RESEARCH REPORT
ENVIRONMENT

Title: Effect of soybean peroxidase activity on phenolic compounds, ammonia, and other organic compounds in swine manure - **NPB #06-115**

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Industry Summary

A simulated manure system was used to evaluate the potential for soybean peroxidase (SoyP) added to swine manure to control phenolic and other odorous compounds in head space air and manure. The SoyP was added to the manure columns (350 mL of manure in 500 mL flasks) at increasing amounts of 7.5, 15, and 30 mg and calcium peroxide (CaP) levels increased from 0, 12.5, to 25 mmoles in a factorial approach of treatments plus 2 controls, a 0-0 SoyP-CaP and 0-25 SoyP-CaP treatments. Manure was collected from a 6 ft deep pit where the pigs had been placed in the facility for approximately 12 weeks. The base manure had no appreciable levels of soybean peroxidase (SoyP) as indicated by the near zero detection in the two control treatments that did not have additional SoyP added to these manure columns. As the SoyP was added to the manure columns at increasing amounts of 7.5, 15, and 30 mg the average analyzed levels of SoyP increased linearly. Adding CaP to the manure resulted in a 14.5% linear decline in the average level of SoyP activity. The average SoyP levels were at their highest levels at the 2 hr sampling and then declined to 48 hrs and remained at this level to 120 hr, maintaining 81% of the initial level of SoyP activity at 120 hr. Manure pH, ammonium N, manure ash content as a % of manure DM were not affect by SoyP levels, however manure DM declined slightly as SoyP increased (7.98, 8.00, 7.87%, respectively). As CaP levels increased, manure pH (6.54, 7.00, and 7.25, respectively), manure DM (7.42, 8.03, 8.40%, respectively) linearly increased and manure ammonium N linearly decreased (3492, 3310, 3293 ppm, respectively). The increasing levels of SoyP reduced manure phenol and manure total phenolic compounds by 27-28% on its own compared to the 0-0 control. The addition of SoyP also had linear numerical reductions in head space air phenol (13.2, 9.0, 6.0 ppm) and total phenolics (16.3, 11.2, 8.0 ppm). As CaP increased, all manure phenolic compounds linearly declined. Total manure phenolic compounds decreased by 30 and 59% as CaP increased to 12.5 and 25 mmoles. Increasing levels of CaP also linearly decreased head space air phenol and total phenolics by 10 and 41% as CaP increased to 12.5 and 25 mmoles. Neither SoyP or CaP had any effect on manure total VFA's however, individual manure volatile fatty acids (VFA) were impacted by the inclusion of SoyP and CaP. Manure acetic acid increased slightly while butyric and isovaleric decreased slightly with increasing SoyP enzyme levels. While increasing levels of CaP increased

manure acetic acid and decreased butyric acid. Increasing levels of CaP also decreased head space air total VFA's by 60%. Careful evaluation of the data clearly shows that it is the combination of SoyP enzyme and CaP that clearly reduces the phenolic compounds. This is best displayed when you compare the 25 mmole level of CaP with no SoyP enzyme having a manure total phenolics level of 15.6 ppm and when any level of the SoyP is added to the 25 mmole level of CaP the total phenolics in the manure are reduced on average by 50% to 7.9 ppm. Also the greatest reduction (63%) in phenols and total phenolics (from 13.1 to 4.8 ppm) in manure column head space air occurred at the highest inclusion rate of SoyP (30 mg) in combination with the highest CaP (25 mmoles). This research proves that the soybean peroxidase enzyme can survive in swine manure up to 5 days. The use of soybean peroxidase in combination with a catalyst (calcium peroxide) may be a viable option to reduce both manure content and air emissions of phenolic compounds, but by its self soybean peroxidase only can reduce phenolic compounds by 27-28% compared to up to 63% with a catalyst. The increased losses of ammonium N with the increased manure pH as calcium peroxide was added to the manure is a potential negative result of this research. This research indicates there may be potential to evaluate either a spray application system that may be applied weekly or potentially even less frequently to help control phenolic compounds from swine manure storage systems. Additionally, potential research evaluating the feeding of soybean hulls (the source of the soybean peroxidase) high in the enzyme through the pig may have similar results if the enzyme can remain intact during the digestion process or sequester phenolic compounds in the gut of the pig. There appear to be many potential future research and applications to this technology that may help the swine industry meet its goal of being a good, responsible neighbor in their rural community.

Scientific Abstract

An in vitro manure system was used to evaluate the efficacy of soy peroxidase (SoyP) in swine manure for the control of phenolic and other odorous compounds in head space air and manure. The SoyP was added to the manure columns at increasing amounts of 7.5, 15, and 30 mg (200 u/mg) and calcium peroxide (CaP) levels increased from 0, 12.5, to 25 mmoles in a factorial approach of treatments plus 2 controls, a 0-0 SoyP-CaP and 0-25 SoyP-CaP treatments. Manure was collected from a 6 ft deep pit where the pigs had been placed in the facility for 8 -12 weeks during this experiment. The manure was thoroughly mixed and subsampled in 350 mL aliquots into 500 mL flasks that served as bench top manure containers. Rubber stoppers with a hole in the top were placed on the flasks and covered with parafilm for 2 hr prior to sampling of headspace air samples. A solid-phase micro extraction fiber (SPME; Supelco, Inc. Bellefonte, PA) was inserted through the parafilm and hole in each rubber stopper on each flask and the fiber exposed for 1 hr to absorb volatile organic compounds in the headspace of each flask. After the SPME fibers were removed, the flasks were mixed and a 50 mL manure sample was obtained and frozen for later analysis of ammonium nitrogen, short chain volatile fatty acids, pH, dry matter, and peroxidase enzyme activity analysis. Sampling of the manure flasks and air head space was at 0 (initial manure slurry); 2, 24, 48, and 120 hr. In between sampling times the manure flasks were left open to naturally vent inside a laboratory hood. The soybean peroxidase activity was determined using a monoclonal-antibody assay specific for soybean peroxidase. The air head space GC fibers and manure samples were analyzed on a GC for phenolic and VFA compounds. All data were analyzed using the GLM procedures of SAS. The base manure had no appreciable levels of SoyP as indicated by the near zero detection in the two control treatments that did not have additional SoyP added to these manure columns. As the SoyP was added to the manure columns at increasing amounts the average analyzed levels of SoyP increased linearly ($P<0.001$; 0.49, 0.68, and 0.78 purpurogallin units/µL, respectively). Adding CaP to the manure resulted in a linear decline ($P<0.01$; 0.69, 0.66, 0.59 purpurogallin units/µL, respectively) in the average level of SoyP analyzed levels of activity. The average SoyP levels were at their highest levels at the 2 hr sampling and then declined to 48 hrs and

remained at this level to 120 hr (Time P<0.01; 0.74, 0.65, 0.60, 0.60 purpurogallin units/µL, respectively). Manure pH, ammonium N, manure ash content as a % of manure DM were not affect by SoyP levels (P>0.31), however manure DM declined slightly as SoyP increased (P<0.02; 7.98, 8.00, 7.87%). As CaP levels increased, manure pH (P<0.001; 6.54, 7.00, and 7.25, respectively), manure DM (7.42, 8.03, 8.40%) and ash as a % of DM (16.7, 18.2, 20.0%) linearly increased (P<0.001) and manure ammonium N linearly decreased (P<0.001; 3492, 3310, 3293 ppm). Increasing levels of SoyP achieved only non-significant changes in most manure phenolic compounds on its own. Manure phenol declined slightly (14.7, 11.3, 13.2, respectively) compared to the 0-0 control at 18.1 ppm with increasing SoyP. The manure compounds indole and 4-ethyl phenol combined declined (P<0.04; 1.35, 1.05, 0.61 ppm, respectively) along with total phenolic compounds (16.0, 12.4, 13.8 ppm) with increasing SoyP. The addition of SoyP had linear numerical reductions in head space air phenol (13.2, 9.0, 6.0 ppm, respectively; P<0.16), total phenolics (16.3, 11.2, 8.0 ppm respectively; P<0.14) as SoyP levels increased in the manure columns. By contrast, as CaP increased, all manure phenolic compounds linearly declined. Phenol (P<0.002), 3 and 4 methyl phenol (P<0.001), indole and 4-ethyl phenol (P<0.06), and total phenolic compounds (20.7, 13.6, 7.9, respectively; P<0.001) were all reduced with increasing CaP. Increasing levels of CaP also linearly decreased head space air phenol (14.5, 8.1, 5.7 ppm, respectively; P<0.09), total phenolics (16.0, 11.8, 7.7 ppm, respectively; P<0.13); while decreasing and then slightly increasing in a quadratic fashion indole and 4-ethyl phenol (Quad., P<0.05). Manure acetic acid (P<0.15) increased slightly while butyric (P<0.14) and isovaleric (P<0.09) decreased slightly with increasing SoyP enzyme levels. While increasing levels of CaP increased manure acetic acid (P<0.001), decreased butyric (P<0.001), and increased and then decreased isobutyric (Quad., P<0.04) and isovaleric acids (Quad., P<0.005), However, neither Soy P nor CaP had any effect on manure total VFA's. The addition of SoyP had a quadratic response in head space air Total VFA's (926, 621, 913 mmoles; P<0.08) as SoyP levels increased in the manure columns. Increasing levels of CaP linearly decreased head space air combined acetic and valeric acids (461, 67, 74 mmoles, respectively; P<0.001) and total VFA's (1365, 528, 566 mmoles/g, respectively; P<0.001) while decreasing and then slightly increasing in a quadratic fashion isovaleric acid (110, 43, 79 mmoles respectively; P<0.03). Careful evaluation of the data clearly shows that it is the combination of SoyP enzyme and CaP that clearly reduces the phenolic compounds. Comparing the 25 mmole level of CaP with no SoyP enzyme, having a manure total phenolics level of 15.6 ppm, and when any level of the SoyP is added to the 25 mmole level of CaP the total phenolics in the manure are reduced on average by 50% to 7.9 ppm (P<0.10). Also the greatest reduction in phenol and total phenolics (from 13.1 to 4.8 ppm) in manure column head space air occurred at the highest inclusion rate of SoyP (30 mg) in combination with CaP (25 mmoles). The use of soybean peroxidase in combination with a catalyst (calcium peroxide) may be a viable option to reduce both manure content and air emissions of phenolic compounds, however the increased losses of ammonium N with the increased manure pH as CaP was added to the manure is a potential negative result of this research. Further research is needed for application of this technology to be viable for the swine industry to use to control phenolic compounds in swine manure storage systems.

Introduction

Manure produced from livestock operations creates odorous compounds and gaseous emissions into the atmosphere. These emissions have resulted in increasing neighbor complaints often resulting in law suits and blocking expansion or development of new livestock operations. New technologies are needed to mitigate the odor and gas production from manure storages. Predominant odor compounds are phenols, volatile amines, volatile fatty acids, sulfurous compounds and ammonia.

Soybean peroxidase, an environmentally friendly substitute, for several manufacturing and processing technologies is the first truly value added trait from the soybean. Currently soybean

peroxidase is a commercial product in several markets. Soybean peroxidase is a superior substitute for horseradish peroxidase in medical diagnostics and is being used by diagnostic companies in both the U.S. and Japan. Peroxidase also is an environmentally friendly substitute for formaldehyde-chlorine chemistry in resin production and is currently being used in both the U.S. and Ireland. The Israeli baking industry is adding peroxidase to breads, which allows for higher quality bread that is still kosher.

There are currently two sources of peroxidase available to use in a variety of applications, soybean peroxidase and horseradish peroxidase. Early research in the 1980's and early 1990's used the horseradish peroxidase in human wastewater treatment research to reduce phenolic compounds. However, this enzyme is more expensive than the soybean peroxidase and is not as environmentally stable. Soybean peroxidase has a working pH range of 3 to 9 with the optimum pH being approximately 6.4 and the optimal pH range of 6-9 (Wright and Nicell, 1999). Kamal and Behere (2003) reported that the optimal pH for maximal catalytic activity for soy peroxidase is 5.5 and they also reported that the specific activity of the soybean peroxidase was twice that of the horseradish peroxidase at pH 6.8 and 4 times greater at pH 5.0, indicating that the soybean peroxidase is a more robust and efficient enzyme than horseradish peroxidase. This pH range is very close to most swine manure pH's and the total range is within the range of the swine gastro-intestinal tract.

The optimal level of soybean hulls to provide the peroxidase enzyme and hydrogen peroxide as an oxygen group donor for the reaction under experimental conditions was determined by Flock et al. (1999). They reported that the optimal hydrogen peroxide level was 10 mmol/1000 mL and that approximately a 4% inclusion of soybean hulls would provide the optimal amount of soybean peroxidase. This combination provided for a conversion of over 60% the phenol compounds after 60 minutes. However, this enzyme reaction can be greatly inhibited by natural organic matter. Lindsay and Tarr (2000) reported that adding 3% of humic acid to the water to simulate organic matter in a field condition, reduced the conversion rate of phenols from nearly 90% to less than 10% in a 40 minute incubation. They further indicated that solid natural organic matter may even further reduce the efficiency of the enzyme to sequester phenolic compounds. This gives rise to the question of how effective the peroxidase enzyme will be in swine manure with its high organic matter.

One study was recently reported (Govere et al., 2005) that looked at adding horseradish root as a source of peroxidase to swine manure to minimize odors and phenolic compounds. They found that the olfactometry panel reported a 50% reduction in odor intensity. Their GC results also reported that there was nearly 100% removal of all phenolic odorant compounds after 72 hr when the combination of horseradish root and calcium peroxide were used. However, when hydrogen peroxide was used with the horseradish root, the phenolic compound reductions were not as great and were less consistent.

The activity of peroxidase in waste systems to control phenolic compounds from being released and potentially reducing ammonia emissions has promise. Specific strains of soybeans with enhanced peroxidase activity have been developed. Therefore, if specific soybeans or more likely soybean hulls (majority of soy peroxidase activity is in the hull) can be fed to pigs with the benefit of reducing the evolution of odorous compounds in manure, this technology could readily be adopted and significantly minimize the threat of neighbor disturbances for pork production in Indiana and the nation. Subsequently, use of this technology could provide the opportunity for growth of the pork industry.

This research took the first step needed to determine the efficacy of using soy peroxidase in manure systems for controlling phenolic, VFA, and possibly ammonia emissions from anaerobically-stored manures.

Objectives of Research Project:

1) To determine the effect of different rates of soybean peroxidase enzyme activity and calcium peroxide on phenolic compounds and other volatile organic compounds (VOC's) in pig manure.

2) To determine the effects of soybean peroxidase enzyme activity and calcium peroxide levels have on manure volatile fatty acids, pH, ammonium N, and DM.

Materials and Methods

An in vitro manure system was used to evaluate the efficacy of soy peroxidase in swine manure for the control of phenolic and other odorous compounds in head space air and manure. Manure was collected by a vacuum core sampling system from a 6 ft deep pit where the pigs had been placed in the facility for 8 -12 weeks during the weekly manure samplings for this experiment and were fed a typical corn-soybean meal based diet with 0.10% synthetic lysine. Fresh manure was collected at the start of each replicate from the same barn/manure pit over the experimental period with the same set of pigs in the facility for the duration of the trial. Manure was transported from the Purdue Animal Sciences Research and Education Center to the campus laboratory for conducting the weekly replicates of the manure in vitro system.

The manure was thoroughly mixed in a 5 gal. bucket and then subsampled in 350 mL aliquots into 500 mL flasks that served as bench top manure containers. Rubber stoppers with a hole in the top were placed on the flasks and covered with parafilm for two hours prior to sampling of headspace air samples. A solid-phase micro extraction fiber (SPME; Supelco, Inc. Bellefonte, PA) was inserted through the parafilm and into the hole in each rubber stopper on each flask and the fiber exposed for 1 hr to absorb volatile organic compounds in the headspace of each flask. After the SPME fibers were removed, the flasks were mixed and a 50 mL manure sample was obtained and frozen for later analysis of ammonium nitrogen (Chaney-Marbach method), short chain volatile fatty acids, pH, dry matter (AOAC, 2003), and peroxidase enzyme activity analysis. Prior to freezing the manure samples, 5 mL was transferred to a 10 mL conical tube for centrifugation. Following centrifugation, the supernatant was transferred to a 2 mL vial and frozen to be used for the soybean peroxidase activity assay at a later date. Sampling of the manure flasks and air head space was at the following times: 0 (initiation of the experimental replicate, in triplicate from the mixed initial manure slurry); 2, 24, 48, and 120 hr after the initiation of the experiment from each flask. In between sampling times the manure flasks were left open to naturally vent inside a laboratory hood.

Fibers were frozen after sampling until they could be analyzed on the GC. Fibers were analyzed using an HP 5890 GC with manual injection using an HP-1 column (19091Z-413: 30 m, I.D. 0.32, Film 0.25) with an FID detector. Fibers were thawed for 1 hr prior to inserting the fiber directly into the injector of the GC. Fibers remained in the injector through the entire 29 min. cycle of temperature ramp up to fully desorbed the fiber of compounds and prepare the fiber for reuse. Volatile fatty acid concentrations of manure samples were determined using a Varian 3900 GC with a Varian Carbowax 20M 80/120 Carbopack B DA column following the extraction procedures outlined by Playne (1985). Manure dry matter and ash were determined after drying a sample overnight (12 + hr) at 100° C and then ashing overnight (12 + hr) at 600° C in a muffle furnace. Manure pH was measured with a calibrated glass electrode pH meter (WTW pH 340). Total N (Nelson and Sommers, 1972) and NH_4 (Bremner and Keeney, 1965) were determined by micro-Kjeldahl procedures. The soybean peroxidase was provided by Dr. Rick Vierling and all peroxidase assays were conducted at Purdue University in Dr. Rick Vierling's lab using a monoclonal-antibody assay specific for soybean peroxidase (Vierling et al., 2000). All manure samples were analyzed in duplicate. The air head

space GC fibers were analyzed as individuals due to time limitations for exposure and run times on the GC. Manure samples with a CV greater than 10% between duplicates were reassayed in duplicate again.

The standards used in the analysis of the phenolic compounds with the fibers and manure are in Table 1 and were based on standards used for swine manure by Gralapp et al. (2001). Prior to initiation of the experiment, each individual compound in the mix was assayed at 5, 10, 25, 50, and 100% of the concentrations in Table 1 to determine the linear curve of concentrations that could be detected by our GC using the HP-1 column. Then every 10 samples the standard mix was exposed to a fiber at the 20% level to serve as a confirmation the fibers, column, and GC were still operating within normal ranges. To determine the phenolic compound levels from the manure samples the procedure of Le et al. (2007) was followed with a slight modification. Briefly, 2.5 g of manure were extracted with 10 mL of methanol for 2 hr. The sample was then centrifuged and the supernatant was transfer to a 2.5 mL vial and frozen until analysis on the GC. The same standards in Table 1 and GC column and conditions used for the fibers were used for the manure phenolics, just directly injected into the column.

All data in the research study were analyzed using the GLM procedures of SAS (SAS Inst. Inc., Cary, NC). The data were analyzed as randomized complete block with the effects on level of soy peroxidase, calcium peroxide, and their interaction along with incubation time were evaluated to determine effects of combinations of treatments using single degree of freedom contrasts. Additional contrasts were used to test for linear and quadratic effects of soybean peroxidase and calcium peroxide. Means were also separated using the Duncan's multiple range test to aide in the evaluation of the data.

The initially proposed treatments for this study are listed below. When we conducted the first replicate problems were immediately identified with these levels that were initially proposed. As is presented in the picture in Figure 1 there was excessive foaming at nearly all levels of calcium peroxide. The following day new levels were attempted that were 25, 50, and 100 mmoles calcium peroxide. These levels were also problematic. The 100 level still foamed too much and contaminated the SPME fibers. The 50 mmole level had a plastic like distinfected smell but the foaming was not as significant. These conditions led us to believe the levels were too high and were simply killing the manure column. Therefore new lower levels of both the calcium peroxide and soybean peroxidase were used for the remaining 3 replicates of this experiment.

Initial levels of calcium peroxide and soybean peroxidase proposed in the grant:

1) Control: no calcium peroxide; no soy peroxidase
2) Control: 350 mmole calcium peroxide; no soy peroxidase
3) Control: no calcium peroxide; 70 mg soy peroxidase
4) 50 mmoles calcium peroxide; 7 mg soy peroxidase
5) 200 mmoles calcium peroxide; 7 mg soy peroxidase
6) 350 mmoles calcium peroxide; 7 mg soy peroxidase
7) 50 mmoles calcium peroxide; 35 mg soy peroxidase
8) 200 mmoles calcium peroxide; 35 mg soy peroxidase
9) 350 mmoles calcium peroxide; 35 mg soy peroxidase
10) 50 mmoles calcium peroxide; 70 mg soy peroxidase
11) 200 mmoles calcium peroxide; 70 mg soy peroxidase
12) 350 mmoles calcium peroxide; 70 mg soy peroxidase

Final treatment levels for calcium peroxide and soybean peroxidase used in the experiment:

1. Control: no calcium peroxide; no soy peroxidase
2. Control: 25 mmole calcium peroxide; no soy peroxidase
3. 0 mmoles calcium peroxide; 7.5 mg soy peroxidase
4. 12.5 mmoles calcium peroxide; 7.5 mg soy peroxidase
5. 25 mmoles calcium peroxide; 7.5 mg soy peroxidase
6. 0 mmoles calcium peroxide; 15 mg soy peroxidase
7. 12.5 mmoles calcium peroxide; 15 mg soy peroxidase
8. 25 mmoles calcium peroxide; 15 mg soy peroxidase
9. 0 mmoles calcium peroxide; 30 mg soy peroxidase
10. 12.5 mmoles calcium peroxide; 30 mg soy peroxidase
11. 25 mmoles calcium peroxide; 30 mg soy peroxidase

Figure 1. Excess foaming of the initial levels of calcium peroxide tested.

Figure 2. Final levels of calcium peroxide and soybean peroxidase used in the experiment with fibers being exposed to measure head space gases.

Results:

Manure Composition (Table 2)

The base manure had no appreciable levels of soybean peroxidase (SoyP) as indicated by the near zero detection in the two control treatments that did not have additional SoyP (Table 2) added to these manure columns. As the SoyP was added to the manure columns at increasing amounts of 7.5, 15, and 30 mg the average analyzed levels of SoyP increased linearly ($P<0.001$; 0.49, 0.68, and 0.78 purpurogallin units/µL, respectively). Adding calcium peroxide (CaP) to the manure also resulted in a linear decline ($P<0.01$; 0.69, 0.66, 0.59 purpurogallin units/µL, respectively) in the average level of SoyP analyzed levels of activity. The average SoyP levels were at their highest levels at the 2 hr sampling and then declined to 48 hrs and remained at this level to 120 hr (Time $P<0.01$; 0.74, 0.65, 0.60, 0.60 purpurogallin units/µL, respectively)

Manure pH was not affect by SoyP levels (Table 1; $P>0.55$). Manure DM declined slightly as SoyP increased ($P<0.02$; 7.98, 8.00, 7.87%), but had no effect on manure ash content as a % of manure DM ($P>0.8$, avg. = 18.3%) or manure ammonium N ($P>0.31$ avg. = 3365 ppm). However, as CaP levels increased from 0, 12.5, to 25 mmoles, the manure pH increased linearly ($P<0.001$; 6.54, 7.00, and 7.25, respectively). There was also an interaction ($P<0.001$) with time and CaP addition on manure pH, the 0 level of CaP had virtually no change in manure pH across all time points, but the 12.5 and 25 CaP levels had a significant rise in manure pH at 2 hr and then a gradual decline as time elapsed to 24, 48 and 120 hr. Manure DM (7.42, 8.03, 8.40) and ash as a % of DM (16.7, 18.2, 20.0%) linearly increased ($P<0.001$) and manure ammonium N linearly decreased ($P<0.001$; 3492, 3310, 3293 ppm) as CaP increased in the manure. As the sampling time reached 120 hr, manure ash as a % of DM increased 4% above the other time points ($P<0.05$). The level of CaP had an interaction with time as it affected ammonium N levels in the manure ($P = 0.05$). This interaction was an order of magnitude response in that the 0 CaP level had 6.3% decline in manure ammonium N levels from 2 hr to 120 hr, while the 12.5 and 25 CaP levels had 8.4% and 12.3% decline by 120 hr, respectively.

Manure Volatile Fatty Acids and Phenolic compounds (Table 3)

The increasing levels of SoyP achieved only non-significant changes in most manure phenolic compounds on its own (Table 3). Manure phenol declined slightly (14.7, 11.3, 13.2, respectively) compared to the 0-0 control at 18.1 ppm. The compounds indole and 4-ethyl phenol declined ($P<0.04$; 1.35,1.05, 0.61 ppm, respectively) along with total phenolic compounds (16.0, 12.4, 13.8 ppm) with increasing SoyP, but the combined compounds of 3 and 4 methyl phenol increased slightly with increasing SoyP (0.0011, 0.0017, 0.0034 ppm, respectively). By contrast, as CaP increased in the manure, all phenolic compounds linearly declined. Phenol ($P<0.002$), 3 and 4 methyl phenol ($P<0.001$), indole and 4-ethyl phenol ($P<0.06$), and total phenolic compounds (20.7, 13.6, 7.9, respectively; $P<0.001$) were all reduced with increasing CaP. But careful evaluation of the data clearly shows that it is the combination of SoyP enzyme and CaP that clearly reduces the phenolic compounds. When one compares the 25 mmole level of CaP with no SoyP enzyme having a manure total phenolics level of 15.6 ppm and when any of the SoyP levels are added to the 25 mmole level of CaP the total phenolics in the manure are reduced on average by ½ to 7.9 ppm ($P<0.10$).

Individual manure volatile fatty acids (VFA) were similarly impacted to a greater extent by the inclusion of CaP than by the SoyP enzyme. Acetic acid ($P<0.15$) increased slightly while butyric ($P<0.14$) and isovaleric ($P<0.09$) decreased slightly with increasing SoyP enzyme levels. While increasing levels of CaP increased acetic acid ($P<0.001$), decreased butyric ($P<0.001$), and increased and then decreased isobutyric (Quad., $P<0.04$) and isovaleric acids (Quad., $P<0.005$).

However, neither Soy P or CaP had any effect on total VFA's. There was a time effect for all VFA's that followed a similar pattern of being relatively constant until the 120 hr sampling time point. At 120 hr, acetic, propionic, and total VFA's declined while isobutyric, butyric, isovaleric, and valeric fatty acids increased at 120 hr.

Manure column head air space samples Volatile Fatty Acids and Phenolic compounds (Table 4)

The addition of SoyP had linear numerical reductions in phenol (13.2, 9.0, 6.0 ppm, respectively; $P<0.16$), total phenolics (16.3, 11.2, 8.0 ppm respectively; $P<0.14$), and a quadratic response in Total VFA's (926, 621, 913 mmoles; $P<0.08$) as SoyP levels increased in the manure columns. Increasing levels of CaP linearly decreased phenol (14.5, 8.1, 5.7 ppm, respectively; $P<0.09$), total phenolics (16.0, 11.8, 7.7 ppm, respectively; $P<0.13$); butyric (506, 221, 180 mmoles/g, respectively; $P<0.001$), combined acetic and valeric (461, 67, 74 mmoles, respectively; $P<0.001$), and total VFA's (1365, 528, 566 mmoles/g, respectively; $P<0.001$) while decreasing and then slightly increasing in a quadratic fashion indole and 4-ethyl phenol (Quad., $P<0.05$) and isovaleric (110, 43, 79 mmoles respectively; $P<0.03$). The time effect for the VFA's isobutyric, butyric, isovaleric, valeric and acetic, and total VFA's all peaked at 24 hr then declined in the head space air to 48 and 120 hr. It is interesting to observe that the greatest reduction in phenols and total phenolics in manure column head space air occurred at the highest inclusion rate of SoyP (30) in combination with CaP (25).

Discussion

The first levels of calcium peroxide (CaP) attempted in this experiment were too high and created excess foaming in the flasks, especially the 200 and 350 mmole levels (See Figure 1). Even the 50 mmole level had a significant level of foaming with levels getting high enough in the flasks to contaminate the SPME fibers used to sample air for compounds. In addition to the high level of foaming, these levels of CaP altered the flasks to have a plastic like smell and changed color to almost a tan color from the normal dark green/black color manure has. When one considers that the CaP was acting much like hydrogen peroxide would on a cut on a person's hand, much of this made sense. There is a significant microbial load in manure and the CaP was acting on that population, creating the foaming and the plastic like smell as it killed the manure microbial population. So a lower level of CaP was used for the rest of the experiment at 0, 12.5, and 25 mmoles. As can be seen in Figure 2, the manure columns did not foam at these levels and the color remained fairly consistent. The final levels of CaP used in this experiment are lower than the levels used by Govere et al. (2005), who used between 34-68 mmoles of either hydrogen peroxide or CaP in 30 mL of manure. Our lower levels of 12.5 or 25 mmoles were added to 10X that amount of manure, 350 mL. Given our observations, one has to wonder if the Govere et al. (2005) experiment did not simply kill their manure with such high levels of peroxide and therefore created their reductions due to these significant changes in the manure chemistry.

The base manure had no appreciable levels of soybean peroxidase (SoyP) as indicated by the near zero detection in the two control treatments that did not have additional SoyP added to these manure columns. As the SoyP was added to the manure columns at increasing amounts of 7.5, 15, and 30 mg (1,500, 3,000, 6,000 purpurogallin units) the average analyzed levels of SoyP increased linearly. However, it was expected to see a larger increase with each doubling of the enzyme, but each additional level did result in additional enzyme being detected by the antibody assay. The average SoyP levels were at their highest levels at the 2 hr sampling and then declined to 48 hrs and remained at this level to 120 hr, maintaining 81% of the initial level of SoyP activity at 120 hr. Adding calcium peroxide (CaP) to the manure resulted in a 14.5% linear decline in the average level of SoyP activity. Why the addition of CaP decreased detectable levels of SoyP is unknown. Potentially it could relate to more of the enzyme being bound with the catalyst (CaP) in a reaction with the phenolic

compounds and therefore not being detectable by the assay. Also, there is the potential of the increased manure pH with additional CaP decreasing the enzyme activity and potential viability as identified by Wright and Nicell (1999) and Kamal and Behere (2003).

Manure pH, ammonium N, manure ash content as a % of manure DM were not affect by SoyP levels, however manure DM declined slightly as SoyP increased. As CaP levels increased, manure pH, manure DM linearly increased and manure ammonium N linearly decreased. As manure pH increased with increasing CaP, the pH level increased above 7.0, became more basic. When this happens we start to drive off more ammonium N from the manure as the more acidic we can maintain manure the more likely we will be able to maintain the manure N and reduce losses to the atmosphere. This pH change is a negative to adding too much catalyst to help drive the reaction of the SoyP to reduce the manure phenolic compounds. While the increased DM content can be a positive if it holds true with more organic nutrients to land apply per gallon of manure hauled, reducing hauling and application costs. However, it may have less N, potentially countering this field application cost benefit. This experiment's data indicates we may want to target a balance near the 12.5 mmoles of CaP per 350 mL of manure to keep the manure pH neutral to slightly acidic to reduce the potential to volatilize of too much N from the manure. The study by Flock et al. (1999) reported that the optimal hydrogen peroxide level was 10 mmol/1000 mL of human waste water. However, the DM is much lower in human waste water than in swine manure. The study by Govere et al., 2005 suggested that CaP is a better catalyst than hydrogen peroxide in swine manure and so changing the catalyst may not be the best option.

Neither SoyP or CaP had any effect on manure total VFA's, however individual manure volatile fatty acids (VFA) were impacted by the inclusion of SoyP and CaP. Increasing levels of CaP did decrease head space air total VFA's by 60%. While manure total VFA's were not affected, the reduction in head space VFA's is likely a strong indicator that a significant amount of the microbial population in manure was killed with the initial addition of the CaP, decreasing the generation rate and volatilization of the VFA's as detected in the manure column head space air. This is also interrelated to the linear increase in manure DM as the decrease in microbial population will decrease the amount of organisms to also metabolize the manure DM into microbial protein and by products (VFA's). While the decrease in the air of the VFA's would decrease the odor from the manure during storage, one question that may need addressing is will there be an increase in DM and sludge in the manure storage structure that may need to be addressed long term.

The increasing levels of SoyP reduced manure phenol and manure total phenolic compounds by 27-28% on its own compared to the 0-0 control. The addition of SoyP also had linear numerical reductions in head space air phenol (13.2, 9.0, 6.0 ppm) and total phenolics (16.3, 11.2, 8.0 ppm). By contrast, as CaP increased, all manure phenolic compounds linearly declined. Total manure phenolic compounds decreased by 30 and 59% as CaP increased to 12.5 and 25 mmoles. Increasing levels of CaP also linearly decreased head space air phenol and total phenolics by 10 and 41% as CaP increased. Encouragingly, there appears to be a strong relationship to the reductions in manure phenolic compounds to the reductions observed in the head space air in this study. Govere et al. (2005) looked at adding horseradish root as a source of peroxidase to swine manure to minimize odors and phenolic compounds. Their GC results reported that there was nearly 100% removal of all phenolic odorant compounds after 72 hr when the combination of 10% horseradish root and 26 mmoles of calcium peroxide were added to 30 mL of swine manure. They also found that the olfactometry panel reported a 50% reduction in odor intensity from head space air off the manure that had horse radish root and CaP. Govere et al. (2005) levels of CaP were much higher than levels used in this experiment in addition they used the whole horse radish root which contributed many other components to the slurry experiment other than just peroxidase.

Careful evaluation of the data clearly shows that it is the combination of SoyP enzyme and CaP that clearly reduces the phenolic compounds. This is best displayed when you compare the 25 mmole level of CaP with no SoyP enzyme having a manure total phenolics level of 15.6 ppm and when any level of the SoyP is added to the 25 mmole level of CaP the total phenolics in the manure are reduced on average by 50% to 7.9 ppm ($P<0.10$). Also the greatest reduction (63%) in phenols and total phenolics (from 13.1 to 4.8 ppm) in manure column head space air occurred at the highest inclusion rate of SoyP (30 mg) in combination with the highest CaP (25 mmoles). This level of phenolic reduction is similar to the levels reported by Flock et al. (1999) where there was over 60% the phenol compounds removed after a 60 minute incubation of soybean hulls with hydrogen peroxide in water, however, not as great as that reported by Govere et al. (2005).

Summary

This research proves that the soybean peroxidase enzyme can survive in swine manure up to 5 days. The use of soybean peroxidase in combination with a catalyst (calcium peroxide) may be a viable option to reduce both manure content and air emissions of phenolic compounds, but by its self soybean peroxidase only can reduce phenolic compounds by 27-28% compared to up to 63% with a catalyst. The increased losses of ammonium N with the increased manure pH as calcium peroxide was added to the manure is a potential negative result of this research. This research indicates there may be potential to evaluate either a spray application system that may be applied weekly or potentially even less frequently to help control phenolic compounds from swine manure storage systems. Additionally, potential research evaluating the feeding of soybean hulls (the source of the soybean peroxidase) high in the enzyme through the pig may have similar results if the enzyme can remain intact during the digestion process or sequester phenolic compounds in the gut of the pig prior to excretion in the manure. There appear to be many potential future research and applications to this technology that may help the swine industry meet its goal of being a good, responsible neighbor in their rural community.

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Table 1. Phenolic and VFA compound standards mix at Purdue University

Analyte	Concentration, ppm
Acetic Acid	1365
Proprionic acid	358
Isobutyric acid	604
Butyric acid	236
Isovaleric acid	301
Valeric acid	90
Phenol	20
4-methylphenol (p-cresol)	62
3-methylphenol	62
4-ethylphenol	4
Indole	6

Based on Gralapp, Powers and Bundy, 2001.

This standard stock solution mixture was used at 100, 75, 50, 25, 10, and 5% concentrations to develop the standard curve for the compounds on the GC.

Table 2. Effect of soybean peroxidase (SoyP) and calcium peroxide (CaP) on overall average manure composition.

SoyP[1]	0	0	7.5			15			30				Trt
CaP[2]	0	25	0	12.5	25	0	12.5	25	0	12.5	25	SE	P <
Peroxidase activity[3], purpurogallin units/µL	0.01^{f}	0.00^{f}	0.48^{e}	0.52^{de}	0.46^{e}	0.75^{ab}	0.68^{bc}	0.60^{cd}	0.86^{a}	0.77^{ab}	0.71^{bc}	0.041	0.001
pH[3,4]	6.64^{c}	7.33^{a}	6.57^{c}	6.97^{b}	7.20^{a}	6.53^{c}	7.02^{b}	7.27^{a}	6.53^{c}	7.02^{b}	7.28^{a}	0.059	0.001
DM,%	7.39^{de}	8.43^{a}	7.58^{d}	8.00^{bc}	8.36^{a}	7.39^{de}	8.17^{b}	8.44^{a}	7.29^{e}	7.91^{c}	8.40^{a}	0.059	0.001
Ash, % of DM[3]	17.0^{c}	20.2^{a}	16.6^{c}	18.3^{b}	20.1^{a}	16.8^{c}	17.9^{b}	20.1^{a}	16.8^{c}	18.3^{b}	19.8^{a}	0.15	0.001
Amm. N, ppm[3]	3467^{ab}	3376^{bc}	3446^{ab}	3317^{c}	3294^{c}	3516^{a}	3308^{c}	3266^{c}	3514^{a}	3305^{c}	3319^{c}	34.0	0.001

[1] SoyP = Soybean Peroxidase level in mg added to 350 mL of grow-finish manure. Each mg had 200 units of peroxidase activity. One unit of peroxidase activity is defined as the amount of enzyme required to catalyze the production of 1 mg of purpurogallin from pyrogallol in 20 seconds at 20°C.

[2] CaP = Calcium Peroxide level in millimoles added to 350 mL of grow-finish manure (0, 0.9, and 1.8 g, respectively).

[3] Effect of time (2, 24, 48, and 120 hr), $P < 0.05$.

[4] Interaction between time and treatment, $P < 0.05$.

[a,b,c,d,e,f] Means with different superscripts differ by $P < 0.05$ based on means separation using the Duncan's multiple range test.

Table 3. Effect of soybean peroxidase (SoyP) and calcium peroxide (CaP) on overall average volatile fatty acids (mmol) and phenolic (ppm) compounds per gram of manure.

SoyP[1]	0	0	7.5			15			30				Trt
CaP[2]	0	25	0	12.5	25	0	12.5	25	0	12.5	25	SE	P <
Acetic[3]	9.10^{e}	11.46^{a}	9.73^{de}	9.17^{e}	10.50^{bcd}	9.82^{de}	9.65^{de}	11.11^{ab}	9.89^{cde}	9.80^{de}	10.76^{abc}	0.30	0.001
Propionic[3]	4.54	4.56	4.63	4.51	4.61	4.71	4.55	4.64	4.56	4.59	4.60	0.13	0.99
Isobutyric[3]	0.63	0.52	0.63	0.62	0.58	0.55	0.68	0.55	0.58	0.67	0.65	0.05	0.20
Butyric[3]	7.64^{a}	5.81^{d}	7.09^{ab}	7.32^{ab}	6.32^{cd}	7.13^{ab}	7.03^{abc}	5.93^{d}	6.95^{abc}	6.88^{bc}	6.10^{d}	0.24	0.001
Isovaleric[3]	1.05^{a}	0.79^{c}	0.87^{bc}	1.00^{ab}	0.86^{bc}	0.84^{c}	0.91^{abc}	0.80^{c}	0.85^{bc}	0.88^{bc}	0.81^{c}	0.05	0.004
Valeric[3]	1.52^{a}	1.26^{c}	1.31^{abc}	1.49^{ab}	1.29^{bc}	1.34^{abc}	1.33^{abc}	1.27^{c}	1.32^{abc}	1.33^{abc}	1.27^{c}	0.06	0.06
Total VFA's[3]	24.48	24.40	24.27	24.11	24.17	24.40	24.15	24.29	24.15	24.14	24.19	0.17	0.85
Phenol	18.1^{xyz}	14.3^{xyz}	19.6^{xy}	17.2^{xyz}	7.2^{z}	17.7^{xyz}	8.1^{yz}	8.2^{yz}	20.0^{x}	12.0^{xyz}	7.5^{z}	4.21	0.16
3 and 4 methyl phenol	0.0040	0.0003	0.0032	0.0003	0.0000	0.0040	0.0000	0.0011	0.0075	0.0011	0.0011	0.0012	0.001
Indole and 4-ethyl phenol	1.33	1.29	1.41	1.95	0.70	1.72	1.42	0.00	1.84	0.00	0.00	0.837	0.62
Total phenolics	19.4^{xy}	15.6^{xyz}	21.0^{xy}	19.2^{xy}	7.9^{z}	19.4^{xy}	9.6^{yz}	8.2^{z}	21.8^{x}	12.1^{xyz}	7.5^{z}	4.28	0.07

[1] SoyP = Soybean Peroxidase level in mg added to 350 mL of grow-finish manure. Each mg had 200 units of peroxidase activity.
[2] CaP = Calcium Peroxide level in millimoles added to 350 mL of grow-finish manure (0, 0.9, and 1.8 g, respectively).
[3] Effect of time (2, 24, 48, and 120 hr), $P < 0.05$.
[a,b,c,d,e] Means with different superscripts differ by $P < 0.05$ based on means separation using the Duncan's multiple range test.
[x,y,z] Means with different superscripts differ by $P < 0.10$ based on means separation using the Duncan's multiple range test.

Table 4. Effect of soybean peroxidase (SoyP) and calcium peroxide (CaP) on overall average manure column air head space volatile fatty acids (mmol/g) and phenolic compounds (ppm).

SoyP[1]	0	0	7.5			15			30				Trt
CaP[2]	0	25	0	12.5	25	0	12.5	25	0	12.5	25	SE	P <
Isobutyric[3]	219.6	227.5	241.6	223.0	298.3	326.3	299.4	160.8	319.2	275.2	246.3	96.51	0.98
Butyric[3]	324.7^{bc}	316.2^{bc}	718.9^{a}	192.5^{c}	203.0^{c}	213.9^{c}	254.0^{bc}	166.7^{c}	586.4^{ab}	216.7^{c}	169.9	109.7	0.006
Isovaleric[3]	78.4^{ab}	104.1^{ab}	150.3^{a}	38.8^{b}	75.0^{ab}	53.8^{ab}	52.5^{ab}	103.5^{ab}	125.1^{ab}	39.2^{b}	56.9^{b}	31.36	0.25
Valeric and Acetic[3,4]	352.1^{ab}	260.2^{ab}	507.3^{a}	68.1^{b}	59.1^{b}	329.4^{ab}	9.5^{b}	99.2^{b}	547.3^{a}	120.7^{b}	60.9^{b}	106.61	0.002
Total VFA's[3]	975^{abc}	908^{abc}	1614^{a}	522^{c}	635^{bc}	924^{abc}	406^{c}	530^{c}	1549^{ab}	652^{bc}	528^{c}	235.1	0.005
Phenol	10.9^{ab}	8.9^{ab}	26.8^{a}	5.8^{b}	7.1^{b}	9.8^{ab}	10.8^{ab}	6.4^{b}	6.8^{b}	7.6^{b}	3.6^{b}	5.77	0.35
Indole and 4-ethyl phenol	2.2	3.3	2.7	3.1	3.3	0.8	4.4	1.4	1.0	3.9	1.3	1.49	0.73
Total Phenolics	13.1^{ab}	12.2^{ab}	29.5^{a}	8.9^{b}	10.4^{ab}	10.6^{ab}	15.2^{ab}	7.8^{b}	7.8^{b}	11.5^{ab}	4.8^{b}	6.21	0.39

[1] SoyP = Soybean Peroxidase level in mg added to 350 mL of grow-finish manure. Each mg had 200 units of peroxidase activity.
[2] CaP = Calcium Peroxide level in millimoles added to 350 mL of grow-finish manure (0, 0.9, and 1.8 g, respectively).
[3] Effect of time (2, 24, 48, and 120 hr), $P < 0.05$.
[4] Interaction between time and treatment, $P < 0.05$.
[a,b,c] Means with different superscripts differ by $P < 0.05$ based on means separation using the Duncan's multiple range test.