Enzymatic Activity of Xylaria digitata

And

The Cultivation of Penicillium pinophilium in Nutrient Salt Solutions.

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Enzyme Activity of Xylaria Digitata.

This paper is a report of the results of an experimental study of the engymatic activities of the fungus Xylaria digitata which causes the black root rot of the apple tree.

A pure culture of the fungus was secured by transfering a portion of a culture, previously isolated, to a petri dish allowing the fungus to develope and then transfering a portion of the outer edge of the growth to a tube of agar. The growth was examined at times and when no contamination developed it was then ready for culturing in sawdust.

Sawdust of an apple tree was placed in seven Erhlenmyer flasks of 250 cc. capacity. The sawdust was then moistened with distilled water and all the flasks and contents were sterilized at twenty-five pounds pressure for about 45 minutes. The flasks and contents were then cooled and inoculated with the fungus from the pure cultures mentioned above. At first it was very hard to distinguish any growth in the flasks but after a few weeks a whitish growth appeared on the surface of the sawdust and finally the mycelium could be seen scattered throughout the sawdust. At the end of about seven weeks the flasks were emptied and the sawdust mycelium were dried out over a radiator taking care not to run the temperature over about 30° C. After drying, the material was ground in a mortar with a pestle, care being taken not to cause too much heat to be generated by friction. This ground, dry material will be termed, in this paper, "Mycelial meal". From this "mycelial meal" were made extractions known as "mycelial dispersions". It consisted of adding

50 cc warm (35° - 40° C) distilled water to ten grams mycelial meal, allowing it to digest, usually over-night with toluene added to prevent bacterial action. The suspended matter was then filtered off and the filter washed with 50 cc warm (35° - 40° C) distilled water. The filtrate was the "mycelial dispersion".

Invertase and Diastase

First 10 cc of the mycelial dispersion was placed in 50 cc of a 1% starch (Merk's soluble) and 1% Sucrose solution, the starch and Sucrose solution being used as a substrate. A check was also prepared using 10 cc of mycelial dispersion in 50 cc distilled water. The three above solutions were tested after about an hour and then after standing over night. The Fehling's test, as used, consisted of adding 3 cc Fehling's A, 3 cc Fehling's B, 6 cc water and about 6 cc of the substance to be tested and heating for about five minutes. A red ppt. for Starch solution and a reddish-yellow ppt. for sucrose solution indicated the presence of invertase and diastase. The check gave no reduction.

Cystase

Test for cellulose splitting enzyme was made as follows:- 25 cc mycelial dispersian were placed in small flask and a few cc of heavy cellulose suspensian was added and also some toluene. This was tested for reducing sugars by Fehling's test at 24 hr., 48 hr. and weekly intervals for about three weeks. The reaction of the solution was also determined by titrating against N/10 H_2SO_4 and N/10 NaOH. Strong reduction was shown in all examinations but the solution showed no change in reaction, the solution being about neutral. This shows absence of any organic acids in the dispersion and the presence of reducing sugars.

Maltase

A test for the presence of the enzyme maltose was next made. To 20 cc of a 1% maltose solution 10 cc "mycelial dispersian" was added and 8 tubes were prepared. The 1% maltose solution was added to each of four tubes and these were placed in boiling water for five minutes, 5 cc mycelial dispersion added and heating continued for five more minutes. The tubes were cooled and toluene added as an antiseptic. These tubes were marked "checks". Maltose and mycelial dispersian, prepared at beginning of paragraph, was added to each of four tubes, toluene added and checks and regulars were incubated at 35-40° C. Osazone test using phenylhydrazine-hydrochloride at first showed only maltose crystals present. Tests were made at five day intervals. A very slight formation of dextrose crystals (glucosazone) at the end of the fifth day was evident in the regular but not in the control. The 3d and 4th (10th and 15th day) test showed the glucosazone to be more pronounced in the regular but none were present in the control. This showed that the enzyme maltose, which has the power of converting maltose sugar to dextrose sugar, was present in the dispersion.

Emulsin

The test for emulsin was next carried out. Four tubes each of amagdylin, arbutin and salicin were prepared as follows: Ten cc of a 1% solution of each of the above substances were placed in each of 4 test tubes making a total of 12 tubes for the 3 substances. To three tubes of each set was added 4 cc of "mycelial ~~enzyme~~ dispersion" and one tube was boiled for about five minutes. To the other tube of each set 5 cc of distilled water was added as a control. To all the tubes toluene was added. The above tubes were incubated at about 30° C. and were tested at about weekly intervals with Fehling's solution. After

the first week the two regulars of amagdylin and arbutin reduced Fehling's but salicin showed no reduction at this time. The boiled and control tubes showed no reduction. At the end of three weeks incubation the salicin tubes gave reduction with Fehling's. These tests show that the enzyme emulsin is present in the Xylaria Digitata Fungus.

Oxidase.

A test for the enzyme oxidase was next made. To 100 cc of .01% vanillin solution 20 cc of mycelial dispersian were added and toluene was added to prevent bacterial action. This was tested immediately and at 3 day intervals, for about three weeks, for the presence of oxidase. The test was made as follows: A few cc of the above solution was poured in a test tube some acid nitrate of mercury reagent was added and the tube and controls heated for a few minutes. If oxidose is not present the solution turned a red color showing that the vanillin had not been oxidized to vanillic acid; on the other hand, if the solution did not show the red color the presence of oxidase which oxidizes the vanillin to vanillic acid is shown. In all the tests the presence of vanillin was shown by the red color developing. This shows the oxidase was not present in the fungus.

The action of the enzymes upon Proteins and their cleavage products was next tested.

Protease

The presence of this enzyme can be shown by its action on fibrin previously stained with cargo red and fixed in boiling water. If the enzyme is present a red color is liberated into the solution. Four tubes (50 cc capacity) were prepared as follows:

1- control, 25 cc H_2O + red fibrin.
2- 10 cc mycelial dispersion, 15 cc H_2O + red fibrin.

3- 10 cc. mycelial dispersion, 15 cc H_2O, 1 cc N/10 H_2SO_4 + red fibrin

4- 10 cc " " 15 cc H_2O, 1 cc N/10 NAOH + " "

At five day intervals an attempt was made to estimate the amount of protealysis occurring in the different tubes, by comparing the color density. The tubes, however, did not show any differences due perhaps to the excess strain not having been thoroughly washed out of the fibrin before preparing the tubes, so the presence of this enzyme could not be determined.

Erepsin

The method of making the test for Erepsin is to demonstrate the presence of tryptophane in the dispersian. 10 cc mycelial dispersion was added to 50 cc of a 1% solution of peptone. To this solution toluene was added and the solution was placed in incubation. Tests, for tryptophane at two day intervals, for about three weeks were made. This was done by adding a few drops of acetic acid to the few cc of the solution, boiling it and then adding a few drops of bromine water. The solution gave no test for tryptophane. Tests were also made upon casein. Two grams casein added to 15 cc N/10 NaOH, the suspensian thoroughly stirred diluted to 60 cc and filtered. 10 cc enzyme dispersian was added to the filtrate, toluene added and incubated as above for peptone. Tests were made at two day intervals as in peptone solution and at the end of three weeks a pink color, showing the presence of tryptophane, was evident. This shows the presence of the enzyme erepsin.

Amidase

For testing the presence of this enzyme the action of the dispersion an ~~adamin~~ alanine and asparagin was tested. An experiment was set up using four large tubes prepared as follows:

1- .2 gr. alamin + 30 cc H_2O

2- .2 gr. alamin + 30 cc H_2O + 10 cc mycelial dispersian

3- .2 gr. asparagin + 30 cc H_2O

4- .2 gr. " + 30 cc H_2O + 10 cc mycelial dispersian.

These tubes were tested with Nessler's reagent at four day intervals for the formation of ammonia from alamin and asparagin. None of the tests gave evidence of the presence of amidase.

Summary.

Tests carried out to determine the enzymatic activity of the fungus Xylaria Digitata showed the presence of

Diastase, Invertase, Cytase, Maltase, Emulsin and Erepsin.

Oxidase, protease and Amidase were not found to be present.

The Cultivation of Penicillium pinophilium in Nutrient Salt Solutions.

A triangular system for testing out the possible ratios for fertilizer experiments has been well described by Schreiner and Skinner in vol. 10, No. 6 of the Journal of the American Society of Agronomy. It is the purpose of this paper to give a report of work done along this line, using, instead of fertilizers, nutrient salt solutions and growing the common mould Penicillium instead of plants as was done by Schreiner and Skinner. In other words work similar to that carried out in the field with plants was carried out in the laboratory with the common mould Penicillium.

In the following discussion the triangle system is fully explained and outlined.

Three nutrient stock solutions were prepared based upon the essential elements N,K, & P. For P. a stock solution of $CaH_4(PO_4)_2 . H_2O$ was prepared by dissolving 1.776 grams of the salt in 1000cc distilled water. The resultant solution carried phosphate (P_2O_5) 1000 ppm. For N a stock solution of $NaNO_3$ was prepared by dissolving 5 grams of the salt in 1000 cc, this gives a concentration of nitrogen (in terms of NH_3) of 1000 ppm. In like manner as a source of potash (K_2O) 1.852 grams of K_2SO_4 was dissolved in 1000 cc of water giving a concentration of 1000 ppm K_2O. These stock solutions were diluted to ten times giving a concentration of 100 ppm of the nutrient element. The solutions were graded in 10 percent stages giving a total of 66 tests in the experiment. To bear in mind all these different ratios is almost an impossibility so a diagram showing the different points etc., has been prepared, (Fig. 1).

This triangle represents all the possible ratios of 10% stages. Each extreme point of the equilateral triangle represents 100 % of

P_2O_5, NH_3 or K_2O. The term "100 percent" refers to the maximum quantity of solution used, I.E. 100 cc of a 100 p.p.m. solution. The sides of the triangle are divided into 10 equal parts and lines connecting these points have been drawn. For convenience the intersections of these lines have been numbered. Table 1 gives the ratios for the three constituents. By reference to the constituents of points in table 1 and studying Figure 1 the ratios for any portion of the triangle can be determined.

Suppose we consider the line representing the base of the triangle, then the point which represents 100% K_2O (Point 56) will have no NH_3 nor P_2O_5, likewise the points representing 100% NH_3 (Point 66) and 100% P_2O_5 (Point 1) will have neither of the other constituents present. It is plainly seen that the extreme points of the triangle represent 100% solutions and that as the lines go toward the opposite base they decrease in 10% stages.

Suppose we take points nearer to either of the corners, there will be a higher percentage of one constituent and a lower percentage of the others. Take point 58 for example, we have, 80% K_2O and 20% NH_3; No. 3 gives 90% P_2O_5, 10% NH_3; On the other hand if points are taken near the center of the triangle a more equal ratio is shown. Point 33 gives 30% P_2O_5, 40% NH_3 and 30% K_2O.

For the sake of convenience it was decided in making this test to use small Erlnmeyer flasks (150cc). An apparatus connecting flasks containing the three properly diluted (100 PPM) salt solutions to buerettes was prepared. The different amounts of the various solutions were then added directly to the flasks and these were numbered consecutively. 100cc of these solutions represented 100%. Therefore, if (as for point 5) it was desired to get a mixture of 80% P_2O_5, 10% NH_3 and 10% K_2O it was simply a case of drawing 80 cc of the P_2O_5 solution, 10 cc of the NH_3

solution and 10 cc of the K_2O solution into the flask. This system of using 100 cc as representing 100% facilitated matters considerably.

After the 66 flasks had been prepared, numbered and plugged with cotton they were then sterilized in the autoclave at a 15 pounds pressure for about 15 minutes. After allowing them to cool they were all inoculated with the Penicillium pinophilium. Examinations and notes as to the rapidity, character, color etc. of growth were taken at daily intervals but only the final weight of growth will be given in this paper.

In the meantime a total of 66 weighing bottles had been prepared, properly numbered, weighed and the weights recorded on a suitable form. After the Penicillium had grown for seventeen days the contents of the flasks, which in some cases consisted of almost a solid mass of growth, were filtered. The fungus growth and filter paper were then placed in their respective weighing bottles and the bottles heated in a drying oven. Heat was applied until no more moisture was present in the tubes and then they were placed in dessicators. Weighings were then made of the bottles and contents and these weights were recorded in the form mentioned above, in their respective places. The difference between the weights of the empty tubes and the tubes containing the dry mycelium gave the weights of fungus for the respective flasks. Figure 2 gives the weights of mycelium developing in the different flasks, in form of the triangle shown in Figure 1.

To summarize the results of the experiment it is necessary to make a careful study of Figure 2. In order to determine which legs of the outside triangle gave the best growth, averages of the fungus weights of each of the three outer legs were made and the weights were as follows: Side 1 to 56 gave average of .0075 grams fungus growth, side 56 to 66 gave average of .0075 grams fungus growth, side 66 to 1 gave average of .0381 grams fungus growth. This showed that the leg of the triangle cont-

aining P_2O_5 and NH_3 and lacking in K_2O gave the best growth of the single and two salts combination. The weights of the legs of the next triangle within were then averaged and the weights were as follows: Side 5 to 47 gave .0708 grams fungus growth, side 47 to 54 gave .1435 grams fungus growth. Side 47 to 54, which contains higher percentages of K_2O and NH_3 and lower percentages of P_2O_5, gave decidedly the best growth in this case. This latter average gives a little better working basis as the triangle contains a mixture of all three constituents and therefore gives a better test.

Points 40,48 and 49 (with one exception) shows the highest weight of the fungus and are enclosed in dotted lines in Figure 2. This shows that the area of the best growth is nearest the K_2O corner of the triangle and that the solutions for these points contain higher percentages of K_2O and NH_3 and less P_2O_5. Point 54 shows the highest weight but some unaccounted mistake has been made and is not included in the comparisons.

Schreiner and Skinner found that the normal growth of wheat in nutrient solutions (on basis of green weight) was best in numbers 41,50 and 51 where P_2O_5 is 10%-20% and K_2O and NH_3 in higher percentage.

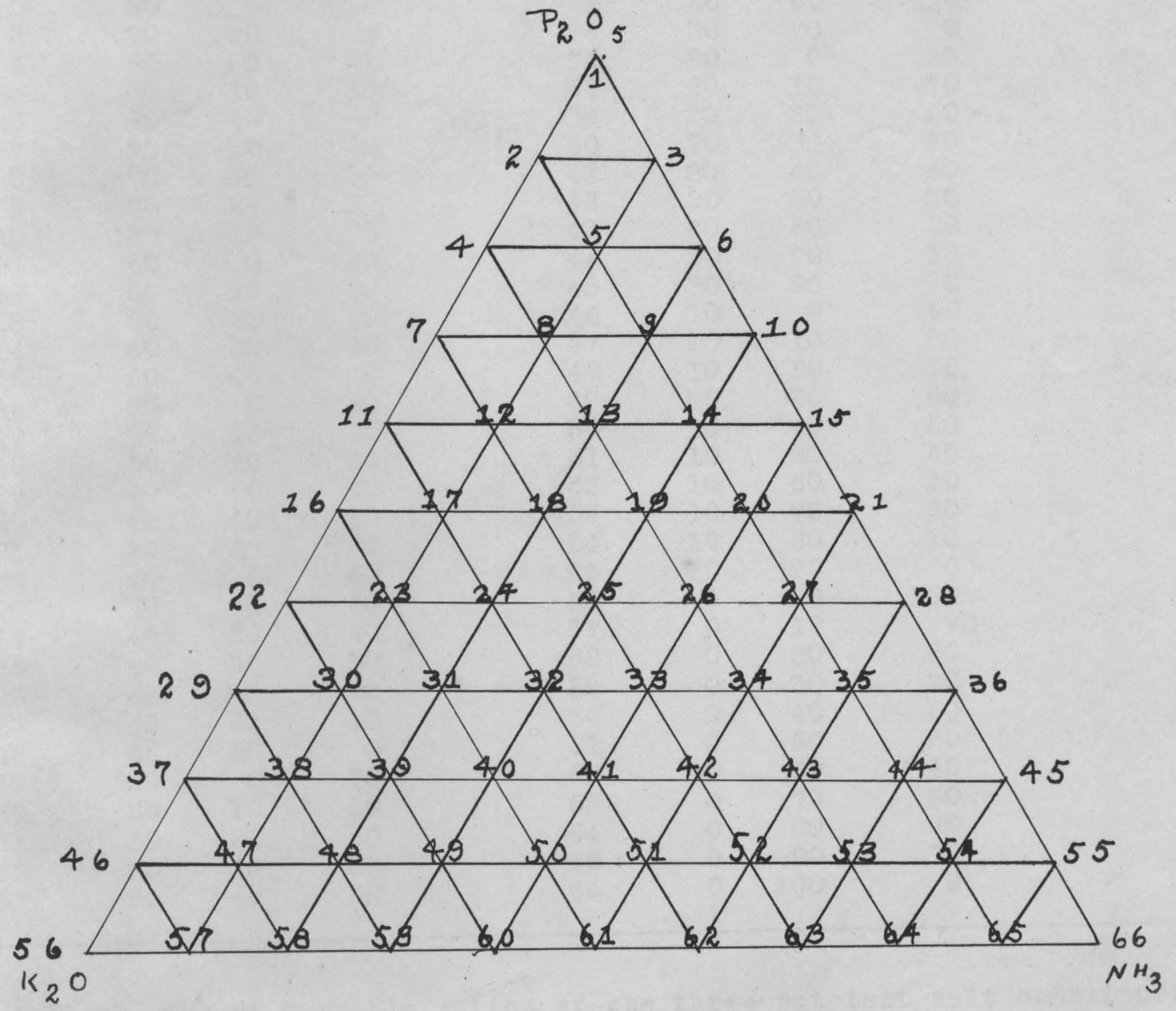

Figure 1- Equilateral triangular diagram showing points numbered; representing the 66 nutrient salt combinations in 10 percent stages.

Point No.	Ratio or percentage composition P_2O_5	NH_3	K_2O	Point No.	Ratio or percentage Composition. P_2O_5	NH_3	K_2O
1	100	0	0	34	30	50	20
2	90	0	10	35	30	60	10
3	90	10	0	36	30	70	0
4	80	0	20	37	20	0	80
5	80	10	10	38	20	10	70
6	80	20	0	39	20	20	60
7	70	0	30	40	20	30	50
8	70	10	20	41	20	40	40
9	70	20	10	42	20	50	30
10	70	30	0	43	20	60	20
11	60	0	40	44	20	70	10
12	60	10	30	45	20	80	0
13	60	20	20	46	10	0	90
14	60	30	10	47	10	10	80
15	60	40	0	48	10	20	70
16	50	0	50	49	10	30	60
17	50	10	40	50	10	40	50
18	50	20	30	51	10	50	40
19	50	30	20	52	10	60	30
20	50	40	10	53	10	70	20
21	50	50	0	54	10	80	10
22	40	0	60	55	10	90	0
23	40	10	50	56	0	0	100
24	40	20	40	57	0	10	90
25	40	30	30	58	0	20	80
26	40	40	20	59	0	30	70
27	40	50	10	60	0	40	60
28	40	60	0	61	0	50	50
29	30	0	70	62	0	60	40
30	30	10	60	63	0	70	30
31	30	20	50	64	0	80	20
32	30	30	40	65	0	90	10
33	30	40	30	66	0	100	0

Table 1- The 66 possible ratios of the three nutrient salt constituents, P_2O_5, NH_3 and K_2O given in 10 percent stages.

P_2O_5

.0078

.0038 .0283 (3)

.0046 .0596 .0358 (6)

.0028 .0582 .0784 .0706 (10)

.0069 .0752 .1083 .0482 .0491 (15)

.0105 .0523 .0905 .1030 .1175 .0411 (21)

.0098 .0669 .1252 .1406 .1351 .1320 .0385 (28)

.0154 .0819 .0974 .1187 .1301 .1393 .0537 .0414 (36)

.0112 .0808 .1105 .1567 .1465 .1396 .1378 .0512 (45)

.0094 .0913 .1496 .1609 .1179 .1481 .1454 .1423 .1926 .0676 (55)

.0007 .0391 .0069 .0016 .0047 .0012 .0057 .0066 .0007 (66)

K_2O NH_3

Figure 2: Showing weights of penicillium in the various nutrient solutions. Weights given in grams.