PENICILLIN

II. Natural Variation and Penicillin Production in Penicillium Notatum and Allied Species[1,2]

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INTRODUCTION

The mold which Professor Alexander Fleming isolated at St. Mary's Hospital in London in 1928, and which Dr. Charles Thom subsequently diagnosed as most closely related to *Penicillium notatum* Westling, is a remarkable organism. It was used in all of the early work on penicillin and penicillin production, and even now the bulk of the work in progress in many different laboratories still centers around cultures derived directly from it. Penicillin production, however, is not a unique property of this mold, and we now recognize that this capacity is present in varying degree in almost all strains of the *Penicillium notatum-chrysogenum* group. Reid reported antibacterial activity by *P. notatum* and *P. chrysogenum* in 1933, and subsequently (1934 and 1935) by a mold closely related to *P. notatum*. In October, 1942, Smith demonstrated that a strain of *P. chrysogenum* Thom produced penicillin or some similar antibacterial substance. Waksman and Horning (1943) isolated green penicillia from nature and noted antibiotic activity but failed to identify specific strains as belonging to the *P. notatum-chrysogenum* group. In the early work on penicillin at the Northern Regional Research Laboratory initiated in July, 1941, other workers had investigated a number of strains belonging to this group and found that penicillin was produced in varying amounts by all cultures examined. None were found to be as good for surface production as the specially selected strain of Fleming's cultures which we were then using, but one was discovered which gave substantially better yields in submerged culture than did the Fleming strain. Today, this culture, *Penicillium notatum* NRRL 832, is generally used for the production of penicillin in submerged or tank culture. Insofar as we know, it is not genetically related to the strain isolated by Fleming.

With this background of experience with *Penicillium notatum* and its allies and with the knowledge that in other groups of molds the production of particular products is not limited to particular strains (e.g., *Aspergillus niger* and the

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[3] This is one of four regional laboratories operated by the Bureau of Agricultural and Industrial Chemistry, Agricultural Research Administration, U. S. Department of Agriculture.

production of citric acid), we set about in June, 1943, to isolate from nature cultures of *P. notatum*, *P. chrysogenum*, and other closely related species and to test these for penicillin production. Our goal was to find superior cultures. It was necessary in this search to handle large numbers of isolates since the types for which we were searching represent only a small fraction of the total number of blue-green species encountered. Cultural and morphological characteristics were relied upon to exclude species belonging to other groups, and a simple screening test was devised for effectively eliminating the very low and non-producing members of the *P. notatum-chrysogenum* group. All cultures which appeared promising in this screening test were subsequently grown in surface culture upon a combination of nutrients developed at the Northern Regional Research Laboratory and successfully employed in industry for the production of penicillin by the surface culture method. Isolates which gave yields approximating, equaling, or exceeding those of strain NRRL 832 when tested by this method were subsequently tested in shaken flasks to determine their capacity to produce penicillin in submerged culture.

SOURCE AND ISOLATION OF CULTURES

For this investigation, molds belonging to the *P. notatum-chrysogenum* group were isolated from a wide variety of sources including (1) moldy food products such as bread, cheese, cured meats, home-canned fruits and vegetables, etc.; (2) fresh fruits and vegetables in the earlier stages of spoilage; and (3) fertile cultivated soils collected from various stations in the United States and from foreign countries, including Newfoundland, England, Mexico, Panama, Cuba, Brazil, Australia, and India. Some forest soils were examined, but generally these were found less productive of the desired strains and, in addition, were more difficult to analyze because of the abundance of such rapidly growing forms as *Trichoderma* and various members of the *Mucoraceae*.

Isolations from food products and fruits were generally made by transferring spores directly from moldy areas to agar plates and subsequently recultivating them until the purity of the cultures was established. In making isolations from soil, two types of cultures were employed:

1. Dilution cultures. Samples of soils were diluted with approximately 10 volumes of sterile water and shaken vigorously for a period of 10 to 15 minutes. Serial dilutions in steps of 1:10 were then made from the resulting suspensions and plated. Dilutions employed ranged from 1:1,000 to 1:1,000,000 depending upon the source and type of the sample—the higher dilutions being used for what appeared to be the more fertile soils. For each sample three dilutions were plated, and, as a rule, two media were employed: (*a*) a Czapek's solution agar of the composition recommended by Thom (1930) for cultivating the penicillia, and (*b*) an acid glucose nitrate agar of the type employed and recommended by Smith and Humfeld (1930) for the isolation of fungi from soil. Dilution plates were incubated at room temperature (24 to 26 C) and desired forms were isolated within 5 to 10 days as they appeared and sporulated.

2. Streak cultures. Plates of hay infusion agar, of the type employed by

Raper (1937) for isolating slime molds from soil, were streaked with from 1 to 4 loopfuls of the initial 1:10 soil suspension. These plates were likewise incubated at room temperature and were examined with a low-power binocular at 7 to 10 days. No molds grew luxuriantly upon this medium, but a large number and variety of forms made a limited development. As it was impossible to differentiate between most penicillia upon this medium, it became necessary to isolate upon Czapek's solution agar, or some other suitable medium, all blue-green forms which might possibly represent members of the *Penicillium notatum-chrysogenum* group. Forms not belonging to this group were subsequently eliminated upon cultural and morphological bases.

GROUP CHARACTERIZATION

Isolates thought to represent members of the *P. notatum-chrysogenum* group were planted upon Czapek's solution agar plates for comparative examination. Incubation was at room temperature. Plates containing from 2 to 4 colonies equally spaced proved most satisfactory for this comparative study since they developed on a scale large enough to show characteristic colony features, and at the same time produced sufficient fructifications in the marginal areas between adjoining colonies to permit examination with the low powers of a compound microscope. The principal characters which distinguish the group are as follows:

1. Colonies at 10 to 12 days, velvety or loose-textured, plane or radially furrowed, with sporing areas in blue-green shades approximating celandine green to artemisia green (Ridgway, 1912, pl. XLVII), pistachio green to American green (pl. XLI), or storm green to Russian green (pl. XLII).

2. Colonies generally characterized by abundant yellow exudate, often collecting in conspicuous droplets, ranging in color from light to rich yellow.

3. Colony reverse in shades of yellow, commonly becoming brown in age; the agar medium surrounding the colonies usually yellow in color, often conspicuously so.

4. The penicillus, viewed under low magnifications, commonly appears to consist of from 2 to 5, more or less divergent, columns of spores; viewed under oil immersion, it is found to represent an asymmetric, biverticillate structure with smooth walls throughout.

5. Conidia (spores) smooth-walled, varying from globose and subglobose in some strains (e.g., typical strains of *P. notatum*) to definitely elliptical in others (e.g., typical strains of *P. chrysogenum*), and ranging in size from approximately 2.5 to 4.0μ in diameter.

Isolates possessing the above characters were presumed to represent members of the *Penicillium notatum-chrysogenum* group (see Thom, 1930) and were therefore investigated for penicillin production.

SCREENING TEST

New isolations possessing the cultural and morphological characteristics of the *P. notatum-chrysogenum* group were subjected to a preliminary screening test to eliminate strains characterized by low penicillin production. This test

represents essentially the opposite of a method reported by Fleming in 1942. He removed undetermined amounts of agar to form pits and gullies in plates uniformly seeded with *Staphylococcus* and subsequently filled these depressions with the cultures or solutions to be tested. In our work we have removed blocks, or plugs, of agar of constant dimensions from the mold cultures being tested and placed these on the surface of plates uniformly seeded with *Staphylococcus*. In both cases the *Staphylococcus* plates were incubated at 37 C and the amount of penicillin present was gauged by the size of the resulting zones of inhibition. Both tests are essentially qualitative.

In the screening test, a modified Czapek's solution agar, termed "E" medium, was employed as a substratum for the molds. This was prepared as follows: 1 per cent by volume of corn steeping liquor (55 per cent solids) was added to a standard Czapek's solution (Thom, 1930, p. 42). This solution was then adjusted to pH 7.0 and to it was added 2.0 instead of the usual 1.5 per cent agar. Accurately measured 20-ml portions of this medium were sterilized in individual tubes and subsequently poured into sterile, flat-bottom petri dishes 100 mm in diameter. Plates for this test were selected with great care to insure that the agar layers in different cultures were of uniform depth. In addition, uniform inoculations were made in order to secure a reliable comparison of different strains. In all cases, single colonies were established in the centers of the plates, cultures of this type being obtained in the following manner: Spores of the cultures to be tested were suspended in melted agar at 45 C and the suspension was allowed to solidify. Very small amounts of this solidified suspension were then lifted out with an inoculating needle and transferred to the centers of the "E" plates. With each series of unknowns, two cultures of *P. notatum* known to be good producers of penicillin were included as controls; namely, NRRL 1249.B21,[4] the most satisfactory strain for surface production, and NRRL 832,[5] the standard strain for submerged production (figure 1). The plates were incubated at 24 C for 6 days.

On the sixth day a radial series of 4 or, if possible, 5 plugs was removed from the agar as illustrated in figure 2, the first plug in every case being adjacent to the colony margin. A no. 2 cork borer with an inside diameter of 5.5 mm was employed for cutting the plugs. Prior to use, this was dipped in alcohol, flamed, and cooled in sterile water. The plugs were lifted out with a flat-pointed inoculating needle and transferred to seeded *Staphylococcus* plates of the type used in the standard cup assay method as described by Schmidt and Moyer (1944). These plugs were arranged on the surface of test plates as shown in figure 2.

[4] Strain NRRL 1249.B21 is generally used in industry for the production of penicillin in surface culture. It was selected and developed at the Northern Regional Research Laboratory from a culture obtained by us from Dr. George A. Harrop of the Squibb Institute for Medical Research. The parent, or "Squibb," strain is maintained in the culture collection of the Northern Regional Research Laboratory as no. 1249.

[5] Strain NRRL 832 came from the Thom Collection and was one of the cultures brought to the Northern Regional Research Laboratory in 1940. It was isolated, or recultivated, in 1936 from a tube labeled "*lacticus* Mazé" brought from the Biourge collection by Dr. Paul Simonart.

The assay plates were incubated overnight at 37 C, and zones of inhibition appeared which were roughly proportional to the concentrations of penicillin present in the mold cultures at the sites from which the plugs were removed. Zones of inhibition were measured to the nearest 0.25 mm with the aid of a large

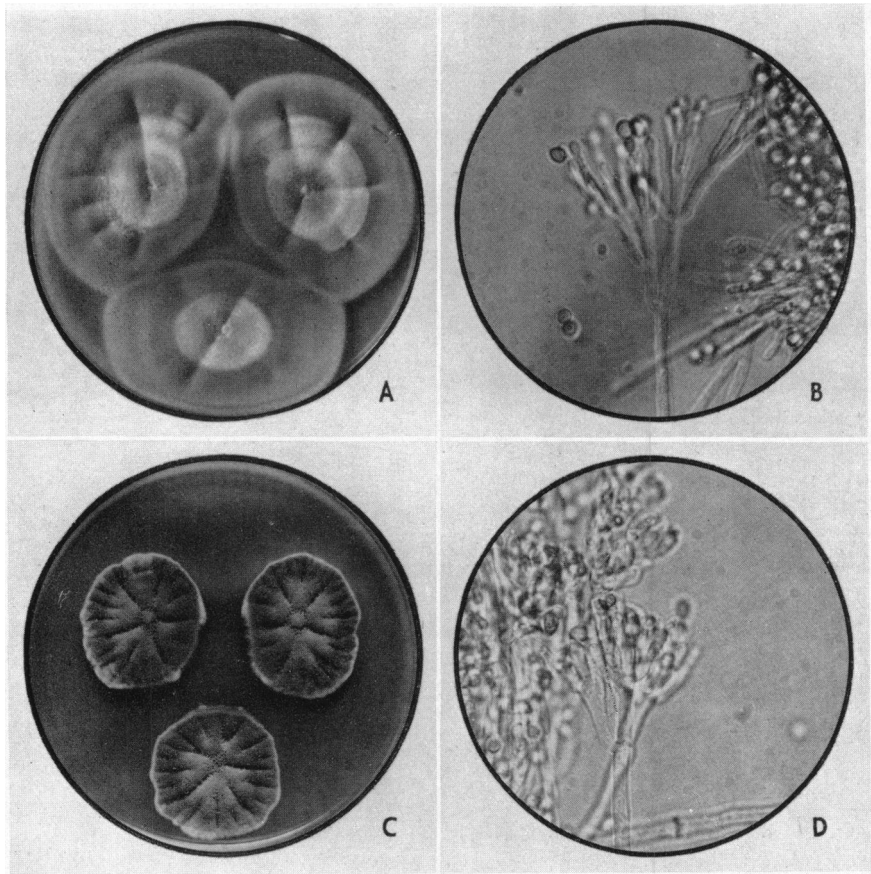

FIG. 1. GOOD PENICILLIN-PRODUCING STRAINS OF PENICILLIUM NOTATUM

A, NRRL 1249.B21 on Czapek's solution agar; incubation at 24 C, age 10 days; × $\frac{2}{3}$. *B*, single penicillus from the same culture showing details of the conidial apparatus, × 550. *C*, NRRL 832 on Czapek's solution agar; incubation at 24 C, age 10 days; × $\frac{2}{3}$. *D*, penicillus from the same showing details of the conidial apparatus, × 550. More restricted colonies and more compact penicilli consistently characterize the latter strain.

reading glass. The plug adjacent to the colony always showed the greatest amount of inhibition, with succeeding plugs showing progressively less. When these tests were first made, they were initiated on the fourth day of incubation and continued through the tenth. This procedure, however, yielded little information not obtained in a single test at 6 days; hence, the latter procedure was adopted as a standard practice.

Cultures which produced circles approximately equal to or greater than those

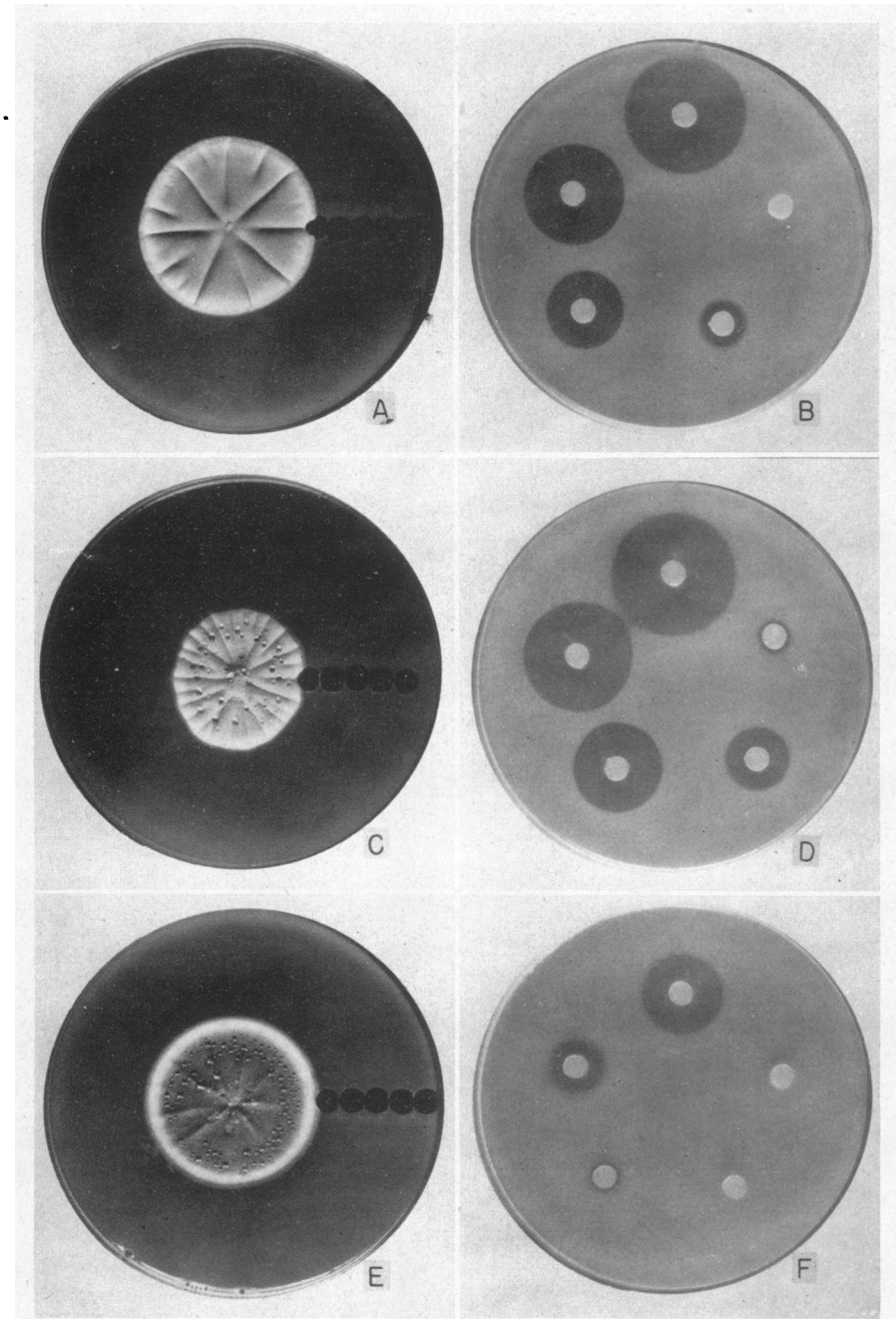

FIG. 2. SCREENING TEST

A, Control culture, *Pencillium notatum* NRRL 1249.B21, on "E" medium; incubation at 24 C; radial series of plugs cut at 6 days; × ⅔. *B*, Agar plug assay plate showing zones of inhibition of *Staphylococcus* developed after agar blocks removed from *A* have been incubated for 16 hours at 37 C, × ⅔. *C*, New isolate, good penicillin-producing strain, incubation and test as in *A*. *D*, Assay plate for same. *E*, New isolate, poor penicillin-producing strain, incubation and test as in *A*. *F*, Assay plate for same.

produced by the control strains, NRRL 1249.B21 and 832, were considered potentially valuable and were retained for more detailed study. Cultures which showed zones of inhibition definitely and consistently smaller than those of the controls were found, by experience, to be low producers, and hence of little or no value. The results obtained in a representative test are graphically shown in figure 3 where the diameters of zones of inhibition are plotted against the position of the test blocks, or plugs.

The screening test is essentially qualitative and is of particular value as a simple means of separating out low-producing or nonproducing strains (e.g.,

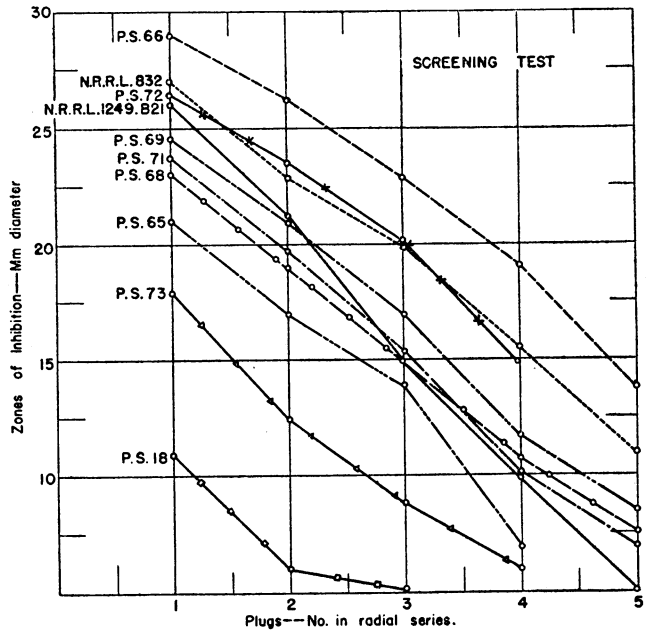

FIG. 3. SCREENING TEST

Graph showing comparative inhibition of *Staphylococcus* by radial series of agar plugs removed at 6 days from eight new isolates (Penicillin survey, or P.S. numbers) and the two control strains of *Penicillium notatum*, NRRL 1249.B21 and 832.

strains P.S. 18 and 73 of figure 3). It is, however, quantitative to a limited degree. This is clearly evident when one compares the relative positions of the curves in this figure with the yields of penicillin actually produced by the same strains upon a surface production medium as shown in table 1. It should be noted that the screening test medium does not reflect accurately the penicillin-producing capacity of NRRL 1249.B21 in surface culture, as in actual production tests this strain is capable of producing higher yields than any of the other strains listed. By adding known amounts of penicillin to melted agar (at 45 C), it has been determined that a maximum penicillin concentration of about 6 o u/ml (Oxford units per milliliter) can be measured by this method. Strains which produce an amount approaching, equaling, or exceeding this amount in the area

adjacent to the colony cannot be differentiated satisfactorily (cf. figure 3 and table 1). Despite this shortcoming, it serves satisfactorily the purpose for which it was developed.

Although we have not studied the matter particularly, there is evidence that such a screening test can be used to detect other molds capable of producing antibacterial substances such as patulin and citrinin. We have cut plugs from cultures of *P. patulum* Bainier and *P. expansum* Link (reported by Anslow, Raistrick, and Smith, 1943, to produce patulin) and found these to produce conspicuous zones of inhibition. The same is true of cultures believed to represent *P. citrinum* Thom. In neither case were the zones as clean-cut as those surrounding plugs taken from penicillin-producing cultures.

PRODUCTION TESTS

Cultures which appeared promising in the screening test were studied under conditions in which the known good cultures, NRRL 1249.B21 and 832, consistently produce good yields of penicillin. It was realized at the outset that conditions and nutrients which had been developed for these two strains might not, in all cases, represent the optimum for new isolates. On the other hand, it was recognized that to investigate different conditions and nutrient combinations for each strain would be an interminable task, without assurance of commensurate results. Hence, it was decided to search first for new cultures which would equal or exceed the known good strains upon substrata developed for them, and then to study such cultures more exhaustively, if they were believed to show promise of superior results.

Surface Production

All new isolates which gave zones of inhibition exceeding, equaling, or approaching those of NRRL 1249.B21 and NRRL 832 in the screening test were tested in surface culture under conditions and upon liquid substrata in which NRRL 1249.B21 usually produces from 110 to 140 o u/ml, and NRRL 832 produces approximately 55 to 65 o u/ml in from 5 to 6 days. For these tests a medium of the following composition, developed and recommended by Dr. A. J. Moyer of this laboratory, was employed:

Lactose	40.0	g	(monohydrate or some crude grade)
$MgSO_4 \cdot 7H_2O$	0.25	g	
KH_2PO_4	0.50	g	
$NaNO_3$	3.00	g	
Zinc (as $ZnSO_4$)	0.01	g	
Corn steeping liquor	90	ml	
Distilled water to make	1,000	ml	

The medium was dispensed in 50-ml quantities in 200-ml Erlenmeyer flasks and sterilized for 20 minutes at 15 pounds steam pressure. Triplicate flasks, A, B, and C, were seeded with dry spores obtained from 5- to 6-day-old sporulation plates. Spores were removed from the plates by means of a large loop, floated on the surface of the nutrient solution, and scattered by violent lateral

agitation with the inoculating loop. A sufficient quantity of spores was added to each flask to provide a thin, but complete, covering film. Spores for these inoculations were obtained by heavily seeding plates of a sporulation medium of the general formula recommended by Dr. Moyer. The essential thing is to secure a heavy crop of dry spores which can be easily removed and uniformly distributed over the surface of the production medium, and any substratum

TABLE 1

Tests of new isolates in surface culture—a representative series

CULTURE DESIGNATION	4TH DAY FLASK A		5TH DAY FLASK B		6TH DAY FLASK C		7TH DAY FLASK A		8TH DAY COMPOSITE	
	pH	Penicillin	pH	Penicillin	pH	Penicillin	pH	Penicillin	pH	Penicillin
		o u/ml		*o u/ml*		*o u/ml*		*o u/ml*		*o u/ml*
NRRL 832.A2 (control)	7.6	41	7.75	58	8.0	56	8.25	38	8.27	21
NRRL 1249.B21 (control)	6.6+	103	7.45	110	7.9	129	8.25	88	8.25	58
P.S.* 60	7.5	61	7.63	82	8.0	79	8.3	58	8.2	52
61	6.75	24	7.05	31	7.45	37	7.82	21	7.87	14
62	5.9	10	6.85	19	7.4	21	7.25		7.57	25
63	6.55	tr.	7.45	10	7.7	10	8.15	14	8.0	10
64	6.15	23	6.95	46	7.12	55	7.75	73	7.7	73
65	6.15	<8	7.17	14	7.27	13	8.02	13	8.1	15
66	7.2	61	7.77	73	7.92	95	8.25	57	8.25	33
67	5.6	12	6.77	23	7.15	41	7.02	33	7.35	51
68	5.1	8	7.1	24	7.4	31	7.92	31	8.1	26
69	5.65	26	6.85	43	7.22	68	7.6	73	7.8	55
70	7.1	27	7.55	30	7.65	30	8.0	31	8.0	27
71	7.3+	49	7.75	50	7.95	53	8.25	44	8.25	32
72 (NRRL 1950)	6.9	52	7.65	91	7.72	75	8.15	69	8.12	55
73	6.1	0	7.0	4	7.6	5	7.9	5	7.8	0
74	7.15	12	7.5	19	7.72	28	7.95	30	7.9	32
75	7.0	10	7.7	24	8.05	24	8.1	24	8.2	21
76	7.65	65	7.8	65	8.15	62	8.35	32	8.4	23

* P.S. (= penicillin survey), a designation given to cultures studied in this survey but not made a part of the permanent culture collection of the Northern Regional Research Laboratory.

yielding spores of this character should prove satisfactory. To speed mat formation the inoculated flasks were generally incubated for approximately 36 hours at 26 to 28 C and then, prior to the beginning of penicillin formation, were removed to a 24 C incubator where they remained throughout the entire production cycle. In each series of new isolates, cultures NRRL 1249.B21 and a substrain of NRRL 832, designated 832.A2, were included as controls.

Each culture tested was assayed on the fourth through the eighth day, the following procedure being generally employed: Flask A was tested on the fourth day. A sample of approximately 5 ml of the culture liquor was removed; from

a portion of this original sample a pH determination was made, while the remainder was used for a standard cup assay of the penicillin present (Schmidt and Moyer, 1944). On the fifth day flask B was similarly sampled. On the sixth day the two values previously obtained were reviewed, and, if possible, duplicate or triplicate flasks were assayed for each strain showing yields equal to or exceeding strain NRRL 832. Assays of duplicate or triplicate flasks were commonly repeated on the seventh day. On the eighth day the culture liquors of flasks A, B, and C were pooled and the composite was assayed. The mycelial mats were harvested, dried at 90 C for 24 hours, and weighed.

The results of a typical experiment are presented in table 1.

In most cases, the highest yields of penicillin appeared on the fifth, sixth, or seventh day. In some strains (e.g., P.S. 62, 65, 67, and 74, of table 1), however, higher values were registered at eight days when the composites of all flasks were assayed, whereas in occasional strains maximum yields were registered as early as the fourth day (e.g., P.S. 76 of table 1). Although this difference in rate could result from variations in the amount of inoculum, or in the speed with which mat development occurs, it is believed to represent, for the most part, basic physiological differences between the various strains.

A more accurate comparison could have been made by assaying each flask on each successive day. This, however, was generally impossible as other work simultaneously in progress limited the number of assays that could be run. In some of our latest experiments we have made duplicate cultures in 300-ml. Erlenmeyer flasks containing 75 ml of culture medium, and we have assayed each flask daily from the fourth through the eighth day. On the whole, we believe this procedure to be more satisfactory, but we feel confident that the original method of sampling was adequate to reveal promising cultures. Such cultures, including strains such as P.S. 60, 66, 72 (NRRL 1950), and 76 of table 1 have been included in subsequent tests and studied more exhaustively.

In the present survey, 241 cultures of different origin have been investigated for penicillin production in surface culture. Included in this number were 189 new isolates, 36 cultures from the Thom collection, and 16 cultures from other collections in this country and in England. Thirteen of the new cultures were contributed by collaborators, and the remaining 176 were isolated from natural sources at this laboratory during the present investigation. The variety of sources from which these cultures were secured is shown in table 2, wherein cultures are grouped according to source, as well as the amount of penicillin produced in surface culture.

A summary of results obtained with the 189 new isolates is graphically presented in figure 4. Yields for individual strains were calculated as the average of the highest assay value for each of the 3 flasks, A, B, and C, or the assay of the composite sample taken at 8 days, whichever was the higher. By plotting numbers of strains against yields of penicillin, the distribution chart presented in figure 4 was obtained. It should be borne in mind that the strains have been grouped upon penicillin yields derived for the most part from triplicate cultures and a limited number of assays, and that when the same strains are recultivated,

TABLE 2

Tested strains of Penicillium notatum and allied species grouped according to source and amount of penicillin produced in surface culture

SOURCE	OXFORD UNITS OF PENICILLIN PER ML											TOTAL
	0	1–10	11–20	21–30	31–40	41–50	51–60	61–60	71–80	81–90	91–100	Over 100	
Soil	8	8	10	14	10	3	7	12	11	4	3	2	92
Bread	1	1	2	7	2	1	3	2	1	0	1	0	21
Cheese and other dairy products	0	0	1	4	1	2	2	2	1	0	0	0	13
Fruits and vegetables	1	0	2	5	3	2	1	5	0	0	0	0	19
Meat and meat products	0	0	1	3	2	0	0	0	2	0	0	0	8
Miscellaneous	5	1	3	8	7	2	4	5	1	0	0	0	36
Total—new isolates	15	10	19	41	25	10	17	26	16	4	4	2	189
Thom Collection (NRRL 807-843)	3	18	3	2	2	2	1	5	0	0	0	0	36
Other collections	6	3	1	2	2	1	0	1	0	0	0	0	16
Total of all strains tested	24	31	23	45	29	13	18	32	16	4	4	2	241

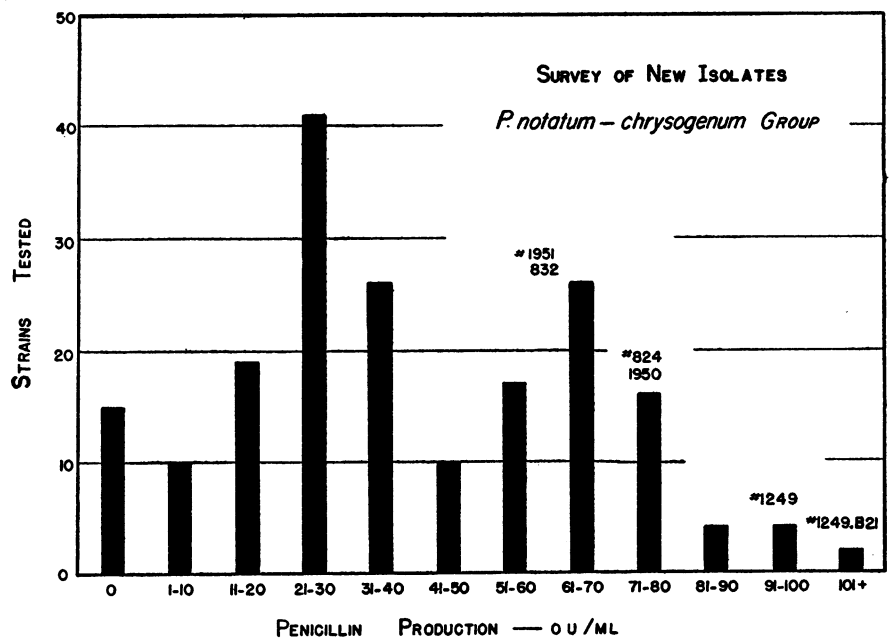

FIG. 4. DISTRIBUTION CHART OF THE NEW ISOLATES TESTED SHOWING THE RELATIVE NUMBERS OF STRAINS PRODUCING DIFFERENT AMOUNTS OF PENICILLIN WHEN TESTED IN SURFACE CULTURE

The positions in this chart of industrially important strains and other cultures commonly referred to in the text are indicated.

somewhat different values are to be anticipated. Based upon our experience, however, we would not expect marked deviation from the present yields except in isolated cases, with the net result that a second chart would probably show the same general pattern as the present one.

Most of the strains investigated fell into two fairly well defined and apparently natural subgroups. The first of these embraced strains producing maximum yields of penicillin ranging from 15 to 40 o u/ml, whereas the second included strains producing maximum yields of from 50 to 80 o u/ml. While it was not universally true, differences in penicillin yield were commonly associated with differences in cultural appearance and in details of microscopic structure. So often have basic cultural characters been associated with certain levels of penicillin formation that in actual experiments we frequently have been able to predict with reasonable accuracy the amount of penicillin that would be produced by a particular strain when tested in surface culture. It would appear, therefore, that there are within the *Penicillium notatum-chrysogenum* group two principal aggregates of strains which differ both physiologically and morphologically.

The majority of the cultures comprising the first of these subgroups (i.e., producing yields ranging from about 20 to 35 o u/ml) may be characterized as follows: Colonies loose-textured, comparatively deep, heavily sporing, at first pale blue-green, nearly stone green (Ridgway, pl. XLII), or celandine green (pl. XLVII), but becoming darker in age, commonly approximating Russian green (pl. XLVII), often deeply furrowed in a conspicuously radial pattern, producing more or less abundant exudate which varies in color from pale to very intense yellow; reverse of colonies and underlying agar in yellow shades; conidiophores often comparatively long and rather coarse, bearing large penicilli; conidia typically subglobose to definitely elliptical, and ranging in size from 2.5 to 3.5 or even 4μ in long axis. On the whole these strains present the cultural and microscopic picture which has come to be associated with the species designation, *P. chrysogenum* Thom. They are common in soil and also occur in considerable abundance in a wide variety of other natural substrata (see table 2). It should be noted, however, that the type strain of this species, NRRL 807 (Thom 26), produces yields of penicillin up to 50 to 60 o u/ml and is characterized by colonies which are more strictly velvety and somewhat more blue-green than the above description would indicate.

Some of the strains producing from 40 to 50 o u/ml fit the group description above reasonably well. Others are slower growing and produce rather compact, velvety colonies differing little in appearance from those of the more productive strains which constitute the bulk of the second large subgroup still to be considered. Strains producing yields of penicillin not exceeding 15 to 20 o u/ml commonly differ from the foregoing group description by showing relatively less green in conidial areas, by producing abundant but pale-colored exudate, and by showing less color in the colony reverse and the surrounding media. Strains producing yields below 10 o u/ml regularly appeared as poor-producing cultures in the screening tests and were included in the surface production tests primarily

for the purpose of checking the usefulness of the screening technique as a means of eliminating poor and nonproducing strains.

Most of the strains comprising the second large subgroup (i.e., producing from 50 to 80 o u/ml) possess the following characteristics: Colonies fairly compact or even close-textured, often restricted, velvety throughout, or with central areas more or less floccose but with marginal area velvety and heavily sporing, ranging in color from celandine green to artemisia green or Andover green (pl. XLVII) in some strains, and from pistachio green to dark American green (pl. XLI) in others, often furrowed in a conspicuously radial pattern, producing more or less abundant yellow exudate; reverse of colonies in yellow to brown shades with the underlying medium often highly colored; conidiophores ranging from comparatively short in some strains to long in others, but generally bearing penicilli somewhat smaller than those of the preceding group; conidia in most strains globose to subglobose, rarely exceeding 3μ in diameter, in other strains subglobose to definitely elliptical, and occasionally up to 3.5μ in diameter. Most of the strains comprising this group seem to fit the description of *P. notatum* Westling reasonably well, although different strains vary materially in their rate of growth, colony appearance, and details of microscopic structure. Culture NRRL 832 falls within this subgroup, as does also NRRL 824 (Thom no. 5112.1), the "Fleming strain" received and diagnosed by Dr. Thom in 1930.

The higher producing strains among the new isolates, ranging up to 100 o u/ml and above, were varied in character, but for the most part conformed with most of the strains comprising the group just considered. The two cultures yielding penicillin in excess of 100 o u/ml bore a close resemblance to strain NRRL 1249.B21. Although they were obtained from soil dilution plates and appeared to constitute a part of the mycoflora of the soils examined, the possibility that they may represent contaminants from the laboratory air is not excluded.

Strains NRRL 1249.B21 and NRRL 832.A2 were regularly included as controls in the survey of new isolates in surface culture. The latter represents a substrain of NRRL 832 which was selected at the beginning of the survey because it appeared to be somewhat more productive than the parent stock. It has been used throughout the work, although it now appears that this choice was scarcely justified (see table 4). The average maximum penicillin concentration for NRRL 1249.B21 was 122 o u/ml in 20 separate tests with individual values occasionally ranging up to 150 to 160 o u/ml. For the 832.A2 controls the average maximum value was 58 o u/ml in 15 separate experiments with assays ranging up to 75 to 80 o u/ml in isolated cases. No new strain has been isolated from natural sources which surpasses strain 1249.B21 for the surface production of penicillin, and only a very limited number have been isolated which approach it closely. A large number of strains, however, were found which gave surface yields equal to, or exceeding, those of strain 832.A2.

Submerged Production

Strains which showed a concentration of penicillin in surface culture exceeding, equaling, or approaching NRRL 832 in surface culture flasks were studied

in submerged (continuously shaken) culture. For this part of the survey, a nutrient solution recommended by Dr. A. J. Moyer, and having the following composition, was employed:

Lactose	20	g
Corn steeping liquor	40.	ml
$MgSO_4 \cdot 7\ H_2O$	0.25	g
KH_2PO_4	0.50	g
$NaNO_3$	3.0	g
Zinc (as $ZnSO_4$)	0.01	g
Distilled water to make	1,000	ml

The solution was dispensed in 125-ml quantities in 300-ml Erlenmeyer flasks and sterilized at 15 pounds steam pressure for 20 minutes. Just prior to inoculation, 1 gram of sterile $CaCO_3$ was added to each flask. Cultures were usually run in duplicate; often in triplicate. As a general practice, each flask was seeded with 1 ml of a heavy (dark green) aqueous suspension of spores. Spores for this purpose were produced upon large slant tubes containing a sporulation agar and were washed off with sterile water containing 1 part in 10,000 of sodium lauryl sulfonate to secure uniform suspensions. Immediately after seeding, the culture flasks were placed on Ross-Kershaw shakers which are provided with slightly enlarged drive pulleys so that they rotate at a speed of 200 to 210 rpm. In addition to a rotary motion, these shakers have a slight vertical pitch due to the asymmetric character of the rotating bearing. Since it was impossible to secure exactly the same agitation with each of the shakers employed, control flasks inoculated with the stock strain of NRRL 832 were included on each shaker. Incubation was at 24 C. Under the conditions of these experiments, yields of approximately 45 to 60 o u/ml were generally obtained in 4 to 5 days with strain 832.

Cultures were assayed on the third through the seventh day, each flask being assayed daily throughout this period. Samples of approximately 5 ml were removed aseptically and, as with the surface culture material, a portion was used for pH determination, and the remainder was used for a standard cup assay of the penicillin present. Cultures which showed yields equal to, or exceeding, that of NRRL 832 were regularly included in one or more subsequent experiments for confirmation of results.

On shakers of the type used in these experiments, the mycelium invariably develops into rounded or oblong pellets of variable size, depending primarily upon the type and amount of the inoculum. Since pellet size is more or less inversely proportional to the quantity of spores contained in the inoculum, and since there is some indication of a relationship between the size of the pellets formed and the amount of penicillin subsequently produced, attempts have been made to secure spore suspensions of approximately the same density for all strains tested. Although it appears to vary somewhat with different strains, an inoculation of 1 ml per flask of a heavy (dark green) spore suspension generally yields pellets of approximately 1 mm diameter. A series of cultures con-

taining pellets of this size can be satisfactorily evaluated by comparing penicillin yields of the unknowns with the controls of NRRL 832.

The results of a representative experiment are shown in table 3. The data presented refer to cultures grown on the same medium, seeded with as nearly as possible the same quantity of spores, and incubated under uniform conditions on the same shaker. Agitation and aeration can be considered constant for all cultures. In this particular list it would appear that strain P.S. 69 is approximately equal to NRRL 832; P.S. 71 is markedly inferior; whereas P.S. 72 (NRRL 1950) appears definitely superior. Such superiority under the conditions of our experiments has been confirmed by repeated tests in which the last strain has been compared with NRRL 832. In 10 separate tests in which the two cultures

TABLE 3

Tests of new isolates in submerged culture—a representative series

CULTURE	FLASK	3RD DAY		4TH DAY		5TH DAY		6TH DAY		7TH DAY	
		pH	Penicillin	pH	Penicillin	pH	Penicillin	pH	Penicillin	pH	Penicillin
			o u/ml		*o u/ml*		*o u/ml*		*o u/ml*		*o u/ml*
NRRL 832	A	7.7+	36	7.75	46	8.25	44	8.35	37	8.5	19
(control)	B	7.8	24	7.7	41	8.1+	42	8.4	40	8.55	21
	C	7.55	41	7.7	40	8.1+	52	8.25+	41	8.5	34
P.S. 69	A	7.5+	23	7.7	45	8.2+	44	8.35	32	8.55	19
	B	7.35	19	7.55	45	8.2	44	8.3	40	8.45	28
	C	7.35	18	7.6	34	8.1+	32	8.35	33	8.5	24
P.S. 71	A	7.5	6	7.8	11	8.05+	18	8.25	19	8.3	12
	B	7.85+	6	7.85	13	8.1	17	8.2+	14	8.35	13
	C	7.5	tr.	7.75	7	8.05	15	8.3	13	8.4+	12
P.S. 72	A	7.7	36	7.8	61	8.2	51	8.3	43	8.35	42
(NRRL	B	7.45+	39	7.7	73	8.15	70	8.3	65	8.3	47
1950)	C	7.5	45	7.8	78	8.15	74	8.3	58	8.3	50

were inoculated in like manner and incubated on the same shaker, NRRL 1950 (P.S. 72) has consistently produced higher yields than NRRL 832. In some instances, the yields have been more than 60 per cent higher, whereas the average over-all difference represents an increase of approximately 34 per cent (table 4).

Over 50 new strains have been investigated for penicillin production in submerged culture. A summary of the results obtained with many of these organisms is presented in table 4. A number of strains have been discovered which produce yields equal to or exceeding that of NRRL 832. Based upon the repeated experiments which we have conducted, certain of these strains appear to be definitely superior. One of these cultures, NRRL 1950 (P.S. 72), has been noted previously, and it is considered significant that other high-producing strains such as P.S. 142, 165, 176, and 180, although isolated from soils collected

in widely separated areas, all present essentially the same cultural picture. They are considered to represent strains of *Penicillium notatum*. Other isolates, such as P.S. 43, 47, 66, and 76, resemble NRRL 832 in rate of growth and in colony appearance, and produce penicillin in approximately the same quantities

TABLE 4

Comparison of penicillin production in submerged culture by strains generally showing surface yields in excess of 60 o u/ml with those showing surface yields less than 50 o u/ml

Where more than one test was performed, average values are presented and the number of tests indicated

GOOD PRODUCING STRAINS—>60 O U/ML			LOW PRODUCING STRAINS—<50 O U/ML		
Culture designation	Penicillin production		Culture Designation	Penicillin production	
	Surface culture max. yield*	Submerged culture max. yield†		Surface culture max. yield	Submerged culture max. yield
	ou /ml	*o u/ml*		*o u/ml*	*o u/ml*
NRRL 832	64 (av. 7)	51 (av. 10)	NRRL 821	6	3
832.A2	58 (av. 15)	57 (av. 2)	P.S. 99	15	17
1950 (PS 72)	74 (av. 7)	68.5 (av. 10)	117	19	31
1951 (PS 46)	66	54 (av. 6)	79	20	32
807	55	30	98	20	15
824	78 (av. 6)	30	114	21	18
1249.B21	122 (av. 20)	26 (av. 2)	100	22	24
P.S. 44	62	34	118	23	16
52	64	31	124	26	20
165	64	72 (av. 6)	68	29	19
76	64	47 (av. 2)	115	29	23
47	67	51 (av. 4)	108	31	34
161	67	59 (av. 2)	106	34	21
43	68	47	103	37	8
81	69	22	149	38	31
136	69	9	133	42	49 (av. 2)
180	70	71 (av. 2)	112	42	30
142	73	68 (av. 3)	93	43	18
66	76	50 (av. 3)	82	48	19 (av. 2)
176	78	71 (av. 2)	105	50	33
167	80	64 (av. 2)			
95	85	19			
120	94	59 (av. 3)			

* Average of triplicate flasks.

† Average of duplicate or triplicate flasks.

as this strain. Culture NRRL 1951 (P.S. 46) represents a loose-textured strain of *P. chrysogenum* which has produced satisfactory yields in submerged culture and which appears to offer considerable promise as the possible source of more productive substrains for both the surface and submerged fermentations.

Many strains which showed good yields in surface culture proved disappointing when studied in a submerged condition. Included among these were such new isolates as P.S. 81, 95, and 136, together with NRRL 824 (the "Fleming strain") and NRRL 1249.B21 (derived from it), which were already known to be poor producers when grown submerged. Strain P.S. 136 is of particular interest since it was isolated from soil collected in Cheshire, England, and in cultural appearance closely resembles the Fleming strain. As a general rule, strains producing low penicillin yields in surface culture showed correspondingly low yields when grown submerged. Only in isolated cases were submerged culture yields greater than those in surface culture, and in these instances the increase could not be considered significant (table 4).

DISCUSSION

In conducting this survey we have endeavored to control as completely as possible all environmental and nutritional factors and to maintain in a uniform state of productivity the two control cultures with which new isolates have been compared. To insure constancy of the nutrient solutions, the same batch of corn steeping liquor was used throughout the entire survey, U.S.P. lactose has been consistently employed, and salts have been of cp grade. To hold natural variations and the possibility of diminished productivity at a minimum, small agar tube cultures of the two control strains were made up in large numbers and allowed to develop at room temperature and partially to dry out, after which they were stored in a refrigerator at 1 to 3 C. Single tubes of each strain were used as spore sources for the control cultures in each succeeding experiment. During the 9 months since the survey was initiated, only two such series of cultures have been required. For each strain, penicillin concentrations have varied appreciably in different experiments, but there have been no indications of decreased yields or of any deterioration in the control cultures. Yields of penicillin have remained at the same general levels throughout the work. We, therefore, feel that limited comparisons between cultures tested in many different experiments are wholly justified.

The variety of sources from which cultures have been isolated in this survey is shown in table 2. Examination of this table clearly reveals that no close correlation can be shown between the source of culture, i.e., the type of substratum from which isolated, and penicillin production. Good strains have been isolated from soil, from cheese, from fruits, and from bread; but from all of these sources poor cultures have been obtained in even greater numbers. Representatives of the *P. notatum-chrysogenum* group occur with reasonable frequency in fertile cultivated soil, and we have devoted considerable attention to the isolation of strains from soils collected from many parts of the United States and from several widely separated foreign countries. Up to the present there is no evidence that soils from one area are more outstandingly productive of desired types than soils from other areas. This lack of specificity in point of isolation is not surprising when we consider that these molds are propagated

by air-borne spores that are produced in enormous numbers and that are strictly nonselective in the substrata whereon they lodge and grow. In searching for members of this group, all materials upon which miscellaneous molds occur should be considered as potential sources.

To establish whether penicillin production is, in fact, a characteristic of the whole *P. notatum-chrysogenum* group (see Thom, 1930), or whether it is limited to representatives of two or three widely distributed species such as *P. notatum* Westling and *P. chrysogenum* Thom, a survey was conducted on all strains belonging to the group brought from the Thom Collection (NRRL 807 to 843). All but 3 of the 36 cultures produced some penicillin, but the yields in most cases were low. In part, this may have resulted from a loss of penicillin-producing capacity during many years of laboratory cultivation, but, in the main, it is believed due to the character of the cultures themselves since certain of these historic strains produced good yields ranging from 50 to 75 o u/ml when tested. Good producing strains include the type of *P. chrysogenum*, NRRL 807 (Thom 26)—and this has been maintained in laboratory culture for more than 35 years (table 4); *P. chrysogenum*, NRRL 811 (Thom 5034.11), the gluconic acid strain investigated by May, Herrick, Moyer, *et al.*; *P. notatum* NRRL 824 (Thom 5112.1), the "Fleming strain" diagnosed by Thom as *P. notatum*; and *P. notatum* NRRL 832 (Thom B69), the standard strain for submerged culture. A single strain of *P. baculatum* Westling, NRRL 843, produced fair yields both in surface and in submerged culture. Among the 18 strains producing from 1 to 10 o u/ml (see table 2) were represented such species as *Penicillium chlorophaeum* Biourge, *P. fluorescens* Laxa, *P. griseo-roseum* Dierckx, *P. citreo-roseum* Dierckx, *P. cyaneo-fulvum* Biourge, and *P. brunneorubrum* Dierckx. All of these are characterized by loose-textured colonies that are rather pale in color and of a general cultural type which we have come to associate with low penicillin yields. As a rule, forms similar to these species, when isolated and investigated in this survey, have been eliminated by the screening test.

Not only do different isolates vary greatly in their capacity to produce penicillin, but substrains derived from the same isolates commonly show marked variation in productivity. The so-called Fleming strain represents a case in point. Strain NRRL 824 (Thom 5112.1) represents the strain received and diagnosed by Thom in 1930. It has been observed by one of us (K. B. R.) throughout this period and has remained essentially constant in culture appearance. It is believed to represent fairly closely the original Fleming isolate. In the present survey it has produced an average of 78 o u/ml in 6 experiments when tested in surface culture. Strain NRRL 1249 represents a substrain developed at the Squibb Laboratories from Thom's culture (personal communication) and forwarded to us by Dr. George A. Harrop, Director of Research, Squibb Institute for Medical Research, in December, 1941. In 5 separate trials in the present survey, this strain, as received from the Squibb Institute, has produced an average penicillin yield of 94 o u/ml. Strain NRRL 1249.B21 represents a substrain developed from the Squibb culture at the Northern Regional Research Laboratory which produces substantially higher yields of penicillin and is cur-

rently used in industry for the production of penicillin in surface culture. With this culture, an average yield of 122 o u/ml has been obtained in 20 experiments. Test conditions have remained constant. Thus, in this case, materially increased yields have been obtained from a single basic strain by careful selection and propagation, first at the Squibb laboratories and subsequently at the Northern Regional Research Laboratory.

Members of the *P. notatum-chrysogenum* group, like all cosmopolitan and widely distributed molds, vary greatly in their cultural and morphological characters. Particular strains can be selected which fit with reasonable exactness the original published description of such species as *P. notatum* Westling, *P. chrysogenum* Thom, *P. baculatum* Westling, etc. Many other strains, however, cannot be so easily classified. They may agree with one species in certain characters, and in other characters fit one of the other species just as satisfactorily; or they may have, as they often do, certain unique characteristics of their own. No attempt has been made to assign a specific name to each and every strain studied. Rather we have endeavored to view the multitude of isolates as "individual" strains which vary one from another, and in so doing reveal different facets of the single natural group which they comprise. We have noted how the *P. notatum-chrysogenum* group contains two reasonably well defined subgroups, or species aggregates, and how the names *P. chrysogenum* and *P. notatum*, when interpreted in a broad sense, can be applied to these. One should not assume from this, however, that all strains of *P. notatum* are good producers, or that all good producing strains represent the species *P. notatum*. Neither is true. To illustrate, the type strain of *P. notatum* Westling, received in 1911 by Dr. Thom and now maintained in the NRRL collection as 821 (Thom 2541), produces penicillin in the amount of only 5 to 6 o u/ml in surface culture and 3 to 4 o u/ml submerged. On the other hand, the type strain of *P. chrygenum*, NRRL 807 (Thom 26), produces yields of approximately 50 to 60 o u/ml in surface culture and 30 o u/ml submerged. Both cultures were verified by Dr. Thom in November, 1943. With information now available, we can safely say that penicillin production is a strain, rather than a species, characteristic. While it can be correlated to some degree with cultural and morphological characters, it is not associated with these in any strict sense. Most of the good strains investigated can best be assigned to the species *P. notatum*, but it is definitely erroneous to assume that penicillin production and *P. notatum* are synonymous, as is so often done in certain scientific reports and in the daily press.

From the results obtained with the Thom cultures, as cited above, together with the much more extensive results obtained with new isolates, we believe the following general conclusions are warranted: (1) penicillin production is characteristic of the entire *P. notatum-chrysogenum* group; (2) strains vary greatly in their capacity to produce penicillin just as they vary in cultural and morphological characters; (3) production of penicillin can often be correlated with particular cultural and structural characteristics, but physiological and morphological characteristics are not strictly interdependent; (4) good penicillin production is generally limited to strains belonging to one of two species aggregates,

typified by *P. notatum* Westing and *P. chrysogenum* Thom; and (5) most of the better-producing strains can most satisfactorily be assigned to the species *P. notatum* Westling.

SUMMARY

A total of 241 different cultures belonging to the *Penicillium notatum-chrysogenum* group have been investigated for penicillin production. The majority of these strains represent new isolates made at the Northern Regional Research Laboratory during the present study.

All but 24 of the strains investigated produced measureable quantities of penicillin.

A simple screening test has been devised which effectively differentiates between low-producing and promising strains.

No strains were isolated from nature which exceeded *P. notatum*, NRRL 1249.B21, for the production of penicillin in surface culture. Approximately 25 per cent of all strains studied, however, produced yields of penicillin equal to or exceeding that of *P. notatum*, NRRL 832, when this was grown in surface culture.

Several new strains were discovered which produced yields of penicillin equal to that of NRRL 832 when grown in submerged culture, and a limited number produced yields somewhat in excess of that obtained with this strain.

Comprehensive studies on the more productive strains found are in progress and will be reported subsequently.

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