[Journal of the Arkansas Academy of Science](https://scholarworks.uark.edu/jaas)

[Volume 17](https://scholarworks.uark.edu/jaas/vol17) [Article 6](https://scholarworks.uark.edu/jaas/vol17/iss1/6)

1963

Further Studies on an Antibiotic Substance Produced by Rhizopus nigricans Ehrenberg

Kenneth D. Mace
University of Arkansas, Fayetteville

Delbert Swartz
University of Arkansas, Fayetteville

FURTHER STUDIES ON AN ANTIBIOTIC SUBSTANCE PRODUCED BY RHIZOPUS NIGRICANS EHRENBERG

Kenneth D. Mace[1] and Delbert Swartz
University of Arkansas

INTRODUCTION

The antibiotic actions of fungus extracts have been known for many years. In 1877 Pasteur (4) found that the growth of **Bacillus anthracis** was inhibited by other bacteria in a contaminated culture. Ward (4) in 1899, proposed the name antibiosis for microbial antagonism. Between 1900 and 1928 the few antibiotics that were isolated were not refined sufficiently to permit tests of their efficiency on humans. Fleming (4) in 1928 made his epochal discovery of penicillin, but it was not thoroughly studied until its potential as a significant treatment for war wounds was realized. The astounding properties of penicillin served to stimulate a wide-spread search for additional substances. Many molds, both common and rare species, were tested.

Broth filtrates of **Rhizopus nigricans** were reported as having no inhibitory power (7). However, Williams (8), working in this laboratory found that filtrates derived from growing this fungus on especially enriched media yielded an inhibitory substance. His tests showed the inhibition of **Bacillus anthracis, Shigella sonnei, Micrococcus aureus, Salmonella typhosa, Salmonella shottmuelleri,** and **Corynebacterium bovis.**

Williams (8) determined that neither lactic, kojic, nor any of the phenolic acids were present in the broth filtrates. The inhibitory substance withstood boiling in air for a period of twenty minutes without appreciable decomposition. Due to lack of time he was unable to examine the details of some of the more interesting chemical and physical properties of the unknown substance.

This work was undertaken to explore further the properties of the substance and to check some of the work reported by Williams.

METHODS

The culture of **Rhizopus nigricans** used in this study was the same as the one used by Williams, and was provided by Dr. Delbert Swartz of the Department of Botany and Bacteriology, University of Arkansas.

---

[1]Graduate Teaching Assistant in Botany and Bacteriology, University of Arkansas.

The fungus was grown on V-8 agar slants at 24°C for six days to obtain spores for inoculation of the flasks of media used in the experiments. One milliliter of spore suspension was placed into the culture medium aliquoted in 125 milliliter Erlenmyer flasks or in one liter Fernbach flasks. These inoculated flasks were incubated at 24°C for seven days, either as stationary surface cultures or as shaken submerged cultures. At the end of this period dry weights of the mats and the submerged mycelial growths were determined. Submerged cultures in 125 milliliter Erlenmyer flasks were agitated on a shaking machine 216 times per minute.

The method of extraction used was similar to that reported by Williams (8), except the metabolized solutions from the broth cultures and the water extracts from the solid media cultures were evaporated in a rotating flask at 60°C. The temperature of the hot ether and hot chloroform extractions was lowered to 40°C. The dried mats were broken up after weighing and extracted with acetone in a Soxhlet apparatus. The extract obtained was then combined with the acetone extract of the evaporated metabolized solutions.

Experimental Procedures and Results

The method of testing solutions for inhibiting ability was a modification of the standard cylinder plate method of assay (5, 6). Controls consisted of uninoculated culture media to determine the possibility that they might contain some substance or substances that would be inhibitory to the test organism **Micrococcus aureus.** None of the media used in this study exhibited inhibitory activity.

The effect of agitation of broth cultures of **Rhizopus nigricans** on the growth of the organism as reflected in mat weights and also on the production of the inhibitory substance was explored. Erlenmyer flasks containing 65 milliliters of non-enriched medium 2* or enriched medium 3* were inoculated with a spore suspension of **Rhizopus nigricans** and incubated at 24° C as submerged cultures and as surface cultures for seven days. At the end of seven days dry weights of the mats were obtained, and the metabolized solution tested for inhibiting ability. Greater mat weights were produced by submerged cultures than those obtained from surface cultures (Table 1). However, the production of inhibitory substance was not increased by agitation (Table 1). Metabolized solutions from aerated cul-

*See Table 6 for formula.

tures of **Rhizopus nigricans*** grown at 24°C for seven days in Czapek Dox broth showed greater inhibition of **Micrococcus aureus** than either surface or submerged cultures (Table 1).

TABLE 1
Mat Weights and Inhibitory Activity of Metabolized Solutions from Surface, Submerged and Aerated Cultures

Type Culture	Average Mat Weight	Test Organism	Diameter of Zone of Inhibition
1 surface	0.189 gm	E. coli	21 mm
		M. aureus	26 mm
		B. mycoides	23 mm
2 surface	0.198 gm	E. coli	22 mm
		M. aureus	26 mm
		B. mycoides	24 mm
3 submerged	0.290 gm	E. coli	17 mm
		M. aureus	27 mm
		B. mycoides	24 mm
4 submerged	0.370 gm	E. coli	17 mm
		M. aureus	27 mm
		B. mycoides	24 mm
5 aerated	—	M. aureus	30 mm

Cultures 1, 3 and 5 grown in non-enriched medium 2
Cultures 2 and 4 grown in enriched medium 3

The effect of light on the growth of **Rhizopus nigricans**, as reflected in mat weights and in the production of the inhibitory substance, was studied. Clear Erlenmyer flasks containing 65 milliliters of medium 6 were inoculated with a spore suspension of **Rhizopus nigricans.** Red glass Erlenmyer flasks containing 65 milliliters of medium 6** were also inoculated with **Rhizopus nigricans.** All of these cultures were incubated seven days at 24°C under a fluorescent lamp. Cultures in clear glass flasks and red glass flasks were wrapped in brown paper and incubated in the dark for seven days at 24°C. Results of these experiments as given in Table 2 show mat weights from cultures grown in the light were less than mat weights from cultures grown in the dark. The same table shows, when tested against **Micrococcus aureus,** zones of inhibition from metabolized solutions taken from cultures grown in the dark were 50% larger than zones of inhibition from metabolized solutions taken from cultures grown in the light. Although the

*Metabolic solution obtained from L. R. Delaney of the Department of Botany and Bacteriology, University of Arkansas.
**See Table 6 for formula.

growth of **Rhizopus nigricans** was slightly inhibited by light after passing through the red glass flask as compared to growth of cultures exposed to light in clear glass flasks, the production of inhibitory substance, as reflected in diameter of zones of inhibition, remained approximately the same.

TABLE 2
Mat Weights and Inhibiting Ability of Metabolized Solutions from Cultures Grown in Different Light Conditions

Culture Number	Average Weight	ph of Solution	Diameter of Zone of Inhibition
1	1.095 gm	2.5	30 mm
2	1.045 gm	2.5	31 mm
3	0.341 gm	2.6	20 mm
4	0.408 gm	2.6	18 mm

Culture 1—Clear glass flasks in absence of light.
Culture 2—Red glass flasks in absence of light.
Culture 3—Red glass flasks in presence of light.
Culture 4—Clear glass flasks in presence of light.

Metabolized solutions taken from media used in this paper were tested in order to determine if enriched media would increase the production of the inhibitory substance. The results of this study as given in Table 3 show that enriched medium 6 does increase the production of the inhibitory substance as reflected in the size of the zones of inhibition.

Dialysis of the inhibitory substance was made by placing 100 milliliters of the metabolized solutions from cultures of **Rhizopus nigricans** in a cellophane dialysis bag and suspending the bag with its contents in a cylinder containing 500 milliliters of distilled water. The solutions were kept at room temperature. Tests for inhibiting ability of the solution inside the bag and from the surrounding distilled water were made at 24 hours and 48 hours. At the end of 24 hours no inhibition was obtained from the distilled water surrounding the dialysis bag.

TABLE 3
Influence of Medium on Production of Inhibitory Substance by Rhizopus nigricans against Micrococcus aureus

Medium Number	Diameter of Zone of Inhibition
1	15 mm
2	27 mm
3	26 mm
4	none
5	20 mm
6	30 mm
7	25 mm

However, at the end of 48 hours zones of inhibition averaging 22 mm were obtained from the solution surrounding the dialysis bag showing the inhibitory substance had dialyzed.

The electrical charge of the inhibitory substance was tested by taking 100 milliliter samples of metabolized solutions from cultures of **Rhizopus nigricans** and passing these through cation resin exchange columns and anion exchange columns. Twentyfive milliliter samples were collected after passing through the columns and evaporated to 10 milliliters and tested for inhibitory ability. The solutions that passed through the cation exchange columns exhibited inhibiting ability, and those that passed through the anion exchange column did not. This indicated the inhibitor substance possessed a negative charge.

The crystals obtained from extracts of metabolized solutions were of two types. The first was crystallized from the cold ether extract of the solutions and was a small rectangular shape with a clear white color, slightly translucent. The second crystal was recovered from the hot chloroform extract and had long needle shapes with yellow coloring.

Metabolized solutions from cultures of **Rhizopus nigricans** and solutions of crystalline inhibitory substances were adjusted to ph values of 4.0 to 8.0. The results as given in table 4 show no reduction in inhibitory ability over the ph range tested.

Fumaric, indol acetic, oleic, succinic, L-glutamic, alpha-ketoglutaric and malic acids were tested for inhibiting ability. The results showed, that although the ph of the acid solutions tested were similar to those obtained from metabolized and crystalline solutions, there was no inhibition exhibited.

Results of tests as given in table 5 showed the inhibiting substances are unstable as a calcium or sodium salt at ph 7.0 in a physiological saline solution and can not be stored in this form. However, in an aqueous solution with a ph of 2.0 to 3.0, or as a dry calcium or sodium salt the substances can be stored for a period of several weeks without impairing their inhibiting ability. This seems to be a function of the ph of the solutions rather than an effect of the physiological saline.

The inhibiting ability of 0.2% aqueous solutions of the two types of crystals obtained from extracts of metabolized solutions was shown to be equal when tested against **Micrococcus aureus** (Plates 1,2,3).

The white crystalline substance had a melting point of 94-95°C. The yellow crystalline substance had a melting point of 86-87°C. Both substances, using standard tests (1,2,3), gave negative results for the presence of peptide bonds, presence of a benzene ring, presence of tyrosine, phenylalanine, tryptophane,

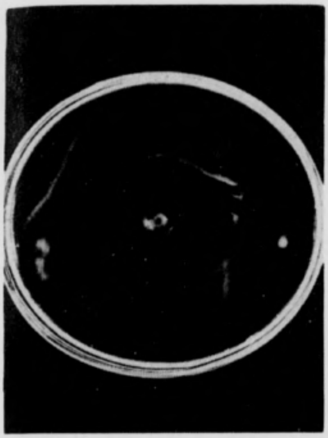

Plate No. 1
Zone of Inhibition Produced by a Metabolized Solution from a Culture of Rhizopus nigricans
(Test Organism-Micrococcus aureus)

Plate No. 2
Zone of Inhibition Produced by a 0.2 % Solution of White Crystalline Substance
(Test Organism - Micrococcus aureus)

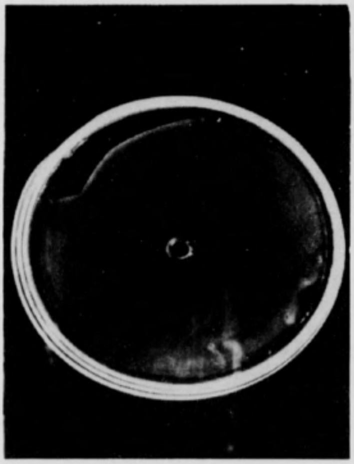

Plate No. 3
Zone of Inhibition Produced by a 0.2 % Solution of Yellow Crystalline Substance
(Test Organism - Micrococcus aureus)

presence of peptides, presence of reducing sugars and the presence of primary and secondary amines. Both substances gave positive tests for carbohydrates and the presence of divalent sulfur.

Both substances were soluble in water, acetone, and hot chloroform. The white crystalline material was also soluble in cold and hot ethyl ether, while the yellow crystalline substance was not soluble in either of these solvents.

Preliminary results indicated that the inhibitory substances were not toxic to animals when administered to living animals in physiological saline solution with the pH adjusted to 7.0.

SUMMARY and CONCLUSIONS

The existence of an inhibitory substance produced by **Rhizopus nigricans** Ehrenberg was confirmed. Two inhibiting substances were recovered.

It was shown that an enrichment medium containing an extract of **Maclura pomifera** fruit, 5% dextrose, and neopeptone increased the production of the inhibitory substances to some degree. Agitation of the type used in this paper did not stimulate the production of the inhibitory substances. Light whether from a fluorescent lamp or after passing through red glass of an Erlenmyer flask had an inhibiting effect on the production of the inhibitory substances. At the present time it is not known what action the light has on the cultures to give the adverse effect.

The two inhibitory substances were found to be similar in several ways:

1. Neither substance was colloidal.
2. They both possessed negative valences.
3. The inhibitory activity of the substances was not due to pH.
4. Both gave positive tests for the presence of carbohydrates and SH groups.
5. Both of the substances were soluble in water, acetone, and chloroform.

The substances differed in that the yellow crystalline material was not soluble in ethyl ether.

Preliminary experiments indicate the substances are not toxic to animals, however more work is needed in this area before positive conclusions can be made.

TABLE 4
Inhibiting Ability of pH Adjusted Metabolized and Crystalline Solutions

Type of Solution	pH	Diameter of Zone of Inhibition
Metabolized* Solution	5.5	20 mm
Metabolized* Solution	7.0	21 mm
White Crystals	4.0	25 mm
Yellow Crystals**	7.0	22 mm
Mixture of*** Crystals	7.0	31 mm
Metabolized** Solution	7.1	25 mm
Metabolized** Solution	7.2	23 mm
Metabolized** Solution	8.0	24 mm
Normal Saline	6.9	none

Test organism—**Micrococcus aureus**
*NaOH used to adjust pH, solution in distilled water.
**Na_2CO_3 used to adjust pH, solution in normal saline.
***$CaCO_3$ used to adjust pH, solution in normal saline.

TABLE 5
Effect of Storage on Inhibiting Ability of Salt Forms

Type of Stored Material	pH of Solution Tested	Time of Storage 6 hrs.	12 hrs.	24 hrs.	1 wk.	3 wks.
Aqueous sol. yellow cryst.	3.0	+	+	+	+	+
Aqueous sol. white cryst.	2.0	+	+	+	+	+
Na salt of yellow cryst. in phys. saline	7.0	+	—	—	—	—
Ca salt of yellow cryst. in phys. saline	7.0	+	—	—	—	—
Na salt of white cryst. in phys. saline	7.0	+	—	—	—	—
Ca salt of white cryst. in phys. saline	7.0	+	—	—	—	—
Yellow cryst. extract (dry)	3.0	+	+	+	+	+
White cryst. extract (dry)	2.0	+	+	+	+	+
Na salt of yellow cryst. (dry)	7.0	+	+	+	+	+
Ca salt of yellow cryst. (dry)	7.0	+	+	+	+	+
Na salt of white cryst. (dry)	7.0	+	+	+	+	+
Ca salt of white cryst. (dry)	7.0	+	+	+	+	+

+: Solution tested exhibited inhibiting ability.
—: Solution tested did not exhibit inhibiting ability.

TABLE 6

List of Media Used in This Paper

Medium Number	pH	Ingredients	
1	5.5	Water extract of 200 grams of Bell Pepper	
		Bacto agar	20 grams
		Dextrose	40 grams
		Distilled water	1000 grams
2	7.4	Czapek Dox Broth (Stock)	
		Bacto agar	20 grams
		Distilled water	1000 grams
3	5.75	Water extract of cortex of Maclura pomifera root	95 grams
		Dextrose	40 grams
		Distilled water	1000 grams
4	5.5	Water extract of 200 grams of Bell Pepper	
		Distilled water	1000 grams
5	5.5	Water extract of 95 grams of cortex of Maclura pomifera root	
		Dextrose	50 grams
		Distilled water	1000 grams
6	4.5	Water extract of 200 grams of Maclura pomifera fruit	
		Neopeptone	20 grams
		Dextrose	50 grams
		Distilled water	1000 grams
7	4.5	Neopeptone	10 grams
		Dextrose	50 grams
		Distilled water	1000 grams

BIBLIOGRAPHY

1. Anderson, Arthur K., Essentials of Physiological Chemistry 4th Edition, John Wiley & Sons Inc., New York, Chapman & Hall, Limited, London, 1953.
2. Anderson, Arthur K., Laboratory Experiments in Physiological Chemistry, 2nd. Edition, John Wiley & Sons Inc., New York, 1954.
3. Feigle, Fritz, Spot Tests, Vol. II, Organic Applications, Elsevier Publishing Co., New York, 1954.
4. Gray, George W., The Antibiotics, Scientific American, Vol. 181, No. 2, pp. 26-35, Aug. 1949.
5. Grove, Donald C., Assay Methods of Antibiotics, N. Y. Medical Encyclopedia, Inc., New York, 1955.
6. Prescott, Samuel C., Dunn, Cecil G., Industrial Microbiology, 2nd Edition, McGraw-Hill Book Co., Inc. pp. 708-797, 1949.
7. Wilkins, W. H., Harris, G.M.C., Investigations into the Production of Bacteriostatic Substances by Fungi; Preliminary Examination of 100 Fungal Species, Brit. J. Expt. Path., Vol. 23, 1942.
8. Williams, B. J., An Antibiotic Substance Produced by **Rhizopus nigricans** Ehrenberg, M. S. Thesis, University of Arkansas Library, 1952.