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Studies on the biological control of Fusarium.

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STUDIES ON THE BIOLOGICAL CONTROL OF FUSARIUM

A Thesis Presented

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To my wife

Donna

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TABLE OF CONTENTS

INTRODUCTION

Plant pathogenic fungi cause numerous wilts and root rots which seriously diminish the output of food. Effective control over the fungal diseases would greatly increase our food production potential. Most disease control measures in the past required the use of large doses of chemical fungicides. However, with the increasing awareness of the harm to the environment that may be caused by the application of chemicals, possibilities for the biological control of disease producing organisms are being increasingly studied.

In the present investigation an attempt was made to control Fusarium solani f. phaseoli, the causal agent to the bean root rot, by biological means. According to Alexander (1961) and others (Garrett, 1963; Sanford and Broadfoot, 1931) soil-borne plant pathogens are more destructive to their host in sterile than in non-sterile soil. Therefore, it appears that an antagonistic microflora is always present in normal soil which helps limit the spread of the disease-producing microorganisms. In this light an attempt was made to modify the pathogen-antagonist equilibrium under test conditions in favor of the antagonist in the hopes of diminishing the incidence of disease. Principally, organic

amendments of chitin-rich lobster shell were used as a means of selectively supporting pathogen-lysing antagonists. In turn, consideration was given to the actual lytic mechanism whereby bacteria attacked Fusarium cell walls.

LITERATURE REVIEW

The soil-borne fungus, Fusarium solani (Mart.) Appel. & Wr. f. sp. phaseoli (Burk.) Snyd. & Hans., attacks the bean plant (Phaseolus vulgaris L.) causing a serious root rot disease. Studies have shown that the fungus enters the plant through both the roots and hypocotyl (Chatterjee, 1958; Christou and Snyder,1962). It directly penetrates the plant by producing a small thallus which enters through mechanical or natural wounds, but most commonly through the stomata of the hypocotyl. The mycelium invades the cortex intercellularly but usually does not penetrate the pericycle. In the early stages of infection there appears to be no disturbance of invaded tissues. In the later stages, however, the cell walls and contents turn a characteristic brown color. Phenolic compounds have been detected in the discolored tissues. In later stages of the disease cycle numerous chlamydospores are formed on the surface and within the outer cortex of the necrotic lesions. Under high moisture conditions, conidia are also produced on sporodochia which emerge from the stomata at the soil level.

In natural soil Fusarium solani exists cheifly in the form of chlamydospores (Nash, Christou, and Snyder, 1961).

Toussoun and Snyder (1961) demonstrated that the exudate from excised bean plants greatly stimulated the germination of chlamydospores in unsterilized soil. From the seed and root exudates of the bean 22 amino acids and several sugars were identified by Schroth, Toussoun, and Snyder in 1963. They also demonstrated the need for exogenous supplies of carbon and nitrogen for chlamydospore germination. Schroth and Hendrix (1962) showed that chlamydospores of Fusarium solani germinate and produce mycelium in close proximity to seeds and roots of many nonsusceptible plants as well. Their data suggested that survival of Fusarium solani may be enhanced by some diffusates and crop residues from nonsusceptible plants which enable the fungus to vegetate briefly and form new chlamydospores.

Numerous studies have been performed which make use of the application of organic amendments to reduce the severity of the bean root rot disease. Snyder, Schroth, and Christou (1959) reported control of fusarium root rot by amending the soil with ground barley straw, wheat straw, corn stover, or pine shavings. Maier (1959) showed reduction of fusarium root rot by amending soil with barley, sorghum, and corn residues. According to Papavizas, Lewis, and Adams (1968) maltose, dextran, starch, and cellulose controlled fusarium root rot in acid soils, but only cellulose was effective in alkaline soil. Chitin and laminarin amendments have also been shown to significantly reduce the disease (Mitchell, 1963).

Recently, Adams, Lewis, and Papavizas (1968) performed greenhouse studies which indicated that spent coffee grounds are effective in reducing fusarium root rot.

The beneficial effects of adding amendments with high C:N ratio (such as barley straw, wheat straw, cellulose, etc.) were reduced by adding nitrogen to the soil (Maier, 1968; Maurer and Baker, 1965; Wienke, 1962). Wienke (1962) suggested that high C:N ratio amendments stimulated microbial multiplication to such an extent that soluble nitrogen was immobilized. This resulted in increased competition for nitrogen. This hypothesis was supported by studies performed by Bytherin 1965. His results indicated that Fusarium roseum could utilize inorganic nitrogen under less than optimal conditions more successfully than F. solani. Also, Marshall and Alexander (1960) working with sterile soil systems found that a variety of bacteria could suppress F. oxysporum as a result of biologically induced nitrogen deficiency. Other explanations are possible however. In 1960 Toussoun, Nash, and Snyder observed that nitrogen favored early penetration of the host. Organic sources of nitrogen were more effective than inorganic sources. Glucose was fourd to delay penetration and pathogenesis. Wienke (1962) also observed that the pathogen was more aggressive when supplied with an external source of nitrogen. Nitrogen increased the development of an aggressive superficial thallus of the pathogen on the bean hypocotyl.

Papavizas in 1963 found that application of nitrogen (200 ppm from NH_4NO_3), either alone or with straw to produce a C:N ratio of 10, substantially reduced the number of antagonists to Fusarium in the bean rhizosphere. The supression was greater in strongly acid soil (pH 4.7) than in slightly acid and neutral to alkaline soil. In a study in 1968, Adams, Lewis, and Papavizas suggested that cellulose controlled bean root rot by raising the fungistatic level of the soil. This prevented the germination of chlamydospores of F. solani adjacent to the host tissue.

The mechanism suggested for the control with spent coffee grounds was quite different. It was found that initially the spent coffee grounds stimulated chlamydospore germination of F. solani. Germination, however, was soon followed by lysis due to increased fungistatic activity in the amended soil compared with the control (Adams, Lewis, Papavizas, 1968). A similar explanation was presented by Sequeira in 1962. He found that certain organic amendments (such as sugar cane and tropical knudzu) reduced the numbers of F. oxysporum in the soil. Using buried slide techniques he demonstrated that the amendments stimulated the chlamydospore germination. This permitted exposure of hyphae and thin walled spores to lysis by unidentifed soil bacteria. Further chlamydospore formation was inhibited in amended soils.

Lysis of fungi by bacteria has received considerable attention in the last few years. In 1958 Horikoshi and Iida successfully isolated from soil a strain of Bacillus circulans which caused visible lysis of Aspergillus oryzae mycelia. When this strain was inoculated into a flask culture of A. oryzae, which had been previously shaken for 24 hours at 30 C, almost all the mycelia disappeared after 80 hours. A crude enzyme was prepared from the supernate of this culture which could lyse living mycelial cells of A. oryzae. In a later study (Horikoshi, Koffler, and Arima, 1963) a B(1-3)glucanase was prepared from the culture filtrate of Bacillus circulans. The enzyme was purified by ammonium sulfate precipitation followed by CG-50 resin column chromatography and DEAE-cellulose column chromatography.

Mitchell and Alexander (1963) isolated a number of bacteria from the soil capable of lysing Fusarium oxysporum. All were found to be species of Bacillus and Pseudomonas. This does not imply that these are the only genera which can digest soil fungi since the technique employed for isolation tended to favor rapidly growing microorganisms. A heavy inoculum of F. oxysporum in unsterilized soil resulted in a relatively rapid increase in the abundance of bacilli, pseudomonas, and actinomycetes coupled with the rapid decline of F. oxysporum. The results lend weight to the possible importance of these genera in mycolysis in nature.

Mitchell and Alexander (1963) and Mitchell (1963) showed that the lysis of several soil fungi is associated with

chitinase and laminarinase activity of the mycolytic bacteria. One of these, a strain of Bacillus cereus, was tested for lytic activity against a range of hosts. Species of Pythium, Streptomyces, Agrobacterium, and Pseudomonas were not lysed by B. cereus. None of these species, however, contained chitin in their cell walls. To obtain more information on the actual lytic mechanism, B. cereus was grown on dead fusarium. The culture was freed of bacterial and fungal cells by filtering with ultra-fine sintered glass filters. It was found that the filtrate ws capable of causing lysis of living and dead fungal hyphae. Lytic activity was destroyed when culture filtrates were heated for 15 minutes at 60 C. In a more recent study (Skujins, Potgieter, and Alexander, 1965) fungal cell walls of Fusarium solani were digested by a streptomycete B(1-3)glucanase and chitinase. The products of the digestion were N-acetylhexosamine and glucose. No significant lysis was produced when the chitinase and B(1-3) glucanase were used seperately. However, the final 20% of the walls (which chemical analysis revealed to be free of glucose) were hydrolyzed by chitinase but not by glucanase, indicating that there probably exists a chitin-containing core. Wall layering has also been observed by Aronson and Preston (1960) who studied many of the lower fungi.

Some fungi spores and chlamydospores are relatively resistant to digestion by B(1-3)glucanase and chitinase,

even though they contain large amounts of glucose and chitin. Kuo and Alexander in 1967 found that the resistance of Aspergillus nidulans hyphae to digestion was correlated with the melanin content of the mycelium. A melanin-less mutant was highly susceptible to hydrolysis by the enzyme mixture. The obvious ecological significance of the findings is that fungal species with high melanin content may be quite resistant to attack by mycolytic bacteria.

Biological control of plant-pathogenic fungi by mycolytic bacteria has met with some success. It is an axiom in ecology that the flora and fauna of a habitat will be selected by the environment. When an antagonistic microorganism is inoculated into the soil in the hope of controlling a plant pathogen, the biological equilibrium will be upset only temporarily. If the environment were changed in some way (such as with organic amendments) to favor the antagonistic microflora then control of the pathogen may be possible. An important study using this basic premise was carried out by Mitchell in 1963. The addition of either chitin or laminarin (which are fungal cell wall components of fusarium) to soil infested with F. solani was tested as a means for controlling this pathogen. Control of diseases caused by F. solani f. phaseoli and F. oxysporum f. conglutinans was statisticly significant. Both of these fungi contain chitin in their cell walls. No suppression of either Pythium debaryanum or Agrobacterium tumefaciens was observed. Chitin

is not known to be in the cell walls of either of these pathogens. Natural sources of chitin and laminarin were also tried in controlling fusarium. Ground lobster shell which contains 12-20% chitin and the brown alga, laminaria, which contains about 40% laminarin were used. Control of fusarium was established with 250, 500, and 750 lbs. of lobster shell per acre. When lobster shell amendments were increased to 3,000 lbs. per acre the disease was severely increased. Similarly, 1,000 lbs. per acre of laminaria resulted in control, but very high concentrations increased the disease. In a previous study on the effect of chitin amendments on microbial populations (Mitchell and Alexander, 1962), the number of fungi was reduced for the first two weeks after applying the amendment while the number of bacteria and actinomycetes was greatly increased. By the fourth week the fungi had recovered. Laminarin had a similar effect on the fungal population.

Another biological control mechanism that has met with some success is to make use of the selective effect of the plant rhizosphere. Numbers of microorganisms are much greater in this zone than in soil devoid of roots. It might be expected, therefore, that antagonistic reactions would be intensified in this region (Katznelson, 1965). Koths and Gunner (1967) made use of this principle in obtaining a degree of control over Fusarium roseum, the causal agent of fusarium stem rot of carnations. Mitchell and Hurwitz

(1965) isolated from the tomato rhizosphere two strains of Arthrobacter which lysed mycelia of Pythium debaryanum. The tomato disease caused by this pathogen was suppressed in aseptic culture by inoculation with the lytic bacterium. In nonsterile soil seed inoculation with the lytic bacteria protected the young seedlings against damping-off caused by P. debaryanum. Plate counts indicated that the lytic inocula predominated in the root zone for approximately 14-21 days. An attempt was made to achieve similar disease control by drenching pythium infested soil with a suspension of a lytic arthrobacter at planting. No disease control was observed, and the arthrobacter could not be detected in the soil 48 hours after addition.

MATERIALS AND METHODS

Effect of Lobster Shell on the Distribution of Soil Microflora

The soil used in this study was a Berkshire loam soil consisting of 50% fine sand, 39% silt, and 11% clay. It was taken from a cultivated plot from the University of Massachusetts farm at Amherst. The soil solution was slightly acid at a pH of 6.5. The Morgan quick test* for nutrient availability produced the following results:

Nitrate Nitrogen	Medium High	or	40 lbs/acre
Phosphorus	Medium High	or	100 lbs/acre
Potassium	Very Low	or	10 lbs/acre
Calcium	Very High	or	3200 lbs/acre
Magnesium	High	or	250 lbs/acre

Lobster shell was obtained from the Maritime Packers, Division of National Sea Products, Ltd., Pictou, Nova Scotia. The partially ground lobster shell was air dried for 48 hours and ground in a Wiley mill until it could readily pass through a 60 mesh sieve. The finely ground lobster shell was added to 1500 gm of soil in plastic pots at rates of 0, 100, 500, and 3000 ppm. Each treatment was replicated in four pots. After adding the lobster shell the soil was thoroughly mixed. The moisture level was kept at 2/3 the

*The soil analysis was performed by Dr. Louis F. Michelson, Plant and Soil Department, University of Massachusetts, Amherst.

soil moisture holding capacity by sprinkling distilled water on the pots every other day.

The pots were sampled at 1, 2, 4, and 8 week intervals. A core was removed with a half-inch cylindrical probe from each of the four replicate pots. The cores were then mixed together and weighed. A 10 gm sample was added to a dilution bottle containing 95 ml of sterile distilled water. The bottle was shaken vigorously for 5 minutes. Subsequent serial dilutions were made by removing 10 ml of suspension from the first bottle and adding it to the next, which contained 90 ml of sterile distilled water, and so on until a 10^6 dilution was made. From the appropriate dilution a 1 ml sample was removed and added to a sterile Petri dish. Pour plates were made by adding about 20 ml of agar preparation kept at 47 C.

For assaying bacteria, nutrient agar was used at a pH of 7.4. Actinomycetes were enumerated on glycerol agar (see Appendix), pH 7.0. Fungal counts were made on glucose-peptone agar (see Appendix) with the pH adjusted to 4.0. The abundance of chitinase-producing organisms was estimated on double-layer plates. The lower layer (15 ml) consisted of minimal media A (see Appendix). The upper layer (10 ml) into which 1.0 ml of the appropriate dilution was added, consisted of minimal salts agar plus sufficient colloidal chitin to give the plate an opaque appearance. Colloidal

chitin was prepared according to the method of Lingappa and Lockwood (1962). Chitin was obtained from the Sigma Chemical Company, St. Louis, Missouri. Lytic organisms were estimated on double layer plates as well. The plates were prepared exactly as the chitin agar plates except that the upper layer contained ground Fusarium cell walls rather than chitin.

The Fusarium cell walls were prepared in the following manner. The fungus was grown on the above minimal media with sucrose (40 gm/l) as the carbon source. The cultures were incubated 4 to 5 days at 22 C on a rotary shaker. After the incubation period the mycelium was killed by steaming for 20 minutes and harvested by filtering through muslin. It was washed several times with distilled. The wet mass, together with a small amount of distilled water, was added to a Sorvall Omni-mixer and ground for 5 minutes. The walls were then centrifuged at 800 G and washed several times with distilled water. Finally, the cells were suspended in 5% KOH to undergo alkaline digestion according to the method of Aronson and Machlis (1959).

The method described by Herr (1959) which primarily indicates the number of antibiotic-producing organisms was used to estimate the population of actinomycetes antagonistic to Fusarium.

Population of Actinomycete Antibiotic-Producers Against *Fusarium* in Soil

Sixty actinomycetes were isolated from soil by using the dilution technique and plating onto glycerol agar plates. After 5 days incubation they were picked off the glycerol agar and streaked onto nutrient agar slants. To test for their antibiotic activity each actinomycete was streaked down the center of a nutrient agar plate. After 3 days incubation *Fusarium* was cross streaked 3 times against each actinomycete. The plates were then incubated another 7 days and any inhibition of *Fusarium* was measured in millimeters.

The Dry Root Rot of The Bean

In order to establish that fusarium root rot could in fact be readily induced in the bean plants, test experiments were conducted with a culture of *Fusarium solani* f. *phaseoli* (Burk.) Snyd. and Hans. which was generously provided by Dr. William Manning of the Waltham Experimental Station, Waltham, Massachusetts. The *F. solani* was grown in Roux culture bottles containing potato dextrose agar (PDA) for approximately 10 days. Five hundred ml of distilled water was added to the culture which was shaken to produce a spore suspension. One hundred ml of the spore suspension were

added to each of four replicate pots. The pots contained a mixture of soil and vermiculture at a 1:1 ratio. They had been previously seeded with 10 seeds of a common variety of bush beans (Phaseolus vulgaris L.). Another series of seeded pots treated only with 100 ml of distilled water was included as a control. The plants were grown under greenhouse conditions with periodic watering to maintain optimal growth. After two weeks the plants were thinned to 5 seedlings per pot. During the course of the experiment the plants were periodically inspected for disease symptoms. After 5 weeks the plants were uprooted and examined for pathogenic lesions.

From randomly selected plants small portions of Fusarium-infected stems were removed with a razor blade. The stem portions were surface sterilized by soaking in a 2.5% Na hypochlorite solution for about 5 minutes. They were then washed in sterile distilled water and aseptically placed on the surface of PDA plates. After one week incubation growth from the infected stems was examined under the microscope.

Effect of Lobster Shell on Bean Root Rot Disease

The soil was mixed with vermiculite in a ratio of 1:1 and approximately 1500 gm of the mixture distributed in replicate 6" plastic pots. Ten pots were seeded (10 seeds

per pot) with bush bean at periods of 4 weeks, 2 weeks, and 1 week respectively, before application of lobster shell. In a parallel series 10 pots were seeded at 1 week and 2 weeks after lobster shell application. The soil was inoculated with a Fusarium spore suspension prepared as previously described. The spore suspension was applied simultaneously with lobster shell ground to 60 mesh. The ground lobster shell was applied to the surface of each pot at a concentration of 1,000 ppm.

Two weeks after planting the seedlings were thinned to 5 seedlings per pot. At this time 1/2 of the plants were wounded by a small cut (ca 0.5 cm) at the base of each plant with a razor blade.

Seven weeks after seeding, the plants were examined to determine the extent of disease. A disease index from 0 to 100 was adopted as a measure of the severity of the root rot. A zero rating reflected no disease and a value of 100 indicated that the plant was killed. Intermediate ratings are included in the following scale:

Rating	Size of lesion
0	-
20	0.75 cm
40	1.50 cm
60	2.25 cm
80	3.00+ cm

In a second series of experiments 5 seeds of Phaseolus vulgaris were planted per pot. The ground lobster shell was applied at a rate of 500 ppm and it was mixed thoroughly with the potting mixture rather than applied only at the surface. Fusarium was again added to the soil as a spore suspension in sterile distilled water. The Fusarium spores and lobster shell were added immediately prior to planting the seeds. Ten replica pots were prepared for each treatment — lobster shell plus Fusarium, Fusarium alone, lobster shell alone, and a control with neither Fusarium nor lobster shell. The plants were thinned to 3 seedlings per pot 1 week after germination. The disease index was determined after seven weeks of growth.

Lysis of Fusarium Cell Walls by Bacterial Isolates

In order to isolate bacteria lytic to Fusarium from the bean rhizosphere, bean plants were grown for about three weeks in pots containing the previously described loam soil. The plants were carefully removed from each pot and shaken or scraped lightly to remove the adhering soil from the roots. Ten grams of soil from the rhizosphere were diluted to 10^6 by the dilution technique. One ml of the 10^4, 10^5, and 10^6 dilution was mixed with 10 ml of Fusarium cell wall agar at 47 C. The Fusarium cell wall agar was then quickly

spread on a plate containing minimal salts media in the double overlay technique previously described.

From the Fusarium cell wall agar plates 18 bacteria were picked and streaked onto the surface of another Fusarium cell wall agar plate. This procedure was repeated once more to insure that the isolates were pure. Each isolate was examined under the microscope and Gram stained. The 18 isolates were also tested for motility and growth on chitin agar and laminarin agar (Laminarin was obtained from the K & K Laboratories, Inc., Plainview, N.Y.). These carbon sources were added to the minimal salts media at a rate of 0.5%.

Each isolate was also tested for its ability to lyse living Fusarium cell walls. Both Fusarium spores and each of the bacteria were inoculated into 18 separate flasks, each containing 50 ml of Morris media (Morris, 1960) (see Appendix). Glucose (0.2%) was used as the carbon source in all of the flasks. The flasks were incubated on a rotary shaker for 7 days. Controls containing each bacteria alone and Fusarium alone were also included. A comparison was made between the control Fusarium and the isolate-Fusarium mixture to determine fungal growth.

Four of the isolates were randomly chosen to test their effectiveness in controlling the bean root rot disease in pure culture. The four chosen were Nos. 1, 8, 17, and 20.

The bacteria were grown on nutrient agar slants for two days. The Fusarium was grown on PDA slants for seven days. Viable counts were made of each suspension by the dilution plate technique. The bacteria were plated onto nutrient agar and the Fusarium spores were plated onto PDA.

Bean seeds were surfaced sterilized for 15 minutes with sodium hypochlorite (2.5%) and germinated on water agar. The seeds were transferred aseptically from the agar plates to large test tubes (2 cm in diameter and 25 cm long) containing 10 ml of Hoagland's solution (Hoagland and Arnon, 1938) (see Appendix). Each test tube was inoculated with 1.0 ml of a mixture containing 0.5 ml of each isolate and 0.5 ml of Fusarium spore suspension. Five replicate test tubes, each containing one plant, were used for each treatment. Controls containing the bacterial isolates and Fusarium alone were also included. The disease index was read 10 days after inoculation with the pathogen.

An experiment was performed to assess the lysis of dead Fusarium cell walls by bacterial culture supernatants presumably containing lytic exoenzymes. Isolates, Nos. 6, 8, and 12, were grown in Morris media with dead Fusarium cell walls (0.5%) as the carbon source. The cultures were incubated for four days on a rotary shaker at 25 C. After the incubation period the cellular debris was removed from each culture by centrifuging at 9,000 G for 15 minutes. This left a clear, slightly yellowish supernatant. A portion of each supernatant was heated to 60 C for 30 minutes for use as a control.

A standard Fusarium cell wall suspension (2.3 mg/ml) was washed and sterilized by autoclaving at 15 lb. pressure and 121 C temperature for 15 minutes. The suspension was then aseptically divided into 30 ml portions and the cell walls were pelleted by centrifugation. The pellet was retained and the liquid discarded. Thirty ml of the culture supernatants were added and shaken with the pelleted cell walls and the mixture was aseptically introduced to nephloflasks. This technique carefully controlled the amount of Fusarium cell wall material which was added to each sample. Lytic activity was measured by recording, at various intervals, the decrease in turbidity in a Klett-Summerson photoelectric colorimeter with a red filter (625 mμ).

The supernatant from isolate No. 8 was sterilized by passing it through a millipore filter (0.22). The supernatant was then tested for the presence of glucose with Glucostat, an enzymatic reagent obtained from the Worthington Biochemical Corporation, Freehold, N. J. The test was performed according to the instructions which accompanied the reagent.

In each of 5 replicate test tubes 2.5 ml of sterilized bacterial culture supernatant were mixed with 2.5 ml of Fusarium cell wall preparation (2.3 mg/ml). The tubes were incubated at 25 C and individual tubes were assayed for glucose and N-acetylglucosamine at hourly intervals for 5

hours. A heated control (60 C for 30 minutes) was also included. Glucose was assayed by means of Gucostat reagent. An estimation of the N-acetylglucosamine released was determined with p-dimethylaminobenzaldehyde (DMAB) reagent by the method of Reissig, Strominger, and Leloir (1955) (see Appendix for standard curve).

Further experiments were conducted on the lysis of Fusarium cell walls with a concentrated enzyme preparation. Isolate No. 8 was grown for 3 days in 3 liters of Morris media with Fusarium cell walls (0.2%) as the carbon source. The cellular debris was removed by centrifugation at 9,000 G for 15 minutes. Proteins were precipitated with 90% ammonium sulphate at pH 7.0. The precipitate was collected by centrifugation and then dissolved in a small amount of 0.01 M phosphate buffer at pH 7.0. The solution was then dialyzed in the cold in deionized distilled water for 24 hours. After dialysis the final solution was brought to a volume of 50 ml. Protein content was obtained by biuret analysis (Gornall, et al., 1949) (see Appendix for standard curve).

The solubilization of Fusarium cell walls by the concentrated enzyme preparation was tested. To each of 8 test tubes (15 x 100 mm) the following additions were made:

Test tube No.	Cell wall suspension	Distilled water	Phosphate buffer 0.5 M pH 6.5	Enzyme prep-ation	Incubation time
1	4 ml	0.25 ml	0.5 ml	0.25 ml	30 min
2	"	"	"	"	60 min
3	"	"	"	"	90 min
4	"	"	"	"	120 min
5	"	"	"	"	180 min
6	"	"	"	"	240 min
7	"	0.50 ml	"	0	"
8	"	0.25 ml	"	0.25 ml*	"

* Heat treated, 15 minutes at 70-75 C.

After each test tube completed its incubation period it was heated for 10 minutes in a boiling water bath to halt the reaction. In preliminary experiments it was found that the Fusarium cell walls tended to clump together as soon as the enzyme preparation was added. This made optical density readings difficult and inaccurate. To counteract this effect each sample was treated for 3 minutes in a Bronwill ultrasonicator to disperse the remaining cell wall material. After taking readings on the Klett-Summerson photoelectric colorimeter each sample was filtered through No. 40 Whatman Filter Paper and assayed for glucose and N-acetylglucosamine. The tests were performed in the same manner as before except that Glucostat was dissolved with

0.1 M phosphate at pH 7.0.

The influence of pH on laminarin activity was also measured. To a series of test tubes numbered 1 to 11 the following additions were made:

Test tube No.	Buffer	pH	Laminarin solution	Enzyme preparation
1	0.5 ml	3.6	4.0 ml	0.5 ml
2	"	3.9	"	"
3	"	4.4	"	"
4	"	5.0	"	"
5	"	5.5	"	"
6	"	6.2	"	"
7	"	6.5	"	"
8	"	7.0	"	"
9	"	7.4	"	"
10	"	7.8	"	"
11	"	7.0	"	0.5 ml*

* Enzyme preparation was heated at 70 C for 15 minutes.

The test tubes were incubated for 80 minutes. Citrate-phosphate buffers (1.0-0.5 M) were used for pH values ranging from 3.6 through 6.2; phosphate buffers (0.5 M) were used for pH values ranging from 6.5 through 7.8 (Colowick and Kaplan, 1954). The laminarin solution was prepared at a concentration of 10 mg/ml.

A similar study was performed to determine the effect of pH on chitinase activity. The experiment was conducted in the same manner as above except that 4 ml of colloidal chitin (6.7 mg/ml) was used rather than 4 ml of laminarin solution. Incubation time was increased to 100 minutes. Chitinase activity was measured by the decrease in optical density in Klett units on the Klett-Summerson photoelectric colorimeter.

A unit (U) of glucanase was defined as the quantity of enzyme required to catalyze the formation of 1 μmole of glucose per minute at 25 C and pH 6.0 from laminarin. A unit of chitinase was defined as the quantity of enzyme required to catalyze the formation of 1 μmole of N-acetyl-glucosamine per minute at 25 C and pH 5.5 from colloidal chitin.

RESULTS

Effect of Lobster Shell on the Distribution of Soil Microflora

As will be seen from TABLE 1 the application of lobster shell was associated with a general increase with bacterial numbers which appeared to be proportional to the concentration of lobster shell applied; the most significant increase was obtained at 3,000 ppm. This effect rapidly fell off, however, at the lower concentrations and after 4 weeks no appreciable difference in numbers was observed between the control pots and pots receiving 100 and 500 ppm lobster shell. After 8 weeks there appeared to be a general levelling of populations even at the maximum rate of application.

More significant, however, was the selective effect on population change which appeared to be associated with lobster shell application. This was most strikingly reflected in the appearance of enhanced numbers of actinomycetes as shown in TABLE 2. Within 1 week in the presence of 3,000 ppm lobster shell there was virtually a tripling in the numbers of actinomycetes isolated. This effect was sustained in a manner consistent with the general population shift, i.e., for some 4 weeks, and by 8 weeks was largely dissipated.

A further population shift was observed to be operative among the fungi. After 1 week the number of fungi associated

with maximum application of lobster shell rose significantly (TABLE 3). It was found ,too, that these were largely restricted to the genus Penicillium. The particular strain present was not found, however, to be causally associated with the redistribution of bacteria noted. When it was spotted onto pour plates of nutrient agar seeded with bacteria no antibiotic effect was observed. An antibiotic interaction was, however, associated with the actinomycete population which appeared to be stimulated by the application of lobster shell. This stimulation was maintained for the duration of the general period of lobster shell activity, some 4 weeks (TABLE 6).

Since chitin was hypothesized to be the operative element in lobster shell,which in fact exercised the major selective action, an attempt was made to establish whether lobster shell-selected populations were in fact chitinase producing organisms. As will be seen in TABLE 4, there was a striking increase in chitinase producers which follows the general curve of lobster shell effect on population selection. It was also significant that these chitinase producers were almost exclusively actinomycetes. It was evident, therefore, that the presence of lobster shell preferentially stimulated chitin-using-actinomycetes rather than other microbial respondents to this compound.

The rationale for the application of lobster shell as a soil amendment was to induce a population of chitinase producers. After the depletion of lobster shell chitin, these organisms would presumably attack Fusarium for the chitin in its mycelium, thereby bringing about the lysis of the fungus. An attempt was therefore made to establish whether lobster shell would in fact generate a significant number of organisms capable of lysing Fusarium. In TABLE 5 it will be seen that a large increase in the number of such lytic organisms was in fact achieved and that this pattern followed the general population trend induced by the lobster shell amendment. In Figures 1 and 2 are shown typical chitinase producing organisms on both pure chitin as well as on Fusarium cell walls.

TABLE 1 -- The influence of lobster shell on numbers of bacteria in soil.

	Nos. of Bacteria x 10^3/gm of soil			
	Lobster Shell ppm			
Weeks	0	100	500	3,000
0	8,900	-	-	-
1	8,100	10,600	12,300	16,300
2	9,900	10,100	11,100	13,600
4	9,000	9,300	8,500	16,300
8	10,200	10,600	9,500	12,100

TABLE 2 -- The influence of lobster shell on numbers of actinomycetes in soil.

	Nos. of Actinomycetes x 10^3/gm of soil			
	Lobster Shell ppm			
Weeks	0	100	500	3,000
0	2,800	-	-	-
1	3,500	4,200	4,200	12,000
2	3,700	5,100	7,500	9,100
4	5,700	5,200	5,700	10,200
8	8,000	8,800	10,600	10,300

TABLE 3 -- The influence of lobster shell on numbers of fungi in soil.

	Nos. of Fungi x 10^3/gm of soil			
	Lobster Shell ppm			
Weeks	0	100	500	3,000
0	93±6*	-	-	-
1	80±5	110±6	90±8	480±2
2	77±5	93±6	120±7	93±5
4	93±6	63±5	100±5	110±4
8	56±2	65±4	77±7	70±5

* Numbers indicated after ± are standard deviations.

TABLE 4 -- The influence of lobster shell on numbers of chitinase-producing organisms in soil.

	Nos. of Chitinase-Producing Org. x 10^3/gm of soil			
	Lobster Shell ppm			
Weeks	0	100	500	3,000
1	750	700	1,700	11,200
2	470	430	1,200	4,100
4	1,500	2,400	2,300	4,300
8	480	740	490	690

TABLE 5 -- The influence of lobster shell on numbers of organisms lytic to *Fusarium*.

	Nos. of Lytic Organisms x 10^3/gm of soil			
	Lobster Shell ppm			
Weeks	0	100	500	3,000
1	130	130	630	990
2	47	42	73	800
4	97	250	260	830
8	220	320	520	320

TABLE 6 -- The influence of lobster shell on numbers of antibiotic-producers against *Fusarium*.

	Nos. of Antibiotic-Producers x 10^3/gm of soil			
	Lobster Shell ppm			
Weeks	0	100	500	3,000
1	120	90	1,000	2,500
2	66	130	710	2,300
4	50	170	320	550
8	80	120	180	160

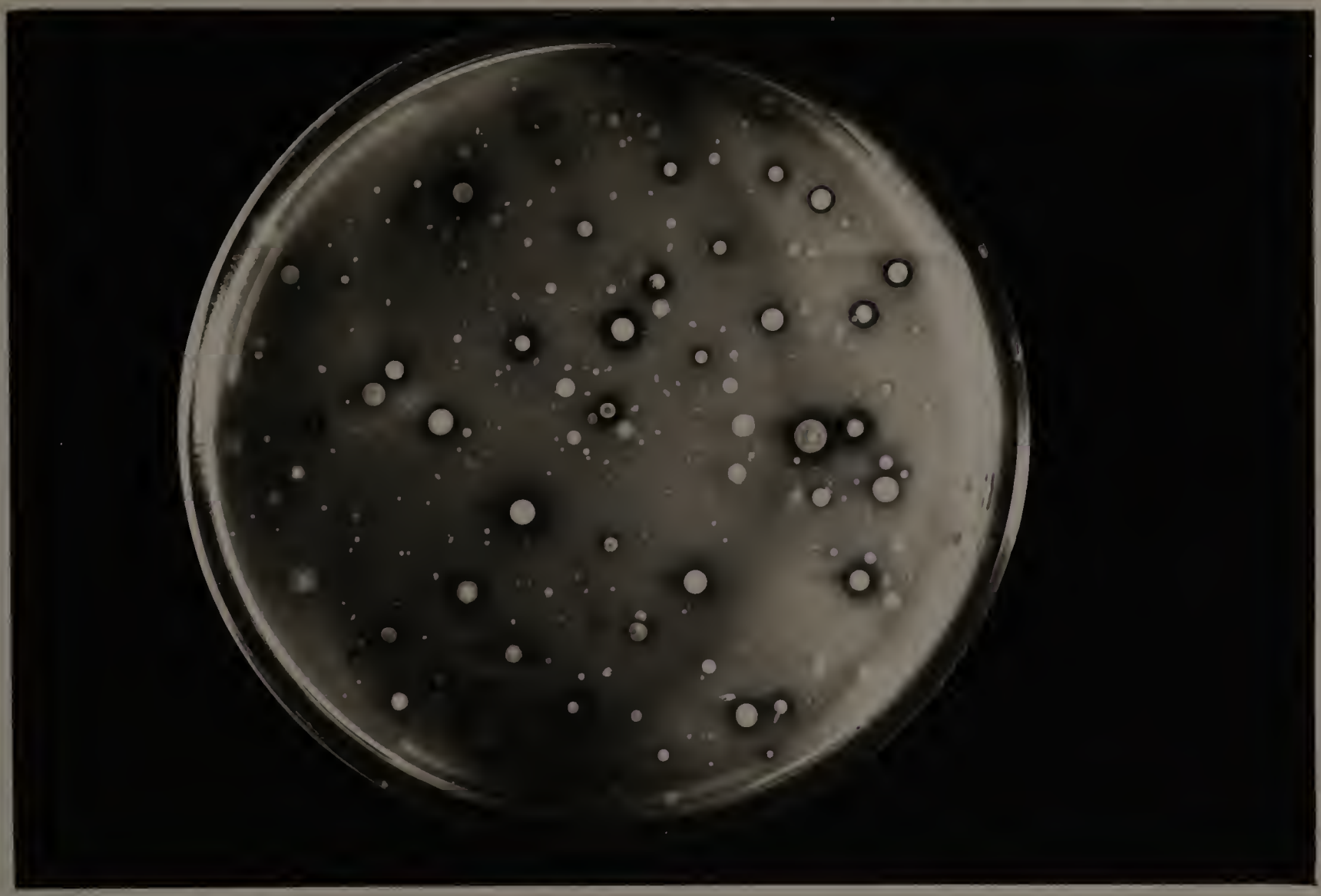


Fig. 1. A typical chitin agar plate with clear areas around many of the colonies indicating chitinase activity. Most of the colonies were actinomycetes.

Fig. 2. An agar plate seeded with Fusarium cell wall homogenate. Clear areas around colonies demonstrates lysis of cell walls. The population consisted largely of actinomycetes.

Population of Actinomycete Antibiotic-Producers Against *Fusarium* in Soil

The portion of the actinomycete population which produced antibiotics against *Fusarium* on nutrient agar plates was found to be 30% (TABLE 7). A significant proportion (39%) of these organisms were regarded as strong inhibitors, i.e., they inhibited the growth of *Fusarium* 10 mm or more from the actinomycete colony. It can be seen from Fig. 3 the various degrees of inhibition displayed by different actinomycete isolates.

TABLE 7 -- Proportion of actinomycetes which produce antibiotics against *Fusarium*.

Total Number of Actinomycetes Tested	% of Strong[a] Inhibitors	% of Weak[b] Inhibitors	Total % of Inhibitors
60	12%	18%	30%

[a] Inhibition of 10 mm or greater
[b] Less than 10 mm inhibition

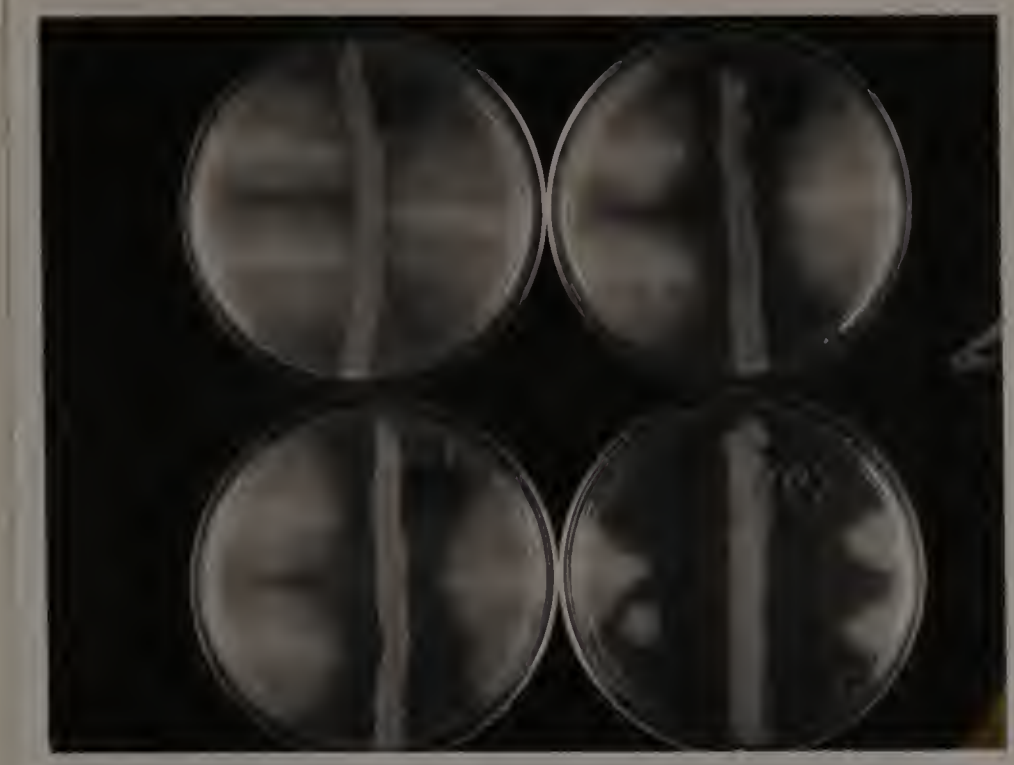


Fig. 3. Different actinomycete isolates exhibiting various degrees of inhibition against Fusarium.

The Dry Root Rot of The Bean

The plants which were artificially infected all showed some degree of disease (TABLE 8). A number of plants became severely infected shortly after germinating. These plants had reddish-brown lesions extending from the root crown to a height of 2-3 cm along the stem within 7-14 days. A short time afterwards they became stunted, turned brown and died. Most of the plants, however, lived through the 5 weeks of experiment. The root rot did not seem to impair the growth of these plants to any great extent. This observation is in agreement with Burkholder (1924) who noted that the disease

TABLE 8 -- Susceptibility of Phaseolus vulgaris to Fusarium solani inoculation.

Time in Weeks	Disease Rating (0-5) Average of 5 Plants/Replicate Pots			
	1	2	3	4
1	0	0	0.4	0.8
2	0.2	0.2	1.0	1.6
3	0.8	1.0	1.8	2.0
4	1.6	1.8	2.0	3.2
5	2.0	2.4	2.8	3.2

reduced yield by about 50% in a dry season but only 10% in a wet season. Since the plants were kept reasonably moist in this experiment, few adverse effects were produced by the disease despite an observed reduction in the size of the root system. Also, unlike many other species of Fusarium, in this instance wilting was seldom produced. This has led to the belief that the fungus does not produce substances which, when carried to plant parts above ground, prove toxic enough to cause noticeable injury (Burkholder, 1920).

The symptoms observed in the diseased plants were in some instances only a few distinct reddish-brown lesions on the taproot. Usually, however, there was a more or less uniform reddish discoloration of a major portion of the taproot extending a few centimeters above the surface of the ground (Fig. 4) and to the larger fibrous roots. The root system of the infected plants was usually severely reduced by more than 50%. The infected roots became dry and shriveled and were easily broken off. This left only a few surface roots for the plants. These symptoms correspond very closely to those described by Burkholder (1920) and Weimer and Harter (1926).

Fungal growth extended from the majority of the stems placed on the PDA plates. Upon examination under the microscope 90% of the infected stems were shown to be exclusively

inhabited by F. solani.

Effect of Lobster Shell on Bean Root Rot Disease

The results are summarized in TABLE 9. In general the application of lobster shell at 1,000 ppm tended to increase the disease or make it just as severe (Fig. 4). Disease was most severe when the lobster shell and Fusarium spores were applied 1 week after planting the bean seeds. The disease seemed to be least severe when the seeds were planted 1 week or 2 weeks after the application of lobster shell and Fusarium spores. There appeared to be little difference in disease index between wounded and nonwounded plants.

When lobster shell was applied at 500 ppm there was a significant reduction in disease severity (Fig. 5). Plants infected with Fusarium in pots untreated with lobster shell showed significantly larger and more frequent lesions at the stem base. The presence of lobster shell was correlated with fewer lesions; this was the only distinct difference in the appearance of the plants. In height, leaf mass, and leaf color little difference could be discerned between treated and untreated plants.

TABLE 9 -- Mean disease index[a] of bean plants treated with *Fusarium* alone and *Fusarium* plus lobster shell.

Time of treatment in weeks after or before planting of beans	Disease Index			
	Lobster Shell 1,000 ppm Nonwounded	Control Nonwounded	Lobster Shell 1,000 ppm Wounded	Control Wounded
4 wks after planting	46	35	39	40
2 wks after planting	58	58	58	43
1 wk after planting	69	41*	64	63
0 same time as plant.	36	20*	52	33
1 wk before planting	29	52	23	16
2 wks before plant.	20	40	21	30

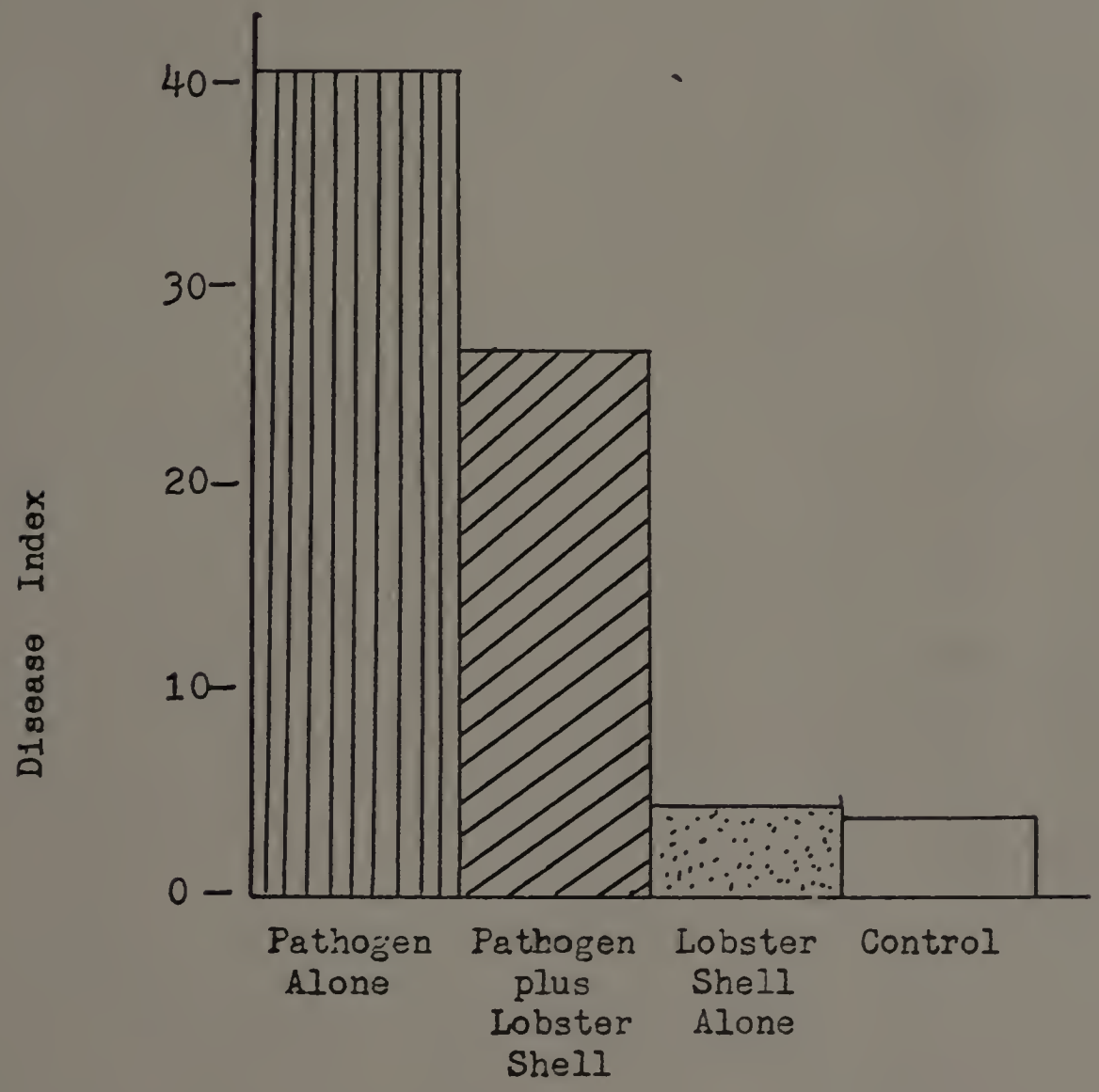
* Statistical difference at the 99% confidence level

[a] Means were compared either by the Student t test or the rank sum test. Only those with an asterisk showed a significant statistical difference. The rest of the means were not significantly different from the controls even at the 95% confidence level. This was due to large variations within the means and not enough replica samples.

Fig. 4. The effect of high applications of ground lobster shell (1,000 ppm) applied to the surface of the pot. Pot A was treated with lobster shell plus Fusarium. Pot B was treated with Fusarium alone.

Fig. 4 continued. Pots C and D were the controls. Pot C was treated with ground lobster shell alone. Pot D was untreated.

Fig. 5. The decrease in mean disease index* of Fusarium root rot of beans in soil mixed with 500 ppm lobster shell.



* Significant difference at the 99% confidence level according to the Student t test.

Lysis of *Fusarium* Cell Walls by Bacterial Isolates

Eighteen bacteria were isolated from the bean rhizosphere which were capable of lysing dead *Fusarium* cell walls. In Fig. 6 is shown the digestive action of a lytic bacteria when streaked across the surface of a *Fusarium* cell wall agar plate. A clear area appears where fungal cell walls are lysed. All the bacteria isolated were Gram negative. The isolates were all motile rods of varying lengths and were therefore judged to be pseudomonads. All of the isolates exhibited good growth on chitin and laminarin agar plates. This was accepted as inferential evidence for chitinase and laminarinase activity.

None of the bacterial isolates possessed the ability to lyse the living fungus. *Fusarium* growth in the presence of lytic bacteria was consistently less than *Fusarium* grown in pure culture. This appeared to be due merely to competition between the fungus and the bacterium for the available carbon source.

Four of the isolates, Nos. 1, 8, 17, and 20, were tested for their ability to control the bean root rot disease in aseptic culture. None of these isolates were effective in controlling the disease.

The culture supernatants of isolates 6, 8, and 12 grown

Fig. 6. Shown above is the lysis of dead Fusarium cell walls by lytic bacterium No. 12. The bacterium was streaked across the surface of a Fusarium cell wall agar plate. The plate was incubated at room temperature for 3 days.

in a medium containing dead Fusarium cell walls as the sole carbon source appeared to effect the lysis of the hyphae (Fig. 7-9). It should be noted that immediately upon addition of the cell walls to the supernatant a certain amount of clumping was observed, an effect which was later achieved by the addition of crude lytic enzyme complex. Furthermore, this was not evident in the flasks containing the heated supernatants, suggesting that the clumping was enzymatically activated. Since the supernatants were not sterile the large increase in lysis observed after the first hour of incubation was probably due to new bacterial growth rather than to enzymes already present in the supernatant. Indeed, the turbidity produced by the large bacterial growth masks any further decrease in optical density after 4 hours. The pH remained between 7.1-7.2 throughout the entire experiment.

When the supernatant of isolate No. 8 was sterilized no lysis of dead Fusarium cell walls was evident. Even after 5 hours incubation there was no decrease in turbidity and no glucose or N-acetylglucosamine released. Evidently, the sterilization process either removed or deactivated the enzymatic activity of the supernatant.

Lytic components released by the bacteria were obtained from the culture supernatant of isolate No. 8 by ammonium sulfate precipitation. This resulted in approximately a

60-fold increase in activity per ml. Chitinase activity of the preparation was 218 mU/ml and the laminarinase activity of the preparation gave a value of 206 mU/ml. The amount of protein per ml of the enzyme preparation was 2.5 mg.

The lysis of Fusarium cell walls by the concentrated enzyme preparation is described in Fig. 10. After 5 hours incubation there was a decrease in turbidity of about 200 Klett units and a release of 34 µg/ml of glucose and 28 µg/ml of N-acetylglucosamine.

The optimum pH for laminarinase and chitinase activity was determined using laminarin and colloidal chitin respectively as the substrates. The pH optimum for laminarinase was found to be 6.0 (Fig. 11); the pH optimum for chitinase was determined to be approximately 5.5 (Fig. 12).

Fig. 7. Lysis of dead Fusarium cell walls by bacterial culture filtrate No. 6.

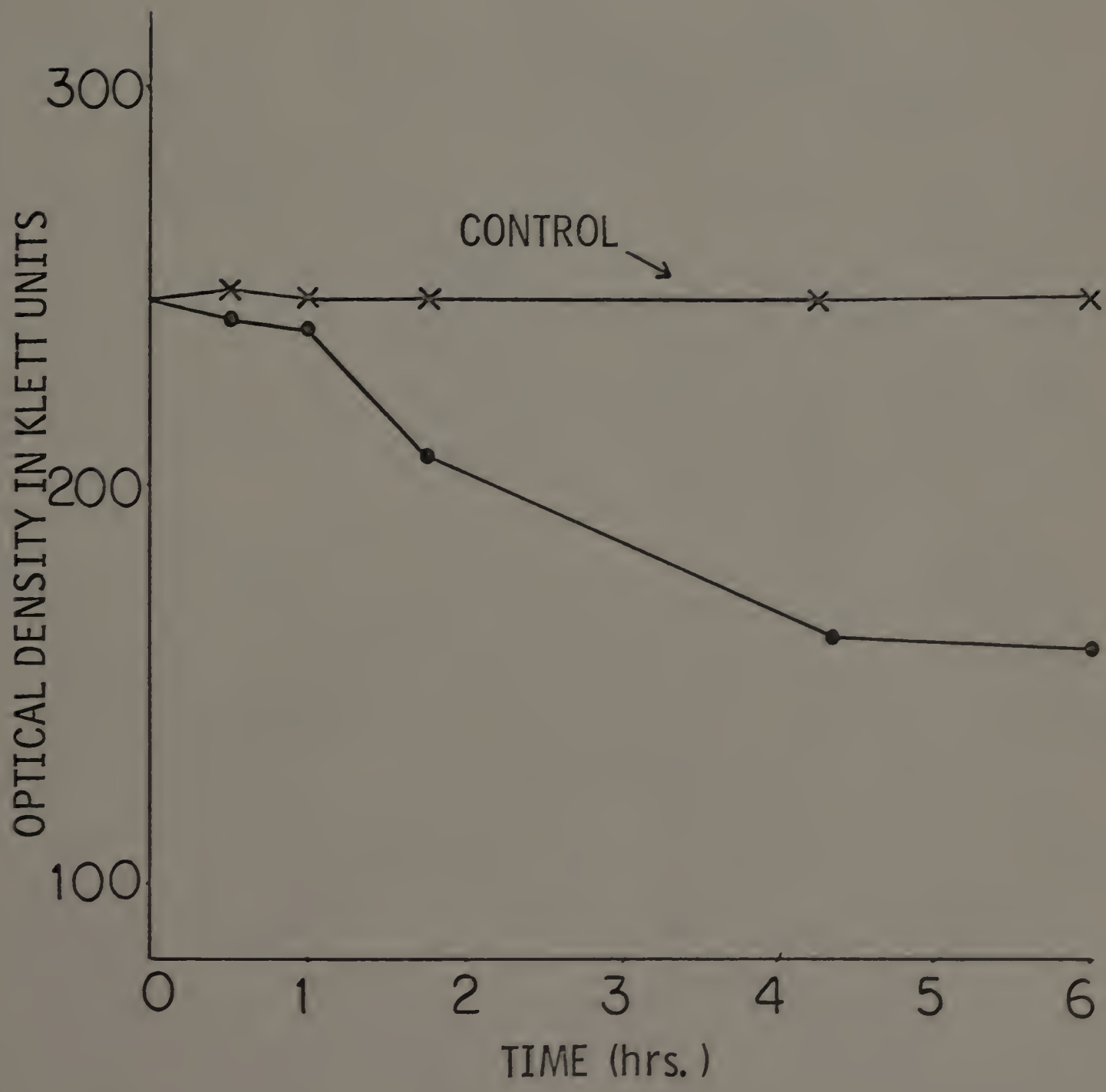


Fig. 8. Lysis of dead Fusarium cell walls by bacterial culture filtrate No. 8.

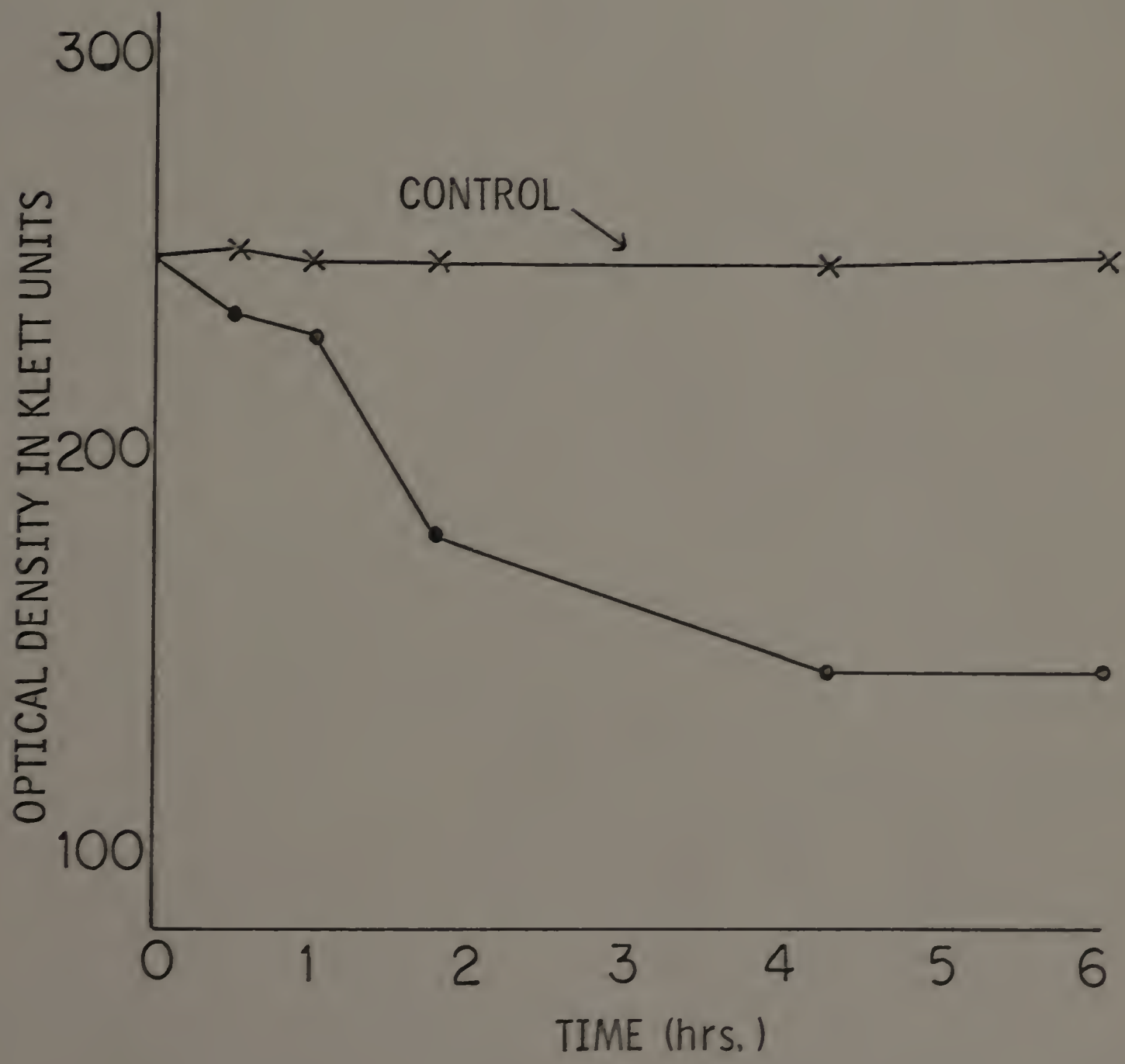


Fig. 9. Lysis of dead Fusarium cell walls by bacterial culture filtrate No. 12.

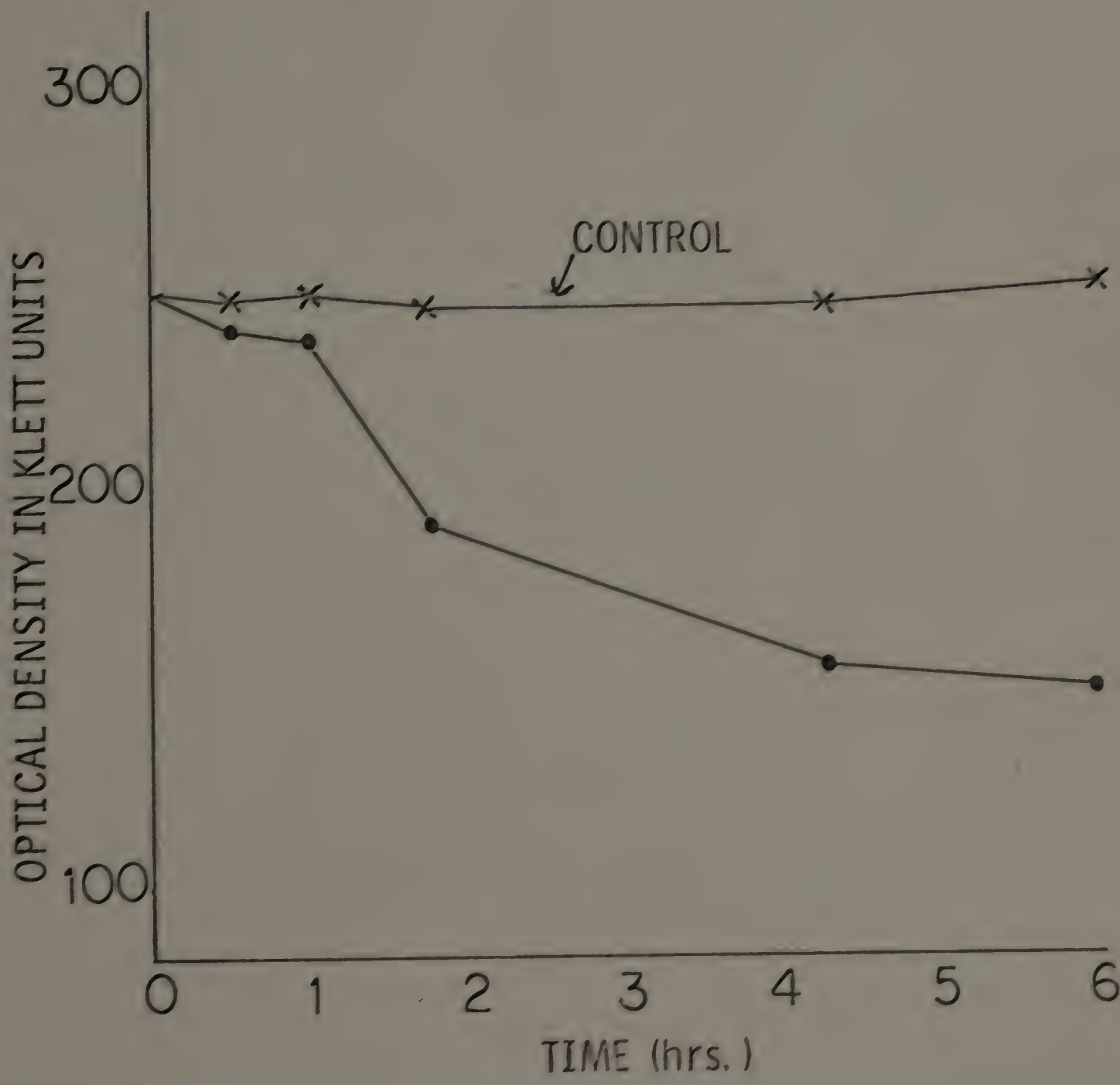


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Fig. 7. Decrease in turbidity (1) and release of glucose (2) and n-acetylglucosamine (3) in the digestion of Fusarium solani cell walls by a concentrated lytic preparation obtained from the supernatant of bacterial culture No. 8.

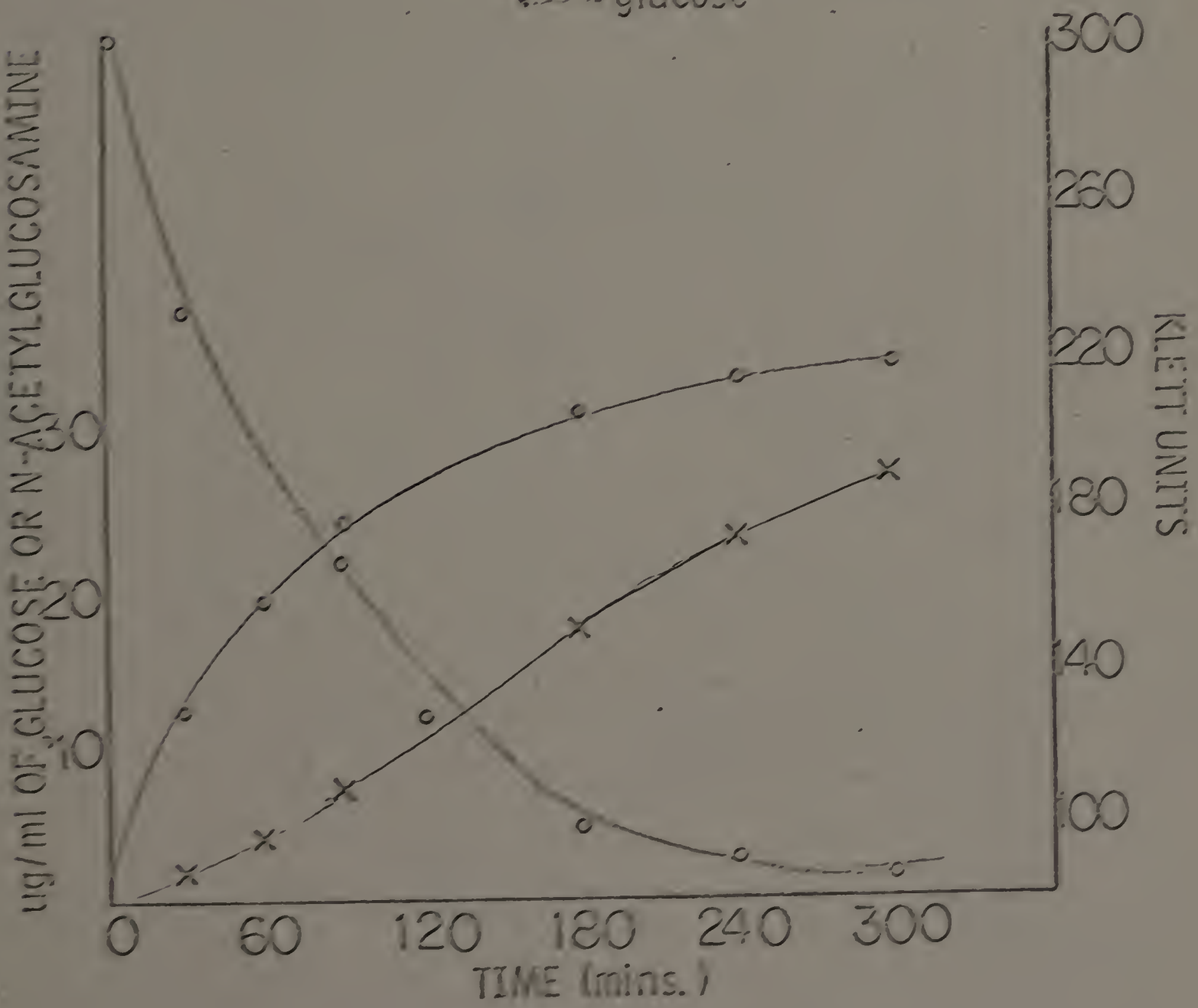


Fig. 11. Optimum pH for laminarinase obtained from the supernatant of bacterial culture No. 8. with laminarin as substrate.

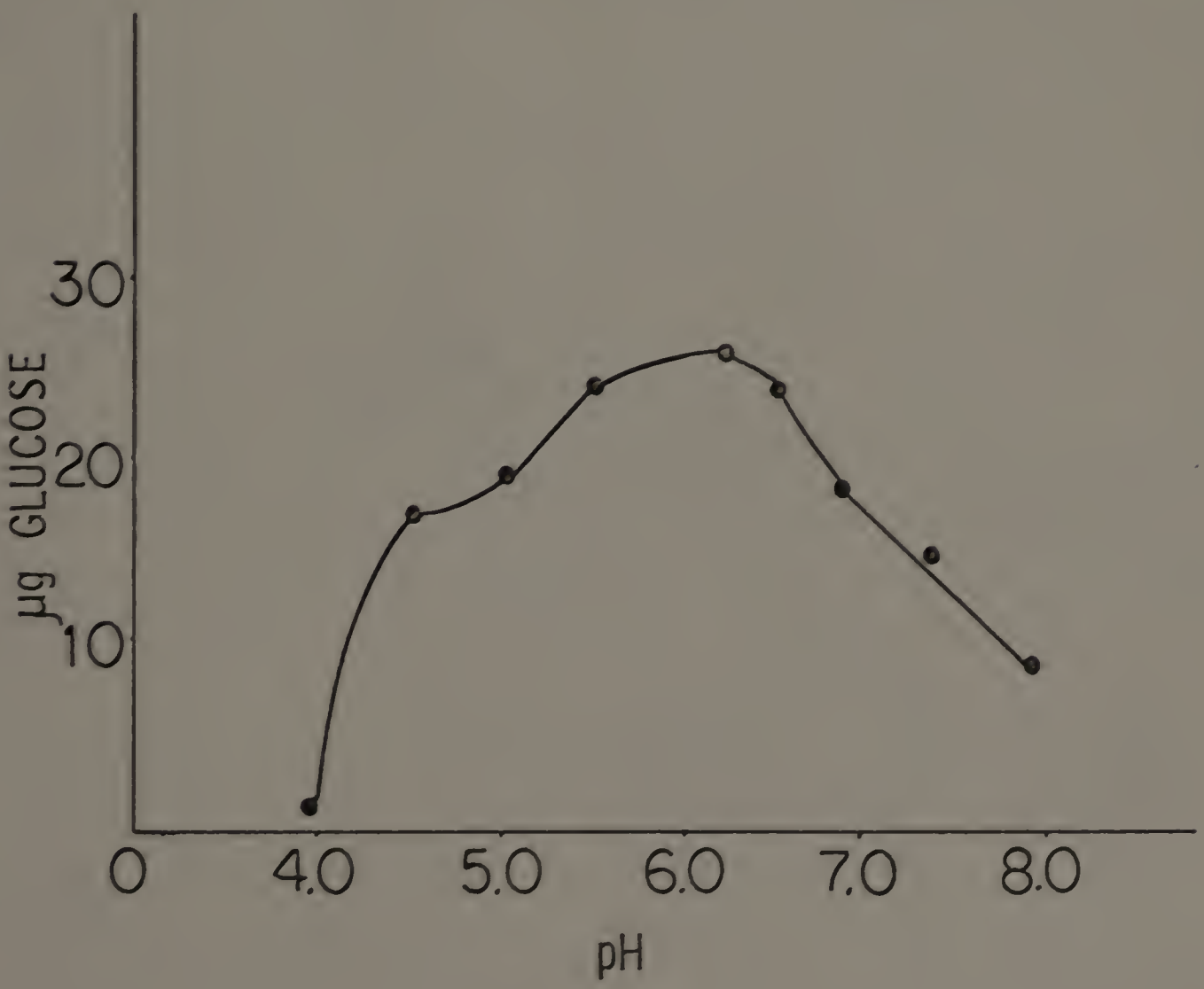
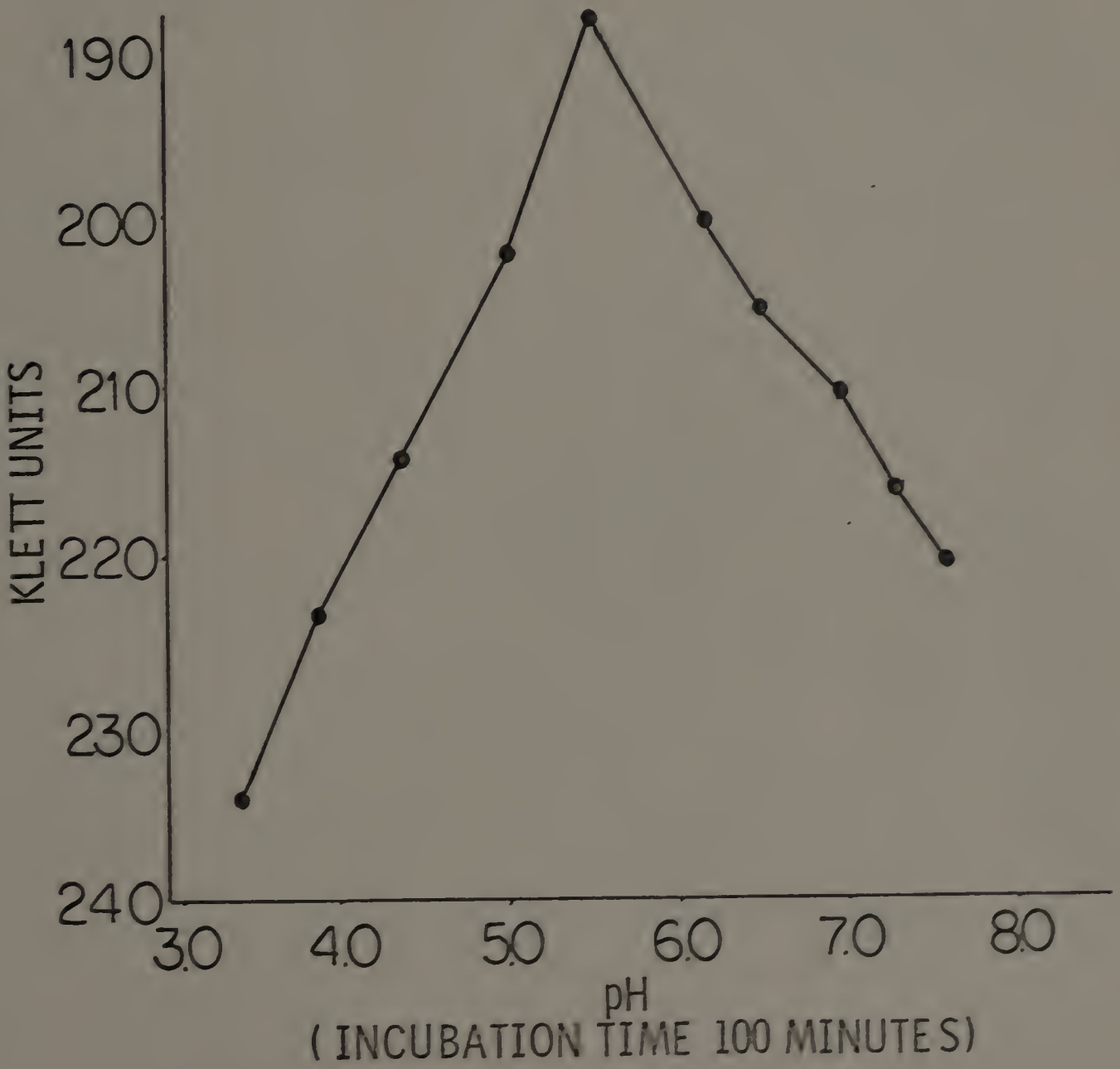


Fig. 12. Optimum pH for chitinase obtained from the supernatant of bacterial culture No. 8 with colloidal chitin as substrate.



DISCUSSION

Four phases have been described which are necessary for the pathogenesis of Fusarium solani. First, the chlamydospores must be stimulated to germinate. Second, the hyphae must be able to successfully compete with other microorgansms until they have reached a susceptible host. Third, the fungus must be able to penetrate the host. Fourth, the fungus must be able to grow and multiply within the host. Control over F. solani can be gained by killing or stopping the growth of the fungus in any one of the four phases.

It has been shown that exogenous supplies of carbon and nitrogen or combinations of other organic nutrients cause the germination of chlamydospores (Schroth, Toussoun, and Snyder, 1963; Toussoun and Snyder, 1961). Adams, Lewis, and Papavizas (1968) demonstrated that chlamydospore germination was inhibited when cellulose was added to soil. It seems unlikely, however, that this mechanism was responsible for the reduction in disease severity when the soil was amended with lobster shell. Lobster shell is a rather rich organic substrate consisting of chitin and protein embedded in a calcium carbonate matrix (Richards, 1951). Therefore, lobster shell may add to the amount of available nutrients

and possibly stimulate chlamydospore germination.

The fourth phase of pathogenesis can also be ruled out as the control mechanism since no strain of beans is known to be resistant to the fungus once it gets inside the tissues. This leaves two possibilities. Either the fungus is killed before it enters the host or the fungus is prevented from penetrating the host. Toussoun, Nash, and Snyder (1960) demonstrated that high concentrations of glucose on root surfaces delayed penetration. However, glucose did not prevent ultimate penetration by the fungus. An excess of nitrogen, on the other hand, was found to increase penetration by the fungus. Since lobster shell has a low C:N ratio, it seems more likely that nitrogen is more available than glucose. It is dubious that the application of lobster shell liberated sufficient glucose for this to become a factor in reducing Fusarium attack. Therefore, one must infer that a mechanism for disease control with lobster shell application would more likely be associated with the killing of the fungus once it has germinated.

It was noteworthy that the number of actinomycetes in relative terms showed the greatest increase. This corresponded with the substantial increase in the lytic and chitinivorous capacity of the population which in effect was largely due to its actinomycete component. What, therefore, appears to be operative at one level is a lobster shell stimulus to a

population of lysing and chitinase-producing actinomycetes. It is justifiable, therefore, to propose that actinomycetes would appear to play a dominant role by direct physiological assault on the fungus tissue. At another level may be the role which actinomycetes may play as a result of their well known capacity to produce a wide variety of antibiotics. Lockwood and Lingappa (1963) have demonstrated that certain of the fungistatic properties of a sterilized soil can be restored by inoculating with a species of Streptomyces. But more significant, however, may be the climination or restriction by actinomycete antibiosis of critical strains of bacteria which may compete with Fusarium. This might explain why applications of 500 ppm lobster shell retrict Fusarium while 1000 ppm appear to enhance it. It could be postulated that at the lower level, while actinomycetes are encouraged, they are not present in sufficient numbers to restrict bacterial populations by their antibiotic excretions. At the high level of lobster shell the concentration of antibiotics may be sufficient to eliminate large segments of bacterial flora which by competition with, or lysis of, Fusarium play a critical role in limiting the fungus. In the absence of these bacterial populations the limited mass of even the enhanced numbers of actinomycetes is insufficient to effectively limit the fungus. Uninhibited by bacterial predation or competition for substrate Fusarium proliferates.

It is of interest that a similar phenomenon is reported from Nova Scotia[1]. Here, where lobster shell is applied seasonally to cereal crops, there is a tradition that though the application of lobster shell seems to be a guarantor of good yields and root rot free plants - "Once a lobster shell ran, always a lobster shell ran". To omit even 1 years application is to invite a violent resurgence of root rot. The implications were clear. Repeated applications of lobster shell stimulate the emergence of high numbers of actinomycetes which in turn restrict bacterial competitors to Fusarium. However, when lobster shell is not applied, few actinomycetes arise in an area where the bacterial competitors and predators have over the years been kept low. The root rot organisms, now unhampered either by actinomycetes or bacteria, proliferate and attack the crop without restrictions.

Mitchell and Alexander (1962) suggested that the lytic mechanism was responsible for reducing bean root rot when chitin was mixed with soil. Since Fusarium contains chitin in its cell walls, they proposed that a functional relationship between lobster shell induced chitin-users and Fusarium cell wall destruction was involved. Thus, damping off caused by Pythium debaryanum was not controlled by chitin amendments, and Pythium, in fact, does not contain chitin

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1. Oral communication, Mr. S. Malkin, Atlantic Canners Inc., Nova Scotia.

in its cell walls.

Alexander and his colleagues did isolate bacteria which can lyse live Fusarium. However, it has not been proven that organisms such as there play a dominant role in limiting Fusarium in natural soils. The simple fact that enrichment cultures were necessary to isolate these bacterial types indicates that they may not be widely prevalent in soil. Indeed, all eighteen of the lytic bacteria isolated in this study appeared to be merely saprophytic. It would appear that most of the lytic bacteria are merely saprophytic rather than parasitic in nature. However, saprophytic bacteria may play an important role in limiting Fusarium when functioning in conjunction with other microorganisms.

Moreover, lysis of fungi may also be brought about in ways other than the direct lysis by bacteria or actinomycetes. For example, substances released by an actinomycete may trigger a mechanism in fungi which causes them to autolyse. Antibiotics have been shown by Carter and Lockwood (1957) to cause lysis of the fungus, Glomerella cingulata. Also, Mitchell and Sabar (1965) demonstrated that autolytic activity of a Pythium culture increased with age.

Further, more than the enzyme chitinase is involved. Laminarinase (Mitchell and Alexander, 1963; Skujins, Potgieter, and Alexander, 1965) and other as yet unidentified enzymes

have all been shown to be necessary for Fusarium lysis in vitro. Nor can mycelium alone be considered as an exclusive target for antagonism. Fusarium chlamydospores and other structures in the life cycle must be taken into account.

The apparent discrepancy between dose response relationships in which higher applications of lobster shell stimulate disease may also be related to chlamydospore persistence. Thus, if competition between Fusarium and other microbial populations were to play a part in the lobster shell system one might expect the Fusarium to produce chlamydospores when the nutrients become limited. The data supports this hypothesis to some extent since there appeared to be no decrease in the numbers of fungi where lobster shell is applied at controlling rates, indicating that chlamydospores may in fact have been formed.

High lobster shell applications may also temporarily increase microbial activity, this in turn may cause an increase in CO_2 concentration which stimulates chlamydospore germination. Bourret, Gold, and Snyder (1958) found that in control soils only about 20% of the chlamydospores germinated but at 20% CO_2 concentration about 50% of the chlamydospores germinated. On the other hand, high CO_2 concentrations inhibit chlamydospore formation (Bourret, Gold, and Snyder, 1965).

It has also been found that the addition of soluble

nutrients to soil may delay the onset of mycolysis. It is possible that high lobster shell applications provide enough soluble nutrients to delay lysis. This gives the fungus time to penetrate the host. High lobster shell amendments may also result in an excess of nitrogen around the hypocotyl which may increase the penetration ability of the fungus (Toussoun, Nash, and Snyder, 1960). In any event it is evident that a complex sequence of physiological interactions are initiated by the addition of lobster shell of which the lytic and antagonistic phenomena based on chitin selectivity offer only a partial explanation of the ultimate effect on Fusarium. Nonetheless, it is not premature to suggest that these insights offer sufficient grounds for continuing efforts to seek out and magnify the natural exclusion mechanism present in the environment, so that an effective measure of the biological control of soil-borne plant disease may be realisticly achieved.

APPENDIX

1. Glycerol Agar for Actinomycetes

Glycerol	10.0 gm
Sodium asparaginate	1.0
K_2HPO_4	1.0
Agar	15.0
Tap water	1.0 L

Adjust to pH 7.0 before adding the agar.

2. Glucose-Peptone Agar for Fungi

KH_2PO_4	1.0 gm
$MgSO_4.7H_2O$	0.5
Peptone	5.0
Glucose	10.0
Agar	20.0
Tap water	1.0 L

Adjust to pH 4.0 with sterile N/10 H_2SO_4just prior to pouring plates.

3. Minimal Media A

KH_2PO_4	0.03 gm
KNO_3	4.00
$MgSO_4.7H_2O$	0.60
$FeCl_3.6H_2O$	0.05
$ZnSO_4.7H_2O$	0.03

Agar	15.0 gm
Distilled water	1.0 L

Adjust pH to 7.0.

4. Morris Media

K_2HPO_4	7.0 gm
KH_2PO_4	3.0
KNO_3	2.0
$MgSO_4.H_2O$	0.2
$FeCl_3$	1.0 cc (1 gm/ml)
$CaCl_2$	1.0 cc (1 gm/ml)
Distilled water	1.0 L

Trace Salts -- 0.2 cc of each of the following:

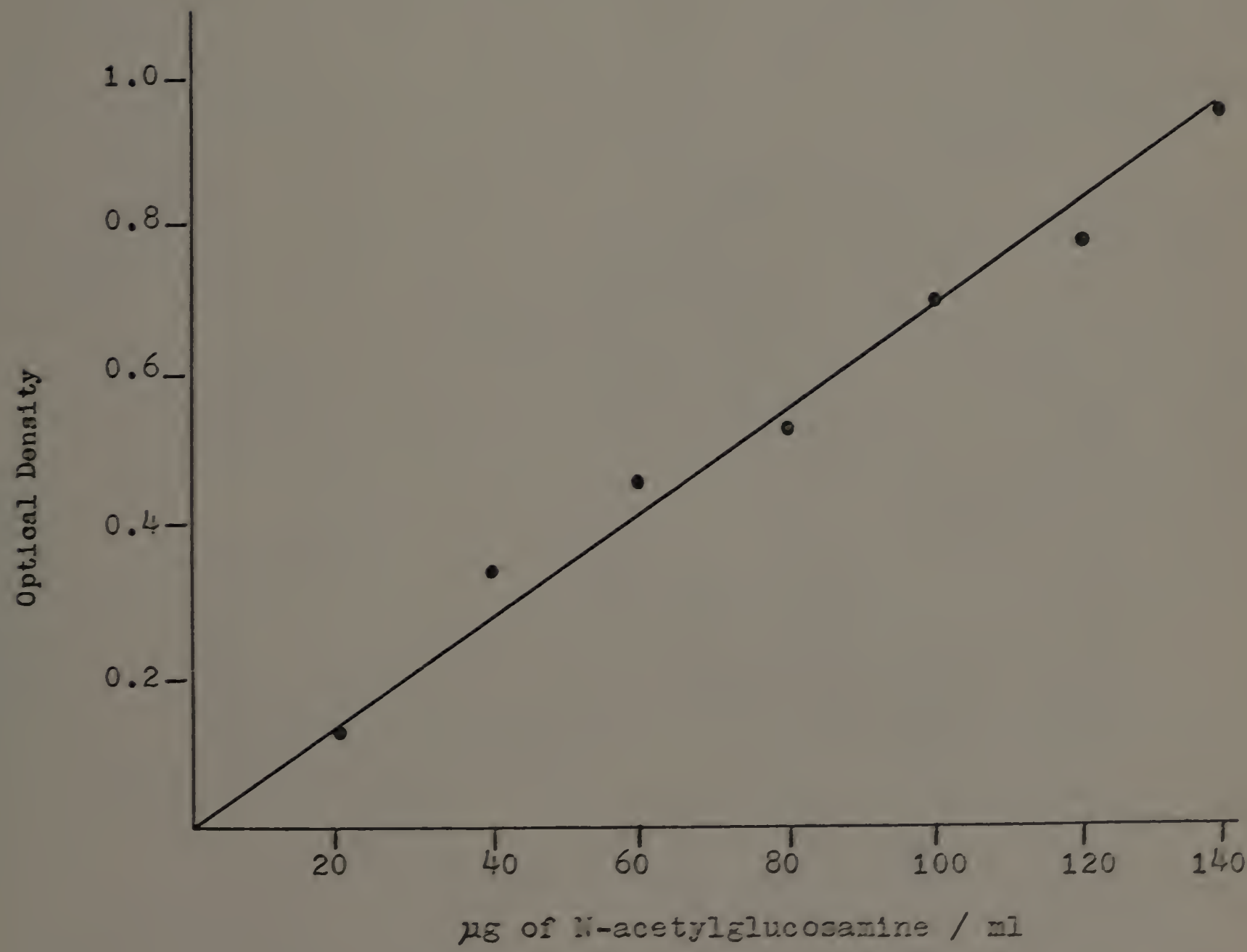
H_3BO_3	2.80 gm/ml
$MnCl_2.4H_2O$	1.86
$CuSO_4.5H_2O$	0.20
$NaMoO_4.2H_2O$	0.75
$CoCl_2.6H_2O$	0.37
$ZnSO_4.2H_2O$	0.25

5. Hoagland's Solution

Macronutrients	Moles per Liter
KH_2PO_4	0.001
KNO_3	0.005
$Ca(NO_3)_2$	0.005
$MgSO_4$	0.002

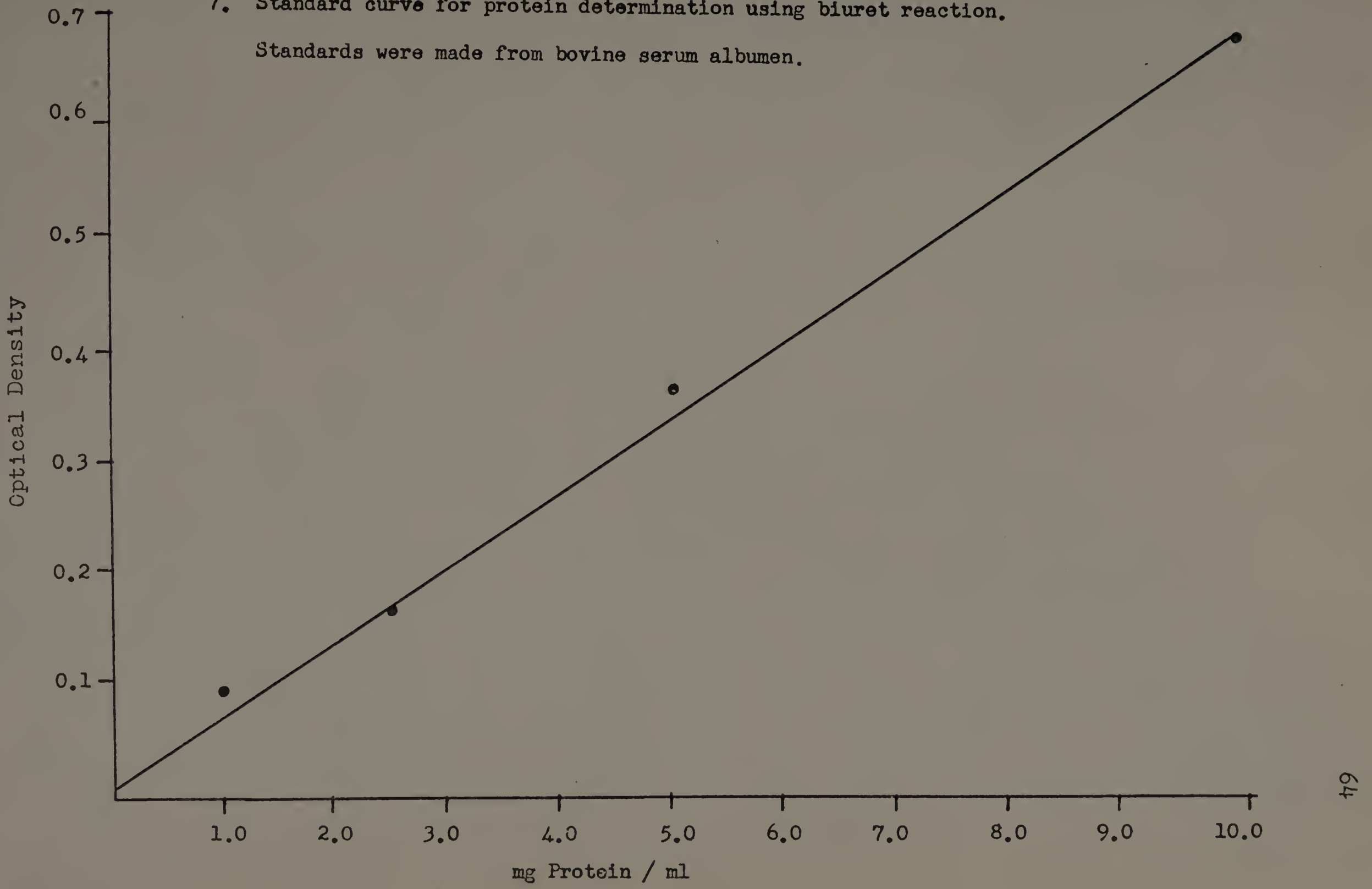
Micronutrients	ppm
H_3BO_3	0.50
$MnCl_2.4H_2O$	0.50
$ZnSO_4.7H_2O$	0.05
$CuSO_4.5H_2O$	0.02
$H_2MoO_4.H_2O$	0.05
Fe tartrate	1.37

6. Standard curve for N-acetylglucosamine



7. Standard curve for protein determination using biuret reaction.

Standards were made from bovine serum albumen.



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