THE ENZYMS OF SPHAEROPSIS MALORUM AND SCLEROTINIA CINEREA.

by

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INTRODUCTION.

This problem was undertaken at the suggestion of Dr. A. J. Mix of the University of Kansas. Such an investigation is justified by the fact that a large part of the work concerning the physiological relations of fungi has been carried out with such common, saprophytic forms as Penicillium and Aspergillus. In vestigators have found the various species of these genera to produce a large variety of enzyms. Dox (11), who worked particularly on Penicillium camemberti, reports erepsin, nuclease, amidase, lipase, emulsin, amylase, inulase, raffinade, sucrase, maltase, and lactase. The enzyms of Aspergillus terricola were investigated by Scales (9). He found that it produced inulase, diastase, invertase, maltase, alcoholoxydase, emulsin, lipase, protease, and amidase. Such investigations, although interesting and instructive to a certain extent, offer little direct contribution to the knowledge of the physiology of parasitism. The need of work in this very important field is being realized, as is indicated by the fact that much of the most recent work is done on parasitic forms of economic importance. For example, Harter and Weimar (1,2) have carried out several investigations on the parasitism of Rhizopus tritici in relation to decay of sweet potatoes. They demonstrated the presence of pectinase and amylase and worked out some of the conditions under which these enzyms act. Willaman (4) worked on the pectinase of Sclerotinia cinerea; Culpepper, Foster, and Caldwell (5) made

chemical analyses of apples affected with black rot as compared with healthy apples; Hesler (6) made an exhaustive study of the black rot disease; William Brown (8) investigated the parasitism of Botrytis cinerea.

In these studies, the chief line of investigation concerns the enzyms produced by Sphaeropsis malorum, Peck (the imperfect stage of Physalospora cydoniae, Arnaud.), and Sclerotinia cinerea, Bonorden. These two fungi are apparently responsible for most of the decay of apples in northeastern Kansas, in which the fruit remains attached to the tree. Sphaeropsis malorum, the cause of black rot, attacks both green and ripe fruit. Hesler (6) says in describing the rot, " The skin at first becomes brown in a small area, but later darkens, finally turning black. On green fruit the affected part may turn black before enlarging to any extent, whereas on fruit that is ripe or ripening, the whole may be involved before it darkens appreciably. Often concentric bands of uniform breadth and of slightly different shades of color appear about the center of the lesion. The affected area is distinct from the healthy part, and the diseased tissues are not of unpleasant taste as in many fruit decays. Later stages in the development of the rot show a shriveled and much wrinkled surface, which typically becomes covered with black pustules. These characters may be assumed within a month or in less time. Ultimately a dry mummy is produced, which may hang to the tree for a year or more. "

The mycelium is probably entirely intercellular. (6).

It would consequently be expected that the production of pectinase was essential to the life of the fungus. Invertase, proteolytic enzyms, and other enzyms to digest the cell contents would be expected, and since green fruits are attacked, it would seem probable that amylase was produced.

Hesler (6) states that the brown rot disease, caused by Sclerotinia cinerea, produces a smooth, coal black, and shiny mummy, which is not so wrinkled as the black rot mummy, although the two diseases are sometimes confused. The mycelium of Sclerotinia cinerea is intercellular also, and would be expected to produce the same types of enzyms as Sphaeropsis malorum. However, since it attacks, so far as known, only ripe fruits, it would seem to have little need for amylase.

EXPERIMENTAL METHODS.

The strains of Sphaeropsis malorum and Sclerotinia cinerea which were used in these studies were isolated from rotted apples found in the vicinity of Lawrence, Kansas. In order to insure pure cultures, single spore strains were isolated. Dilution plates of the pycnospores of Sphaeropsis malorum and of the conidia of Sclerotinia cinerea were made, using agar which had been cleared with white of egg. These plates were then examined, inverted, under the low power of the microscope, and single spores alone in the field marked with a dot of India ink. When the colonies became large enough, they were transferred to agar slants. In the case of the Sclerotinia the conidia were very difficult to locate and colonies were picked which apparently came from single spores. The

pure cultures thus obtained were grown on potato-dextrose agar and transferred about every four weeks.

The mycelium for the enzym tests was grown in 1-liter Erlenmeyer flasks containing 200 or 250cc. of medium. At first modified Czapek's nutrient solution plus 1% dextrose was used, but it was found that both fungi grew much better on potato-dextrose broth. When the mycelium had formed a mat over the surface of the broth, it was filtered through cotton and the mycelium treated by the "acetonedauerhefe" method. The mat was first washed in running water 15 minutes, then squeezed dry and treated with an excess of acetone for 10 minutes. During this time the mycelium was constantly kept in motion and picked apart. It was then squeezed dry and acetone added for 2 minutes. After again being squeezed, ether was added for 3 minutes, after which the mycelium was spread on filter paper to dry. When dried, it was ground in a mortar with an equal amount of fine quartz sand and stored in small flasks at 9 C. The filtrate was used immediately for making tests.

EXPERIMENTATION.

Amylase.

Culpepper, Foster, and Caldwell (5) state that Sphaeropsis malorum had no effect upon starch, the starch content of sound and diseased apples being the same. They also maintain that the starch grains of the apple were not eroded and extracts of pure cultures of the fungus had no effect on corn and apple starch. Hawkins (7) found that the starch

content of healthy potatoes and potatoes rotted with Fusarium oxysporum was the same, and that the starch grains were not eroded. However, extracts of the mycelium readily digested soluble starch and starch paste. The results of these investigations failed to confirm the complete absence of amylase in Sphaeropsis malorum, but it has not yet been possible to obtain green apples to test the effect of the fungus on apple starch. (However such an experiment is now in progress.)

Sphaeropsis malorum and Sclerotinia cinerea were grown upon modified Czapek's nutrient solution plus 1% dextrose, also 1% starch and 1% soluble starch. Both fungi grew upon the starch media, although the Sphaeropsis produced a much better growth than the Sclerotinia. The dextrose medium was more favorable for growth in both cases. At the end of two weeks the solution from one flask of each was tested for starch with iodine and for reducing sugar with Fehling's solution. The starch solutions upon which both fungi grew gave a negative starch test with iodine and a red precipitate with Fehling's, showing that all of the starch had been converted into reducing sugar.

The two fungi were also grown on starch agar plates. The agar was prepared with 1½% agar and ½% starch. After 3 days the Sphaeropsis colonies were fairly good-sized and two plates were tested with Gram's iodine. The area occupied by the colony was light brown extending slightly beyond the growth of the fungus. This area was surrounded by a light purple cir-

cle gradually blending into the deep blue of the rest of the plate. The Sclerotinia grew only very slightly and no evidence of the action of amylase could be detected.

For the purpose of testing the action of the dried mycelium and the solution, the two fungi were grown on nutrient solution plus 1% starch, and also plus 1% dextrose. The flasks of Sclerotinia all became contaminated and could not be used. The cultures were 24 days old when employed for tests. Fifteen cc. of the solutions upon which the Sphaeropsis had been growing were added to 15cc. of 1% starch paste. Starch paste and water was used for a control. Toluene was added as an antiseptic and the flasks incubated at 26° C. Five cc. samples were tested at intervals with iodine. After a little over 18 hrs., the solution which had contained starch had affected complete conversion of the starch, while the dextrose solution gave a purple color with iodine, in contrast to the deep blue of starch and water. In testing the dried mycelium, .25gm. of the powdered mycelium in 25cc. of distilled water were added to 25cc. of 1% starch paste with toluene for antiseptic. At the end of 50hrs. the mycelium grown on starch had produced complete conversion of the starch. The mycelium grown on dextrose still produced a purple color with iodine, indicating only partial conversion, after 197 hrs. of incubation.

The experiment was repeated in order to obtain results for Sclerotinia cinerea. The starch solution upon which the fungus had been grown had absolutely no effect upon starch

paste even after 48 hrs. The solution itself was then tested with iodine, revealing the fact that the starch in it was far from exhausted. Consequently a test of its action upon starch paste could be of little value. The dextrose solution brought about partial conversion of the starch after 119 hrs. However it progressed no farther even up to 287 hrs. The mycelium grown on dextrose produced partial conversion of starch in 167 hrs., but no further change was brought about up to 213 hrs.

It is evident that Sphaeropsis malorum produces amylase which digests potato starch. The enzym is both intra- and extracellular. The results would seem to indicate that the enzym is largely extracellular, but there is no way to determine whether or not the amounts of powdered mycelium and culture solution used in the tests are comparable. The results on Sclerotinia cinerea are not so complete and conclusive but it seems that it produces amylase in rather small quantities, even when grown on starch-free medium.

Pectinase.

In testing for pectinase, the effect of the powdered mycelium and the solution upon which the fungi were grown, upon apple disks, was determined after the method used by Harter and Weimar (2). The mycelium was grown on potatp dextrose broth and was between two and three weeks old when tested. Twenty-five cc. of the solution were put in small Erlenmeyer flasks and 5 disks of Jonathon apple added. The disks were obtained by cutting a cylindrical plug from the

apple with a 12 mm. cork borer. Disks 1 mm. thick were then cut with a razor blade. Steamed solution was used as a control. Toluene was added and the flasks incubated at 26 C. After 72 hrs the Sphaeropsis solution had brought about complete maceration of the disks, that is, theyoffered no resistance to pull. The Sclerotinia solution produced maceration in 18 hrs. Five apple disks were also placed in 25cc. of distilled water containing .25 gm. of powdered mycelium. Five disks in distilled water were used for a control. Both fungi brought about complete maceration in 24 hrs. The disks in water also appeared to be somewhat macerated.

The maceration test was repeated using disks of Jonathon apple, Winesap apple, potato, sweet potato, and carrot, in an attempt to determine whether the pectinase produced was specific. Five disks of each were added to 25cc. of the solution of Sphaeropsis malorum and Sclerotinia cinerea. Disks in water, and solution upon which no fungus had grown were used as controls. The solution of neither fungus had any effect upon the potato, sweet potato, or carrot up to 14 days. The Sclerotinia solution brought about maceration of the Jonathon and Winesap disks in 14 hrs., but in the case of the Winesap, both controls also showed maceration. The Sphaeropsis solution gave similar results, except that no maceration occurred in the Jonathon disks, and maceration of the Winesap disks was brought about in 24 hrs. The Winesap controls were also macerated. The dried mycelium caused slight maceration of the Jonathon disks after 48 hrs. in both cases. The

Winesap disks became completely macerated in experiment and c controls. The vegetable disks were unaffected after a week.

The results obtained with the Winesap apples may have been due to the very ripe condition of the apples used, the experiment having been carried out in March. However, it was decided that the maceration test was not satisfactory for apple tissue and the method used by Paton (3) was employed. Pectin was prepared from the white inner skin of a grape fruit in the following manner: the white was removed, boiled in water and put through a meat chopper; after standing 24 hrs. in cold water, it was boiled from ½ to 1 hr. and strained through cheesecloth; it was then filtered, concentrated over a water bath and the pectin precipitated with absolute alcohol and dried in an oven.

For tests .25 gms. of powdered mycelium in 25 cc. of distilled water were added to 5cc. of pectin solution (.30 gm. in 25 cc. of water) in small Erlenmeyer flasks. Boiled mycelium and water plus 5 cc. pectin solution were used as controls For the solution tests, it was necessary to allow the fungi to grow until the dextrose in the medium was exhausted. This was determined by extracting a few cc. of solution by means of a sterile pipette and testing it with Fehling's solution. For the tests 15 cc. of solution were added to 15 cc. of pectin solution in small flasks. Boiled solution and water plus pectin solution were used for controls. Toluene was added to each flask and the flasks incubated at 35° C. It was thought that this temperature was probably closer to the

optimum than 26° C. Three cc. samples were tested with Fehling's. The tubes were heated in a water-bath about ½ hr. and the precipitates filtered onto oven-dried, weighed filter paper. The papers were washed with hot water to remove the Fehling's solution, dried in an oven and reweighed.

The results are given in the following table:

1. Test on dried mycelium:

	Wt. of paper.	Wt. paper & ppt.	Gain.
Sphaeropsis malorum-	.5472 gm.	.5544 gm.	.0672 gm.
Boiled mycelium	.5708 "	.5748 "	.0040 "
Sclerotinia cinerea-	.4488 "	.4562 "	.0074 "
Boiled mycelium	.4680 "	.4732 "	.0052 "
Water	.5536 "	.5574 "	.0038 "

2. Test of solution:

	Wt. of paper.	Wt. paper & ppt.	Gain.
Sphaeropsis malorum-	.4251 gm.	.4322 gm.	.0071gm.
Boiled solution	.5220 "	.5278 "	.0058 "
Sclerotinia cinerea-	.4536 "	.4578 "	.0042 "
Boiled solution	.4596 "	.4632 "	.0036 "
Water	.4192 "	.4220 "	.0028 "

The gain in water is probably due to the fact that a slight amount of Fehling's solution remained in the filter papers.

Both fungi evidently produce pectinase which is intra- and extracellular.

Invertase.

In testing for invertase, .25 gm. of the mycelium of Sphaeropsis malorum and Sclerotinia cinerea were put in small

flasks with 10 cc. of water and 10 cc. of 2% cane sugar. Boiled mycelium and water plus 2% cane sugar were used for controls. Toluene was added and the flasks incubated at 26°C. Three cc. from each flask were tested with three cc. of Fehling's solution, after 24 hrs. The tubes were heated in a water bath ½ hr. The Sphaeropsis and Sclerotinia both gave a red-brown precipitate. The boiled mycelium gave a very slight precipitate and the water none.

Solution from which the dextrose had been exhausted was also tested. Fifteen cc. of solution and 15 cc. of 2% cane sugar were placed in a small flask. As usual, boiled mycelium and water plus 2% cane sugar were set up for controls. Three cc. from each flask were tested with Fehling's and the amount of precipitate determined as in the case of pectinase.

The results are as follows:

	Wt. of paper.	Wt. paper & ppt.	Gain.
Sphaeropsis malorum	.4218 gm.	.4362 gm.	.0144 gm.
Boiled solution	.4999 "	.5121 "	.0022 "
Sclerotinia cinerea	.4732 "	.4808 "	.0076 "
Boiled solution	.5076 "	.5134 "	.0058 "
Water	.5492 "	.5516 "	.0024 "

The above results indicate that Sphaeropsis malorum and Sclerotinia produce a very active intra- and extracellular invertase

Cellulase.

The cellulose used in testing for the enxym cellulase was obtained by dissolving filter paper in cupra-ammonia. This solution is produced by adding copper filings to concentrated ammonia. After the cellulose was dissolved, it was obtained in a pure state according to the method employed by Paton (3). The liquid containing the dissolved filter paper was poured into dilute hydrochloric acid (1:5) to precipitate the cellulose. The precipitate was filtered on a Buchner funnel by suction and washed with water until the filtrate showed no sign of HCl when tested with $AgNO_3$. The cellulose was then boiled with distilled water to form a suspension. Ten cc. of this solution were added to 30 cc. of distilled water containing .25 gm. of powdered mycelium, to the same amount of boiled mycelium and to distilled water alone. For the solution tests, 15 cc. of solution and 15 cc. of cellulose suspension were used. The usual controls were set up. Toluene was added and the flasks incubated at 35°C. Three cc. from each flask were tested with Fehling's solution, thetubes being heated in a water bath about ½ hr. The amount of precipitate was determined as for pectinase.

The results appear in the following table:

	Wt. of paper.	Wt. paper & ppt.	Gain.
1. Test of mycelium:			
Sphaeropsis malorum-	.4999 gm.	.5056 gm.	.0057 gm.
Boiled mycelium ---	.5668 "	.5708 "	.0040 "
Sclerotinia einerea-	.5691 "	.5736 "	.0045 "
Boiled mycelium ----	.4499 "	.4526 "	.0027 "
Water ----------	.5180 "	.5210 "	.0030 "

2. Teat of solution: Wt. of paper. Wt. paper & ppt. Gain.

Sphaeropsis malorum-.5115 gm. -----.5178 gm. ----.0057 gm.

Boiled solution ----.4194 " . -----.4238 " . ----.0044 " .

Sclerotinia cinerea-.5458 " . -----.5480 " . ----.0022 " .

Boiled solution ----.5352 "..------.5384 " .----.0032 " .

Water ----------.4949 " . -----.4996 " . ----.0047 " .

From the foregoing tables, it would appear that Sphaeropsis malorum produces cellulase in very small amounts. It seems doubtful that Sclerotinia cinerea produces the enzym.

Erepsin.

Reed and Stahl (10) found that Sphaeropsis malorum produced tryptophane from Witte's peptone, when grown on Dunham's solution. The action of the dried mycelium of both fungi on a 1/10% solution of Witte's peptone was tested; also the effect of the solution upon which the fungi had been growing. No trace of tryptophane could be detected even after a week's incubation. The incubation was carried on at both 26°C. and 35°C. Small flasks containing Czapek's nutrient solution with Witte's peptone as the only source of Nitrogen, and flasks containing Dunham's solution were then prepared. Five of each were inoculated with Sphaeropsis malorum and with Sclerotinia cinerea. The Sclerotinia grew only in one of the flasks containing nutrient solution plus peptone. At the end of three weeks 5 cc. of solution from this flask were tested for tryptophane by adding a few drops of acetic acid, then bromine water drop by drop. A faint pink color was produced, showing that tryptpphane was present. The solution upon which the Sphaerop-

sis was growing had become a dark brown color, and consequently could not be used. Three flasks of Dunham's solution were therefore inoculated with Sphaeropsis malorum and 5 cc. of solution from one of them tested at the end of a week. A pink color was produced. The controls, solution upon which no fungus grew, remained colorless in both cases.

Evidently the two fungi produce erepsin, and it may be that the reason the dried mycelium and solution had no effect upon peptone is that the enzym is not produced, or is produced in very small quantities when the fungi are grown on a peptone-free medium.

Reductase.

The ability of the mycelium and the solution to reduce methylene blue was tested by adding 1 cc. of methylene blue solution to 15 cc. of water and .25 gm. of powdered mycelium in a test tube; the same amount was also added to 10 cc. of solution plus 10 cc. of water. Controls were set up as usual. Toluene was added and the tubes incubated at 35°C.

In 24 hrs. the results were as follows:

1. Test of mycelium:

Sphaeropsis malorum -- complete reduction.

Boiled mycelium ------ partial reduction.

Sclerotinia cinerea - partial reduction.

Boiled mycelium ------ no reduction.

Water ---------- no reduction.

2. Test of solution

2. Test of solution:

Sphaeropsis malorum -- no reduction.
Boiled solution ------ " " .
Sclerotinia cinerea -- " " .
Boiled solution ------ " " .
Water ---------- " " .

The above results indicate that both Sphaeropsis malorum and Sclerotinia cinerea produce reductase which is entirely intracellular. It appears that the former produces the enzym in larger quantities than the latter.

Catalase.

Catalase was tested for by determining the action of powdered mycelium and solution on H_2O_2. Ten cc. of distilled water containing .25 gm. of mycelium were placed in a test tube. The tube was then filled with hydrogen peroxide diluted with an equal amount of water, and inverted into a small bottle filled with water. Ten cc. of solution were also set up in the same manner, as well as the usual controls. The tubes were left at laboratory temperature.

In 24 hrs. the results were as follows:

1. Test of mycelium:

Sphaeropsis malorum -- $\frac{1}{2}$ tube-full of gas.
Boiled mycelium ------ slight amount of gas.
Sclerotinia cinerea --- $\frac{1}{2}$ tube-full of gas.
Boiled mycelium ------ no gas.
Water ---------- no gas.

2. Test on solution:

Sphaeropsis malorum -- no gas.

Boiled solution ------ " " .

Sclerotinia cinerea -- " " .

Boiled solution ------ " " .

Water ---------- " " .

Upon applying the glowing splinter test, the gas produced was found to be oxygen. Evidently both these fungi produce an intracellular catalase.

Zymase.

Culpepper, Foster, and Caldwell (5) found that the alcohol content of apples rotted by Sphaeropsis malorum was much greater than that of healthy apples. However, this is not necessarily an indication of the production of zymase by Sphaeropsis malorum and it was attempted to discover whether alcohol was produced when the fungus was grown on artificial media containing dextrose. Half a dozen fermentation tubes containing potato-dextrose broth (10% dextrose) were inoculated with Sphaeropsis malorum and the same number with Sclerotinia cinerea. Several tubes without fungus growth were used as controls. After 11 days the contents of one tube of each were filtered and 5 cc. of the filtrate tested for alcohol by the iodoform test. An equal amount of iodine in potassium iodide was added, then just enough caustic potash to decolorize the iodine. If, upon gently warming, the odor of iodoform is recognizable and a yellow precipitate is formed, alcohol is present. It was thought that a slight odor of iodo-

form could be detected in the case of Sphaeropsis malorum, but no precipitate occurred. Subsequent tests were made but the odor did become strong enough for positive recognition. The results of this experiment were considered inclusive.

CONCLUSION.

From the results of the preceding experiments, the following conclusions seem justified:

(1). Sphaeropsis malorum produces intra- and extracellular amylase which digests potato starch. The amount of amylase is increased by growing the fungus on a starch medium. Sclerotinia cinerea also produces amylase but only to a slight extent.

(2). Sphaeropsis malorum and Sclerotinia cinerea both produce pectinase which is intra- and extracellular.

(3). A very active invertase is produced by both fungi.

(4). Cellulase is produced in very small quantities, if at all, by Sphaeropsis malorum, and probably Sclerotinia cinerea does not produce this enzym.

(5). Erepsin is produced by both fungi.

(6). Both fungi produce reductase and catalase which is entirely intracellular.

(7). It is very doubtful that either fungus produces zymase.

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