PENICILLIN

IX. The Laboratory Scale Production of Penicillin in Submerged Cultures by Penicillium notatum Westling (NRRL 832)[1]

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Penicillin was first produced by cultivation of the fungus *Penicillium notatum* Westling on the surface of a liquid nutrient, such as Czapek-Dox glucose medium. Such a method of cultivation is very laborious, costly, and time-consuming when practiced on a large scale. Huge numbers of bottles or pans must be washed and sterilized, relatively small volumes of nutrient media must be dispensed into individual containers, and each container with its allotment of medium must be sterilized and inoculated. The incubation period for such cultures is usually 6 to 12 days, and, at the conclusion of the fermentation, considerable hand labor is required to remove the penicillin-containing liquors from the numerous fermentation vessels and from the fungus mycelium.

It was obvious that a more economical fermentation process would result from growing the mold submerged and uniformly distributed in vats or tanks such as are used in other fermentation industries. Submerged mold fermentation processes have been previously used for the production of gallic acid (Calmette, 1902), gluconic acid (Moyer *et al.*, 1940), and lactic acid (Ward *et al.*, 1938), and the adaptation of this method to the cultivation of *P. notatum* appeared to offer a means of decreasing the labor involved, of decreasing the fermentation time, and of increasing the penicillin yield.

This paper deals with the selection of a strain of *P. notatum* Westling suitable for the production of penicillin in submerged culture, and with investigations of nutrient media and culture conditions as nearly optimal as possible for this procedure.[3] (*P. notatum* NRRL 832 was originally selected and evaluated for submerged penicillin production by the senior author.)

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[3]The results of this work have been communicated monthly and bimonthly since March, 1942, in a series of restricted reports to Dr. A. N. Richards, chairman, Committee on Medical Research, Office of Scientific Research and Development, who, in turn, sent copies to all penicillin producers and to many research groups in this country and abroad. Owing to the strategic significance of penicillin, publication of papers covering this research has been delayed.

METHODS AND MATERIALS

The fungus was grown in the submerged condition in 300-ml Erlenmeyer flasks which were shaken continuously on Ross-Kershaw shaking machines. In this machine the flasks are secured to a flat table which is mounted excentrically and revolved at 200 cycles per minute. This movement imparts a swirling motion to the contents of the flasks and serves both to agitate and aerate the medium. Since the flasks are plugged with cotton, gas diffusion into and out of the flasks occurs readily. All cultures were maintained at 24 C. Samples were withdrawn for penicillin assay and pH determinations by means of a sterile, wide-mouthed pipette.

Assays were made by the cylinder-plate method originated by Abraham *et al.* (1941), and modified by Schmidt and Moyer (1944). Determinations of pH were made electrometrically.

The nutrient salts, unless otherwise specified, were $MgSO_4 \cdot 7H_2O$, 0.25 g; KH_2PO_4, 0.50 g; $NaNO_3$, 3.0 g; $ZnSO_4 \cdot 7H_2O$, 0.044 g; and $MnSO_4 \cdot 4H_2O$, 0.020 g per liter of medium. This concetration of salts will be referred to as the standard nutrient salts. The $CaCO_3$ was always sterilized dry in separate containers and added just prior to inoculation. The corn steep liquor employed contained 50 to 55 per cent total solids, 5 to 6 per cent free sugar calculated as glucose, and an acidity corresponding to pH 4.0.

Two types of inoculum were employed, namely, ungerminated spores used dry or wetted in a 0.1 per cent soap solution, and germinated spores in the form of clumps or tiny pellets. The pellet inoculum was prepared by developing mycelium from spores, in submerged culture, in a medium containing, in addition to the standard nutrient salts, 30.0 g of lactose and 55 ml of corn steep liquor per liter. One gram of sterile $CaCO_3$ and 125 ml of medium were employed for each 300-ml flask. In seeding flasks with spores, a suspension of spores from an agar slant (in a 6- by 1-inch test tube) was made in 60 ml of a 0.1 per cent soap solution; 10 ml of this suspension wree used to inoculate each culture for pellet production. When it was desired to start the fermentation with ungerminated spores, 1 ml of such a spore suspension were used for direct inoculation of production cultures. After 2 to 3 days on the Ross-Kershaw shaker, the pellets were 0.5 to 1.0 mm in diameter, and the solution had a penicillin content of 8 to 10 units per ml and a pH of 7.6 to 7.8. Usually 5.0 to 7.5 ml of this pellet preparation were employed to inoculate a production culture.

EXPERIMENTAL RESULTS

Selection of the organism and sporulation media. The first experiments on penicillin production in submerged culture were made with the following strains: the Fleming strain of *P. notatum* supplied by Dr. Florey of Oxford University, and a strain also derived from the Fleming strain but obtained from the Squibb Institute for Medical Research, the latter now being designated as *P. notatum* NRRL 1249 in our culture collection. Various combinations of nutrients, includ-

ing carbon and nitrogen sources, trace elements, $CaCO_3$, and corn steep liquor, were investigated. The highest penicillin yield obtained was only 14 Oxford units per ml.

A limited survey of other strains of *Penicillium*, which had given promising penicillin yields in surface cultures, was made in submerged culture by using a medium containing lactose and corn steep liquor. It was found that, under these conditions, *P. notatum* NRRL 832 was superior to the other strains for penicillin production. The origin of this strain has been reported by Raper, Alexander, and Coghill (1945).

After improvements had been made in the culture conditions, other tests were made, in which were used various strains of *P. notatum*, *P. chrysogenum*, and allied species which were available. The results of a part of this survey are

TABLE 1

Comparison of penicillin yields obtained with strains of P. notatum and P. chrysogenum in submerged cultures

ORGANISMS*	CULTURE AGE—DAYS				
	3	4	5	6	7
	Units per ml				
NRRL 832	14	27	34	54	53
NRRL 838	6	10	15	16	21
NRRL 824	14	18	28	30	27
NRRL 807	4	8	12	15	17
NRRL 811	4	7	7	6	6

Culture medium: Lactose 20 g; standard salts; corn steep, 40 ml; and distilled water to make 1 L. $CaCO_3$, 1.5 g per culture. All inoculated with ungerminated spores.

* 832, *P. notatum*, obtained from the Thom collection as no. B-69 (not related to Fleming strain).

838, *P. cyaneo-fulvum*, obtained from the Thom collection as no. 5221.

824, *P. notatum*, obtained from the Thom collection as no. 5112.1 from the Fleming strain received by Thom in 1930.

807, *P. chrysogenum*, obtained from the Thom collection as no 26.

811, *P. chrysogenum*, obtained from the Thom collection as no 5034.11.

shown in table 1. Under the conditions indicated, *P. notatum* NRRL 832 again gave the highest penicillin yields, and strain NRRL 824 showed some promise. Because of the apparent superiority of *P. notatum* NRRL 832, it was used in all subsequent studies on submerged penicillin production.

P. notatum NRRL 832 rapidly developed a heavy crop of spores on a fairly wide range of nutrient media. One of the best agar media was the one recommended for *P. notatum* NRRL 1249.B21 by Moyer and Coghill (1945). For the large-scale production of spores, wheat bran moistened with a 2 per cent solution of corn steep liquor was also found satisfactory, as were small cubes of fresh whole-wheat bread. A good crop of spores developed in 4 to 5 days at 24 to 26 C on either the bread or bran medium.

Carbohydrate sources. One of the best media for submerged cultures has the following composition:

Corn steep liquor[4]	40.0	ml
Lactose monohydrate	27.5	g
$NaNO_3$	3.0	g
$MgSO_4 \cdot 7H_2O$	0.25	g
KH_2PO_4	0.50	g
$ZnSO_4 \cdot 7H_2O$	0.044	g
$MnSO_4 \cdot 4H_2O$	0.020	g
Glucose monohydrate	3.0	g
Distilled water to make 1 liter		

Previous studies (Moyer and Coghill, 1945) of the production of penicillin in surface culture showed that sorbitol, glucose, and sucrose were inferior to lactose as carbon sources, despite the fact that the fungus grows readily on media containing any of these compounds. When *P. notatum* NRRL 832 was cultivated (submerged) in media similar to that described above, except that lactose was replaced by similar concentrations of glucose, sorbitol, or sucrose, and that 1.5 g of $CaCO_3$ was added to each flask, only about half as much penicillin was produced as when the lactose medium was employed. At the age of 3 days all of the cultures which had been inoculated with ungerminated spores contained a large number of pellets 1 to 2 mm in diameter. During the first 3 days the pH rose more slowly in the glucose, sucrose, and sorbitol than in the lactose cultures, but thereafter it continued to rise more rapidly in the lactose cultures. After the pH had risen above 8.0, the penicillin content of the broth decreased sharply.

The effect of various concentrations of lactose on penicillin yield is shown in table 2. The optimum lactose concentration under these conditions appeared to be between 2 and 3 per cent. Cultures having lower lactose concentrations showed a rapid rise in the pH of the broth; a more gradual pH rise was observed in cultures containing 3 and 4 per cent lactose. It is believed that the slower rise in pH is more favorable for penicillin production.

Corn steep liquor. Preliminary experiments conducted in media containing varying quantities of concentrated corn steep liquor indicated that at least 40 ml of steep liquor per liter is required to obtain high yields of penicillin. Data obtained from an experiment designed to compare the effects of using 40 and 60 ml of steep liquor per liter of medium and two different lactose concentrations are presented in table 3. The higher yields of penicillin were obtained from media containing the smaller quantity of steep liquor, regardless of lactose concentration. There was heavier growth in the cultures containing the most steep liquor; however, the pellets were not so compact, but more nearly filamentous, so that the entire liquid was very viscous. The pellets produced in the medium containing 40 ml of steep liquor were much more compact.

[4]The commercial product, 30° Baume, containing 50 to 55 per cent total solids was used, unless otherwise noted.

No significant difference in pH change was observed in the cultures containing 20 or 25 g of lactose per liter. The cultures containing 40 ml of corn steep liquor

TABLE 2

Effect of different concentrations of lactose on penicillin yields

LACTOSE	MEASUREMENTS MADE ON SUCCESSIVE DAYS				
	3	4	5	6	7
	Units per ml				
%					
1	23	36	34	27	19
2	26	60	74	61	47
3	26	60	72	80	80
4	26	60	70	73	82
	pH of media				
1	7.8	7.9	8.1	8.4	8.5
2	7.6	7.5	7.8	8.0	8.1
3	7.6	7.5	7.6	7.8	7.8
4	7.5	7.5	7.6	7.8	7.8

Culture medium: Corn steep, 40 ml; and standard salts, with 1.5 g $CaCO_3$ per culture. Inoculated with ungerminated spores of *P. notatum* NRRL 832.

TABLE 3

Effect of concentration of corn steep liquor on penicillin yields

CORN STEEP	LACTOSE	MEASUREMENTS MADE ON SUCCESSIVE DAYS					
		3	4	5	6	7	8
		Units per ml					
%	*g per L*						
4	20	12	20	40	73	80	68
6	20	11	18	27	34	47	
4	25	32	47	59	80	80	
6	25	22	34	37	45	59	
		pH of media					
4	20	7.5	7.8	7.6	7.5	7.8	8.0
6	20	7.5	7.8	7.8	7.85	7.8	
4	25	7.5	7.7	7.5	7.7	7.8	
6	25	7.6	7.7	7.8	8.0	8.0	

Culture medium: Standard salts, with 1.5 g $CaCO_3$ per culture. Inoculated with ungerminated spores of *P. notatum* NRRL 832.

per liter showed a decrease in pH on the fifth and sixth days, but such a decrease was not observed in the cultures containing 60 ml of corn steep liquor. It is believed that the poorer yields of penicillin which resulted when higher concentrations of corn steep liquor were employed were due to an increased supply of

readily available carbon. The heavy viscous growth on such cultures, as contrasted with the cultures having less growth, appears to be correlated with insufficient aeration.

The effect of $CaCO_3$. The effect of $CaCO_3$ on fermentations conducted in media containing varying quantities of corn steep liquor and inoculated with ungerminated spores is shown in table 4. It was found in all cases that cultures containing $CaCO_3$ produced more penicillin than those without it, and that the pH rise during the fermentation was much less than in the cultures lacking it. That better penicillin yields resulted when 6 per cent of steep liquor was used than when a 4 per cent concentration was employed is probably due, at least in part,

TABLE 4

Effect of different concentrations of corn steep liquor with and without $CaCO_3$ on penicillin yields

CORN STEEP LIQUOR	$CaCO_3$ PER CULTURE	MEASUREMENTS MADE ON SUCCESSIVE DAYS		
		4	5	6
		Units per ml		
%	g			
2	0	6	8	15
2	1.0	9	15	20
4	0	8	12	17
4	1.0	17	27	47
6	0	5	11	17
6	1.0	23	36	60
		pH of media		
2	0	6.3	6.5	7.0
2	1.0	7.3	7.2	7.4
4	0	6.2	6.4	6.9
4	1.0	7.2	7.3	7.4
6	0	6.5	6.2	6.4
6	1.0	7.5	7.4	7.6

Culture medium: Standard salts, with 25 g lactose per L. Inoculated with ungerminated spores of *P. notatum* NRRL 832.

to the fact that this particular lot of corn steep liquor contained only 46 per cent total solids instead of the 54 per cent usually present.

The principal function of $CaCO_3$ is probably the partial neutralization of the lactic acid present in the corn steep liquor, resulting in the adjustment of the pH of the medium to a more favorable level. The unneutralized lactose corn steep liquor medium has a pH of about 4.0; this pH is increased to 5.3 and 5.4 upon addition of $CaCO_3$. There appears to be some degree of specificity among neutralizing agents, since media adjusted to pH 5.0 to 7.0 with KOH have not resulted in such good yields as have been obtained on media treated with $CaCO_3$.

A precipitate is always formed upon heat sterilization of corn steep liquor media, the quantity of this precipitate being much increased in media to which $CaCO_3$ is added. In media containing the heavy precipitates and $CaCO_3$,

formation of the pellet type of growth is more pronounced, and within 3 days the pellets contain all of the undissolved $CaCO_3$ within their hyphal meshes and most of the precipitated fraction of the corn steep liquor.

As shown in table 5, the addition of $CaCO_3$ to surface cultures of *P. notatum* NRRL 832 resulted in decreased mycelial growth and decreased penicillin yields. The penicillin yields are much lower than those obtained on the same medium in the shaken flasks. The precipitate in these surface cultures remains on the bottom of the culture vessels and is never in contact with the surface mycelium, whereas in the submerged cultures the mycelial pellets are formed around the

TABLE 5

Effect of $CaCO_3$ on penicillin yields by P. notatum NRRL 832 in surface cultures

LACTOSE	$CaCO_3$ PER CULTURE	MEASUREMENTS MADE ON SUCCESSIVE DAYS				
		3	4	5	6	7
		Units per ml				
%	g					
2	0	7	15	20	23	23
2	1.0	5	8	8	9	10
4	0	7	9	23	27	24
4	1.0	5	7	8	7	7
		pH of media				
2	0	5.3	5.9	6.8	7.3	7.8
2	1.0	6.9	7.0	7.1	7.2	7.5
4	0	5.0	5.5	6.8	7.1	7.3
4	1.0	6.9	6.9	6.7	6.6	6.7
		Dry weight fungus per culture				
2	0	.42	.50	.57	.52	.53
2	1.0	.25	.33	.42	.41	.50
4	0	.45	.59	.75	.77	.79
4	1.0	.24	.34	.44	.44	.45

Culture medium: Standard salts; corn steep, 40 ml per L. Inoculated with ungerminated spores. Culture size: 50 ml in 200-ml Erl. flasks.

precipitated particles. It is not unlikely that the $CaCO_3$ removes from the medium some essential factors or trace elements which are needed for good fungus growth and penicillin formation. In surface cultures containing 8 per cent of corn steep liquor and no $CaCO_3$, much higher penicillin yields have been obtained with this fungus (Moyer and Coghill, 1945).

Other components of the nutrient medium. The concentrations of magnesium sulfate and mono-potassium phosphate have been increased up to four times the amounts specified in the standard submerged nutrient medium without a corresponding increase in penicillin yields. These salts have been omitted in a medium containing 4 per cent by volume of corn steep liquor, with no resultant decrease in penicillin yields. These results indicate that inorganic ions equiv-

alent to these nutrient salts are present in sufficient amounts in the corn steep liquor; therefore the addition of these salts to a corn steep liquor medium is not essential.

Better penicillin yields have been obtained with $NaNO_3$ than with urea or any of the ammonium salts. The concentration of $NaNO_3$ has been decreased to 1.5 g per liter without a decrease in penicillin yields. However, lower yields resulted when $NaNO_3$ was omitted or increased much beyond 3.0 g per liter.

Media containing corn steep liquor, crude lactose, and tap water do not show any superiority after the addition of zinc or maganese ions; hence, the addition of these ions is regarded as optional.

The addition of small amounts of glucose to the culture medium did not result in decrease in penicillin yields; on the contrary, when highly fermented corn steep liquor (which was low in glucose) was employed, significant increases in penicillin yields were obtained by the addition of 3 to 5 g of glucose monohydrate per liter of medium. *P. notatum* will not grow readily in a synthetic medium in which lactose is the sole carbon source. The addition of 2 to 3 ml of corn steep liquor per liter to a synthetic medium will enable the fungus spores to germinate and to attack the lactose. Under such conditions the addition of a small amount of glucose (0.3 to 0.5 per cent) along with the steep liquor markedly increases the growth rate. Thus, the beneficial action of glucose in submerged cultures may be related to the rapid establishment of a vigorous vegetative growth which can elaborate the lactose-attacking enzyme. If the steep liquor employed is not the highly fermented type and if it contains the normal quantity of sugar, the addition of glucose is unnecessary. In a corn steep liquor lactose medium, the readily available carbohydrates are believed to be practically exhausted before the lactose is utilized. A partial carbohydrate starvation may exist before the lactose is utilized.

Addition of nutrients during fermentation. An increase in penicillin yield in surface cultures of *P. notatum* NRRL 1249.B21 can be effected by the addition of nutrients during the fermentation, as demonstrated by Moyer and Coghill (1945). The periodic, or continuous, addition of nutrients to the submerged cultures is easier and more practical than such addition to surface cultures. It is obvious that innumerable combinations of nutrients and feeding schedules are possible, but in the limited time available it has not been practicable to make an exhaustive study of all these factors. However, it was found that the addition of certain nutrient components, principally glucose, was advantageous. The addition of corn steep liquor alone was not sufficient to prevent a rapid rise in the pH of the medium, with its concomitant decrease in penicillin potency, when the alkalinity of the medium became greater than pH 8.0. The effect of adding steep liquor alone, or glucose plus steep liquor, during the course of the fermentation is shown in table 6. Beginning on the fourth day, 5-ml samples were withdrawn daily for assay and pH determination, and 5-ml portions of the specified feed solutions were added. At 4 days, this series of cultures showed a slightly better than average penicillin yield. All cultures showed excellent pellet formation, and there was appreciable foam in the flasks. The formation of yellow pigment

was not intense. On the seventh and eighth days, the cultures receiving the glucose steep liquor feed showed appreciably more growth and slightly larger pellets than did the control cultures. The cultures receiving corn steep liquor alone differed only slightly from the controls, which received distilled water. The penicillin yields in the control cultures and in the cultures receiving the corn steep liquor feed reached a maximum at 6 to 7 days, and dropped steadily thereafter as the pH rose above 8.0. In contrast, those cultures receiving glucose plus steep liquor showed a steady increase in penicillin content until a maximum yield of 127 Oxford units per ml was reached on the ninth day. The pH of this group of cultures did not rise above 8.0 until the eleventh day.

TABLE 6

Effect of adding nutrients during the fermentation on penicillin yields

	MEASUREMENTS MADE ON SUCCESSIVE DAYS						
	4	5	6	7	8	9	11
	Units per ml						
Feed A	51	48	52	59	38	30	11
Feed B	50	70	74	68	42	36	18
Feed C	50	68	78	91	120	127	102
	pH of media						
Feed A	7.5	7.9	8.15	8.25	8.4	8.6	8.7
Feed B	7.6	7.7	8.15	8.2	8.2	8.45	8.6
Feed C	7.6	7.65	7.8	7.9	7.9	7.9	8.1

Culture medium: Lactose, 20 g; corn steep liquor, 40 ml; standard salts; and distilled water to make 1 L; $CaCO_3$, 1.5 g per culture. Inoculated with ungerminated spores of *P. notatum* NRRL 832.

First feed on fourth day, and daily thereafter.

Feed A: 5 ml of distilled water.

Feed B: 5 ml containing 0.2 ml of steep liquor.

Feed C: 5 ml containing 0.9 g glucose and 0.2 ml steep liquor.

The use of a pellet inoculum. When the inoculum used for submerged cultures consisted of ungerminated spores, a large proportion of the tiny colonies, resulting from germination of the spores, washed up on the walls of the flask just above the liquor level. This made it necessary to remove the flasks from the shaking machine several times during the first 3 days of incubation in order to wash the colonies back into the liquid by a vigorous shaking. When this was not done, a solid band of mycelium was formed on the flask walls, and the number of pellets in the broth was greatly reduced. After the pellets had attained a diameter of about 1 mm they were not so readily washed up on the sides of the flasks. In order to prevent this wall growth, it seemed advisable to make the inoculation with a preformed pellet inoculum. It was hoped that the use of this pellet inoculum would also result in greater uniformity within a culture series and would effect a decrease in the time required for the fermentation.

A pellet inoculum may be prepared by heavily seeding ungerminated spores directly into the standard lactose steep liquor production medium, followed by a 2- to 3-day period of shaking. It is advantageous to modify this medium slightly for more rapid growth and pellet formation, as shown in table 9. At 2 days the pH of either pellet inoculum was 7.8, and the broth assayed 8 to 10 Oxford units per ml.

Ten- and twenty-ml portions of a pellet inoculum, grown for 2 days in the standard lactose steep liquor production medium (1.5 g of $CaCO_3$ per 125 ml of medium), were used to seed cultures containing the standard medium found to be optimum when ungerminated spores were used as the inoculum. The tendency for the fungus growth to accumulate on the flask walls was greatly reduced, resulting in uniformity in the culture series with respect to pellet size and total amount of growth. The most conspicuous result was the rapid rise in the pH, which reached 8.1 to 8.3 in 4 days. The highest penicillin yield was 35 units in 5 days.

TABLE 7

Effect of $CaCO_3$ on penicillin yields with a pellet inoculum in glucose and lactose media

CARBON SOURCE	$CaCO_3$ PER CULTURE	MEASUREMENTS MADE ON SUCCESSIVE DAYS			
		3	4	5	6
		Units per ml			
	g				
2% Lactose	0	17	24	61	87
	1.0	18	14	3	0
2% Glucose	0	8	17	26	19
	1.0	20	31	41	22
		pH of media			
2% Lactose	0	7.6	7.65	7.7	8.0
	1.0	8.1	8.35	8.4	8.6
2% Glucose	0	6.3	7.6	8.1	8.5
	1.0	7.2	7.9	8.15	8.5

Culture medium: Corn steep liquor, 40 ml; ½ strength of standard salts; and distilled water to make 1 L. Ten ml of a 3-day-old pellet inoculum used per production culture.

Since this low penicillin yield seemed to be associated with a too rapid increase in the alkalinity of the medium, another series of cultures was prepared, using 2 per cent glucose or lactose, both with and without $CaCO_3$. The inoculum consisted of 10 ml of a pellet suspension, similar to that described above (table 7). Low penicillin yields were again obtained in the lactose cultures containing $CaCO_3$, whereas much better yields were obtained in those cultures lacking it; the pH of the medium did not rise so rapidly in the former cultures. Better penicillin yields were obtained in the glucose cultures when $CaCO_3$ was added to the medium.

It has been observed that the increase in the size of the pellets in the production medium depends upon the quantity of inoculum employed. When it is low, very large pellets (4 to 6 mm in diameter) are formed. The best penicillin yields have been obtained when 5 to 7.5 ml of pellet inoculum has been used per production culture, the pellets attaining a diameter of 2.5 to 3.0 mm. The effect of using 5 and 15 ml of pellet inoculum is shown in table 8. In this series, no $CaCO_3$ was added, and half the standard salt concentration was used. The better

TABLE 8

Effect of different amounts of pellet inoculum on penicillin yields

PELLET INOCULUM PER CULTURE	MEASUREMENTS ON SUCCESSIVE DAYS					
	3	4	5	6	7	8
	Units per ml					
ml						
5	21	39	88	91	99	78
15	18	22	25	41	37	26
	pH of media					
5	7.7	7.5	7.6	7.6	7.7	8.0
15	8.0	7.8	7.7	7.7	7.9	8.2
	Average pellet diameter, mm					
5	3	3	3	3	3	3
15	2	2	2	2	2	2
	Formation of yellow pigment in medium*					
5	2.3	3.5	4	4	4	4
15	2.7	2.5	2.5	2.5	2.5	2.5
	Amount of foam in culture†					
5	3	3	3	3	3	3
15	3	3	1	0	0	0

Culture medium: Standard salts, ½ strength; corn steep, 40 ml; lactose, 27.5 g; and distilled water to make 1 L.

* The figure 4 represents a deep yellow; lower numerals represent correspondingly less.

† Height of foam in inches above liquid level.

penicillin yields were obtained from cultures inoculated with the smaller quantity of pellets, and these cultures also produced a greater amount of yellow pigment and a more persistent head of foam. In these small submerged cultures the appearance of the yellow pigment and a persistent head of foam 3 to 4 cm in height has always been associated with good yields of penicillin.

Penicillin yields and changes in culture appearance which result from a slightly modified pellet medium are shown in table 9. A maximum penicillin yield of 112 units of penicillin per ml was obtained in 6 days. The initial pH of such a medium with 1 to 1.5 g of $CaCO_3$ per culture is 5.2 to 5.3, and without $CaCO_3$, it is

4.0 to 4.2. Under these culture conditions (without $CaCO_3$), the pH rises from 4.0 to 4.2 up to 7.7 to 7.8 in 3 days. From the third to the fifth day there is a decrease in the pH, followed by a rise on the sixth and seventh days.

A penicillin yield of 41 units per ml has been obtained with a pellet inoculum in a medium containing a 2 per cent concentration of glucose, standard salts, 40 ml of corn steep liquor per liter, and 1.5 g $CaCO_3$ per culture. Other experiments showed that when the initial glucose concentration was raised much above 2 per cent, the pH rose too slowly and too heavy mycelial growth occurred. If less than 2 per cent of glucose was employed, the amount of growth was too light and the pH rose rapidly to about 8.2.

A series of cultures containing $CaCO_3$ was prepared with low initial glucose concentration. Different amounts of glucose were added daily, beginning on the

TABLE 9

Penicillin production and culture appearance with a pellet inoculum in submerged cultures of P. notatum NRRL 832*

	MEASUREMENTS MADE ON SUCCESSIVE DAYS					
	2	3	4	5	6	7
Units per ml	12	32	58	90	112	106
pH of medium	7.3	7.7	7.5	7.2	7.8	8.0
Formation of yellow pigment in medium	1.7	2.3	3.5	3.7	4.0	4.2
Average pellet diameter, mm	1.6	2.5	2.5	2.5	2.5	2.5
Amount of foam in culture	2	3	3	3	3	2

Medium for pellet inoculum: Corn steep liquor, 50 ml; standard salts; lactose, 10 g; glucose, 4.0 g; zinc sulfate, 0.044 g; and distilled water to make 1 L. Inoculated with a spore suspension, and 1.3 g of $CaCO_3$ per culture.

Production medium: Corn steep liquor, 40 ml; lactose, 27.5 g; glucose, 2.0 g; ½ strength of standard salts; and distilled water to make 1 L. No $CaCO_3$ added to these cultures.

* 7.5 ml of a 3-day-old pellet inoculum per each production culture.

second day and ending on the sixth day. Penicillin yields and pH changes of the media are shown in table 10. The initial medium contained only 0.5 per cent lactose; hence, glucose was the major carbon source. The daily addition of 0.5 to 1.0 g of glucose to these cultures gave maximum penicillin yields of 72 units per ml. It seems possible that better penicillin yields may be obtained with glucose as the major carbon source if changes are made in the initial concentration of the carbon supply, and if a different schedule of nutrient additions is employed.

The best culture medium for penicillin production by the submerged growth of *P. notatum* NRRL 832 in shake flask cultures contained lactose and corn steep liquor in about one half the concentration found optimum by Moyer and Coghill (1945) for penicillin production in surface cultures of *P. notatum*, NRRL 1249.B21. An increase in nutrients above the optimal levels gave either an unfavorable pH change or a marked increase in fungus growth.

It has not been possible to determine the optimum pH for penicillin accumulation in these submerged cultures, although it is known that the pH increases during the course of the fermentation. Penicillin accumulation, however, appears to be optimum at higher pH levels than in the surface cultures with *P. notatum* 1249.B21. Penicillin potency usually drops rapidly when the pH of the medium increases above 8.0 to 8.2.

The pH change in cultures containing $CaCO_3$ and 4 per cent of corn steep liquor, inoculated with ungerminated spores (table 3), and in pellet-inoculated cultures without $CaCO_3$ (table 9), follows an interesting course. During the first

TABLE 10

Effect of adding glucose during the fermentation, with pellet-inoculated cultures low in initial carbon

	MEASUREMENTS ON SUCCESSIVE DAYS				
	3	4	5	6	7
	Oxford units per ml				
Feed 1	16	26	36	31	22
Feed 2	29	35	54	72	62
Feed 3	29	33	40	61	73
Feed 4	19	22	37	45	54
	pH of media				
Feed 1	7.85	7.9	7.95	8.1	8.4
Feed 2	7.75	7.7	7.75	7.95	8.0
Feed 3	7.6	7.55	7.6	7.7	7.65
Feed 4	7.0	6.8	7.2	7.6	7.5

Culture medium: Commercial glucose, 11 g; lactose, 5.5 g; ½ strength of standard salts; corn steep, 30 ml; and distilled water to make 1 L. $CaCO_3$, 1.0 g per culture. Ten ml of pellet inoculum per culture.

Feed 1, 5 ml containing no glucose.
Feed 2, 5 ml containing 0.5 g glucose.
Feed 3, 5 ml containing 1.0 g glucose.
Feed 4, 5 ml containing 1.5 g glucose.
First feed at 2 days and last feed at 6 days.

2 or 3 days of growth, there is a rapid rise, followed by a slight drop for 2 to 3 days, after which there is a final rise. In these cultures the carbohydrates present in the corn steep liquor support the early growth, after which there is a comparatively slow utilization of the lactose.

The same factors which influence the pH changes in the surface cultures (Moyer and Coghill, 1945) are believed to function in the submerged cultures. The speed of the pH change can be controlled to a large measure by adding glucose during the fermentation. This pH control is believed to be associated with the prevention of carbohydrate starvation, rather than with the production of an organic acid.

The role of corn steep liquor with respect to penicillin production in surface

cultures has been considered by Moyer and Coghill (1945). It is believed that the function of corn steep liquor is substantially the same in both surface and submerged fermentations. It has not yet been established that there are specific compounds in this medium which are required for penicillin synthesis by the fungus. The addition of corn steep liquor to suitable culture media enables the fungus to make a rapid growth, with pH changes which permit the accumulation of penicillin.

The superiority of lactose over glucose, sorbitol, and glycerol for penicillin production appears to be associated with the greater speed at which the latter are assimilated by the fungus. Starch is slowly hydrolyzed but has not been satisfactory for good penicillin production under submerged conditions. There is sufficient carbohydrate with glucose at 3.0 g per liter, in addition to the lactic acid and other reducing substances in the corn steep liquor, to permit the fungus to utilize the lactose. In such cultures, the fact that lactose is not so readily assimilated provides a more favorable pH change and a longer growth period. A rapid and dense fungus growth develops when the more readily assimilated carbon sources are supplied in concentrations corresponding to the optimal concentration of lactose. Because of the heaviness of this growth, and its effect on the motion within the flask, it is possible that these cultures received inadequate aeration. There is also an unfavorable pH change and a shorter period of active growth with these readily assimilated carbon sources. Glucose, as the major carbon source, will give good penicillin yields only if the initial concentration is low and if more glucose is gradually supplied during the course of the fermentation.

The addition of nutrients during the course of the fermentation has given increased penicillin yields. This culture feeding has given some pH control and has prolonged the productive life of the culture. These results suggest the feasibility of commercial application. The time of harvest is not so critical as in the case of cultures which are not fed. The increase in penicillin yield may, under an improved feeding schedule, more than compensate for the cost of the extra time required for the fermentation. The withdrawal of liquor from the partly completed fermentation, and the addition of more nutrients, suggest the possibility of a semicontinuous process.

SUMMARY

It was found that a strain of *Penicillium notatum*, NRRL 832, not related to the Fleming strain, gave higher penicillin yields than any other fungus tested in submerged culture. This strain has been widely used in the industrial production of penicillin in tanks.

The best medium for penicillin production contained lactose and corn steep liquor in about one-half the concentration found optimal for surface culture production.

Penicillin yields of 112 Oxford units per ml have been obtained in 6 days by using a pregerminated inoculum. Some increase in penicillin yields has been obtained by adding nutrients during the course of the fermentation.

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