CULTURAL CHARACTERISTICS OF PENICILLIUM NOTATUM IN RELATION TO THE PRODUCTION OF ANTIBACTERIAL SUBSTANCE

INDICATION OF THE DUAL NATURE OF THE ANTIBACTERIAL SUBSTANCE[1]

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Of the bacterial antagonists thus far known to be produced by microorganisms, the most effective in bactericidal action against *Brucella abortus* is the culture filtrate from a strain of *Penicillium notatum* (Kocholaty, 1942). Another antibacterial substance, penicillin, also obtained from a culture of a strain of *Penicillium notatum*, but with very little potency against *Brucella abortus*, has been obtained and purified by a group of British workers (Abraham, Chain, Fletcher, Florey, Gardner, Heatley, and Jennings, 1941).

The work reported here deals chiefly with the cultural habits of a certain strain of *Penicillium notatum* with special regard to the production of an antibacterial substance against *Brucella abortus*. Distinction is drawn between penicillin and the antibacterial substance active against *Brucella abortus*, because the evidence thus far indicates that those two are not identical. As compared to previous results, it has been possible to increase the production of the antibacterial substance of the mold by about 10 times, so that 0.01 ml. of the crude culture filtrate, added to 10 ml. of tryptose agar will either strongly inhibit or completely suppress the growth of *Brucella abortus*. In addition to this the influence of different media, temperature, vitamins, heavy metal salts, etc., will be discussed.

Five different strains of *Penicillium notatum*, here called PEN 1, 2, 3, 4, 5, have been investigated; all strains with the exception of PEN 4[2] were obtained from Dr. S. A. Waksman's collection.[3]

Cultivation of those five strains on different solid media shows slight divergencies in growth, pigment and spore formation, etc.; on liquid media, all 5 strains show different production of antibacterial substances, PEN 2 being the most active in this respect, followed by PEN 6 and then PEN 4. Those three strains also showed distinctly the secretion of two different antibacterial substances. The other strains, producing less antibacterial substance, were not further investigated. Most of the experiments were carried out with PEN 2.

[1] This investigation is supported by the Thomas H. Dougherty, Jr. Fellowship in Research in Brucellosis Fund.

[2] Thanks are due to the Merck Company, Rahway, N. J., for this strain, which is a transfer of the strain the British workers were using in the preparation of penicillin.

[3] The writer is greatly indebted to Dr. S. A. Waksman for obtaining those strains, and also for the test organisms used in this work.

EXPERIMENTAL

Before discussing the influence of different factors upon growth and production of antibacterial substances, a few procedures, standardized for the sake of uniformity of results, are mentioned.

Inoculation. The mold is grown on glucose agar slants for 6 days at 28°. After this time spore formation is abundant. The spores are scraped off and suspended in about 8 ml. of saline, 1 ml. being used for the inoculation of a volume of about 70 ml. of medium, thus always insuring heavy growth, which results in a complete pellicle within 48 hours at 28°.

Incubation, growth, production of antibacterial substance. As compared with the temperature of 24°, used by the British workers, the incubation of PEN 2 was carried out at 28°, which in our experience gave the best results. Under those conditions an almost constant level of production of the antibacterial substance is reached around the fifth to sixth day at a pH of 3.5 to 4.0, using the modified Czapek-Dox medium, without addition of yeast extract or other vitamin source. After this time there are only slight changes in the activity of the filtrate. After about 10 days of incubation, at which a pH of about 7 is reached, the activity drops sharply and is practically at zero two days later; the pH at this time is approximately 8 or slightly greater. Pigment, spore formation, color of pellicle, etc., were found to be influenced by slight changes in the composition or sterilization of the medium and can therefore not be relied upon as characteristics. These features will be discussed in the respective sections.

Harvesting. At determined intervals, usually daily, the contents of 2 to 3 Erlenmeyer flasks were combined, in order to increase uniformity of result, and the antibacterial values of the crude culture filtrate were determined.

Medium. If not otherwise stated the medium was the modified Czapek-Dox medium, as suggested first by Clutterbuck, Lovell, and Raistrick, (1932). The depth of the medium was 17–20 mm.

The antibacterial test. In preference to the test in liquid medium or the assay method of the British workers, both of which are too awkward if many organisms have to be tested at short intervals, the streak test on solid media was preferred, as more economical in that it allows 4 organisms to be tested on the same plate. The culture filtrate of the mold, containing the antibacterial substance, diluted and adjusted if necessary to pH 5–6, was mixed with 10 ml. of tryptose agar. The test organisms from a 1- to 2-day culture were suspended in saline and streaked out on this medium. Readings were taken after 48 hours at 28° or 37°, depending on the organism tested. The growth was designated 0, 1, 2, 3; 0 meaning no growth, 3 full growth similar to control, and 1 and 2 intermediate stages of poor and fair growth.

Investigation of five different strains of Penicillium notatum for production of antibacterial substance against different test organisms. The five strains of *Penicillium notatum* were cultivated on the modified Czapek-Dox medium, sterilized under pressure. Each day changes in growth, mycelium, pH, production of

pigment, and antibacterial substance were noted. The antibacterial value of the culture fluid was assayed against 8 test organisms, 4 gram-positive, and 4 gram-negative, using the plate method as described.

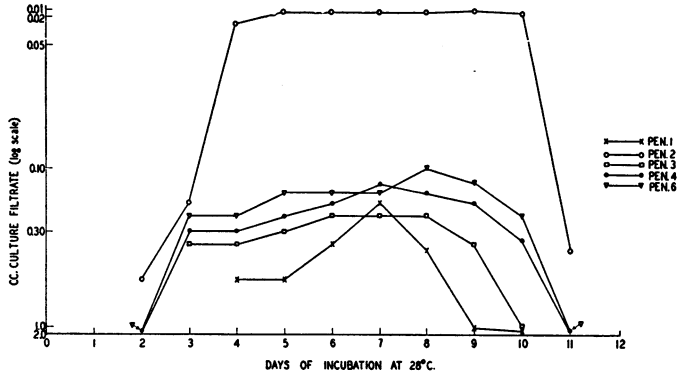

FIG. 1. SECRETION OF ANTIBACTERIAL SUBSTANCE, ACTIVE AGAINST BRUCELLA ABORTUS, BY FIVE DIFFERENT STRAINS OF PENICILLIUM NOTATUM

Abscissa: Days of incubation of five different strains of *Penicillium notatum.*
Ordinate: Points indicate minimum amount of crude culture filtrate (in ml.)—added to 10 ml. of tryptose agar—sufficient to suppress the growth of *Brucella abortus* (streak test).

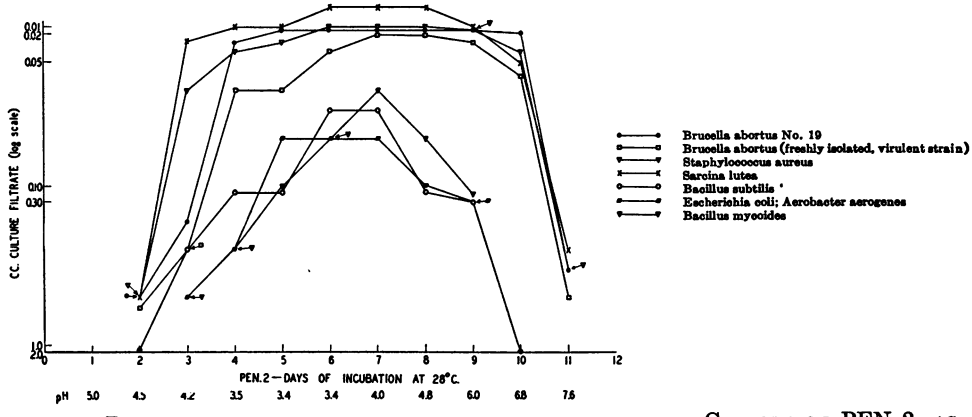

FIG. 2. PRODUCTION OF ANTIBACTERIAL SUBSTANCE DURING THE GROWTH OF PEN 2, AS ASSAYED AGAINST DIFFERENT TEST ORGANISMS

Abscissa: Days of incubation of PEN 2 at 28°; pH of the culture fluid day by day.
Ordinate: Points indicate minimum amount of crude culture filtrate (in ml.)—added to 10 ml. of tryptose agar—sufficient to suppress the growth of the respective test organism, (streak test).

For obvious reasons not all the results can be published here, but figures 1 and 2 will suffice to demonstrate the effect. If one tabulates the results in such a way that all the values from total suppression to complete growth are expressed at correlate intervals, the "curves" give the minimal amount of crude culture fluid in ml. sufficient for complete suppression of the growth of the respective test organism in 10 ml. of tryptose agar (streak test). This way of expressing the

antibacterial activity of the filtrate will be retained for the other figures to follow. (For the sake of uniformity, fractions of ml. are spaced logarithmically.)

Although 8 different organisms were tested against each of the five strains of *Penicillium notatum*, day by day, figure 1 shows only the differences of the antibacterial substances as assayed against *Brucella abortus*, United States Bureau of Animal Industry, strain 19. Figure 2 gives a complete picture of the production of antibacterial substance by PEN 2 against 8 test organisms. Similar results, especially with regard to the relative sensitivity of those test organisms were obtained with the other 4 *Penicillium* strains, except that the absolute amount of antibacterial substance secreted by those strains was considerably less.

Since it was found that PEN 2 surpasses all other strains in the production of antibacterial substance, this strain was used for all the experiments reported here. It is worth mentioning that PEN 2 and PEN 6 both surpass PEN 4, (the strain used by the British workers for the purification of penicillin), in the production of antibacterial substance, not only against *Brucella abortus*, but also against *Staphylococcus aureus*. While all the rest of the strains of *Penicillium notatum* look quite similar to each other, especially on liquid medium, the pellicle of PEN 2 has an immediately recognizable fluffy appearance, an abundance of mycelium formation, strikingly different from the rest; the pellicle exceeds in thickness those of all other strains.

Influence of sterilization of the medium upon production of antibacterial substance. The modified Czapek-Dox medium was sterilized in three different ways: 1) 30 minutes under flowing steam for 3 consecutive days; 2) glucose was sterilized separately from the salt solution as a 56 per cent solution and added after sterilization in corresponding amounts to the salt solution; 3) the whole medium was sterilized under pressure.

The differences between sterilization 1), and 3) are practically negligible with regard to differences in the formation of antibacterial substance. Figure 2 represents the results of methods 1 and 3. If glucose is sterilized separately according to method 2), differences are evident, as shown in figure 3.

Apart from minor changes—shifting of the peak of the production of the antibacterial substance for instance—the formation and disappearance of an antagonistic substance acting against *Escherichia coli* and *Aerobacter aerogenes*—but not acting against *Brucella abortus*—is most striking. While on the fifth day of growth an amount of 0.05 to 0.1 ml. of the culture fluid will suppress the growth of *Escherichia coli*, *Aerobacter aerogenes*, *Staphylococcus aureus* or *Brucella abortus*, 4 days later even 100–200 times the amount capable of suppressing the growth of *Brucella abortus* or *Staphylococcus aureus* is insufficient to cause even a slight inhibition of *Escherichia coli* or *Aerobacter aerogenes*. This fact is only to be explained by the formation of two different antibacterial substances, influenced by the detail of sterilizing the glucose separate from the salts.

Apart from this, other changes are apparent. The pigment production of the medium sterilized under pressure or especially with flowing steam is rather meager, and in color a light yellow-green, becoming darker only in the latest stages of growth, in which the antibacterial substance disappears; in contrast

the pigment production after sterilizing glucose separately is on the fourth day a faint orange, becoming almost brown at the peak of the production of antibacterial substance. The under side of the mycelium if the medium is sterilized with flowing steam or under pressure is first white, in the latter stages brownish; if sterilized according to method 2) it is bright yellow-green.

This difference in the production and activity of antibacterial substance against *Escherichia coli* and *Aerobacter aerogenes* was also possible to demonstrate on strains PEN 4 and PEN 6 to a somewhat lesser extent; the remainder of the strains showed too little antibacterial activity to make such changes detectable.

Influence of heavy metal salts. Substituting for the 10 mg of $FeSO_4 \cdot 7H_2O$ per liter in the modified Czapek-Dox medium equivalent amounts of the sulfates of Zn, Cu, and Mn, the results obtained are represented in figure 4 against *Brucella abortus* as test organism. Also tested were *Staphylococcus aureus*,

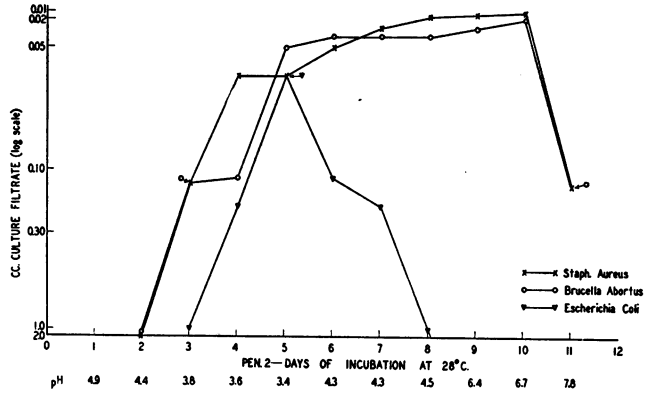

FIG. 3. THE PRODUCTION OF ANTIBACTERIAL SUBSTANCE BY PEN 2 AFTER STERILIZING THE GLUCOSE SEPARATELY

Abscissa: Days of incubation of PEN 2 at 28°; pH of the culture fluid day by day.
Ordinate: Points indicate minimum amount of crude culture filtrate (in ml.)—added to 10 ml. of tryptose agar—sufficient to suppress the growth of the respective test organisms, (streak test).

Sarcina lutea, and *Escherichia coli*, the relative sensitivity of all of them remaining unchanged. The addition of Zn diminished the secretion of antibacterial substance as compared with Fe, but the growth of the mold was superior in its development and heaviness of pellicle as compared with Fe. In contrast to this, the addition of Cu resulted in a very meager growth, resembling in its appearance very much the growth which is obtained when the mold is grown on a slant at 37°. No regular pellicle was formed, only spots of mycelium on the surface and rim of the vessel were obtained, presenting a "crowded" white growth of brittle mycelium at best covering only 10 to 20 per cent of the available surface area. In relation to this meager growth the production of antibacterial substance seems rather high.

The influence of Mn ions is outstanding; Mn not only replaces but surpasses Fe in increasing heaviness of growth and production of antibacterial substance. At the peak of the secretion of antibacterial substance, 0.005 ml. of the crude

culture filtrate was able to suppress the growth of *Brucella abortus* or *Staphylococcus aureus* and 0.001 ml. was sufficient to suppress the growth of *Sarcina lutea* (the most sensitive test organism used), in 10 ml. of tryptose agar.

Temperature, growth and production of antibacterial substance. Clutterbuck, Lovell, and Raistrick (1932) grew the mold at 25°, the other British workers (1941) at 24°, while here the temperature of 28° was used exclusively. The finding of Fleming (1929) that the mold will grow at 37° was confirmed (with PEN 2); the mold will cover within 14 days a Czapek-Dox agar slant, (very much less growth is obtained on glucose agar slant), with a crowded brittle growth similar to that obtained by substitution of Cu for Fe in the liquid medium at 28°. Slow growth is also obtained at refrigerator temperature, a Czapek-Dox slant being covered within 14 days with a fluffy white growth. If a flask of a mold

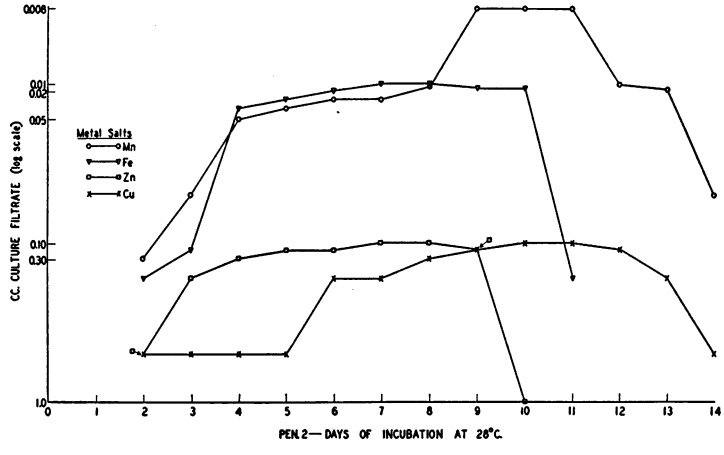

FIG. 4. INFLUENCE OF DIFFERENT HEAVY METAL SALTS UPON PRODUCTION OF ANTIBACTERIAL SUBSTANCE BY PEN 2

Abscissa: Days of incubation of PEN 2 at 28°.
Ordinate: Points indicate minimum amount of crude culture filtrate (in ml.)—added to 10 ml. of tryptose agar—sufficient to suppress the growth of *Brucella abortus* (streak test).

culture on liquid medium after reaching its peak in the production of antibacterial substance is placed in the refrigerator, it is found after some days that the activity of the antibacterial substance is increased. Whether this increase is due to some slight additional growth, or to the diffusion of active material from the pellicle into the culture fluid was not investigated.

Certain signs, however, speak for the possibility that conditions optimal for growth are not necessarily optimal for production of penicillin; (see for instance the influence of Zn, Cu). In one instance the mold was grown at room temperature, (about 20°). Although the pellicle formed was thin and the growth retarded for a longer period of time, the amount of production of antibacterial substance was about the same as if the mold were incubated at 28°. In another case the addition of $CaCO_3$ was tried and although the pellicle obtained after 14 days of growth at 28° was almost paper-thin, with no pigment formation at

all, the production of antibacterial substance again was normal. All these findings seem to indicate that optimal growth, or optimal temperature for growth, or optimal pellicle formation, are no indication for optimal production of antibacterial substance.

If grown at 28° on modified Czapek-Dox agar the mold (PEN 2) forms a complete pellicle within 2 days, at which time the formation of antibacterial substance can be detected in the culture fluid. The changes in pH during the successive stages of growth, at first an increase toward the acid side, later a slow decrease in acidity, and finally a rapid rise in the pH are about the same as reported by other workers. (See also figures 1 and 2.)

Composition of medium. In tryptone medium with the necessary salts, with or without glucose, the pH rises after 6 days to about 8, and production of antibacterial substance is not demonstrable. Substituting ammonium sulfate for $NaNO_3$ as nitrogen source in the Czapek-Dox medium lowers the production of antibacterial substance. However, doubling the nitrogen and carbon source results in some increase in the production of antibacterial substance, resembling very much the results obtained when Fe in the medium is replaced with Mn.[4]

From the few experiments conducted regarding the utilization of glucose, it was found consistently, whether 100 g or 40 g of glucose were used per liter of Czapek-Dox medium, that this source of carbon was rapidly utilized, 90 per cent or more of the glucose being used up at the optimum of the production of antibacterial substance. Determination of acidity carried out at the same time showed no characteristics worth mentioning.

It has been reported by different investigators (Abraham, Chain, Fletcher, Florey, Gardner, Heatley, and Jennings, 1941; Clutterbuck, Lovell, and Raistrick, 1932) that occasionally batches of the culture filtrate of the mold are obtained, apparently free of contamination, without any antibacterial activity. The strain of PEN 2 used for almost all of the experiments reported in this paper for more than 8 months has not given a single batch of material without antibacterial activity, parallel determinations showing great uniformity.

DISCUSSION

Except for the experiments recorded in figure 2, where 8 test organisms were used, there were used 4 test organisms, namely *Brucella abortus*, *Staphylococcus aureus*, *Escherichia coli*, and *Sarcina lutea*. The peak of the production of antibacterial substance always showed the following relationship in sensitivity of these four organisms: *Staphylococcus aureus*:*Brucella abortus*:*Escherichia coli* = 1:1–2:10, i.e., if one ml. of the culture filtrate will suppress *Staphylococcus aureus*, 1–2 ml. are necessary to suppress growth of *Brucella abortus* and 10 ml. *Escherichia coli*. (*Sarcina lutea* is much more sensitive than *Staphylococcus aureus*, its relation being about 0.3.) This relation between these four organisms remained unaltered in all the experiments.

The only evidence brought forth in this paper that two different substances

[4] Thiamin hydrochloride, pyridoxin, or riboflavin, added to the culture fluid were without influence upon either growth of the mold or secretion of antibacterial substance.

are secreted by the mold (recorded in figure 3) is afforded by the substance acting against *Escherichia coli*. If one compares the crude culture filtrate of the mold as found here, however, with the more than 1000 times purified penicillin of the British workers (1941), another difference is apparent. The sensitivity of *Staphylococcus aureus*:*Brucella abortus*:*Escherichia coli* against the highly purified penicillin compares as 1:500:1000, as against the ratio with the crude culture filtrate of PEN 2 of 1:1–2:10. Even assuming slight variations in the sensitivity of the test organisms, these would not suffice to reconcile those discrepancies.

In the purification of the penicillin, the British workers (1941) probably used *Staphylococcus aureus* as a test organism. It might well be conceivable that in using *Escherichia coli*, for instance, a substance with entirely different antibacterial properties against those three organisms might have been isolated. Furthermore, the possibility that two different antibacterial substances might be formed by the mold would fit into the picture of other soil organisms producing two antibacterial substances such as the Gramicidin and Tyrocidine produced by *Bacillus brevis*, (Dubos, 1939), and Actinomycin A and B produced by *Actinomyces antibioticus*, (Waksman, 1941). Regarding the two substances secreted by the mold it must be said that these two substances need not necessarily be quite different. It could be that only slight changes in one and the same substance—for instance during the growth of the mold—might produce the difference in their antibacterial properties. Another possibility is that in the purification of penicillin the original substance might have been slightly altered in its original antibacterial properties. Only the purification of the antibacterial material can bring the desired evidence.

SUMMARY

1. Several strains of *Penicillium notatum* investigated were found to vary widely in their property of producing penicillin.

2. The optimal growth of the mold, or optimal temperature for the growth of the mold does not necessarily coincide with the optimal production of antibacterial substance.

3. The mode in which the (glucose-containing) medium is sterilized shows divergencies in the production of antibacterial substances as well as other differences.

4. The influence of other heavy metal salts than Fe was studied and Mn was found to surpass Fe in production of antibacterial substance.

5. Among five strains of *Penicillium notatum* tested for the production of antibacterial substances, it was found that at least three strains—all varying widely in their production of antibacterial substance,—seem to produce 2 different substances which differ in their antibacterial properties. Using optimal conditions, so far as they are known as yet, a strain of *Penicillium notatum* was found to produce an antibacterial substance, which in amounts of 0.01 ml. of the crude culture filtrate added to 10 ml. of tryptose agar, will prevent the growth of *Brucella abortus*.

6. The difference between purified penicillin and the substance found in the crude culture filtrate is discussed with reference to the possibility of the dual nature of the antibacterial material produced by the mold.

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