A COMPARISON OF PENICILLIN-PRODUCING STRAINS OF PENICILLIUM NOTATUM-CHRYSOGENUM[1]

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Most of the work reported in this paper was done under a government-sponsored co-operative research program. One of the problems at this laboratory involved a comparison of the various strains of the *Penicillium notatum-chrysogenum* group which have been isolated or developed in co-operating laboratories.

EXPERIMENTAL METHODS

Sampling and storage. The fermentations were conducted in the 100-gallon tanks described by Stefaniak *et al.* (1946). Samples were removed periodically into 500-ml flasks and filtered. A 0.5-ml filtered sample was immediately diluted for penicillin assay by addition to previously chilled phosphate buffer in volumetric flasks (4.0 g KH_2PO_4 and 1.0 g K_2HPO_4 per 500 ml solution in distilled water; pH 5.9 to 6.0). These flasks were stored at 10 C until they were assayed for penicillin (usually no longer than 16 hours). Samples kept 2 days showed no change in potency greater than the variations to be expected in the assay, ca. 5 to 10 per cent. A 1.0-ml filtered sample was diluted to 10.0 ml with 0.1 N H_2SO_4 and frozen until sugar determinations and Kjeldahl nitrogen determinations could be made. The remainder of each filtered sample was also frozen and saved for the ammonia determination. The pH values were determined on the original unfiltered samples by means of a glass electrode.

Penicillin assay. The cylinder plate assay for penicillin (Abraham *et al.*, 1941; Schmidt and Moyer, 1944) was used with modifications. *Staphylococcus aureus* 209-P was used as the test organism. Eight plates were set up for each two samples and the results averaged. The standard penicillin was checked against a Food and Drug Administration standard (penicillin G).

Sugar determination. The lactose in diluted samples was hydrolyzed by heating to 120 C for 30 minutes in 0.75 N HCl. Sugar analysis was conducted by the method of Shaffer and Somogyi (1933). Reagent 50 with 5 grams of KI was used, and the samples were heated for 30 minutes in the boiling water bath. Glucose and lactose values were determined from appropriate calibration curves.

Total nitrogen. The total nitrogen of the filtrates was also determined on the diluted samples by the micro-Kjeldahl method described by Johnson (1941).

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Duplicate tubes containing 0.01 ml of filtrate were analyzed. From the values thus obtained were calculated the mycelial nitrogen (by difference from the zero time sample) and the organic nitrogen of the medium (by subtracting the ammonia nitrogen values).

Ammonia nitrogen. Samples of undiluted filtrate were analyzed for ammonia nitrogen by a modification of the aeration procedure described by Umbreit and Bond (1936). A bank of 18-by-150-mm test tubes was constructed in sets of two tubes, one for the sample and one for the acid. The addition of a few drops of water-white caprylic alcohol (practical *n*-octyl alcohol, Eastman Kodak Company) was necessary to prevent frothing of the samples. Since the aeration is considerably slower in this size tube than in those used by Umbreit and Bond, a higher pH was used to hasten the liberation of ammonia. An excess of alkali (1 ml of 10 N NaOH) was used, and aeration at a rate of 40 to 50 ml per minute continued at least 15 hours at room temperature. It is known that this alkalinity liberates easily hydrolyzable ammonia (as in certain amides) as well as ammonia from salts. The results, therefore, represent free and loosely combined ammonia. In the case of the unfermented steep liquor this combined ammonia is roughly 40 per cent of the total ammonia, 60 per cent occurring as free ammonium salts.[2] Two 5.0-ml aliquots of each sample were analyzed. The receiving acid was approximately 0.09 N H_2SO_4 containing methyl red. After aeration the tubes and several blanks were titrated with 0.0357 NaOH (hence, when using a 5.0-ml sample, the titration difference multiplied by 10 represents mg ammonia nitrogen per 100 ml of medium).

Preparation of the inoculum. Soil stocks of the various cultures were kept, and spore plates in 6-oz bottles were made by inoculating small amounts of soil onto the agar surface and moistening slightly with sterile water. The sporulation medium, developed at the Northern Regional Research Laboratory, consisted of the following ingredients (in grams per liter):

Glycerol	7.5
Brer rabbit molasses (orange label)	7.5
Curbay BG (U.S.I.)	2.5
Peptone	5.0
$MgSO_4 \cdot 7H_2O$	0.05
KH_2PO_4	0.06
NaCl	4.0 or 40.0
Agar	25.0

Table 1 shows the effect of temperature and NaCl concentration on the sporulation of several cultures. Peptone is required for mycelium formation, and the NaCl concentration is important for sporulation at 30 C but not at 23 C. The omission of any one constituent, other than peptone, has no effect in retarding sporulation. After incubation at 23 to 25 C for 4 days (or, for certain cultures, 30 C with the high salt medium), the plates were stored at 10 C for as

[2] It has been found that a more exact liberation of ammonia from the ammonium salts alone can be accomplished by 24 hours' aeration at 40 to 50 ml per minute at pH 9.7 to 10.1 (0.5 ml saturated Na_2CO_3 per 5.0 ml sample).

long as 2 weeks before use. The inoculum for tank fermentations was grown in seed tanks as described by Stefaniak *et al.* (1946). Two methods of inoculating this seed tank have been used. The earlier method involved adding an aqueous suspension of spores from two 6-oz bottle plates to the 68 liters of medium and incubating at 23 C for at least 48 hours. The later method made use of 200 ml of vegetative inoculum grown in two 500-ml flasks on a shaker at 23 C. This usually overcame the variable induction period prior to good mycelium formation and, hence, resulted in a more reproducible inoculum.

TABLE 1

Sporulation of cultures

(The constituents of the medium are listed in the text under Preparation of the inoculum)

CULTURE	23 C INCUBATION			30 C INCUBATION		
	Concentration of NaCl			Concentration of NaCl		
	0%	0.4%	4.0%	0%	0.4%	4.0%
832		+	+		+	+
1951-B25		+	+		−	+
1982		−	(+)		(+)	(+)
23248		+	+		−	+
25099		+	+		−	+
35217		+	+		−	+
35347		+	+		−	+
45417		+	+		−	+
R-38		+	+		+	+
J347		+	+		+	+
X-1612	+	+	+	−	−	+

− indicates no formation of green spores.
(+) indicates formation of gray or poor spores.
+ indicates formation of green spores in 3 to 4 days.

Cultures. A list of the cultures mentioned in this report is presented in table 2.[3]

Fermentation equipment. In an attempt to test cultures under conditions similar to those in tanks, 4-liter fermentations were conducted in 9-liter bottles, which were arranged for aeration (17 to 20 liters per minute through 25 to 35 $\frac{1}{32}$-inch holes), for stirring (two propeller blades, 5 inches in diameter, rotating at 250 rpm), for the addition of antifroth (30 to 50 ml of 3 per cent octadecanol in lard oil), and for sampling under aseptic conditions. The spargers, propellers, and sampling tubes were made of aluminum alloy and fitted through an iron cap, which was packed with cotton when placed on the bottles before sterilization.

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The medium was inoculated with 8 to 10 per cent of vegetative inoculum, which was grown in shaken flasks in a liquid medium containing 2 per cent steep liquor solids and 6 per cent dextrin (dextrin 151, Clinton Company, Clinton, Iowa). The bottles were incubated in a water bath at 23 C. The 200-liter fermentations were conducted in the 100-gallon tanks described by Stefaniak *et al.* (1946).

TABLE 2

List of Penicillium cultures tested

DESIGNATION	SOURCE	DESCRIPTION
832	N.R.R.L.	*Penicillium notatum*, isolated from natural source
1951-B25	N.R.R.L.	*P. chrysogenum*, single spore isolated from 1951-B, a sector of 1951 which was isolated from a cantaloupe
1982	Stanford	*Penicillium*, ultraviolet mutant from NRRL1950
3271	Standord	*P. notatum*, ultraviolet mutant from NRRL832
4171	Stanford	*P. notatum*, ultraviolet mutant from NRRL832
23248	Stanford	*P. chrysogenum*, ultraviolet mutant from NRRL1951-B25
25099	Stanford	*P. chrysogenum*, ultraviolet mutant from NRRL1951-B25
35217	Stanford	*P. chrysogenum*, ultraviolet mutant from NRRL1951-B25
35347	Stanford	*P. chrysogenum*, ultraviolet mutant from NRRL1951-B25
45417	Stanford	*P. chrysogenum*, ultraviolet mutant from NRRL1951-B25
R-13	Minnesota	*Penicillium*, isolated from dried soil
15-U-1	Minnesota	*Penicillium*, ultraviolet mutant from R-13
R-38	Minnesota	*Penicillium*, isolated from dried soil
R-1138	Minnesota	*Penicillium*, isolated from soil
R-1139	Minnesota	*Penicillium*, isolated from soil
R-1204	Minnesota	*Penicillium*, isolated from soil
R-1205	Minnesota	*Penicillium*, isolated from soil
R-1262	Minnesota	*Penicillium*, isolated from soil
X-1612	Carnegie	*P. chrysogenum*, X-ray mutant from NRRL1951-B25
J347	Wisconsin	*Penicillium*, isolated from a natural source
Q176	Wisconsin	*P. chrysogenum*, ultraviolet mutant from X-1612
Q176-A8	Wisconsin	*P. chrysogenum*, single spore isolated from Q176

CONDITIONS FOR CULTURE COMPARISON

In order to find mold cultures which produce large amounts of penicillin, it became necessary to test a large number of strains of the *Penicillium notatum-chrysogenum* group. Co-operating workers at this and other universities conducted many preliminary small-scale tests using various techniques to separate poor penicillin-producing cultures from possible good producers. In this laboratory experiments were confined to shaken flasks, 9-liter stirred and aerated bottles, and 100-gallon tanks. The cultures which reached the shaken-flask test were tried under a variety of conditions, but none gave better yields than the control organism (*Penicillium notatum* NRRL 832) in the early tests. It was realized that conditions in flasks were not comparable with those in large tanks (Koffler *et al.*, 1945; Bowden and Peterson, 1946). Fortunately, 9-liter stirred and aerated bottles were found to give good yields in 5 to 6 days, and the conditions were more like those in tanks. A number of cultures were compared

by this method, and the results, shown in table 3, indicate the superiority of some cultures over others. The best strains appeared to be *Penicillium* strains 45417 and X-1612. It was possible to test most of these superior cultures in the 100-gallon tanks.

Conditions for tank fermentations. Some of the characteristics and conditions of tank fermentations have been discussed by Stefaniak *et al.* (1946). The

TABLE 3

Penicillin yields of various cultures in stirred bottle fermentations

CULTURE	MEDIUM I		MEDIUM II	
	Penicillin yield	Age at maximum yield	Penicillin yield	Age at maximum yield
	units/ml	*days*	*units/ml*	*days*
832	90	7	145*	6
1951-B25			156	5
1982	166†	8	183	
3271	153	5		
4171	91	6		
23248	134	6		
25099	58	5	194	5
35217	194†	6	148	6
35347	169†	6	207	6
45417	159†	6	267*	6
R-13	140	5		
15-U-1			203	5
R-38	181	5	158	5
R-1138	127	4	160	4
R-1139			217	4
R-1204	168	4	226	5
R-1205			227	6
R-1262	135	5	102	5
J347	96	5	100	5
X-1612			294	6

Medium I contained 2% lactose, 2% steep liquor solids, and salts (1.5 g $NaNO_3$, 1.5 g $CaCO_3$, 0.25 g KH_2PO_4, and 0.125 g $MgSO_4 \cdot 7H_2O$ per liter).

Medium II contained 3% lactose, 4% steep liquor solids, and 1% $CaCO_3$.

* One bottle; all other figures refer to the average yield of 2 or 3 bottles.

† Medium I with no salts except 2.5 g $CaCO_3$ per liter.

question of deciding upon standard conditions for comparing cultures raises the problem of the suitability of any one medium for all cultures. It is to be expected that certain characteristics (such as temperature range, pH range, general nutrient requirements) of all strains of the *Penicillium notatum-chrysogenum* group would be similar. It is probable, however, that specific differences between strains would cause the strains to differ widely in their optimum conditions for penicillin production. During the early experiments variations were made in the media between parallel tank fermentations in order to determine the most probable optimum conditions. Chemical changes (particularly lactose

and ammonia nitrogen) were determined in order to ascertain roughly the metabolic characteristics of the cultures and the nutrient conditions throughout the fermentations.

The information on carbohydrate metabolism and its relation to the changes in ammonia concentration and pH value, which has been discussed by Koffler *et al.* (1945), was exemplified repeatedly in the tank fermentations. Thus glucose was found to be unsuitable for a comparison of cultures because it was utilized too rapidly; this resulted in a low pH value early in the run, rising too slowly for good penicillin production, a deficiency of available ammonia nitrogen in the penicillin-producing phase, and an early autolysis of mycelium on exhaustion of the carbohydrate. However, the ammonia level in some lactose fermentations tended to rise too high; this condition could be corrected by the addition of a small amount of glucose to the medium in order to stimulate

TABLE 4

Effect of concentration of steep liquor solids on penicillin production by P. chrysogenum X-1612 in tank fermentations

RUN NO.	STEEP LIQUOR SOLIDS	PENICILLIN YIELD	AGE AT MAXIMUM YIELD
	%	*units/ml*	*hours*
138	2*	235	54
139	2*	240	45
118	4†	479	64
119	6†	525	55
116	6†	636	67

* The medium contained 2 per cent steep liquor solids, 2 per cent lactose, and salts (1.5 g $NaNO_3$, 2.0 g $CaCO_3$, 0.25 g KH_2PO_4, 0.125 g $MgSO_4 \cdot 7H_2O$ per liter).

† The medium contained 3 per cent lactose and 1 per cent $CaCO_3$ besides the steep liquor solids.

mycelial growth. The addition of glucose was accomplished by inoculating the tanks with 10 per cent inoculum containing 1 to 2.5 per cent of unfermented glucose. At least 3 per cent lactose, and later 4 per cent, was used in the fermentation medium in order to provide sufficient carbohydrate for a 72-hour fermentation.

The concentration of steep liquor in the medium has a marked effect on the fermentation (table 4). It will be seen that there is a progressive increase in yield with an increase in steep liquor concentration (probably due to better growth). The limit may not have been reached but there are disadvantages in using the higher concentrations (e.g., frothing is an important problem in fermentations of the higher concentrations of steep liquor). It was decided that a comparison of cultures on 4 per cent steep liquor solids would be most useful.

Two types of steep liquor have been tested. Table 5 gives a comparison of the regular steep liquor (Staley Manufacturing Company, Decatur, Illinois) and a fermented steep liquor (Corn Products Refining Company, Argo, Illinois). The major differences between the two steep liquors are in their content of re-

ducing material and lactic acid. The unfermented steep liquor is low in lactic acid and high in reducing sugars. The amount of lactic acid in the fermented steep liquor above that in the regular steep liquor is roughly equivalent to the loss in reducing material calculated as glucose. Thus the loss of sugar does not change the amount of available carbon but results, in the fermentation, in the liberation of more ammonia in the fermented liquor runs than in the regular runs. The subsequent drop in ammonia nitrogen is delayed or incomplete in

TABLE 5

Comparison of corn steep liquors

(*P. chrysogenum* X-1612 grown on 3 per cent lactose, 4 per cent steep liquor solids, and 1 per cent $CaCO_3$)

REGULAR STEEP LIQUOR							FERMENTED STEEP LIQUOR						
Run	Penicillin yield	pH at maximum yield	Ammonia maximum	Age at ammonia maximum	Ammonia minimum	Age at ammonia minimum	Run	Penicillin yield	pH at maximum yield	Ammonia maximum	Age at ammonia maximum	Ammonia minimum	Age at ammonia minimum
no.	*units/ml*		*mg/100 ml*	*hours*	*mg/100 ml*	*hours*	*no.*	*units/ml*		*mg/100 ml*	*hours*	*mg/100 ml*	*hours*
126	558	8.1	34	24	26	48	128	415	8.0	43*	23	7	47
							129	330	7.8	43*	23	7	47
130	405	7.5	39	24	15	48	132	370	7.6	60	24	21	46
131	405	7.3	26	24	15	48	133	375	7.8	51	24	11	77
164	440	7.4	37	25	6	48	162	225	7.9	92	31	40	66
165	365	7.5	35	25	7	48	163	410	7.8	71	31	27	66
206	240	7.8	50	26	7	50	212	295	7.9	86	19	35	48
207	250	7.5	50	20	8	50	213	175	8.2	88	19	39	72
142†	312	7.1	40	50	10	68	144†	239	7.6	91	24	14	72
143†	300	7.4	47	35	10	68	145†	260	7.7	90	24	25	72

Reducing material (as glucose) in regular steep liquor solids, 85 to 140 mg per g; in fermented steep liquor solids, 10 to 15 mg per g. Lactic acid in regular steep liquor solids, 130 to 140 mg per g; in fermented steep liquor solids, 230 mg per g.

* In these runs the inoculum contained 4.5 per cent glucose when used. The ammonia concentrations are therefore lower.

† These fermentations were aerated at 30 liters per minute instead of 200 liters per minute and demonstrate the slower growth and utilization of ammonia at this aeration rate.

the fermented steep liquor runs. Hence, the pH value is usually higher in these runs. The penicillin yields are usually higher in the regular steep liquor fermentations; hence, regular, unfermented steep liquor was chosen for the standard comparisons.

There are many factors which affect the pH of a fermentation. A comparison of NaOH and $CaCO_3$ as neutralizing agents is given in table 6 for several cultures. It will be seen that the $CaCO_3$ medium usually gave higher yields, earlier penicillin maxima, and lower pH values than the NaOH medium. For these reasons $CaCO_3$ at a level of 1 per cent was used regularly.

In temperature experiments with shaken flasks a temperature optimum

around 23 C was observed. A comparison of several temperatures in tank fermentations showed that the optimum range was rather broad. A temperature of 20 C retarded the fermentation but normal yields were eventually obtained. Temperatures from 23 C to 32 C were tested and found to give variable results; sometimes the higher temperatures gave rise to rapid fermentations which resulted in lower yields, but occasionally a good yield was observed even at 32 C. Nevertheless, a temperature of 23 C was used for all standard fermentations.

In brief, the choice of a standard medium for comparison of cultures depended on the ability of the medium to maintain good penicillin-producing characteristics: rapid mycelial growth, presence of a slowly fermentable sugar, presence of ammonia nitrogen during lactose utilization, pH values from 7.0 to 7.8 during the penicillin-producing phase, and late initiation of autolysis. The medium best suited to meet these requirements seems to be 3 to 4 per cent lactose, 4 per

TABLE 6

Comparison of NaOH and $CaCO_3$ as neutralizing agents

CULTURE	NaOH				$CaCO_3$			
	Run no.	Penicillin yield	Age at maximum yield	pH at maximum yield	Run no.	Penicillin yield	Age at maximum yield	pH at maximum yield
		units/ml	*hr*			*units/ml*	*hr*	
R-38	74	72	67	8.3	75	100	92	7.4
R-38	79	93	70	7.3	78	135	55	7.8
1951-B25	81	153	78	7.3	80	174	62	7.4
25099	83	27	72	5.6	82	244	62	7.2
25099	87	85	87	7.4	86	187	68	7.5

The initial pH values of these fermentations were always between 5.6 and 6.1.

cent steep liquor solids, and 1 per cent $CaCO_3$. Ten per cent inoculum grown on 4 per cent glucose, 2 per cent steep liquor solids, 0.5 per cent $CaCO_3$, and salts (Stefaniak *et al.*, 1946) was used. To prevent frothing, 1 to 4 liters of 3 per cent octadecanol in lard oil was automatically added as required. The 200 liters of inoculated medium in each tank was stirred at 270 rpm and aerated at 200 liters per minute. The tanks were kept under a pressure of 20 pounds per sq inch.

COMPARISON OF CULTURES

Because of the variations in yields caused by unknown and uncontrolled factors, it was necessary during the fermentations in which various cultures were compared to intersperse control runs on a known culture. *P. chrysogenum* 1951-B25 was used for this purpose, since it was the highest-yielding culture known at the time the tests were begun. Table 7 presents a summary of all fermentations run under the standard culture comparison conditions. It will

be seen that several of the Stanford cultures were apparently slightly superior to the 1951-B25 control, but none of the isolations from natural sources surpassed this control culture. The ultraviolet mutant from Minnesota (*Penicillium* strain 15-U-1) appears similar to the Stanford mutant from the stand-

TABLE 7

Summary of penicillin yields in culture comparison experiments

(Tank fermentation)

CULTURE	NO. OF FERMENTATIONS	PENICILLIN YIELD		CONCURRENT YIELD WITH 1951-B25*
		Average	Best	
		units/ml	*units/ml*	*units/ml*
832	1		98	214
1951-B25	7	169	245	
25099	2	216	244	184
35217	1		275	245
35347	1		255	245
45417	2	199	229	245
15-U-1	3	157	200	214
R-38	2	109	135	165
R-1139	2	158	160	214
R-1204	2	147	148	214
R-1205	2	160	191	214
X-1612	46	369	558	245
Q176	6	761	904	
Q176-A8	6	286	360	

* This culture was widely used in experimental and early industrial fermentations; hence, control fermentations were run at intervals in order to compare them more accurately with other cultures.

TABLE 8

Loss of activity of P. chrysogenum X-1612

SOIL STOCK	PERIOD OF FERMENTATIONS	NO. OF FERMENTATIONS	AVERAGE OF PENICILLIN YIELDS
			units/ml
I	Jan. through March, 1945	13	421
I	April through June, 1945	16	344
I	July through Sept., 1945	9	290
II	Sept. through Dec., 1945	8	423

point of penicillin yield. However, the X-ray mutant from the Carnegie Institution (*P. chrysogenum* X-1612), which was only slightly better than the other cultures in bottle fermentations, proved to be definitely superior to all the previously tested cultures under tank conditions. This culture was used for further testing of the tank conditions, but a variability and a loss of penicillin-producing activity took place over the course of the experiments. Table 8

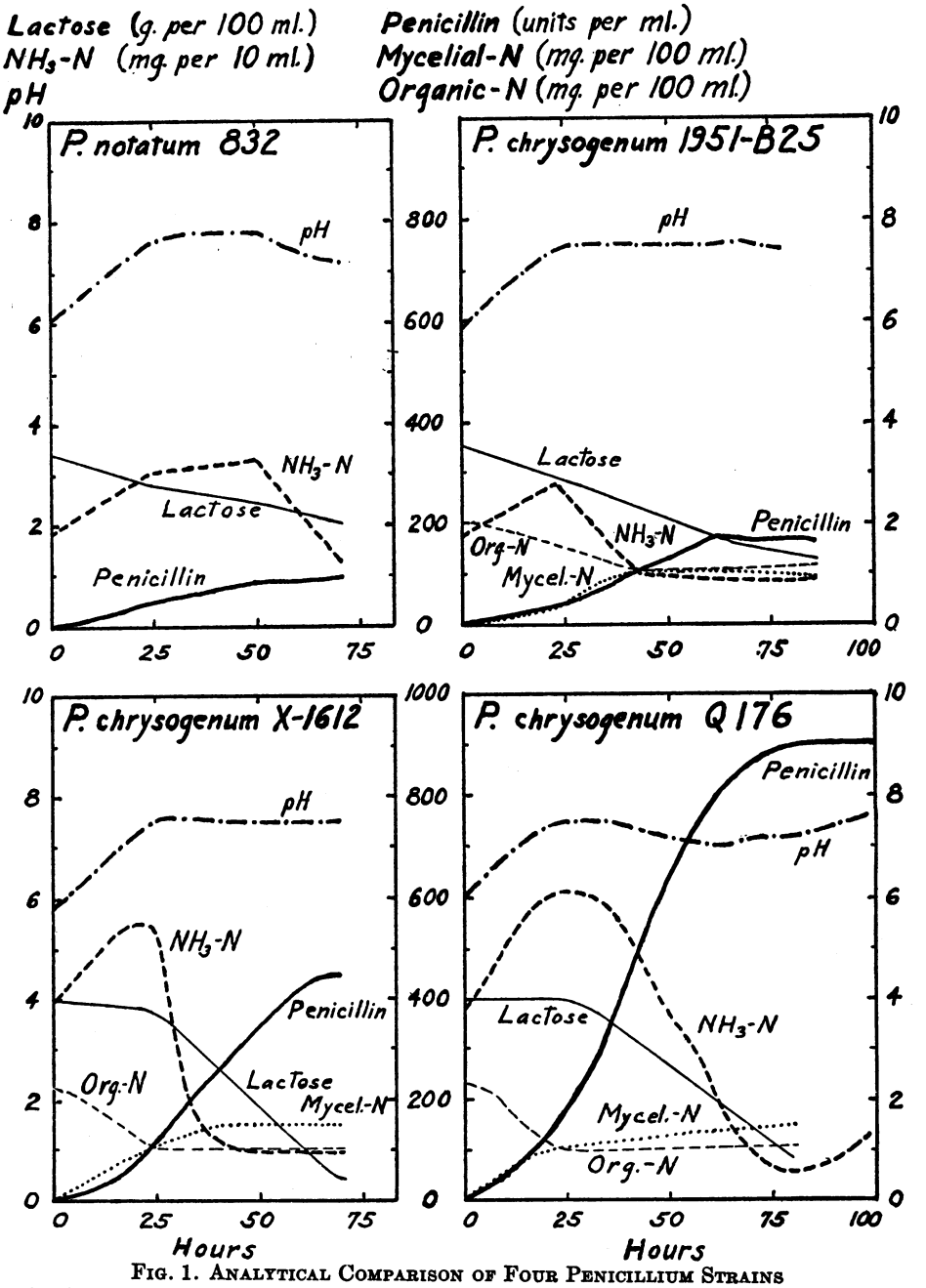

FIG. 1. ANALYTICAL COMPARISON OF FOUR PENICILLIUM STRAINS

Medium: 3 to 4 per cent lactose, 4 per cent steep liquor solids, and 1 per cent calcium carbonate.

shows a separation of the 46 standard fermentations by strain X-1612 into four groups, the first three groups representing the progressive aging of soil

stock I (kept at room temperature). This loss of activity could not be correlated with any other factor, such as differences between lots of steep liquor. Soil stock II was somewhat closer to the original strain of X-1612 (it had been stored in a cool room). Fermentations with it appeared to be similar to the early fermentations with the other soil stock culture.

The ultraviolet mutant from strain X-1612, called Q176, and the single cell isolation from it, strain Q176-A8, gave promising results in shaken-flask experiments, appearing equivalent to each other but superior to the parent strain. The tank fermentations revealed the superiority of strain Q176, but its variability in fermentations indicates that it may be an unstable culture.

It will be seen from table 7 that only a few fermentations were run on the majority of the cultures. Hence, chemical analyses and penicillin values are not available for an accurate comparison of these cultures. Figure 1 is a graphical representation of the analyses of several typical fermentations. *P. notatum* NRRL832 is included for comparison, since it was used widely in early experiments. There is no assurance that this represents its normal behavior in tank fermentations. The other three graphs are more or less representative of the normal or average tank fermentations for these strains of *P. chrysogenum* under our standard conditions. Strain Q176 uses lactose more slowly than strain X-1612. The liberation of ammonia from organic nitrogen during mycelium formation is evident in these fermentations. When the available organic nitrogen is utilized, further growth occurs at the expense of lactose and ammonia nitrogen. Mycelial growth is limited by the amount of organic nitrogen and ammonia nitrogen in the medium (since an increase in mycelial nitrogen ceases upon the exhaustion of available ammonia nitrogen). Because strain Q176 grows more slowly than the strains 1951–B25 and X-1612, it uses ammonia nitrogen more slowly than the parent strains.

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SUMMARY

The penicillin-producing properties and the gross metabolic characterstics of a number of strains of the *Penicillium notatum-chrysogenum* group were compared. The fermentations were conducted in 100-gallon tanks.

The conditions, aside from adequate aeration, apparently necessary for optimum penicillin production are rapid initial production of mycelium, the presence of a slowly fermentable carbohydrate and available nitrogen (e.g., ammonia), and the maintenance of a pH value between 7.0 and 7.8.

The penicillin yield under these conditions varies widely with the culture used. Of the many strains tested, the mutant *P. chrysogenum* strains X-1612 and Q176 have been found to produce the highest yields. Strain X-1612 yields 400 to 500 units per ml, and strain Q176 gives 700 to 900 units per ml.

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