
THE TOXIC ACTION OF CERTAIN THIO ACIDS ON THE
MOLDS OF *PENICILLIUM* AND *ASPERGILLUS* SPP.¹

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INTRODUCTION

The present paper comprises fungicidal studies carried out on the spores of white mold that developed on different silages when exposed to air. The white mold is composed of a mixture of the *Penicillium* and the *Aspergillus* spp. type. The action of bisulfite solutions on *Pencillium* and *Aspergillus* spp. was reported previously by Pasiut and DeMarinis (1958). The fungicidal studies in the present paper contain the use of (A) tetra- and penta-thionic acid solutions, (B) thiosulfuric acid solutions.

¹The fungicidal studies with tetra- and penta-thionic acid mixtures were reported by the authors at the annual meeting of The Ohio Academy of Science, 1958, at Akron, Ohio.

The experiments were carried out on cultures of white molds isolated from old dry silage and are reported in the experimental section.

Young (1922) prepared hydrophilic and hydrophobic sulfur, of which hydrophilic was found to be the most toxic. Young also reported that the toxic property of sulfur is only exhibited when oxygen and water are present. The toxic property of sulfur has been found by Young to be penta-thionic acid. Young and Williams (1928) showed that penta-thionic acid is associated with sulfur. When resublimed flowers of sulfur or ground roll sulfur was triturated with water to effect wetting and then filtered, the clear filtrate had titrable acidity as sulfurous, sulfuric and penta-thionic acids.

Williams and Young (1929) examined the toxicity of sulfuric, dithionic, trithionic, and a mixture of tetra- and penta-thionic acids on the spores of *Sclerotinia cinerea*, and found the mixture of tetra- and penta-thionic acids to be the most toxic.

Oden's colloidal sulfur (1912) with a hydrophilic nature was proven by Freundlich (1926) to contain penta-thionic acid. Weiser and Cunningham (1929) prepared tetra- and penta-thionic acid mixtures by reacting SO_2 and H_2S in aqueous media.

PROCEDURES AND RESULTS

(A) *The Action of Tetra- and Penta-Thionic Acid Mixtures*

The results of Williams and Young (1929), through the application of a mixture of tetra- and penta-thionic acids to the spores of *Sclerotinia cinerea*, induced the authors to examine the action of these two acids on the spores of *Penicillium* and *Aspergillus* spp. isolated from ailing silages.

Preparation of the mixture.—Into a 500 ml flask, 250 ml distilled water were added and then saturated with sulfur dioxide during continuous stirring. Hydrogen sulfide was introduced at a slower rate than the sulfur dioxide, so that sulfur dioxide was in excess at all times. During two hours of treatment the solution became viscous and the temperature rose from 20° C to 30° C. When this temperature was reached, the sulfur dioxide was shut off and only hydrogen sulfide was kept running until the hydrogen sulfide was in excess and the reaction temperature began to drop. The reaction mixture was corked, allowed to stand over night, and degassed the next day from the excess of hydrogen sulfide by a vacuum aspirator for eight hours.

The degassed reaction mixture was placed in a centrifuge and spun for 5 minutes at 4000 rev/min, which separated the mixture into a paste and liquid phase. The paste was divided into two parts. One part was diluted with water to 250 ml and used in the test as fraction A. The other part was dialyzed. The residue after dialysis was diluted to 250 ml and used as fraction A_1 . The dialysate occupied a volume of 400 ml and was used as fraction A_2 . The liquid phase which was separated from the paste was also dialyzed and furnished a liquid residue which was diluted to 250 ml and was used as fraction B_1 . The liquid dialysate occupied 400 ml and was used as fraction B_2 .

Analysis of the different fractions.—Several samples of 5 ml each of A, A_1 , A_2 , B_1 , and B_2 were analyzed for (1) total sulfur as BaSO_4 , (2) free sulfuric acid as BaSO_4 , and (3) active sulfur admixed with colloidal sulfur and determined by the difference of (1) and (2).

Total sulfur determination.—The analysis was based on the oxidation of sulfur and of all the sulfur compounds with concentrated nitric acid and liquid bromine. To establish the accuracy of this method, several samples of sulfur were treated with 3 ml of liquid bromine in 15 ml of concentrated nitric acid, resulting in evolution of heat in the reaction mixture. The reaction vessel was then placed on a water bath and carefully controlled to avoid bumping by vaporization of the excess bromine. When the solution was cleared of bromine, the excess nitric and

hydrobromic acids were evaporated by placing the residue in a large crucible and evaporating the excess water and volatile acids until fuming ceased. The residue represented a dilute sulfuric acid, which was precipitated down with barium chloride as in any standard sulfate determination. The accuracy of the method was determined by using sublimed sulfur; and the values obtained were within 0.5 percent. This method was used to determine the total sulfur as barium sulfate in samples of A, A₁, A₂, B₁, and B₂.

Free sulfuric acid determination.—For determination of the free sulfuric acid, the thionic acids were removed, according to the method of Mellor (1935), by precipitating them out with mercurous nitrate. Mercurous nitrate formed voluminous yellow precipitate with tetra- and penta-thionic acids. The absence of tri-thionic acid was indicated by the lack of black precipitate. The precipitation was carried out until no more yellow precipitate was formed by the addition of mercurous nitrate. Then, it was washed with distilled water on the filters until the wash fluids did not give the sulfate reaction.

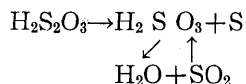
The wash fluids and filtrate were united, evaporated to a smaller volume, and then precipitated with barium chloride as in any standard sulfate determination. The barium sulfate precipitate was heated strongly in the crucible on account of the possible coprecipitation with excess of mercurous nitrate, which evaporates by decomposition.

Colloidal and active sulfur determination.—By subtracting the weight of free sulfuric acid as barium sulfate from the weight of the total sulfur as barium sulfate, the weight of colloidal and active sulfur as barium sulfate was obtained. As seen in table 1, column 1 indicates the total volume of the fraction; column 2, the volume analyzed; column 3, total sulfur content expressed as BaSO₄; column 4, the free sulfuric acid as BaSO₄; and column 5, the colloid and active sulfur which contains the poly-thionic acids obtained by the difference between columns 3 and 4.

Toxicity tests.—The molds for the toxicity tests were isolated from ailing silage by standard methods and grown on Sabouraud's agar media. The mold colonies appeared between the 24 and 48 hour period. The number of colonies is taken as a measure of the effectiveness of the additive agents. The test results in table 2 indicate: (1) that the most active fractions were A and A₁ which should contain the most toxic tetra- and penta-thionic acid mixture. This assumption was based on Williams and Young's finding (1929) which stated that di-thionic acid was slightly more toxic than sulfuric acid and tri-thionic less toxic than the mixture of tetra- and penta-thionic acids; (2) that the toxicity of the fractions reduces with a decrease in concentration; (3) that the active components of A did not readily pass through the membranes by dialysis as shown in fraction A₁; (4) that the dialysate in A₂ is not active; and (5) that the high sulfuric acid content which is observed in fractions B₁ and B₂ did not contribute much to the toxicity, but possibly acted as a peptizing agent for the formation of colloidal sulfur.

(B) The Actions of Thiosulfate Solutions

Roach and Glyne (1928) attributed the toxic effect caused by an acidified solution of sodium thiosulfate to the free thiosulfuric acid. This theory was opposed by Williams and Young (1929) because of the short life of the free acid. It decomposes within a few minutes according to the equation:



from which again penta-thionic acid is formed according to Riesenfeld and co-workers (1928) by the freshly formed colloidal sulfur.

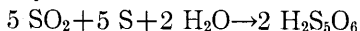


TABLE 1
Analytical data on fractions A, A₁, A₂, B₁, and B₂

Total volume of fraction	Volume analyzed (in ml)	Total S as BaSO ₄	Free H ₂ SO ₄ as BaSO ₄	Colloid S and active S as BaSO ₄	pH of the fraction
Fraction A 250 ml	5	.4470	.0102	.4368	pH 1.5
	1	.0894	.0020	.0874	
	0.5	.0447	.0010	.0437	
	0.25	.0224	.0005	.0219	
	0.1	.0089	.0002	.0087	
Fraction A ₁ 250 ml	5	.4344	.0043	.4301	pH 3
	1	.0869	.0009	.0860	
	0.5	.0435	.0005	.0430	
	0.25	.0218	.0003	.0215	
	0.1	.0087	.0001	.0086	
Fraction A ₂ 400 ml	5	.0130	.0058	.0072	pH 3
	1	.0026	.0012	.0014	
	0.5	.0013	.0006	.0007	
	0.25	.0007	.0003	.0004	
	0.1	.0003	.0002	.0001	
Fraction B ₁ 250 ml	5	1.1749	.3621	.8128	pH 1
	1	.2350	.0724	.1626	
	0.5	.1175	.0362	.0813	
	0.25	.0588	.0181	.0407	
	0.1	.0235	.0072	.0163	
Fraction B ₂ 400 ml	5	.4129	.1318	.2811	pH 1
	1	.0826	.0263	.0562	
	0.5	.0413	.0132	.0281	
	0.25	.0206	.0066	.0140	
	0.1	.0083	.0026	.0057	

TABLE 2
Toxicity tests of the different fractions containing tetra- and penta-thionic acids on mold growth

	Volume of fraction added* (in ml)	pH of fraction	No. of colonies				pH of media after addition of fraction
			6/13 11 AM	6/14 9 AM	6/17 12 PM	6/28 12 PM	
Fraction A paste separated by centrifuge from liquid	1	1.5	0	0	0	0	5
	0.5		0	2	3	4	5
	0.25		0	1	3	4	5
	0.1		0	14	14	14	5
Fraction A ₁ residue of the paste A after dialysis	1	3	0	0	0	0	5
	0.5		0	1	6	8	5
	0.25		0	6	15	15	5
	0.1		0	14	30	30	5
Fraction A ₂ dialyzate of paste A	1	3	0	23	33	33	5
	0.5		0	23	30	30	5
	0.25		0	24	26	26	5
	0.1		0	34	34	34	5
Fraction B ₁ liquid dialyzed, left in residue	1	1	0	15	30	30	4.5
	0.5		0	15	30	30	4.5
	0.25		0	18	35	35	4.5
	0.1		0	32	34	34	4.5
Fraction B ₂ dialyzate	1	1	0	4	17	17	4
	0.5		0	11	30	30	4
	0.25		0	26	34	34	4.5
	0.1		0	38	38	38	4.5
Control	1	4.5	0	96	96	96	4.5
Blank	1	4.5	0	0	0	0	4.5

*All fractions added to 15 ml nutrient agar media.

Also the $\text{H}_2\text{S}_5\text{O}_6$ may react with sulfurous acid forming tri-thionic and thio-sulfuric acid: $\text{S}_5\text{O}_6^{2-} + 2 \text{SO}_3^{2-} \rightarrow \text{S}_3\text{O}_6^{2-} + 2 \text{S}_2\text{O}_3^{2-}$ from which the tri-thionic acid slowly decomposes with H_2O into sulfuric and thiosulfuric acids. Further, it is known that an acidic condition stabilizes the tetra- and penta-thionic acids but not the tri-thionic acids. Thus, there is a chance of accumulation of penta-thionic acid with an expected toxic effect.

TABLE 3
Toxicity tests with thiosulfate solutions*

Volume of $\text{Na}_2\text{S}_2\text{O}_3 \cdot 5 \text{H}_2\text{O}$ (in ml)	Solution I—2.96% $\text{Na}_2\text{S}_2\text{O}_3 \cdot 5 \text{H}_2\text{O}$				pH of media after addition of reagent
	6/12	No. of colonies		6/28	
4	0	26	29	no increase in colonies, but covers	5.5
2	0	28	31	"	5.0
1	0	72	72	"	5.0
0.5	0	120	120	"	5.0

Solution II—1.65% $\text{Na}_2\text{S}_2\text{O}_3 \cdot 5 \text{H}_2\text{O}$ and 0.7% lactic acid						
Volume of lactic acid (in ml)	Volume of $\text{Na}_2\text{S}_2\text{O}_3 \cdot 5 \text{H}_2\text{O}$ (in ml)	6/13	No. of colonies		6/28	pH
4	4	0	0	0	0	4.5
2	2	0	11	34	covers	4.5
1	1	0	50	50	covers	4.5
0.5	0.5	0	68	68	covers	4.5

Solution III—1.65% $\text{Na}_2\text{S}_2\text{O}_3 \cdot 5 \text{H}_2\text{O}$ and 1.4% lactic acid						
Volume of lactic acid (in ml)	Volume of $\text{Na}_2\text{S}_2\text{O}_3 \cdot 5 \text{H}_2\text{O}$ (in ml)	6/13	No. of colonies		6/28	pH
4	4	0	0	0	0	3.5
2	2	0	0	2	extended	4.0
1	1	0	48	48	covers	4.0
0.5	0.5	0	80	80	covers	4.0
Control		0	103	103	covers	5.6
Blank		0	0	0	0	5.6

*All test solutions added to 15 ml agar media.

For studying the possible toxic effect of thiosulfate solutions, three solutions were prepared: Solution I—2.96 percent sodium thiosulfate alone. Solution II—1.65 percent sodium thiosulfate and 0.7 percent lactic acid. Solution III—1.65 percent sodium thiosulfate and 1.4 percent lactic acid.

The lactic acid and sodium thiosulfate were added to 15 ml Sabouraud Agar media and mixed before inoculating with the spores of *Penicillium* and *Aspergillus* spp.

Considering the toxicity test, shown in table 3, we found that for Solution I—Sodium thiosulfate·5 H₂O in 2.96 percent solution did not show complete inhibition of the growth of the colonies at any dilution; for Solution II—Sodium thiosulfate·5 H₂O in 1.65 percent solution mixed with 0.7 percent lactic acid, did show complete inhibition at a concentration of 4 ml of 1.65 percent sodium thiosulfate and 4 ml 0.7 percent lactic acid for a period of 15 days; and for Solution III—Sodium thiosulfate·5 H₂O in 1.65 percent solution mixed with 1.4 percent lactic acid did show a complete inhibition of the growth of the colonies at a concentration of 4 ml of 1.65 percent sodium thiosulfate and 4 ml of 1.4 percent lactic acid. Using 2 ml of thiosulfate and 2 ml of lactic acid, the media kept clean for a period of 4 days, when 2 colonies were counted on the 5th day. Using Solution II under the same conditions, 34 colonies appeared on the 5th day. These results support Riesenfeld theory in the formation of penta-thionic acid. The difference obtained here is not due to a decrease in the pH, as already shown by the present authors (1958).

SUMMARY

Two types of molds, *Penicillium* spp. and *Aspergillus* spp., isolated from ailing silages, were exposed to two types of solutions. One type was a mixture of tetra- and penta-thionic acids, which was prepared and separated into fractions by centrifugation and dialysis. The most toxic active fractions did not pass readily through the membrane and were located in fractions A and A₁, which kept the media free from colonies for 15 days.

The other mixture was thiosulfuric acid which in aqueous solution forms penta-thionic acid. Three solutions were tried; one was the sodium salt as sodium thiosulfate, 2.96 percent; the second was a mixture of sodium thiosulfate, 1.65 percent, and lactic acid, 0.7 percent; and the third was a mixture of sodium thiosulfate, 1.65 percent, and lactic acid, 1.4 percent. The second and third solutions which contained the free acid, kept the media free from colonies for 15 days.

A process was developed for the preparation and analysis of the fractions.

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