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PEER-REVIEWED ARTICLE

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Control of *Aspergillus* and *Fusarium* In Vitro and in Wheat Using Sodium Bisulfate Acidulant

ABSTRACT

We evaluated the efficacy of an acidulant, sodium bisulfate (SBS) against *Aspergillus flavus* and *Fusarium graminearum* in a simulated wheat storage model. The broth microdilution method was used to determine the MIC of SBS against the molds. Sterile wheat samples were inoculated with mold culture, followed by a 30-min attachment and the application of SBS treatment (0, 0.5, 1.0, and 1.5%, w/v). Positive and negative controls were maintained. The final moisture in wheat was consistent with the tempering process (16%). The treatments were incubated at 25°C, and microbiological analyses were performed at predetermined times (0, 6, 12, 18, 24, and 48 h). The MIC of SBS against *A. flavus* and *F. graminearum* was 0.31 and 0.16%, respectively. In wheat, the highest concentration of SBS used (1.5%) reduced ($P \leq 0.05$) ~1.0 and 1.3 log of *A. flavus* after 18 and 48 h, respectively, whereas all three concentrations of SBS lowered ($P \leq 0.05$) *F. graminearum* to a nondetectable level within 18 h. Overall, the addition of SBS in tempering water during the wheat tempering process seemed effective in controlling *F. graminearum* and partly effective against *A. flavus*.

INTRODUCTION

Cereals and cereal products are a good medium for pathogens such as bacteria and molds to survive and grow. The sources of microbial contamination of cereals include air, dust, water, insects, rodents, birds, humans, shipping, transportation, and storage equipment. Other factors that influence microbial loads in cereals are temperature, humidity, frost, wind, use of chemicals, and moisture control systems *(4)*. Along with bacteria, yeast and molds are the main spoilage organisms in cereals and cereal-derived products *(29)*. Over 150 species of molds and yeast, including *Fusarium*, *Aspergillus*, *Alternaria*, *Cladosporium*, and *Curvularia*, have been reported on cereal grains *(25)*. Mycotoxins are secondary metabolites produced by filamentous fungi and are produced mainly by fungal species of *Aspergillus*, *Penicillium*, and *Fusarium (14)*. They can be toxic to both humans and livestock at varying concentrations. Consumption of aflatoxin-contaminated maize resulted

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in 125 deaths and 200 illnesses in Kenya in 2004 *(27)*. Major mycotoxins found in cereals are aflatoxin (produced by *Aspergillus flavus*), deoxynivalenol, zearalenone, and fumonisins (produced by *Fusarium graminearum*) and ochratoxin (produced by *Aspergillus ochraceus*) *(4)*. It is estimated that about 25% of the global food and feed output is contaminated by mycotoxins *(16)*.

Minimizing the introduction of the fungal spores in the grains during preharvest and postharvest periods is the primary approach implicated to minimize the mycotoxin introduction in the food chain *(26)*. In addition to various management practices, physical and physicochemical methods are also used to decontaminate and degrade the already formed mycotoxins in grains *(17)*. However, chemical control of molds in stored grains for human and animal consumption is not very common. The use of plant essential oils such as the terpenoids, eugenol and cinnamaldehyde, were reported to control fungal growth in cereals *(17)*. Valeric acid, propionic acid, and other organic acids have also been known to inhibit mold growth *(19)*. However, the application of organic acids may affect the taste and smell, especially in foods for human consumption. In addition, the cost of organic acids required to treat grains in bulk storage could be a concern. Chlorine compounds are the most commonly used antimicrobials during wheat tempering to control bacterial and fungal pathogens *(13)*. However, due to the occurrence of potentially hazardous residues of chlorine *(24)* and the export restriction of chlorine-treated foods to some countries, safer and widely accepted intervention strategies must be investigated.

Given the breadth and magnitude of this issue and the limitation of the existing intervention strategies, identifying a method that can be deployed in the grain processing industry to mitigate mold infestation and growth could have a profound benefit to human and animal health, reduce grain losses, and have a positive effect on the sustainability of grain-based food for future generations. Though the mycotoxin production in the stored wheat typically happens prior to milling, the presence of the molds during milling and the subsequent transfer of molds and mycotoxins in flour is of concern. In wheat milling, tempering is a premilling stage, when water is added to the raw grain (final moisture of ~16%) for ~18 h to toughen the bran and mellow the wheat endosperm, thereby improving the efficiency of the flour extraction. The occurrence of *Aspergillus* and *Fusarium* in wheat flour *(1)* and hence the presence of mycotoxins in wheat flours *(23)* lead us to design a study for the application of an antimycotic during the premilling stage and attempt to control molds and mycotoxins in postmilling fractions.

Sodium bisulfate (SBS; $NaHSO_4$) is a natural acidulant and a hygroscopic chemical that has known antimicrobial activities against *Salmonella (9–12)* and *Escherichia coli (7, 30)* in foods without negative effects on quality. This is generally recognized as safe for use in human foods and is also approved for use in animal feed as a general purpose additive *(22)*. The effect of SBS as an antimycotic in stored wheat products is not known. Therefore, the objective of this study was to evaluate the postharvest application of SBS in wheat grains against *A. flavus* and *Fusarium graminearum* Schwabe.

MATERIALS AND METHODS

Sources of mold strains, antimycotic, and wheat grains

Stock cultures of *A. flavus* (ATCC 15548) and *F. graminearum* Schwabe (ATCC 46779) were obtained from the American Type Culture Collection. *A. flavus* (ATCC 15548) is known to produce aflatoxin B1 and G1, and *F. graminearum* Schwabe (ATCC 46779) is known to produce deoxynivalenol and zearalenone. The frozen cultures were maintained in potato dextrose broth (PDB): glycerol (7:3) at −80°C. SBS was provided by the Jones-Hamilton Co. (Walbridge, OH). Wheat samples (hard red winter wheat) were procured from Indigo Agriculture (Boston, MA).

Preparation of the working culture of the molds

Before use, the frozen cultures of the molds were grown in PDB overnight. The broth was then streaked on potato dextrose agar (PDA, supplemented with 0.1 g/liter chloramphenicol) plates in duplicate and incubated at 25°C for 72 h. After 72 h, the mold-grown agar lawns were scrapped by dislodging the spores by using sterile L-rod spreaders in 5 ml of 0.1% peptone water *(3, 8)*. The spore suspension was then transferred to a sterile tube and used for the wheat inoculation study within an hour. The mold suspension was used for the MIC, minimum fungicidal concentration (MFC), and wheat inoculation study.

MIC and MFC assays

The MIC of SBS was determined by broth microdilution assay according to the method of the Clinical and Laboratory Standards Institute *(5)* in PDB. A 100-µl aliquot of PDB mold culture containing ~5 log CFU/ml was added to each well of a plate containing 100 µl of decreasing concentrations of SBS for a final volume of 200 µl per well. The positive control consisted of *A. flavus* or *F. graminearum* inoculum only (no SBS), and the negative control consisted of PDB alone. A separately prepared SBS solution with concentrations from 0.25 to 3%, with a 0.25% increase, were tested in the MIC assay. The MIC was defined as the lowest concentration of the antimicrobial agent that inhibited the visible growth of molds after 72 h of incubation at 25°C *(2)*. For the determination of the MFC assay, 10-µL content from the wells showing no visible growth after 72 h of incubations were plated on PDA plates in duplicate. The plates were incubated for 72 h at 25°C. The lowest SBS concentration that caused no visible colony on the agar plate was considered to be the MFC *(28)*. Each experiment for each strain was done in triplicate.

Agar-based disk diffusion assay. Due to the ease of use and low-cost method, we also performed a disk diffusion-based assay to evaluate the antifungal activity of SBS, a method similar to Zabka et al. *(34)*. For this, the PDA plates were supplemented with various concentrations of SBS (0, 0.25, 0.5, 0.75, and 1.0%) by adding filtered sterile SBS solution in agar just before pouring onto the plates. The mold disks were prepared by dipping sterile paper disks (diameter: 0.25 in.; Carolina Biological, NC) in 10 ml of mold broth for 72 h at 25°C. The average concentrations of *A. flavus* and *F. graminearum* used for disk diffusion assay were 4.91 and 4.02 log, respectively. The disks were lifted with sterile forceps and placed on the center of the agar plates, and the plates were incubated for 72 h at 25°C before measuring the diameter. The assay was done in triplicate for each strain.

Wheat inoculation study

A 150-g aliquot of autoclave-sterilized hard red winter wheat for each treatment was transferred to an autoclaved plastic container. Note that the autoclaving of the wheat might change the nutrient composition of the food and possibly behave differently than raw wheat. The moisture of the autoclaved wheat was measured and found to be 10.7%. For each mold type, a total of five containers were maintained: three for treatments (0.5, 1.0, and 1.5% final concentration of SBS in wheat); one for the positive control (mold only and no SBS); and one for the negative control (no mold and no SBS). The various treatments were selected on the basis of the MIC and MFC of SBS. The working culture of molds (~4 to 5 log) was spot inoculated in the wheat and mixed thoroughly by closing the lid of the jars. Due to the nonuniform nature of harvesting mold spores from the lawn, the initial inoculum level of the molds tended to vary for each replication, with 4 to 5 log typical for both *A. flavus* and *F. graminearum*. After the mold inoculation, 30 min at 25°C was allowed for the attachment of molds. After 30 min, aqueous SBS treatments were applied to the mold and mixed uniformly. The volume differential for positive and negative controls was adjusted by adding sterile distilled water. Microbiological analyses were conducted for each of the containers at various predetermined times: 0, 6, 12, 18, 24, and 48 h. From each treatment and control, a 25-g subsample was collected in sterile-filtered Whirl-Pak bags (Nasco, Fort Atkinson, WI), mixed in 225 ml of buffered peptone water, and stomached for 2 min. The mixtures were serially diluted in 0.1% peptone water and plated on PDA. A total of 1-ml volume of the diluent was plated on four PDA plates. The plates were incubated at 25°C for 60 to 72 h, and colonies were counted. The detection limit for the mold was 1 log CFU/g of the sample. The mold colonies were counted, and the results were expressed as log (CFU per gram of the wheat sample). Each experiment was conducted in triplicate.

Statistical analysis

The first experiment was a 5 × 6 factorial arrangement of treatments using three SBS concentrations (0.5, 1.0, and 1.5%), the positive and negative controls, and six sampling intervals. Each treatment consisted of three replicates. Mean values were analyzed by using the general linear model procedure using Tukey's test ($P \leq 0.05$) comparison. All statistical analyses were conducted by using SAS statistical software, version 9.3 (SAS Institute Inc., Cary, NC).

RESULTS AND DISCUSSION

The powdered SBS provided by Jones-Hamilton Co. had solubility of 28.5 g/liter of water at room temperature. Due to this limitation, a concentration higher than 1.5% of SBS was not able to be achieved in the tempered wheat model. To our knowledge, this is the first study of its type using SBS as an antimycotic against molds (*A. flavus* and *F. graminearum*). In this study, we reported the MICs of SBS against *A. flavus* and *F. graminearum* to be 0.31 and 0.16%, respectively, and the MFC of SBS to be 2.5 and 1.25%, respectively. As expected, the MFCs were higher than the corresponding MICs for both organisms. The results indicated that *A. flavus* is much more resistant compared with *F. graminearum*. This observation is consistent with the report by Savi and Scussel *(31)* who also noted a higher resistance of *A. flavus* against ozone (O3) when compared with *F. graminearum*. In another study with organic acids in animal feed, Higgins and Brinkhaus *(19)* also discovered that *Aspergillus* spp. were more resistant than *Fusarium* spp.

Table 1 shows the results of agar-based disk diffusion assay. The diameters of the disk (mold growth) on the PDA plates were measured after 72 h with complete inhibition for the 0.75% concentration against both mold species.

In this experiment, we simulated the common tempering condition for wheat grain during the flour milling process. Though normal tempering is done for an 18- to 24-h period, we extended the evaluation time point further (up to 48 h) to evaluate the potential for reduction during extended time periods. Further, due to the method used for harvesting molds, the concentrations of molds were not uniform for each replication, and an average was taken from the three replications. Similar methods were used in previous studies *(32)* involving mold harvesting. Chlorine and chlorine gas have been traditionally used during wheat tempering to reduce microbial loads *(33)*. However, this poses safety concerns among the consumers, as well as for milling workers. Hence, studies to find safer replacements, such as O3 and other chemicals have been sought. SBS possessed antimicrobial effects against *E. coli* (Shiga toxigenic) in wheat tempering conditions *(7)* and against *Salmonella* in poultry meals *(6)* and rendered chicken fat systems *(10, 11)*.

When *A. flavus*-inoculated wheat was treated with different concentrations of SBS, the initial (0 h) recovery from the control wheat (no SBS) was 4.9 log/g. In the wheat samples

TABLE 1. Inhibition zone diameters of molds in different SBS concentrations[a]

	Disk diameter (cm ± SD):	
SBS (%)	*A. flavus*	*F. graminearum*
0	4.18 ± 0.07	4.28 ± 0.05
0.25	1.18 ± 0.10	1.16 ± 0.09
0.50	0.66 ± 0.07	0.76 ± 0.04
0.75	0	0
1.00	0	0

[a]PDA plates fortified with different concentrations of SBS contained paper disks with molds. The diameters of the disk (mold growth) on the PDA plates were recorded after 72 h.

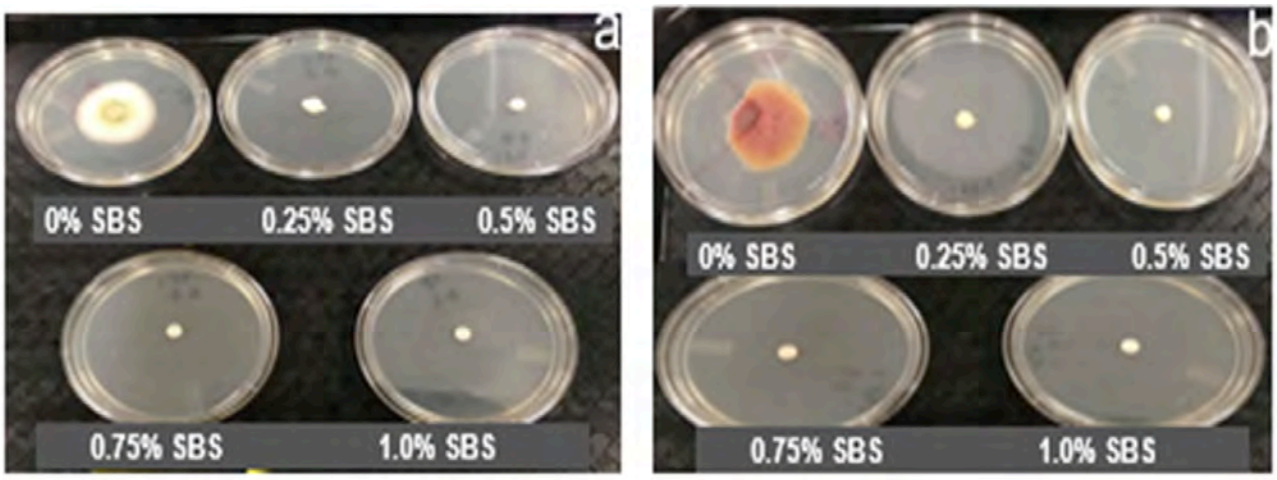

Figure 1. The inhibition of *A. flavus* (a) and *F. graminearum* (b) by various concentrations of SBS on a PDA plate.

treated with 0.5, 1.0, and 1.5% SBS, 0.40-, 1.40- and 1.40-log reductions were observed at 0 h, respectively *(Fig. 2a)*. After 48-h incubation, 0.5% SBS caused only 0.93% reduction of *A. flavus* in wheat, whereas 1 and 1.5% SBS caused 1.60- and 1.30-log reductions, respectively. We observed 0.93- and 1-log reduction[a] ($P < 0.05$) of *A. flavus* after 18 h when treated with 1 and 1.5% SBS, respectively. At the 24-h evaluation time points, 1 and 1.5 % of SBS were effective ($P < 0.05$) in reducing *A. flavus*, whereas at 48 h, all three concentrations were effective ($P < 0.05$) against *A. flavus*. When the reduction was compared across different time points for individual concentrations, only 0.5% SBS led to a significant reduction ($P < 0.05$) at 48 h when compared with 24 h. SBS was found to be more effective against *F. graminearum* than *A. flavus* in wheat. The initial recovery from control wheat (no SBS) was 4.0 log. At 0 h, we observed 1.57-, 1.82-, and 1.53-log reductions ($P < 0.05$) of *F. graminearum* with 0.5, 1.0, and 1.5% of SBS, respectively *(Fig. 2b)*. All three concentrations were able to reduce the mold to a nondetectable level after 18-h incubation. These results from the in vitro study were comparable to the MICs against the two molds.

Though the exact mechanism of action of SBS against mold is not known, on the basis of the proposed hypothesis of the mechanism of actions of organic acids, we believe that this acidulant also acts similarly: the acidulant lowers the pH that then influences the growth by acidifying the cells *(20)*. Another possibility proposed is membrane disruption causing the interruption of the metabolic reactions and accumulation of toxic anions *(18)*. Kang et al. *(20)* proposed a possible decline in the catalase activity of fungus *Colletotrichum* spp. after treatment with acetic acid and

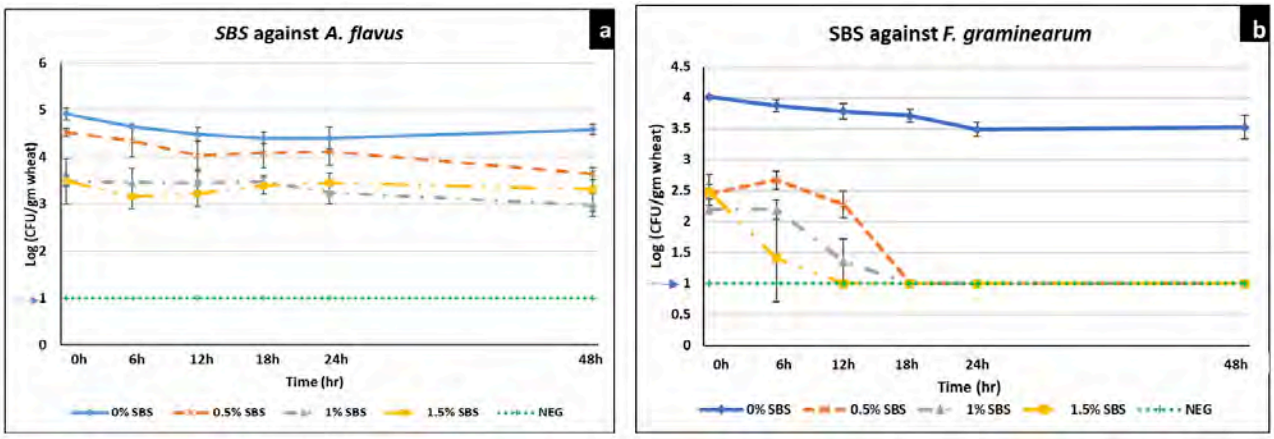

Figure 2. Treatment means ± standard errors for 0% SBS (◆), 0.5% SBS (×), 1% SBS (▲), 1.5% SBS (■), and negative control with no mold or SBS (NEG, +) on wheat grains inoculated with *A. flavus* (a), and *F. graminearum* (b) at 25°C on PDA plates over 72 h, with a detection limit 1.0 log CFU/g (arrow).

hydrogen peroxide, leading to poor scavenging of hydrogen peroxide. Also, combinations of a 2-hydroxy-4-(methylthio) butanoic acid with organic acids, such as fumaric acids, benzoic acid, and lactic acids, were effective against *A. flavus* in dry dog food kibbles with up to 3.8-log reduction after 4 weeks of storage *(8)*.

When the SBS-treated wheat was evaluated for flour quality and baking performance and the results were compared with the nontreated controls, there was not any significant negative impact on flour quality due to the SBS treatment. This part of the research was already published by our lab *(30)*. In brief, significantly higher ($P < 0.05$) protein and carbohydrate content was also observed in wheat flours with added SBS. In terms of flour color, wheat flours from SBS-tempered wheat generally had lower L* and higher a* and b* values, indicating lower brightness, higher red, and yellow tones. No significant differences were observed in terms of the loaf volume and specific bread volume between SBS-treated wheat flours and control (0% SBS) wheat flours. Wheat flours from SBS-tempered wheat yielded bread with significantly ($P < 0.05$) higher cell counts and smaller crumb structure (lower cell diameter and volume) compared with the control. This will further support our approach to mitigating molds in wheat and flour using SBS.

To address health concerns, as well as consumer perception in a positive manner, chemical antimycotics would need to be replaced with natural agents. SBS is a natural acidulant and a nonhazardous acid commonly used in human and animal food *(22)*. The use of SBS as an antimycotic agent in stored grain may help to reduce the cost of antimycotic treatments in bulk grains because SBS is more economical than organic acids or natural plant-based extracts. In addition, the use of organic acids may cause corrosion of the stainless steel containers of the tempering bins. Even though SBS is a stronger acid than organic acids, it is known to cause less damage to stainless steel. This was demonstrated by 10% SBS on stainless steel that caused no or negligible corrosion *(15)*.

In conclusion, the addition of SBS in tempering water during the wheat tempering process was effective in controlling *F. graminearum* and partly effective against *A. flavus*, implying possible inhibition of mycotoxin production. The results also suggest that despite some changes in wheat flour attributes, the use of SBS in wheat tempering did not negatively affect the flour quality and bread loaf characteristics. Various chemicals (sodium bisulfite, sodium hydroxide, and O_3) and enzymes (laccases and peroxidases) are used to detoxify already produced mycotoxins in food *(21)*, but further studies regarding the by-products formed and the effects on food quality need to be conducted. Future work on the potential use of SBS in mycotoxin detoxication and sensory perceptions in foods is warranted. Finally, to achieve effective reduction of *A. flavus* in wheat, additional measures, such as use of organic acids, or combination of SBS with organic acids, need to be tested. Overall, the application of SBS in tempering water could be a viable alternative against synthetic chemicals to improve microbial quality by reducing mold and potential mycotoxin production, while maintaining flour and bread quality.

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