expected that our SPB-specific bacteriophage may be effective against the same species of SPB isolates.

The total transportation and storage time of raw animal material prior to the rendering process is less than 12 h. During this period of time, SPB have enough time to enter the log phase of growth under ambient temperature and then produce H_2S. Therefore, an extended lag phase for SPB growth is significant for maintaining the quality of raw animal by-products. In this study, extension of the lag phase for at least 10 h was achieved by applying phage treatment to five selected SPB strains in TSB at 30°C. Using phages, selected SPB could be reduced during this critical time prior to the rendering process, thereby preventing H_2S production. The growth of SPB mixture after 10 h of phage treatment was detected. However, it is important to note that none of the phage treated samples reached the level of growth of controls within the 24 h sampling period. The re-growth of the bacteria has been reported for several bacterial species during phage treatment. McLaughlin et al. (2006), O'Flynn et al. (2006) and Heringa et al. (2010) all reported the re-growth of host bacteria in studies involving the control of either *Salmonella* or *E. coli* O157:H7 by bacteriophages. This can be explained by the development of bacteriophage-insensitive mutants (BIM) among the host bacteria cells. Heringa et al. (2010) observed the re-growth of host bacteria in broth tests with four of five bacteriophages in 24 h sampling period, and one of them re-grew to a level even greater than the bacterial growth in control sample. O'Flynn et al. (2006) found the bacterial numbers were increased nearly 3 logs when *E. coli* O157:H7 were treated with three-phage cocktail with an MOI between 1 and 100.

All of these results emphasized the importance of optimizing phage cocktails that can prevent the development of BIM's.

On the contrary to the high effectiveness at 30°C, inhibitory effect of bacteriophage against SPB decreased at 22°C. Phage treatment only extended the lag phase slightly by 2 h at MOI of 10, 100 and 1000 as compared with control sample, and there was no extension of lag phase at MOI of 1 or less. Bigwood et al. (2009) obtained similar results in a study of phage inactivation of foodborne pathogens on cooked and raw meat. In that study, no significant effect of phage addition was observed at low host density of 4 log CFU/mL with MOIs of 10 and 100 at 24 °C, but an inhibitory effect occurred at high MOIs of 1,000. This may be explained by the fact that phages were not able to replicate due to the slow growth of bacterial population at lower temperatures. However, at an optimal temperature such as 30°C, host cells multiply quickly which leads to the production of more offspring virions that can infect more host cells. Furthermore, Heineman et al. (2008) reported that the bacterial physiological state affected bacteriophage adsorption. The ineffectiveness in extending the lag phase in the broth test at 22°C may also be explained by the less active state of bacterial cells which leads to a decrease in the occurrence of phage adsorption or no phage replication in the treatment process, especially when MOI was low.

In conclusion, SPB-specific bacteriophages were successfully isolated in abundance from the same sources where the SPB were isolated. Both TEM and restriction enzyme analysis were used to characterize and identify the phage isolates for developing a phage cocktail that could effectively reduce the host bacterial strains. The cocktail of nine selected phages prevented the growth of a five strain mixture of SPB for 10 h with a multiplicity of infection (MOI) ratio of 1, 10, 100 and 1000 in TSB at 30°C, and for 2 h with a MOI of 10, 100 and 1000 at 22°C. Further

study on applying the SPB specific bacteriophage cocktail to rendered animal by products is currently underway.

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Table 1: Summary of hydrogen sulfide producing-bacteria and bacteriophage isolates from various raw materials

Raw materials*	Sources†	Number of SPB isolates	Number of Bacteriophage isolates
Poultry meat	F	24	11
Fish meat	B, F, I	26	23
Pork	B, F, I	22	0
Beef	B, F, I	35	2
Chicken offal	P	6	NT‡
Feather processing water	P	20	16
Chicken Feather	P	9	NT

*Meats purchased from supermarkets were either expired or near expiration date.

†B, F, I are supermarkets in Clemson, SC; P is Carolina By-products in Ward, SC, USA.

‡ NT means not tested.

Table 2: Host range of nine bacteriophages specific to hydrogen sulfide producing-bacteria [*]

Bacteriophage isolates[†]	SPB isolates				
	S12	**S201**	**S203**	**S183**	**S211**
211a	-	-	-	-	++
213a	-	-	-	++	-
214a	-	-	-	-	+
214b	-	-	-	++	-
214c	-	-	-	+++	+++
217a	-	-	-	+	++
218a	-	-	-	+	++
12a	+++	-	-	-	-
201a	-	+++	+++	-	-

[*]A subset of the host range data obtained for 52 bacteriophage isolates screened against host SPB isolates.

[†]Lysis results were recorded as decreasing lysis effect as follows: +++, ++, +; -, no lysis.

Table 3: Morphology of nine bacteriophage isolates specific to hydrogen sulfide producing-bacteria under transmission electron microscope (TEM)

Phage	Morphology				Family
	Plaque*	Head† (nm)	Tail (nm)	Tail Type	
211a	Medium	95 ± 6	217 ± 8	Non-contractile	Siphoviridae
213a	Small	97 ± 6	154 ± 4	Contractile	Myoviridae
214a	Medium	79 ± 3	217 ± 14	Non-contractile	Siphoviridae
214b	Medium	80 ± 3	148 ± 8	Contractile	Myoviridae
214c	Medium	94 ± 3	196 ± 7	Non-contractile	Siphoviridae
217a	Medium	87 ± 7	186 ± 13	Non-contractile	Siphoviridae
218a	Medium	99 ± 5	216 ± 9	Non-contractile	Siphoviridae
201a	Small	101 ± 4	121 ± 5	Contractile	Myoviridae
12a	Large	71 ± 4	191 ± 11	Non-contractile	Siphoviridae

*Size of plaques (diameter): small (0~2 mm), medium (2~4 mm), and large (4~6 mm).

†Head and tail measurements are the average of 10 phage particles ± standard deviation.

Figure 1: Restriction enzyme analysis of nine bacteriophage isolates

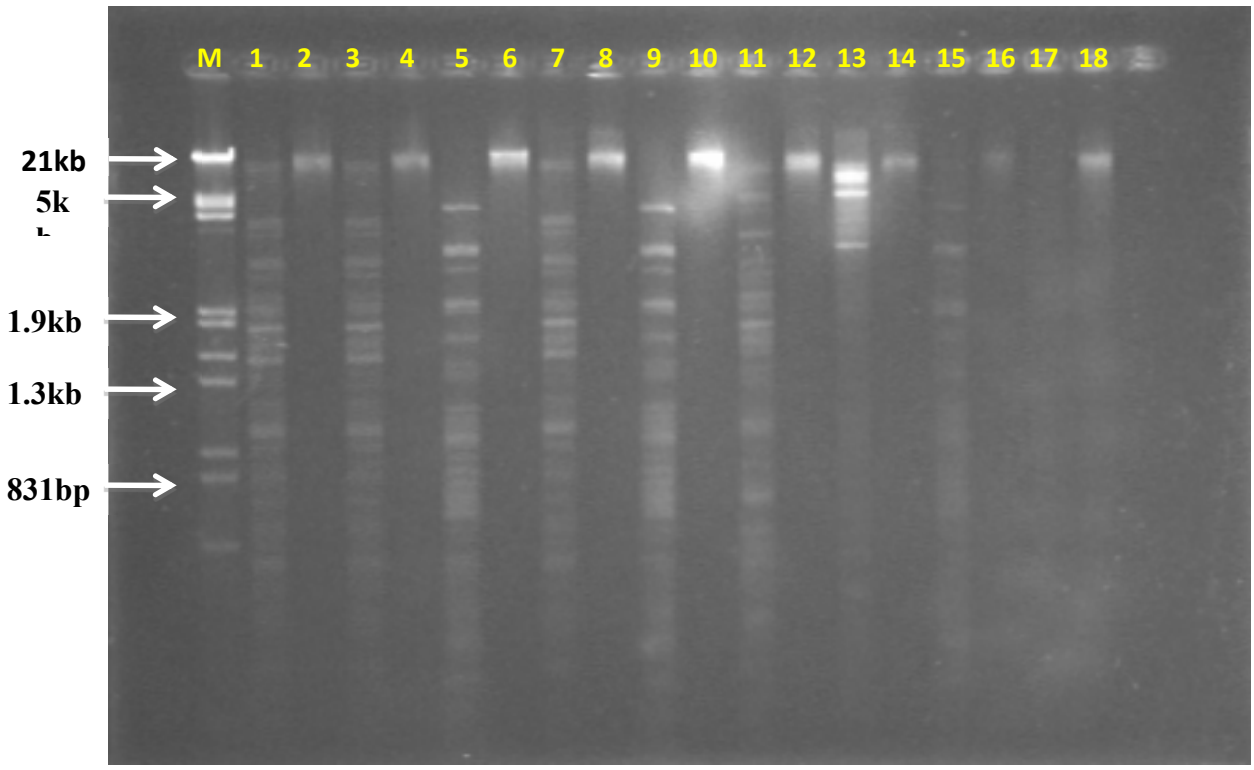


Figure 2:

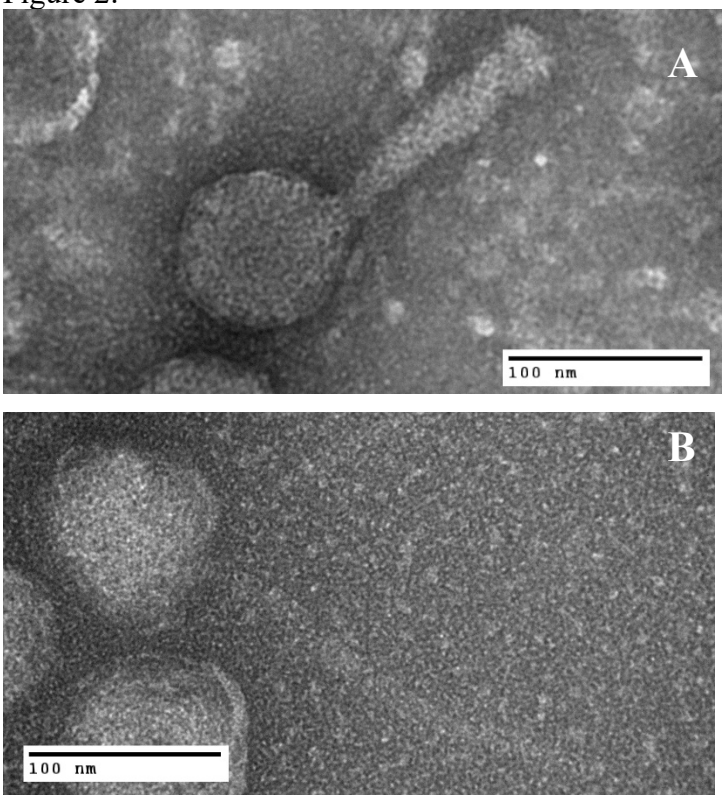
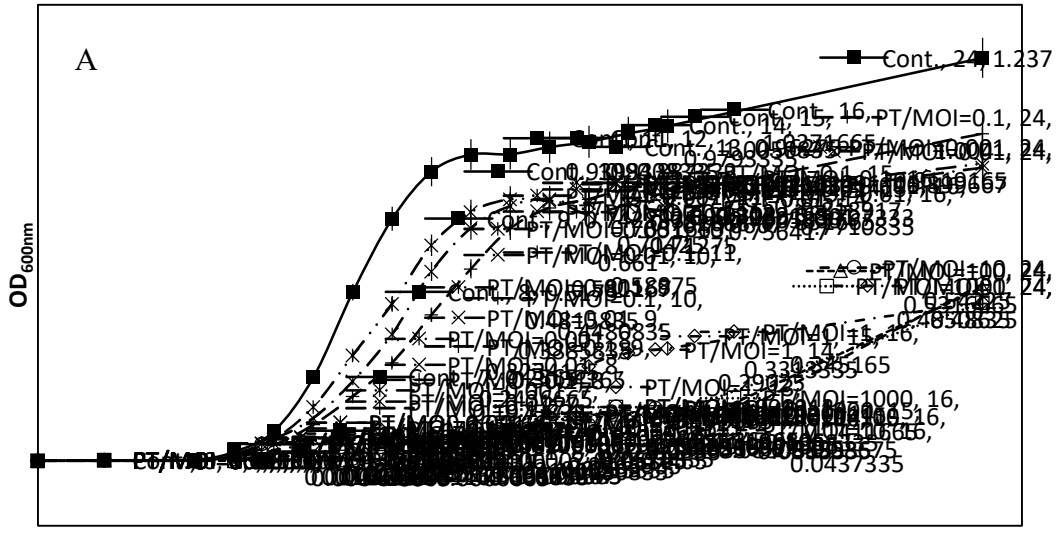


Figure 3:


A
Cont., 24, 1.237
PT/MOI=0.1, 24,
PT/MOI=1, 24,
PT/MOI=10, 24,
PT/MOI=100, 24,
PT/MOI=1000, 16,
OD600nm
Time (h)

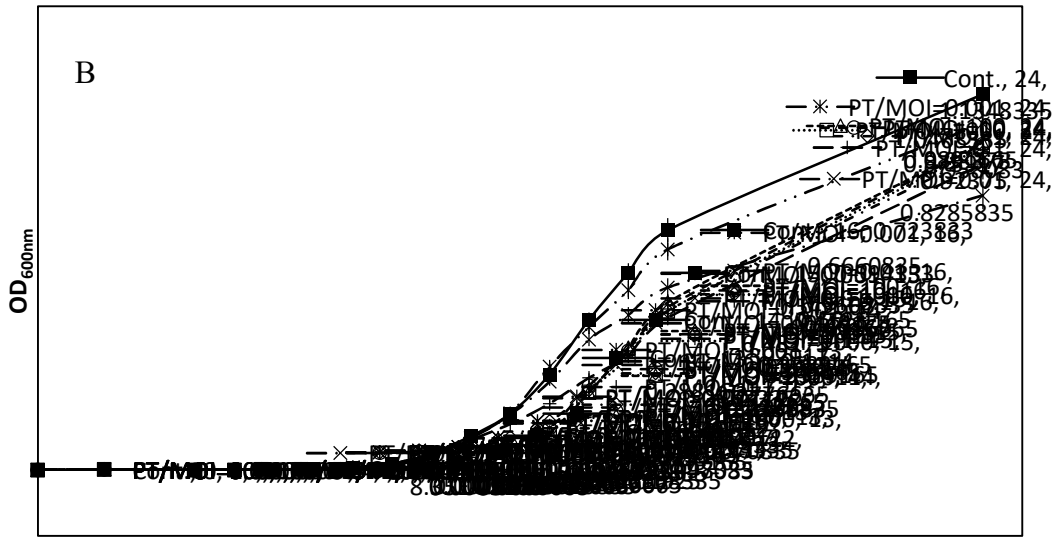

B
Cont., 24,
PT/MOI=0.1, 24,
PT/MOI=1, 24,
PT/MOI=10, 24,
PT/MOI=100, 24,
PT/MOI=1000, 16,
OD600nm
Time (h)

Figure Captions

Figure 1: Restriction enzyme analysis of nine SPB-specific bacteriophage isolates using enzyme DraI. Lane M is Lambda HindIII/EcoRI DNA marker. Lane 1-2, 3-4, 5-6, 7-8, 9-10, 11-12, 13-14, 15-16, and Lane 17-18 are digested and undigested DNA of phages 211a, 214a, 214b, 214c, 213a, 217a, 218a, 201a and 12a, respectively.

Figure 2A-B: TEM images of representative phage 214b belong to family of *Myoviridae* (A), and phage 211a belongs to *Siphoviridae* (B).

Figure 3A-B: Growth inhibition of five selected SPB in TSB medium by phage cocktail with MOI of 0.001 (*), 0.01 (×), 0.1 (+), 1 (◊), 10 (○), 100 (△) and 1000 (□) at 30 (A) and 22°C (B). Symbol " ■ " represents the control sample. Each data point represents the average of six replicates.

Part II

Results reported in the following manuscript were generated between 7/1/2010 and 9/17/2012.

Application of Bacteriophages Specific to Hydrogen Sulfide Producing Bacteria in Raw Animal Materials

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Running Title: Application of bacteriophage specific to hydrogen sulfide producing bacteria

Key Words: Hydrogen sulfide producing bacteria; Hydrogen sulfide; Bacteriophages; Raw animal materials; Rendering process

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ABSTRACT

Aim: Hydrogen sulfide bacteria (SPB) can spoil raw animal materials and release harmful hydrogen sulfide (H_2S). The objective of this study was to apply a SPB-specific bacteriophage cocktail to control H_2S production by SPB in different raw animal materials under laboratory and greenhouse environment by simulating transportation and rendering facility condition.

Methods and Results: The amount of H_2S production was determined using either test strips impregnated with lead acetate or a H_2S monitor. Under laboratory conditions, phage treatment applied to fresh chicken meat inoculated with SPB, spoiled chicken meat, chicken guts and chicken feathers reduced H_2S production by ca. 25% to 69% at temperatures from 20 to 37°C. Under greenhouse conditions, phage treatment achieved ca. 30~85% reduction of H_2S yield in chicken offal and feathers. Among all phage treatments, multiplicity of infection (MOI) of 100 exhibited the highest inhibitory activities against SPB on H_2S production, but was not significantly different ($p>0.05$) from MOIs of 1 or 10. Several factors such as initial SPB level, temperature and contact efficacy between SPB cells and phages were found to affect lytic activities of bacteriophages.

Conclusions: Our study demonstrated the phage cocktail is effective to reduce the production of H_2S by SPB significantly in raw animal materials.

Significance and Impact of the Study: This biological control method can control SPB in raw animal materials at ambient temperatures, leading to a safer working environment and high quality product for rendering industry.

INTRODUCTION

In the United States, over 8 billion animals such as hogs, cattle and chickens are produced, slaughtered and processed annually (Hamilton and Meeker, 2006), resulting in large amounts of inedible by-products such as heads, feet, offal and feathers. Microorganisms from animal hide/skin, gastrointestinal tracts and processing equipment can easily contaminate these by-products and cause spoilage due to rapid multiplication at ambient temperature. Spoilage of these by-products is always accompanied by the production of off-odors and off-colors when the bacteria utilize substrates such as protein and amino acids for metabolism (Gram & Huss, 1996). Some spoilage microorganisms produce hydrogen sulfide gas (H_2S) during metabolism and have been appropriately named as hydrogen sulfide producing bacteria (SPB). When the population of SPB exceeds 7 log CFU g^{-1} in raw animal material, the spoilage becomes sensory detectable (Katikou et al., 2007).

To control the pathogenic and spoilage bacteria in raw animal materials, many preservation methods, such as modified atmosphere packaging, lactic acid bacteria and antibacterial essential oils, have been developed and applied to foods. In addition, chemical agents such as nitrate salts, organic acids, and antibiotics have also been employed (Leisner et al., 1996; Ouattara et al., 1997; Saucier et al., 2000). However, there are no published studies focusing on the biological control of SPB in raw animal by-products.

Bacteriophages were discovered a century ago and have been used for more than 60 years for bacterial control. In recent years, bacteriophage treatment has drawn much attention due to the rapid development of antibiotic resistance by bacteria. Although many factors affect the inhibitory effect of phage treatment such as temperature, multiplicity of infection (MOI), and incident of phage attachment to bacterial cells, some researchers have successfully applied

bacteriophages to raw meat to control spoilage microorganisms and extend shelf life of meat products. For example, Greer (1986) applied bacteriophages to control of *Pseudomonas* spp. in beefsteaks by extending the shelf life from 1.6 to 2.9 days. Greer and Dilts (2002) also demonstrated the control of a pork spoilage organism, *Brochothrix thermosphacta*, with phage treatment. In that study, the shelf life of pork treated with phage was extended from 4 to 8 days compared to control samples. Other studies have demonstrated the successful application of bacteriophages for reducing pathogens in live animals (Smith and Huggins, 1983; Sheng et. al., 2006; Atterbury et. al., 2007), fresh produce (Leverentz et. al., 2003; Pao et. al., 2004), meat products (Greer et. al., 1988; Whichard et. al., 2003) and ready-to-eat foods (Intralytix, 2006). However, there is a lack of research on the application of bacteriophages in controlling spoilage bacteria, especially, SPB in raw animal materials destined for rendering process. Therefore, the objective of this study was to develop a SPB-specific bacteriophage cocktail for application and investigate if such phage treatment can be effective in controlling the growth of SPB and eliminating H_2S production in raw animal products used for the rendering process.

MATERIALS AND METHODS

Bacterial culture and phage stock preparation

Five predominant SPB strains (S12, S201, S203, S183 and S211) isolated from meat and raw animal by-products were grown overnight in tryptic soy broth (TSB; Becton Dickinson, Sparks, MD, USA) at 37°C with shaking (Gong, 2010). Bacterial cells were collected, washed in 0.85% saline, and adjusted to an optical density of 0.5 at a wavelength of 600 nm (approximately 9 log CFU ml^{-1}). Phage stock solutions were prepared according to Heringa et al. (2010). Prior to each experiment, bacteriophage stocks were incubated at 37°C for 30 min and then diluted to the

desired concentrations using SM buffer [100 mmol l^{-1} NaCl, 8 mmol l^{-1} $MgSO_4 \bullet 7H_2O$, 50 mmol l^{-1} Tris-HCl (pH 7.5)]. Phage titer was determined by the double agar layer plaque assay according to Heringa et al. (2010). Based on our preliminary study, nine strains of SPB-specific bacteriophages (211a, 213a, 214a, 214b, 214c, 217a, 218a, 201a and 12a) were selected and mixed as the bacteriophage cocktail (Gong, 2010), which was used at a multiplicity of infection (MOI) of 1, 10 and 100 for lab study and 1,000 for greenhouse study.

Preparation of hydrogen sulfide detection strips

Hydrogen sulfide production was quantitatively detected by a test strip impregnated with lead acetate (PbAc, ACROS, Fair Lawn, NJ, USA, stock of 0.4 g ml^{-1}). A No. 2 sterile filter paper (Whatman, Piscataway, NJ, USA) was cut aseptically into 10 x 90 mm strips, impregnated with PbAc solution at desired concentration and dried inside a biological safety hood (Labconco, Kansas city, MO, USA).

Lead acetate titration for determining the hydrogen sulfide amount

The titration curve obtained in this test was used to convert the blackened length of the test strip to the amount of H_2S absorbed. First, stock solutions of 5 g l^{-1} iodine (I_2, Fisher, Rochester, NY, USA) and 0.1 mol l^{-1} sodium thiosulfate ($Na_2S_2O_3$, Fisher, Rochester, NY, USA) were prepared and diluted to the required concentrations for titrating the test strips impregnated with PbAc. The blackened test strip was reacted with a mixture of I_2 and 5 mol l^{-1} hydrochloric acid (HCl, Fisher, Rochester, NY, USA) until the black color disappeared, indicating that all of the lead sulfide (PbS) had been oxidized. Residual I_2 was titrated by $Na_2S_2O_3$ solution with 0.5% (w/v) starch solution (Difco, Detroit, MI, USA) as an indicator of titration end-point. Control samples of test strips without PbAc were also titrated in the same way. The volumes of I_2 and $Na_2S_2O_3$ solutions were recorded to calculate the amount of H_2S absorbed by each test strip. The

titration curve was constructed with blackened length (mm) of test strip as the x-axis and amount of H_2S (μg) as the y-axis. Correlation factors were determined by regression analysis in Excel (Microsoft, Redmond, WA, USA).

Preparation of raw animal materials

Raw chicken meat near the expiration date was acquired from a local grocery store. Raw chicken offal and feathers were collected from Valley Protein rendering facility (Ward, SC, USA) and Amick poultry processing plant (Batesburg-Leesville, SC, USA). Collected raw materials were stored at -20°C, and thawed overnight at 4°C before use. Raw chicken meat and offal were aseptically homogenized (Blender Model: 51BL30, Waring, Torrington, CT, USA) with 0.85% saline in a ratio of 1:5 (w/v), and chicken feathers were cut aseptically into small pieces.

Relationship between H_2S production and SPB population

Ground chicken offal (1g) was added to each test tube (13 x 100 mm) in which a H_2S detection strip was hung to the screw cap top. Sixteen tubes were incubated at 30°C. The lengths of blackened strips were recorded at selected sampling intervals and converted to the amount of H_2S production based on the titration curve of PbAc at the desired concentration. At the same sampling intervals, the SPB population was enumerated by plating on tryptic soy agar-hydrogen sulfide (TSA-H_2S) medium. For each experiment, two trials were conducted at separate time.

Phage challenge study with different MOIs

A 100 μl mixture of five predominant SPB strains were added into 800 μl of blended chicken meat to a final concentration of 10^4 CFU ml^{-1} that was mixed thoroughly using a vortex mixer (VWR, West Chester, PA, USA). Twenty-four tubes were divided into three groups which were incubated at 20, 30, or 37°C. To each tube, 100 μl of either bacteriophage cocktail with

MOI of 1, 10, and 100 or SM buffer washed from TSA plate was added and mixed with SPB inoculated raw meat. A hydrogen sulfide detection strip was used to measure H_2S production as described above.

Modified Mason jar preparation

To study the phage treatment of raw chicken offal and feathers under greenhouse conditions, modified Mason jars (Mason jar; Ball, Daleville, IN, USA) were constructed to accommodate large sample volume. Briefly, a trimmed 25 ml pipette was fixed onto a 1 L Mason jar and the gas was sealed with Silicon glue (GE, Huntersville, NC, USA). A test tube cap was put on the top of the pipette and sealed with Parafilm® (Pechiney, Chicago, IL, USA) to allow sampling of H_2S at predetermined intervals.

Green house study

To simulate conditions during transportation of raw animal materials, a greenhouse study was performed from April to October 2010. In a greenhouse facility, the maximum temperature was set to 30.5°C (ca. 3-4°C less than outdoor temperatures during the summer), which was also recorded by a remote monitoring system (Argus Control System Ltd., White Rock, British Columbia, Canada) as well as the humidity of the experimental unit. Daily light intensity inside the greenhouse was set to 700 W m^{-2} and monitored by LI-COR pyranometer (Argus Control System) during all experiments. Occasional shading, forced ventilation and evaporated cooling were used to meet the criteria for the greenhouse setting.

To each modified Mason jar, 450 ml blended chicken offal or 90 g cut-feathers was added, and mixed with 50, and 10 ml of phage cocktail for each treatment group, respectively. Equal volume of SM buffer washed from TSA plates was added to the control jars. Plate enumeration on H_2S-TSA plates was employed to determine the SPB population. In the trials of

ground chicken offal and feather, two different H_2S measuring methods, i.e. H_2S monitor (Gasbadge Plus, Industrial Scientific, Oakdale, PA, USA) and PbAc test strip, were used to determine the amount of H_2S production. All the samples in Mason jars were kept in the greenhouse for 12 h.

Statistical analysis

Bacterial count data were converted to $\log_{10}$ CFU ml^{-1} or g^{-1} for statistical analysis. An analysis of variance (ANOVA) for a completely randomized design was conducted to determine if general differences existed between treatment means using the general linear model (GLM) procedure. Specific comparisons among different phage treatments were accomplished with Tukey's test. All statistical analyses were performed using Statistical Analysis System 9.1 (SAS; SAS Institute, Cary, NC, USA).

RESULTS

Lead acetate (PbAc) test strip titration

By titrating the PbS on the test strip, we obtained titration curves and equations of three PbAc concentrations for relating the blackened length of the test strips to the amount of H_2S absorbed (data not shown). For the test strips impregnated with 0.05, 0.5 and 10% (w/v) PbAc, the equations were $y = 0.7111x + 0.2099$, $y = 0.6647x - 0.5576$ and $y = 12.391x - 6.7074$, respectively. The correlation factors for 0.05, 0.5, and 10% PbAc test strips were 0.9992, 0.9905 and 0.9988, respectively.

Relationship between H_2S production and SPB population

Initial SPB population of blended chicken offal sample was determined as ca. 2 log CFU g^{-1} (Figure 1). From 0 h to 2 h, growth of SPB was in a lag phase. From 2 to 8 h, SPB population

began to increase exponentially at the growth rate of 0.67 log CFU g^{-1} h^{-1}. However, there was no H_2S production detected by test strip during log phase. When the growth of SPB entered stationary phase at 10 h, rapid H_2S production was observed. From 10 h to 14 h, H_2S production increased rapidly at the rate of 3.7 μg h^{-1}, while the SPB population maintained at the same level.

Bacteriophage treatment of artificially inoculated SPB in raw chicken meat

Raw chicken meat, inoculated with five predominant SPB strains (10^4~10^6 CFU ml^{-1}), was treated with the bacteriophage cocktail at an MOI of 1, 10, and 100 at 20, 30 and 37°C (Table 1). H_2S production was detected by the test strip assay after 4 h of incubation at 37°C in control samples, and 5 h with phage-treated samples (Figure 2A). After 24 h of incubation, samples treated with phages at different MOI values (1, 10 and 100) produced ca. 65~80% of H_2S as in the control sample and MOI of 100 yielded the least amount of H_2S.

At 30°C, H_2S production was detected by the test strip after 7 h of incubation in controls and total H_2S production was decreased by 57% as compared to the control at 37°C (Figure 2B). With the bacteriophage treatments, H_2S production was delayed by 1 h. Phage treatment with MOI of 100 reduced H_2S amount up to 47% within 24 h as compared with the control sample at 30°C. Similar to phage treatment at 37°C, there was no significant difference ($p>0.05$) among phage treatments with MOIs of 1, 10, 100.

In considering the slow metabolic activity of SPB at lower incubation temperatures, 10^5 CFU ml^{-1} of predominant SPB were inoculated into chicken meat and incubated at 20°C for H_2S measurement. The detectable time for H_2S production in control sample was extended to 10 h at 20°C (Figure 2C), and the maximum H_2S production was decreased by 82 and 58% as compared at 37 and 30°C in control samples with SPB inoculation level at 10^4 CFU ml^{-1}. With the phage treatment, H_2S production was detectable after 11 h. As compared with control, phage treatment

at an MOI of 1, 10 and 100 reduced H_2S production by 55, 69 and 69%, respectively. Even though the initial population of SPB was increased to 10^6 CFU ml^{-1} close to the level in spoiled meat, there was a 55% reduction of H_2S production with phage treatment (Figure 2D). However, the phage treatment could not delay the time that H_2S was first detectable at 8 h of incubation.

To validate if the current phage cocktail was effective against naturally occurring SPB in meat products, the indigenous SPB in chicken meat were allowed to multiply at room temperature to 4~5 log CFU g^{-1} prior to phage treatment (Table 1). In the presence of 4 log CFU g^{-1} of indigenous SPB, the initial time for H_2S detection was after 5 and 8 h of incubation in both control and phage treatment samples at 37°C and 30°C, respectively (Figure 3A-B). However, within 14 h incubation, the total yield of H_2S production was reduced by 25 and 57% in the samples treated with phage cocktail with a MOI of 100 at 37 and 30°C, respectively. By analyzing the H_2S release curves, we found that the H_2S release rates were different between the samples at 37 and 30°C, and also between the samples with or without phage treatment ($p<0.05$). In the log phase of H_2S production, H_2S was produced at 0.8 μg h^{-1} in control samples, which was 2.4 times the average rate of samples treated with phages at 30°C. The H_2S release rate was increased to 4.1 μg h^{-1} in control sample at 37°C, which was 1.2 times of the average rate of 3.3 μg h^{-1} with phage treatment.

For the phage treatment of chicken offal with 4 log CFU g^{-1} of indigenous SPB at 30°C, H_2S production was delayed by 2 h as compared to the control sample (Figure 3C). At the MOI of 100, the phage treatment resulted in a 56% reduction of H_2S production up to 14 h incubation as compared with 38 and 53% in samples treated with MOI of 1 and 10, respectively. For the phage treatment of chicken feathers with 6 log CFU g^{-1} of indigenous SPB at 30°C, H_2S production of both control and treatment samples was detected after 5 h (Figure 3D). At the MOI

of 100, the phage treatment resulted in 62% reduction of H_2S production in 14 h incubation as compared with 40% and 58% in samples treated with MOI of 1 and 10, respectively.

In order to simulate the conditions for transporting raw animal materials to a rendering plant, a greenhouse study was performed using chicken offal and feathers as raw materials (Table 1). In the greenhouse study, environmental parameters including temperature, humidity and global light energy of each trial were recorded by a remote monitoring system every 15 min during the 12 h trial from 9 AM to 9 PM (data not shown). The average temperature in the trials of chicken offal and feathers were 29.0 and 31.0°C, respectively. The average humidity of 34.8 and 59.8% were recorded respectively in the trials of chicken offal and feathers. The average global light energy was 604.8 in the trials of chicken feathers, respectively, but not available in the chicken offal trial. Due to the large sample size required for the greenhouse study, H_2S test strips being used in laboratory experiments were inadequate to measure large amounts of H_2S. Therefore, two H_2S measurements, i.e., H_2S test strip with higher PbAc concentrations (0.5 and 10%) and a H_2S monitor, were evaluated.

For chicken offal with an initial SPB population of 6 log CFU g^{-1}, phage treatment with MOI of 1,000 in 12 h reduced H_2S production by 30 and 85% using the measurements of test strip and H_2S monitor, respectively (Figure 4A). There was a 0.4 log reduction of SPB with phage treatment. Apparently, the amount of H_2S production was measured much differently between test strip and H_2S monitor methods. For phage treatment of chicken feathers with an initial SPB population of 7 log CFU g^{-1}, phage treatment (MOI of 100) in 12 h reduced H_2S production by 54 and 30% using the measurements of test strip and H_2S monitor, respectively. There was a 0.2 log reduction of SPB population with phage treatment.

DISCUSSION

In this study, three methods including PbAc test strips, H_2S monitor and plate enumeration were used to measure either H_2S production or SPB population as affected by bacteriophage treatment. The amount of H_2S produced was dependent on SPB growth phase and population. The earliest time of H_2S detection by test strips and the H_2S monitor was negatively correlated with the increase in SPB population. The lowest SPB population required for H_2S detection was ca. 7.5 log CFU g^{-1} of raw meat (Figure 1). High correlation factors ($R^2 > 0.99$) obtained from lead acetate (PbAc) test strip titration indicated that the blackened length of the test strips detect correct amount of H_2S being absorbed. Therefore, the PbAc test strip method can be considered as a quick, reliable and cheap method for measuring H_2S production.

The H_2S monitor used in this study is a widely used piece of safety equipment in the rendering industry to monitor H_2S level and alert workers when H_2S concentrations increase to unsafe levels. The meter with a minimum detection limit as 0.1 ppm H_2S can provide accurate measurements of H_2S production in the range of 0.1~500 ppm of H_2S. However, in the greenhouse study, the results of H_2S reduction obtained from test strips and the H_2S monitor did not match to each other (Figure 4A-B). The possible explanation is that the test strip may be saturated with H_2S generated in chicken offal samples due to the higher H_2S production of 50~300 μg, whereas for the chicken feather samples, the H_2S monitor may not pick up the H_2S signal because of the low H_2S production of 0~20 μg. These results emphasize the H_2S detection method needs to be carefully chosen based on the level of H_2S released. Although the values of H_2S reduction obtained using test strip and H_2S monitor were not consistent in different trials due to their own limitation in sensitivity, the overall trend of H_2S reduction by phage treatment

has demonstrated the effectiveness of this biological control method, which was confirmed by plate enumeration of SPB population in each trial.

Based on the relationship between H_2S production and SPB population (Figure 1), it is clear that H_2S production started at the beginning of stationary phase of SPB growth. The possible explanation is that at the beginning of incubation, SPB population was as low as 4~5 log CFU g^{-1} and oxygen level was relatively high, therefore those facultative anaerobic SPB respire aerobically. When SPB grew to the stationary phase, SPB population was high (> 7.5 log CFU g^{-1}) and most of oxygen has been used up during log-phase growth of SPB. At this time, low oxygen level created an anaerobic environment for SPB to use sulfur as terminal electron acceptor and produce H_2S rapidly. This relationship between H_2S production and SPB population could also explain the effectiveness of phage treatment in raw animal materials. Phage treatment not only extended the lag phase of SPB growth but also decreased the growth rate in exponential phase. Thus the H_2S production started later and yielded less in phages-treated samples as compared with control samples.

Multiplicity of infection (MOI) is considered to be an important factor determining the effectiveness of phage treatment. Normally, phage treatment with higher MOIs has stronger lytic activities on bacterial cells than lower MOIs. In our study, all tests conducted under laboratory conditions revealed a dose dependent response on lytic activity of bacteriophages. In the trials with raw chicken meat, chicken offal or feather, phage treatment with a MOI of 100 achieved higher reduction of H_2S than samples treated with a MOI of 1 and 10. Many studies focusing on phage application used phage cocktails with MOIs of 1 to 1,000 and also observed a dose response effect of MOI (Leverentz et al., 2003; Sheng et al., 2006; Abuladze et al., 2008; Heringa et al., 2010). Several other studies used high MOIs of 10^4~10^6 to reduce pathogens in

broilers (Huff et al., 2003; Higgins et al., 2005; Wagenaar et al., 2005; Atterbury et al., 2007). However, in a study conducted by Callaway (2008), phage treatment with MOI of 1 was found to be more effective than higher MOI of 10 and 100 in the gastrointestinal tract of sheep. It may be explained by the fact of "lysis from without" (Sulakvelidze, 2005). When a bacterium is infected by multiple phages simultaneously, the bacterial cell would be lysed by cell wall degradation instead of phage replication. Thus phages are unable to maintain a self-sustaining chain reaction when host cells cannot multiply.

Based on results described in Table 1, the incubation temperature was found as a critical factor affecting the SPB growth and lytic activities of bacteriophage. SPB grew faster at 37°C than at 30 or 20°C resulting in reduced effectiveness of phage treatment. For example, in the raw chicken meat inoculated with SPB, phage treatment reduced H_2S production by 35, 47~57 and 71% at 37, 30 and 20°C, respectively. The growth rate of SPB was lower at 20°C as compared with 30 or 37°C, thus, phages have more time to contact and lyse the target bacterial cells. When more bacterial cells are propagated at 37 or 30°C, possible development of bacteriophage insensitive mutant (BIM) can diminish the inhibitory effect by phage treatment. BIM can be induced as long as the phage treatment is applied (Alisky et al., 1998), and sometimes the regrowth of BIM even reached a higher level as compared with control (Heringa et al., 2010). Studies found in most situations that a single phage is not sufficient to eliminate an entire bacterial population in broth, therefore, a phage cocktail is necessary and important to prevent adverse affect from development of BIM (O'Flynn et al., 2004; Mclaughlin et al., 2007).

As compared with phage treatment in artificially inoculated meat (Table 1, Figure 2), the lag phase extension of H_2S production was not observed in the samples with indigenous SPB except for chicken offal sample (Figure 3A-D). Although our results have demonstrated that five

selected SPB strains used in this study were strong H_2S producers in raw poultry products and able to represent the predominant SPB strains for production of H_2S, our phage cocktail may not be able to inhibit all species of indigenous SPB in spoiled meat and animal by products, especially, when a large initial population of indigenous SPB was present in these samples, and H_2S was produced immediately without lag phase extension at 30°C. Similar results in previous studies reported a variety of inhibitory effects ranging from none to 99% reduction (Greer and Dilts,1990; Goode et al., 2003; Higgins et al., 2005), which are close to the results in our study with artificially inoculated raw meat samples ranging 35-69% reduction of H_2S production or SPB population. It was also found that a higher MOI could reduce more *Salmonella* population from inoculated chicken carcass (Higgins et al., 2005). Moreover, the low inhibitory effect of phage treatment on naturally contaminated samples was attributed to the relatively small library of bacteriophages, which also emphasized the importance of developing a bacteriophage cocktail having a broad host range. In agreement with their study, we also observed that phage treatment could not eliminate all SPB cells, especially naturally contaminated SPB, which may not be within the host range of phage cocktail used in this study, thus there was still H_2S production by the end of those trials.

In conclusion, this study applied a bacteriophage cocktail to control the growth of SPB, consequently reducing H_2S production in raw animal materials destined for the rendering process. The effectiveness of phage treatment was positively correlated with the MOI. Due to a variety of indigenous SPB species existing in raw meat materials, the host range of phage is considered as a critical factor for phage treatment and our results demonstrated our phage cocktail was effective against the predominant indigenous SPB in tested raw poultry samples. Although the current bacteriophages cocktail cannot eliminate the production of H_2S by SPB

completely, it can delay and reduce the harmful gas production significantly to a relatively low level, especially in combination with low temperature storage.

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Table 1 Summary of phage treatments of SPB in poultry by-products.

Matrix	Temperature (°C)	SPB* (log CFU ml^{-1} or g^{-1})	MOI†	Minimum time (h) for H_2S detection‡		Reduction of H_2S** (%)	Reduction of SPB (log CFU ml^{-1})
				Control	Phage treatment		
Inoculated chicken meat	37	4	100	4	5	35±0.3	N/A††
	30	4	100	7	8	47±0.9	N/A
	20	5	100	9	10	69±3.0	N/A
	20	6	10	8	8	55±1.2	N/A
Naturally spoiled chicken meat	37	4	100	5	5	25±0.4	N/A
	30	4	100	8	8	57±5.0	N/A
Chicken offal	30	4	100	7	9	56±1.0	N/A
Chicken feather	30	6	100	5	5	62±1.9	N/A
Chicken offal	Greenhouse‡‡	6	1000	4	4	57±39	0.4
Chicken feather	Greenhouse	7	100	4	4	42±17	0.2

* Values are initial populations of SPB (log CFU ml^{-1} or g^{-1}) inoculated or naturally occurring in sample matrix.

† MOI, multiplicity of infection, used in phage treatment.

‡ Values (h) are earliest times of H_2S detection.

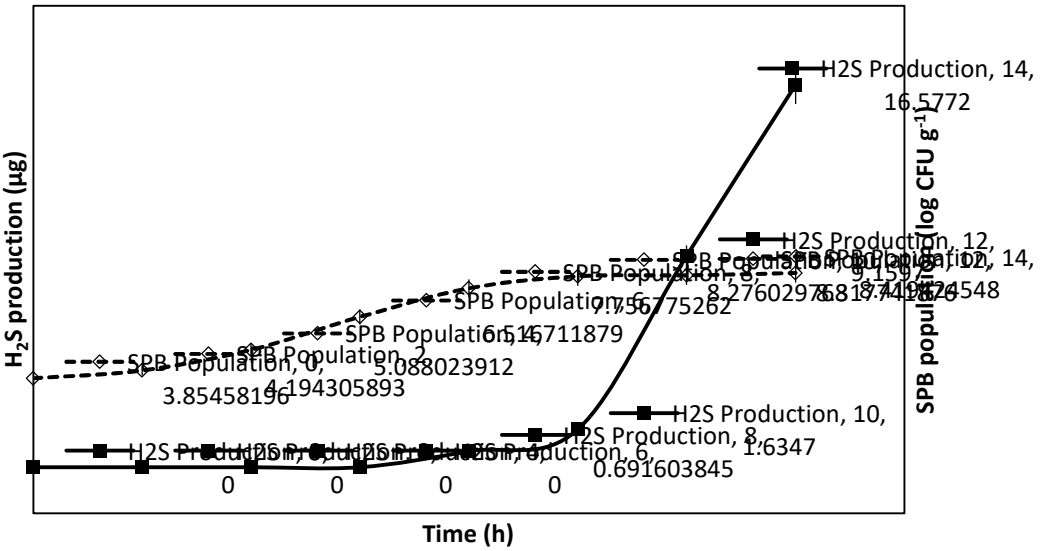
** Values are average reductions of H_2S production with phage treatment as compare with control sample, measured by test strip and H_2S monitor in greenhouse study.

Reduction% = (H_2S in control - H_2S with phage treatment)/ H_2S in control,

†† N/A is not available.

‡‡ The average temperature over 12 h period of experiment for chicken offal and feather were 29 and 31°C, respectively.

Figure 1:


H2S Production, 14, 16.5772
H2S Production, 12,
H2S Production, 10,
H2S Production, 8,
H2S Production, 6,
0.691603845
SPB Population, 0, 3.85458196
SPB Population, 2, 4.194305893
SPB Population, 4, 5.088023912
SPB Population, 6, 6.516711879
SPB Population, 8, 7.756775262
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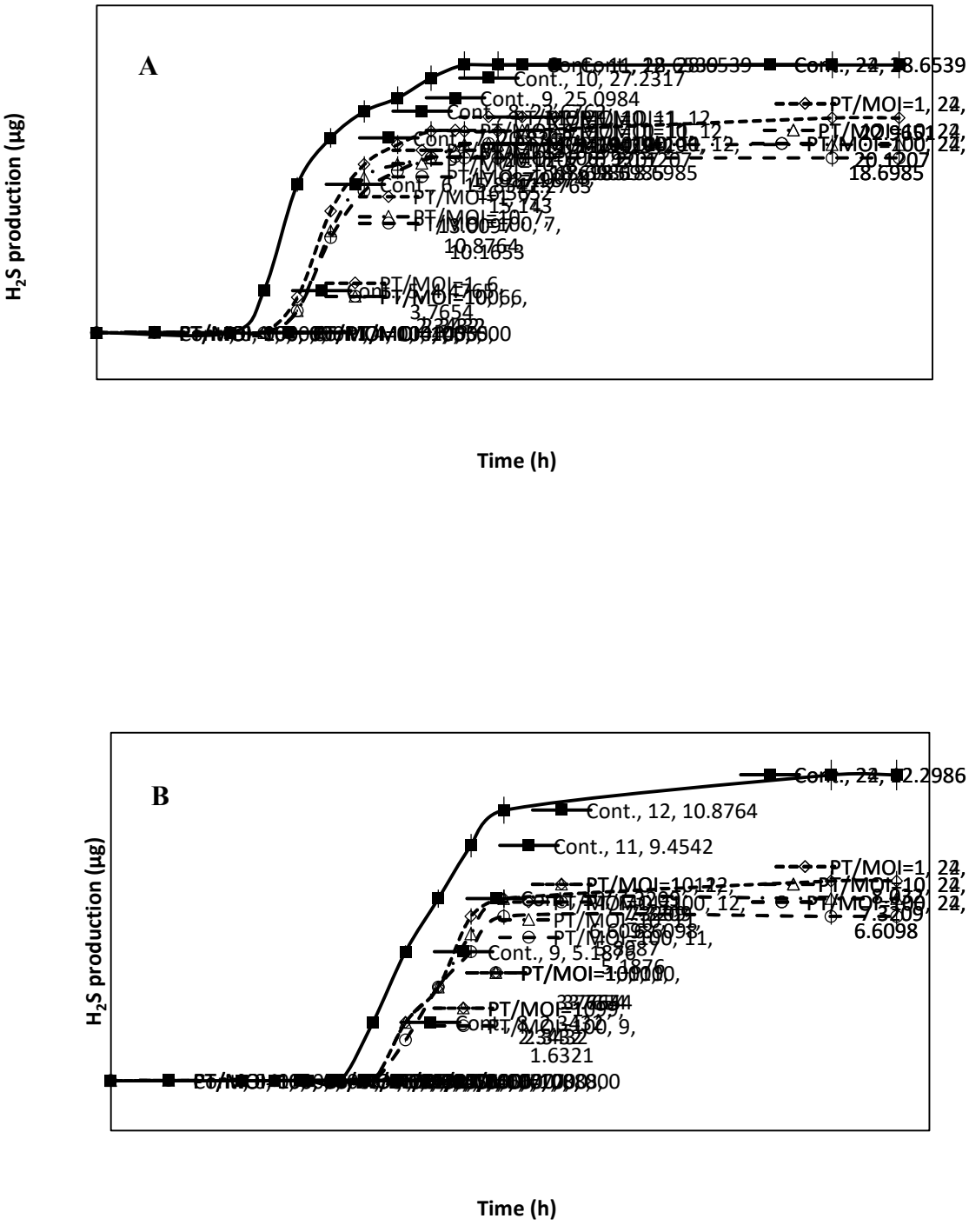
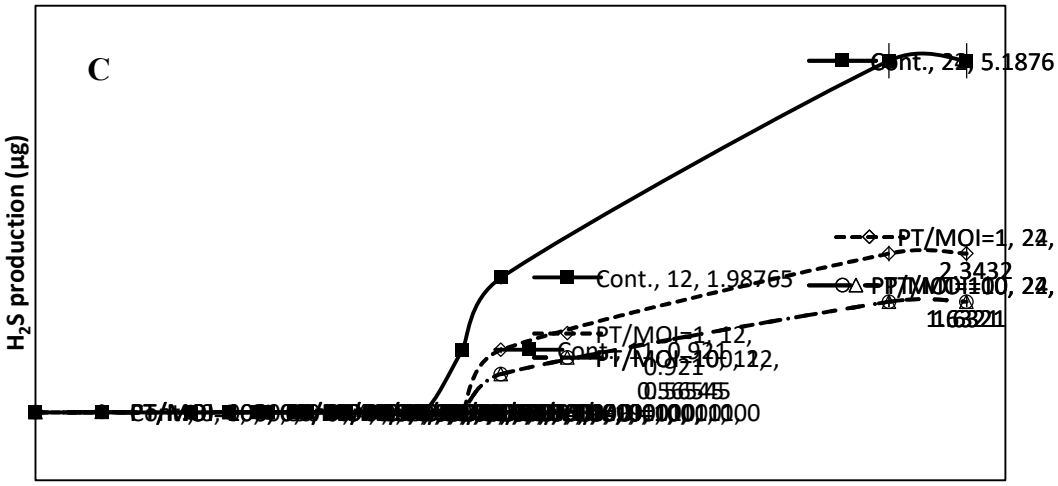
0
0
0
0
H_2S production (µg)
SPB population log CFU g^{-1}
Time (h)

Figure 2:


A
B
H_2S production (µg)
Time (h)
Cont., 10, 27.2317
Cont. 9, 25.0984
PT/MOI=1, 24, 18.6985
Cont., 12, 10.8764
Cont., 11, 9.4542
Cont., 9, 5.1876
PT/MOI=1, 24, 6.6098


C
H2S production (µg)
Cont., 24, 5.1876
Cont., 12, 1.98765
PT/MOI=1, 24,
2.3432
PT/MOI=1, 12,
Cont., 11, 0.921
PT/MOI=10, 12,
0.921
Time (h)

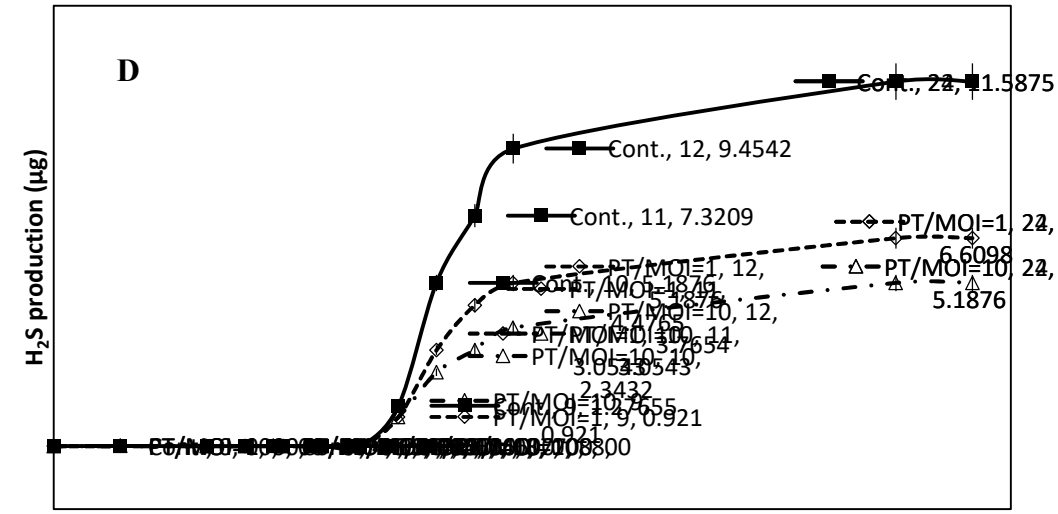
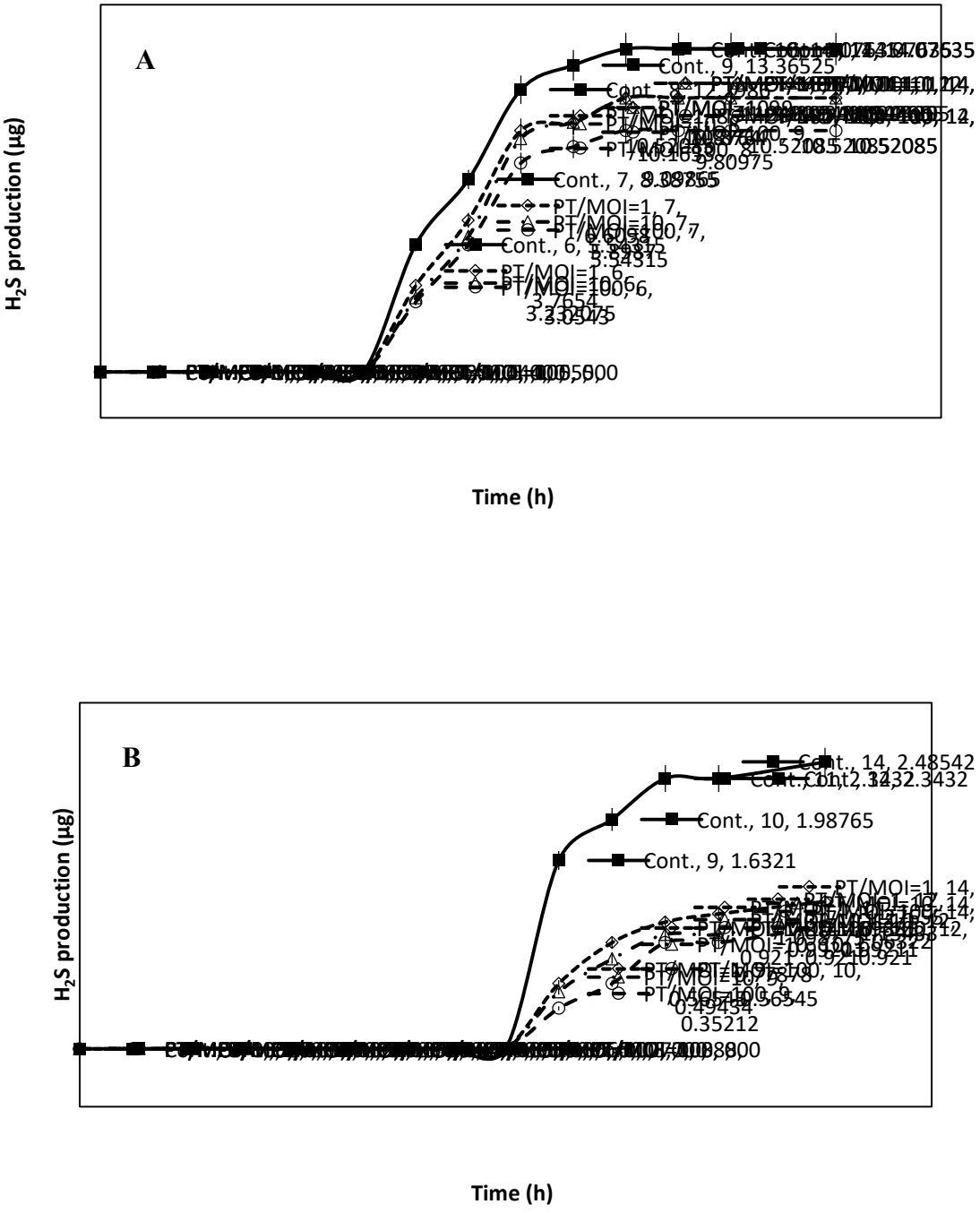
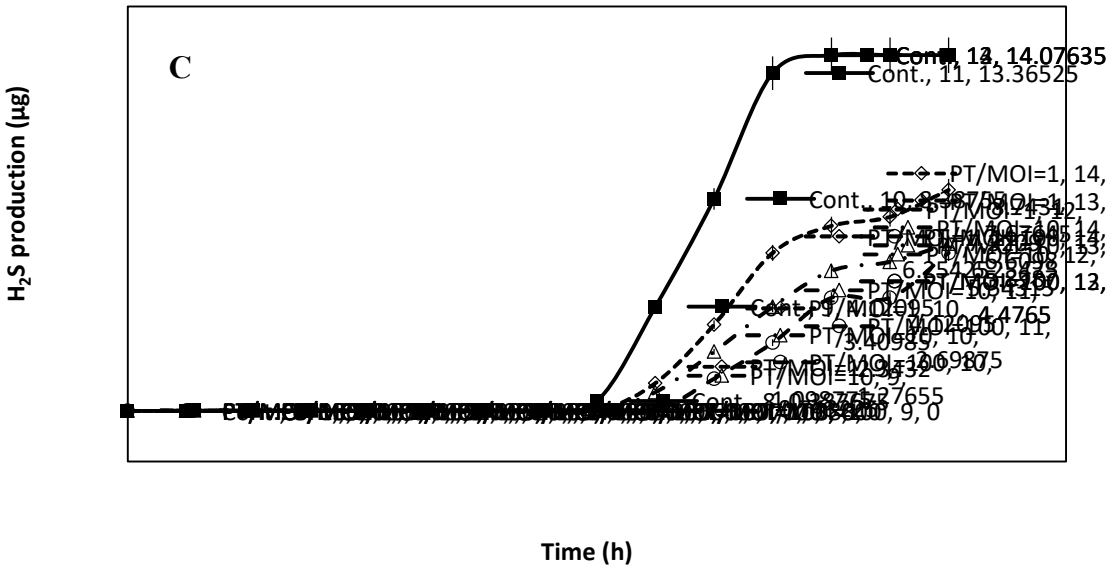
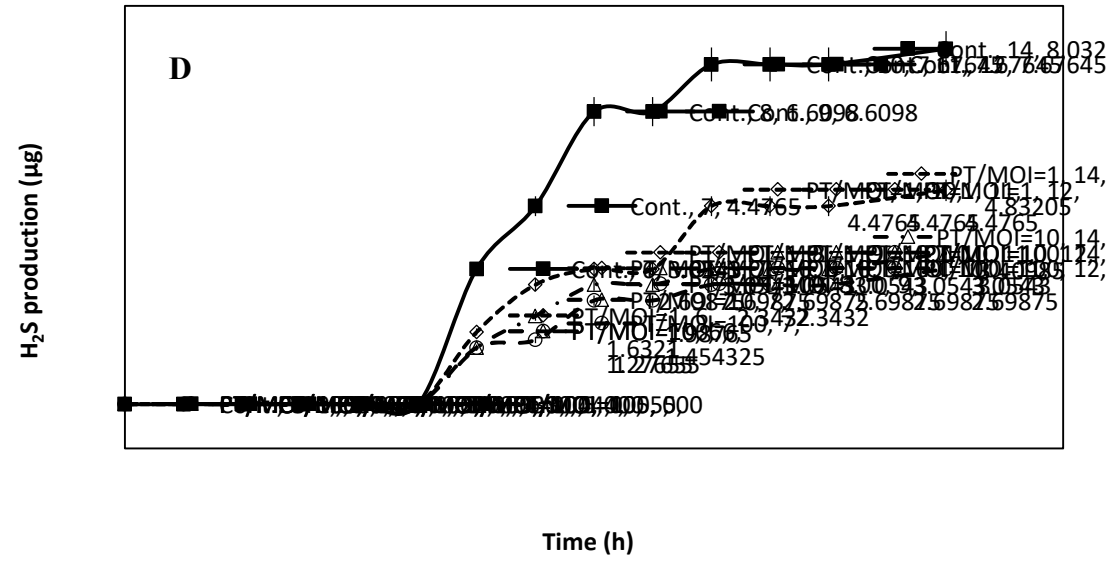

D
H2S production (µg)
Cont., 24, 11.5875
Cont., 12, 9.4542
Cont., 11, 7.3209
PT/MOI=1, 24,
6.6098
PT/MOI=10, 24,
5.1876
PT/MOI=1, 12,
PT/MOI=10, 12,
PT/MOI=10, 11,
2.3432
PT/MOI=1, 9, 0.921
0.921
Time (h)

Figure 3:


A
H_2S production (µg)
Time (h)
Cont., 9, 13.36525
Cont., 7, 8.38755
PT/MOI=1, 7,
PT/MOI=10, 7,
Cont., 6, 5.54315
PT/MOI=1, 6,
PT/MOI=10, 6,
B
H_2S production (µg)
Time (h)
Cont., 14, 2.48542
Cont., 10, 1.98765
Cont., 9, 1.6321
PT/MOI=1, 14,
PT/MOI=10, 10,
0.49434
0.35212


C
H2S production (μg)
Time (h)
Cont., 11, 13.36525
PT/MOI=1, 14,
PT/MOI=10, 11,
PT/MOI=10, 9,
Cont., 9, 0


D
H2S production (μg)
Time (h)
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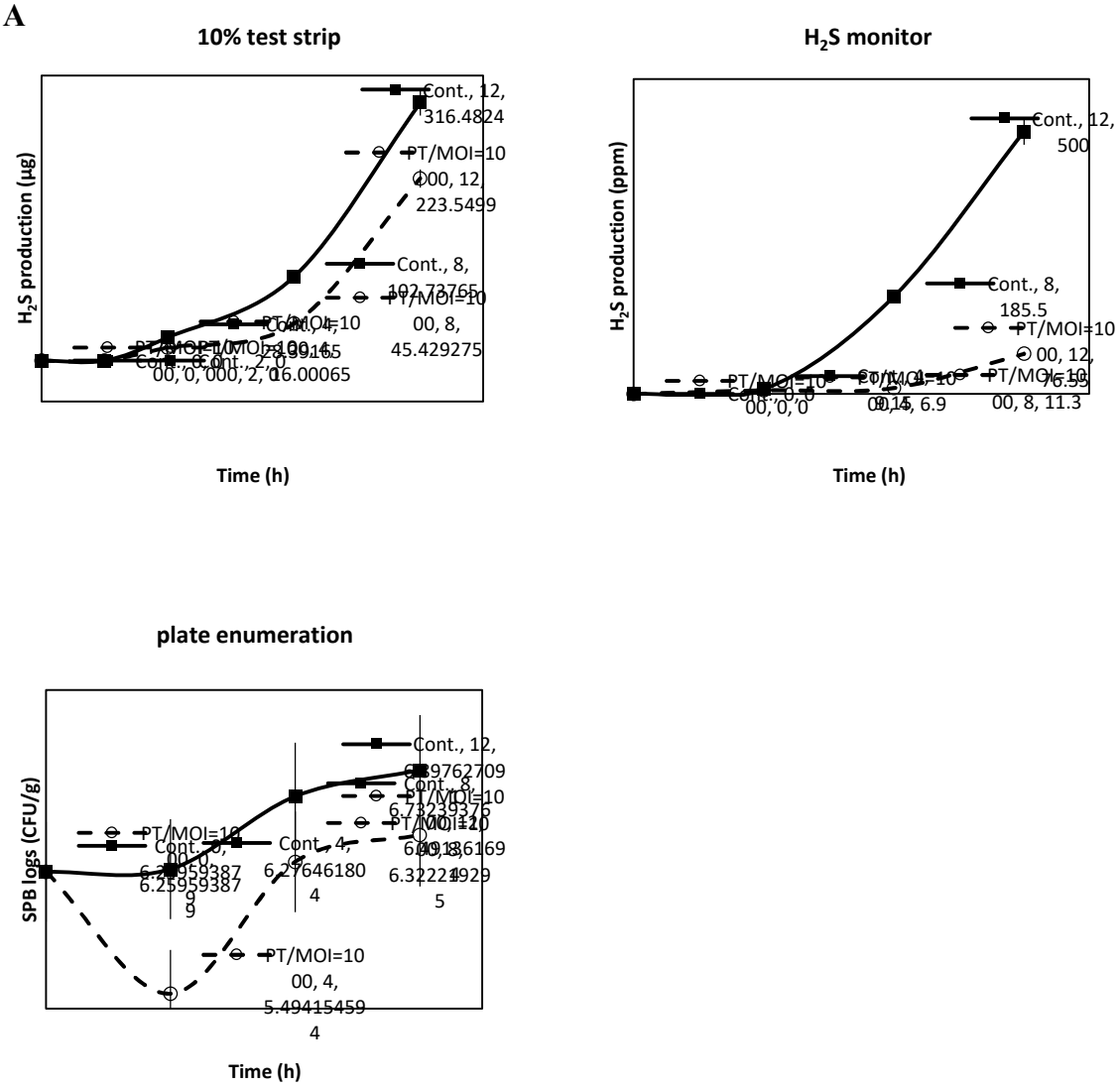
PT/MOI=1, 14,
Cont., 7, 4.4765
4.83205
PT/MOI=10, 14,

Figure 4:

A


10% test strip
H2S production (µg)
Time (h)
Cont., 12, 316.4824
PT/MOI=10 00, 12, 223.5499
Cont., 8, 102.7376
PT/MOI=10 00, 8, 45.429275
H2S monitor
H2S production (ppm)
Time (h)
Cont., 12, 500
Cont., 8, 185.5
PT/MOI=10 00, 12, 76.55
PT/MOI=10 00, 8, 11.3
plate enumeration
SPB logs (CFU/g)
Time (h)
Cont., 12,
Cont., 8,
PT/MOI=10 00, 4, 5.49415459 4

B

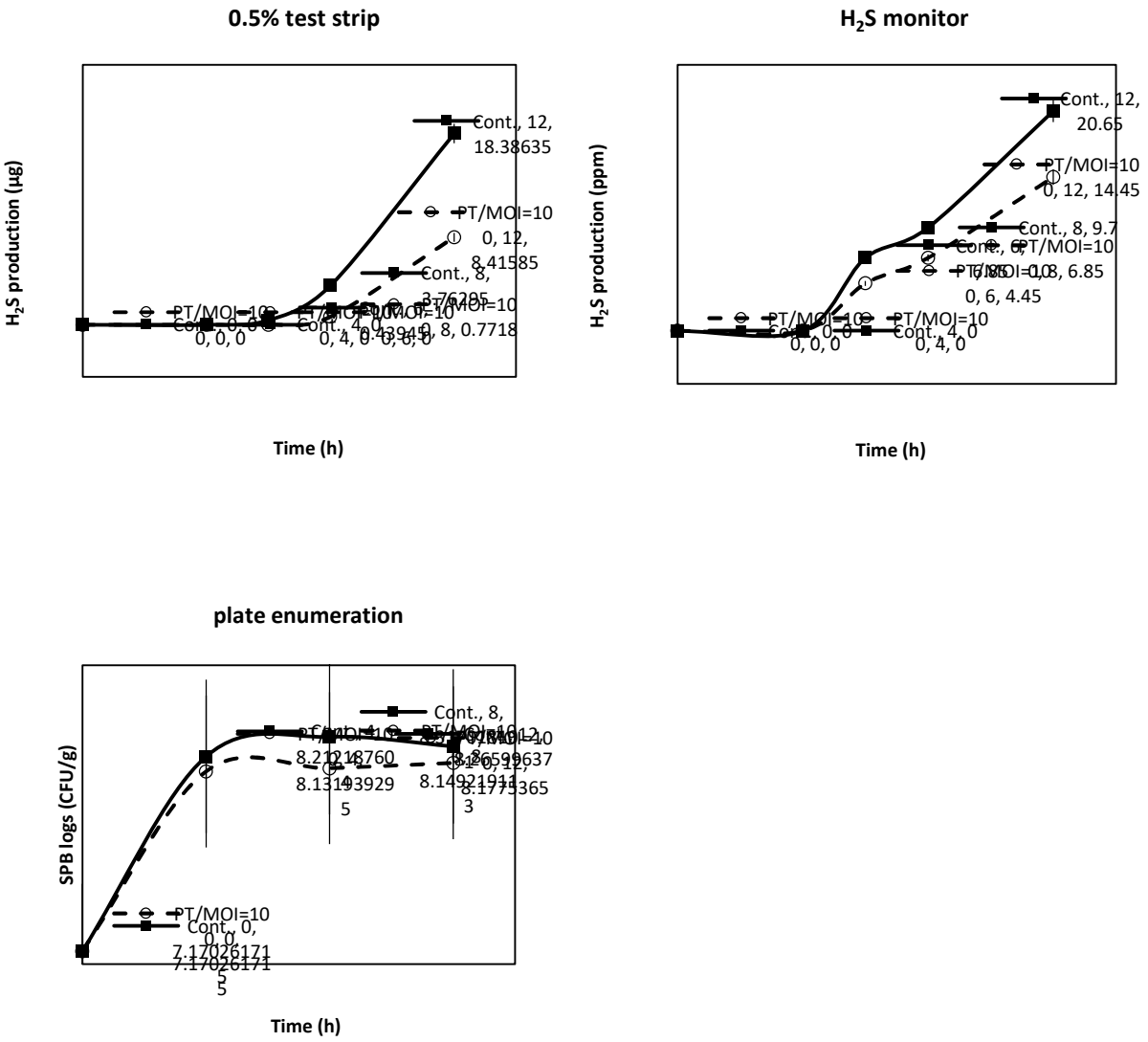

0.5% test strip
H2S production (μg)
Cont., 12, 18.38635
PT/MOI=10
0, 12, 8.41585
Cont., 8, 3.76285
PT/MOI=10
0, 8, 0.7718
PT/MOI=10
Cont., 0, 0
0, 0, 0
Cont., 4, 0
0, 4, 0
Time (h)
H2S monitor
H2S production (ppm)
Cont., 12, 20.65
PT/MOI=10
0, 12, 14.45
Cont., 8, 9.7
PT/MOI=10
0, 8, 6.85
PT/MOI=10
0, 0, 0
Cont., 4, 0
0, 4, 0
0, 6, 4.45
Time (h)
plate enumeration
SPB logs (CFU/g)
Cont., 8,
8.13193929
5
8.14921911
3
8.1775365
PT/MOI=10
Cont., 0,
0, 0
7.17026171
5
7.17026171
5
Time (h)

FIGURE LEGEND

Figure 1: Relationship between H_2S production and SPB population in raw animal materials. Each data point represented the average of two trials. Symbols "■" and "◊" represent H_2S production and SPB population, respectively.

Figure 2A-D: Application of bacteriophage cocktail to raw chicken meat inoculated with 4 log of SPB at 37°C (A) and 30°C (B), with 5 log of SPB at 20°C (C) and 6 log of SPB at 20°C (D). Each data point represented the average of two trials. Symbols "◊ ", "△", and "○ " represent phage treatment with MOI of 1, 10, and 100, respectively. Symbol "■" represents the control sample.

Figure 3A-D: Application of bacteriophages cocktail in raw chicken meat with 4 log of indigenous SPB at 37°C (A) and 30°C (B), in chicken offal with 4 log of indigenous SPB (C) and in chicken feather with 6 log of indigenous SPB (D) at 30°C. Each data point represented the average of two trials. Symbols "◊", "△", and "○ " represent phage treatment with MOI of 1, 10, and 100, respectively. Symbol " ■" represents the control sample.

Figure 4A-B: Greenhouse study of applying bacteriophage cocktail to chicken offal with MOI of 1,000 (A), chicken feather with MOI of 100 (B) using test strip, H_2S monitor and plate enumeration methods. Each data point represented the average of two trials. Symbols " ■ " and "○" represent control and phage treatment, respectively.