

RECENT ADVANCES IN PENICILLIN FERMENTATION

MARVIN J. JOHNSON, Ph.D.

*Professor of Biochemistry, College of Agriculture,
University of Wisconsin, Madison, Wis., USA*

During the second World War, a great deal of work was done on penicillin fermentation. Much of that work, however, was directed towards procedures for the industrial production of penicillin. At the end of the war, procedures for carrying out a practical fermentation were known, but much of the knowledge was empirical. The production of penicillin was definitely an art rather than a science. Unfortunately, there is still a great deal that is unknown about the factors influencing penicillin synthesis by moulds. The mechanism of the synthesis itself is a complete mystery. Although the conditions conducive to rapid penicillin synthesis are gradually being defined, the reasons for the superiority of a certain set of conditions are entirely unknown.

However, a certain amount of progress has been made in recent years in knowledge of the fermentation. A little more is known about the selection of suitable cultures and about the procedures for producing high-yielding mutant strains, and also about the mechanism by which the proper amount of oxygen is supplied to the fermentations. There is a better understanding of the role of precursors and of the type of chemical structure that a good precursor must have. Studies have been made of the effect of environmental conditions on the growth of the mould mycelium, and on the capability of that mycelium to produce penicillin. It is some of these modest advances in knowledge that I shall discuss in this paper.

Production of High-Yielding Mutant Strains

Logically, the first subject of discussion should be the culture used in the fermentation. All the cultures used today are derived from a culture (no. 1951) isolated at the Northern Regional Research Laboratory, Peoria, Ill., USA. An abridged genealogy of the newer penicillin-producing cultures is given in fig. 1.

It will be noted that spontaneous variation, and mutations induced by ultra-violet light and by nitrogen mustard, have played a part in the series of selections. Methyl-bis(β -chloroethyl)amine (MBA), called nitrogen mustard, is an excellent mutagenic agent.

It might not be out of place to say a word about some of the experiences of F. R. Roegner and Professor J. F. Stauffer of the Department of Botany of the University of Wisconsin in irradiation studies on these *Penicillium chrysogenum* cultures. First, they found that the percentage of mutants (among surviving cultures) reached is more or less the same whether ultra-violet light, x-rays, or nitrogen mustard is used as the mutagenic agent.

FIG. 1. ORIGIN OF PENICILLIUM MUTANTS

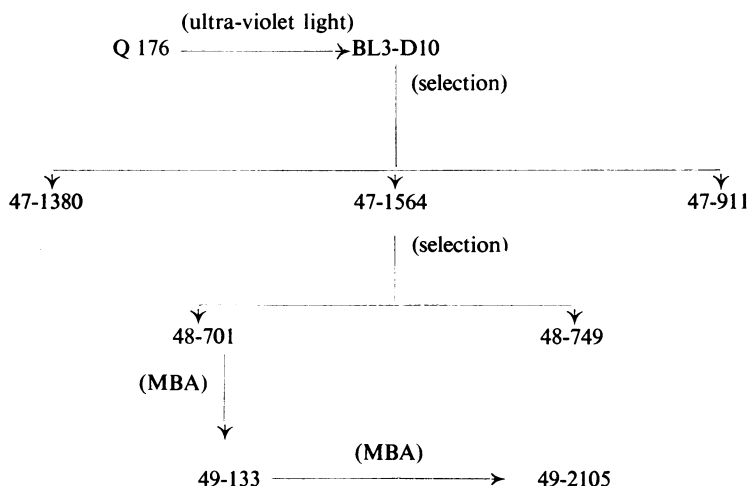
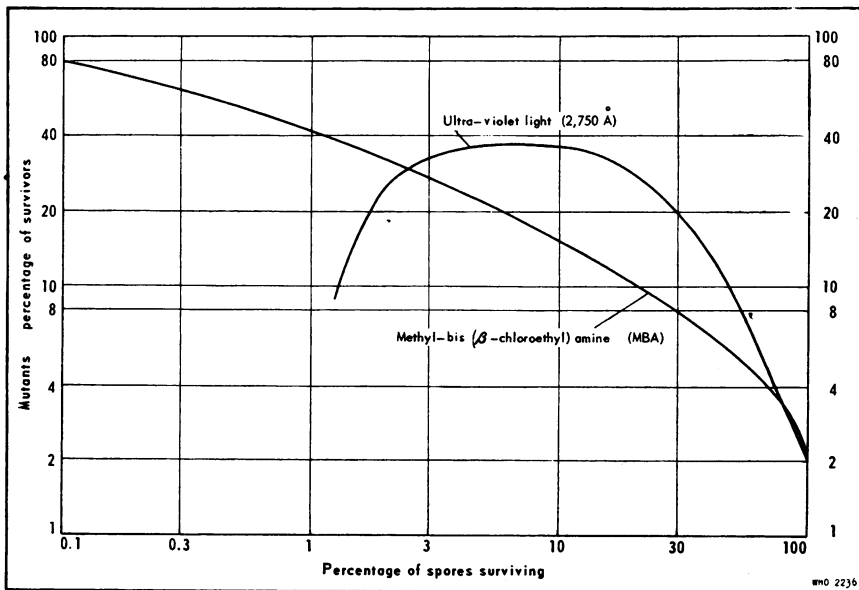


Fig. 2 compares the mutagenic activity of ultra-violet light (2,750 Å) with that of nitrogen mustard. It will be noted that when the dose is sufficient to give 3% to 30% survivors, the percentage of mutants among survivors is greater with ultra-violet light. However, at very low survival levels, a higher percentage of mutants is obtained with nitrogen mustard than with ultra-violet light. The figure is based on morphological mutants, but other studies have shown that no significant difference between the two mutagenic agents exists with regard to the percentage of the total mutants which are mutants with regard to penicillin production, nutritional requirements, or morphological characteristics.

One of the practical problems involved in a search for high-yielding mutant strains is the selection of a suitable testing technique. It is obviously true that selections made by the use of a given testing technique will tend to give cultures particularly adapted to the conditions under which the test was made. Not enough is known about the biochemistry of the mould and its method of synthesizing penicillin for the inherent penicillin-synthesizing ability of even one culture to be assessed. All that can be done is to set up a set of test conditions, and try to obtain, by blind selection from

thousands of mutants, those which synthesize most penicillin under the test conditions. In other words, each worker chooses those cultures which have mutated in such a way that they are better able to overcome the obstacles to penicillin synthesis that his particular test conditions have imposed upon them. Needless to say, this is not an ideal method of culture selection. In our laboratory, and in others, it has repeatedly been found

FIG. 2. COMPARISON OF ULTRA-VIOLET LIGHT AND MBA AS MUTAGENIC AGENTS



To the spore suspension in sodium-bicarbonate solution, 1 g of MBA per litre was added every five minutes until the desired mortality was obtained.

that the cultures selected are often able to give better performance under the test conditions set up, but not under other conditions. The ideal test method would be to check each mutant under a very large number of conditions. The only practical method is to make the test procedure as much as possible like the procedure in which the culture is ultimately to be used.

The cultures selected by Professors M. P. Backus and J. F. Stauffer of the Department of Botany of the University of Wisconsin have, in a large number of cases, been checked in our laboratory for performance under conditions other than those used in selecting them. Table I shows yields, most of them averages of a large number of runs, of various cultures run under three sets of conditions. The shake-flasks run in the mutation programme in the Department of Botany are run on a reciprocating shaker, and precursor (phenylethylamine) is added only at the beginning of the

TABLE I. RELATIVE YIELDS OF NEW CULTURES TESTED AT THE UNIVERSITY OF WISCONSIN

Culture	Comparative yield (Q 176 = 100)		
	Shake-flasks (Department of Botany)	Shake-flasks (Department of Biochemistry)	30-litre fermenters (Department of Biochemistry)
47-911	104	50	65
47-1380	122	57	72
47-1564	149	69	74
48-701	227	80	74
48-749	246	55	76
49-133	352	141	83
49-2105	371	140	105
Q 176	350*	735*	1,400*

* Actual potency in units per ml

fermentation. This is done for convenience, since hundreds of flasks are run simultaneously. The shake-flasks run in the Department of Biochemistry are incubated on a rotary shaker, which gives somewhat better aeration, and the precursor (phenylacetic acid in this case) is added every 12 hours. The 30-litre fermenters are run in the Department of Biochemistry, and are equipped with agitators. Precursor is added at intervals. It will be noted that the yields of Q 176, the reference culture, are best in the 30-litre fermenters, and lowest in the shake-flasks of the Department of Botany. The other cultures, which are all free of the yellow pigment characteristic of Q 176, were selected by the Department of Botany as being equal to or better than Q 176 in performance. It will be noted that the cultures selected as being much better than Q 176 were certainly very much better under the conditions used for the selection, but were not necessarily better under different conditions.

It will be noted, however, that the various strains seem to rank in roughly the same order regardless of the method of test. The exception to this statement is Q 176 itself, and the reason for this exception is probably that the tests run in the Department of Biochemistry were run in a laboratory which has been engaged for a few years in devising fermentation conditions optimal for Q 176. Hence, the other cultures were tested under conditions optimal for Q 176. It is interesting to note that, under commercial conditions, cultures such as 47-1564, 48-701, and 49-133 have in some places been found greatly superior to Q 176, in other places similar, and in many places somewhat inferior.

Another property of the newer cultures, which again reflects the conditions under which they were selected, is their efficient utilization of precursor.

The role of precursor will be discussed later, but I will mention now the behaviour of the new cultures toward precursor. For optimal yields, phenylacetic acid or other precursor should be added at intervals, but in a testing programme where large numbers of flasks are run, this is not convenient. When precursor is added at the beginning, the amount added is limited by toxicity, and may rapidly disappear through oxidation. It might, therefore, be predicted that high-yielding cultures selected in such a testing programme would be better able to give high yields of penicillin at low precursor concentrations than the parent culture. This has been found to be true. For example, in one series of experiments, culture 49-133 produced, on a synthetic medium, 85% penicillin G at a precursor level of 50 mg of phenylacetic acid per litre per day, while Q 176, the parent culture, produced, at ten times this precursor level, only 75% penicillin G. The new cultures produce 100% penicillin G at precursor levels of 200 mg per litre per day.

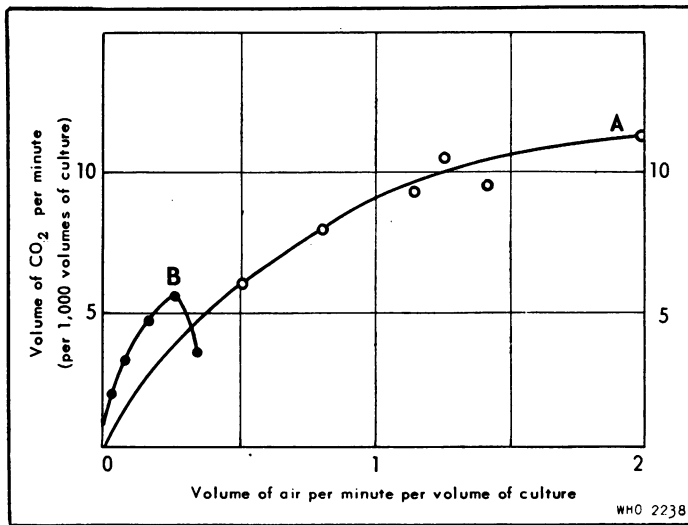
Aeration and Agitation

The fact that the penicillin-producing abilities of various cultures vary greatly depending on the exact conditions of the fermentation is a good indication of the fact that all the factors influencing penicillin synthesis by a given culture are not known. A number of factors that are known to some extent will now be discussed. One of these factors is aeration. It is impossible to speak of aeration and agitation separately since, in penicillin fermentation, the chief function of agitation is the dispersal of air and the mixing of the medium to bring dissolved oxygen to the cell surface. It is very difficult, in a medium in which mycelium is growing as a thick suspension, to bring an adequate air-supply to that part of the liquid in contact with the cell.

The subject of aeration and agitation may be introduced by describing some simple experiments done on fermentations in a 100-gallon (380-litre) tank¹¹ in our laboratory. Fig. 3 shows, in curve A, the results of an experiment performed a number of years ago. When the culture was about 24 hours old, and its oxygen utilization (as measured by carbon-dioxide production) was at a maximum, the aeration-rate was varied at 15-minute intervals, and the rate of carbon-dioxide production at each aeration-rate was measured. The results show that the rate of CO₂ production at first rose, but then became fairly constant. At the time, we came to the tentative conclusion that, at the highest aeration-rates, the mould mycelium was being supplied an excess of oxygen, and that it was producing as much carbon dioxide as it would under the optimal aeration conditions. Curve B represents a similar experiment performed in a 5,000-gallon (19,000-litre) tank in a commercial installation. It will be noted that the carbon-dioxide

production decreased after the aeration reached a critical value. Since the electrical energy required by the agitator decreased greatly at the same time, it was concluded that at high aeration-rates the impellers became "flooded" with air; in other words, that they were rotating in air rather than water, and required little energy, but caused but little agitation. This condition was later corrected, and resulted in increased penicillin yields.

FIG. 3. RELATION BETWEEN AERATION-RATE AND CARBON-DIOXIDE PRODUCTION



A = 100-gallon (380-litre) tank
 B = 5,000-gallon (19,000-litre) tank

Returning to curve A, I should like to present some data correlating the behaviour of this fermentation with the aeration behaviour of the tank as measured by the sulfite-oxidation procedure. In this procedure, the fermenter is filled with an aqueous solution of sodium sulfite, either neutral or slightly alkaline. A little copper is added as an oxidation catalyst, and aeration and agitation are begun. Samples are removed, usually at 5-minute intervals, and the remaining sulfite is titrated with iodine. Since the rate of oxidation of the sulfite is found to be independent of sulfite concentration over a wide range, it can be concluded that what is being measured is the rate of transfer of oxygen from the air-bubbles into the medium. In an actual fermentation, the oxygen must be further transferred from the part of the medium close to an air-bubble to the part of the medium close to the cell. One of the important questions in any fermentation is which of these two processes limits the amount of oxygen available to the cell. When the aeration efficiency of the fermenter used in the experiment of the slide was

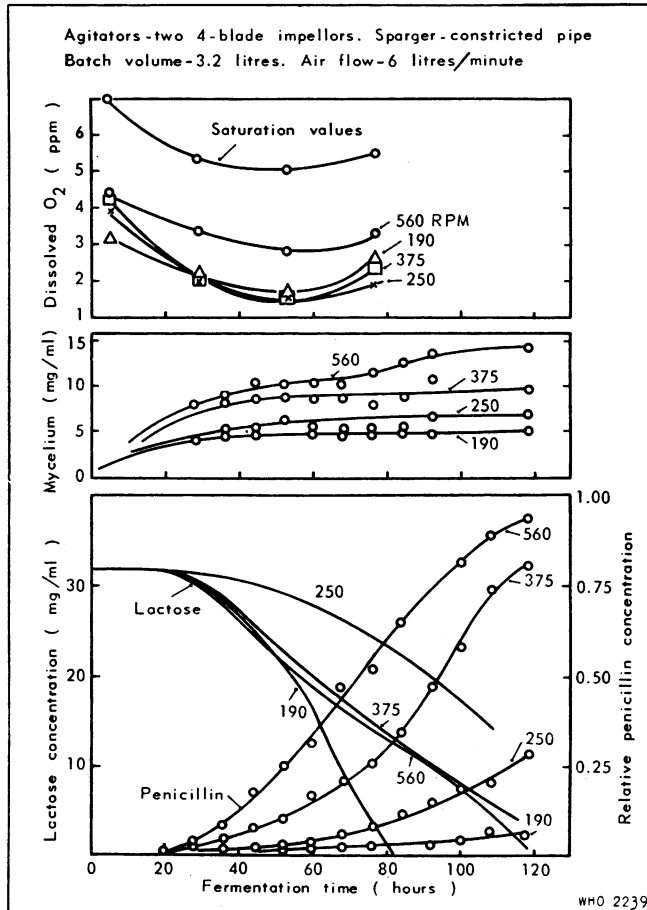
evaluated about four years after the experiment was done, it was found that from 25% to 40% of the oxygen capable of being transferred across the air-liquid interface was actually available to the mould. Whether this disparity was caused by the difficulty of transfer through the medium to the cell, or was caused by the difference of behaviour of the aeration system when the tank was filled with a suspension of mycelium in steep liquor medium rather than with water, the experiment does not reveal. We have found, however, a fair correlation of the aeration efficiency of various fermenters, as measured by the sulfite-oxidation method, with their performance in actual fermentations.

We have never found, in penicillin fermentations, that it was possible to give the fermentation too much effective air. In some fermentations, for example, streptomycin, it is possible to have too large an air-supply. We have repeatedly observed increases in penicillin yield with increased aeration efficiency, but we have never observed a decrease in yield with increased aeration efficiency.

Work from other laboratories has contributed greatly to our knowledge of the effect of agitation and aeration in penicillin fermentations. The dropping-mercury electrode has been used to determine the dissolved oxygen concentration in penicillin fermentations by Bartholomew and co-workers,¹ and by Hixson & Gaden,⁴ in the USA, and by Wise¹³ in England. An interesting experiment performed by Bartholomew and co-workers at the Merck laboratories, Rahway, N.J., USA, is shown in fig. 4. Penicillin fermentations were run at four different agitation-rates. Previous experiments had shown that effectiveness of oxygen transfer from bubbles to medium increased greatly with increased agitation-rate. The dissolved oxygen content of the medium reached a minimum, at the end of the growth phase of the fermentation, which was low, and was almost identical for all agitation-rates except the highest. The oxygen concentration was determined by rapidly filtering a sample of medium into de-aerated phenol solution, and measuring the dissolved oxygen polarographically. Since a small amount of oxygen probably unavoidably enters the sample during filtration, and since reduction of the agitation-rate did not reduce this apparent minimum oxygen content, it probably actually represents almost zero concentration. That is, at all agitation-rates except the highest, the mycelium must have been oxygen-starved. Even at the highest rate, where there was a considerable amount of dissolved oxygen, it must be remembered that what is measured is the oxygen concentration in the bulk of the medium, not the oxygen concentration in the part of the medium close to the cell. It will be seen that the amount of mycelial growth bears a direct relation to the rate of agitation. Also, as might be expected, the penicillin production depends on the rate of agitation. The rate of lactose utilization of the fermentation agitated at 190 revolutions per minute (r.p.m.) is anomalous (for unknown reasons, conceivably contamination). At the other agitation-

rates, however, there is a correlation. The general conclusion would seem to be that at all agitation-rates, including probably the highest, oxygen-supply limited growth and penicillin production.

FIG. 4. INFLUENCE OF AGITATION ON THE COURSE OF PENICILLIN FERMENTATION

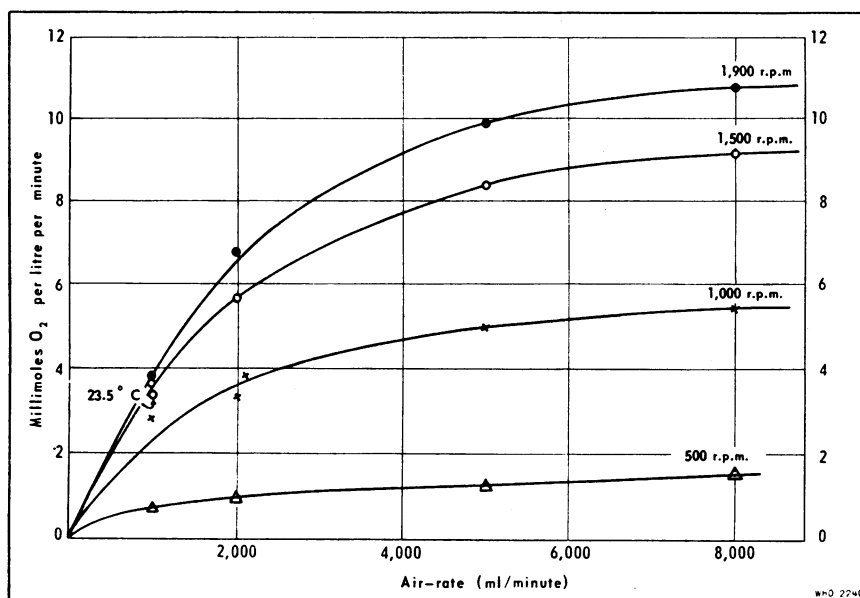


The same conclusion can be reached from more general considerations. In a given fermentation, it is obvious that the penicillin yield can be doubled if the concentration of mycelium can be doubled while all other factors are kept constant. If the level of nutrients is raised in order to double the amount of growth, not only does the high level of mycelium require more oxygen, but also even the same amount of oxygen is more difficult to transport to the cell, because of the increased apparent viscosity of the medium. Hence, anyone carrying out experiments in penicillin production tends to

arrive at a medium in which the nutrient concentration is such that the air-supply limits penicillin yield.

It should be mentioned that in practical fermenters, high effective aeration-rates are achieved by agitation rather than by simply blowing large amounts of air into the medium. This is illustrated by fig. 5, which is typical of the behaviour of many fermenters. The data of the figure were taken on a small laboratory fermenter (3.5 litres) designed for work on yeast. Oxygen-transfer rates were determined by the sulfite-oxidation method. It will be noted that much higher levels of effective aeration could be secured by increasing the agitation than by increasing aeration.

FIG. 5. INFLUENCE OF AGITATION AND AERATION ON OXYGEN ABSORPTION

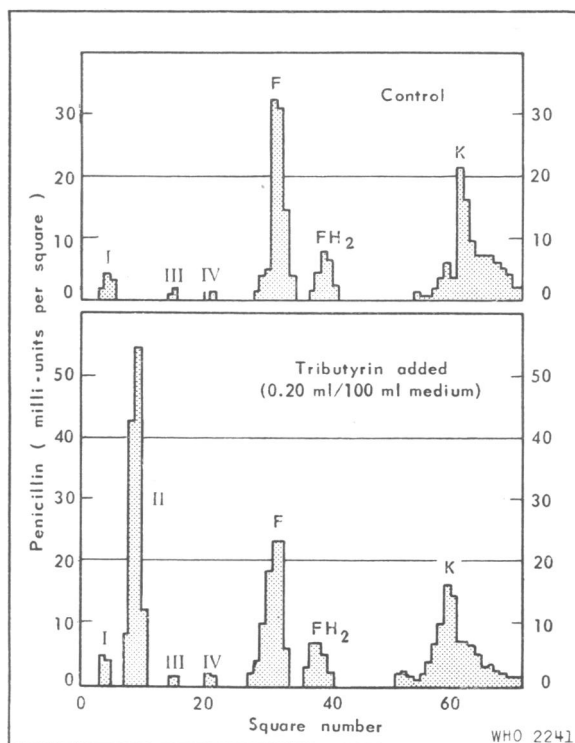


Precursors

An important factor influencing penicillin synthesis by moulds is the presence of a proper precursor. Any penicillin may be considered as a substituted amide of a carboxylic acid $R.COOH$. If this R acid is phenylacetic acid, penicillin G, the only penicillin now produced commercially, is formed. If the R acid is an unsaturated β -carbon-atom acid— β,γ -hexenoic acid—penicillin F, the first penicillin isolated, is formed. Any one of a large number of penicillins may be made simply by addition of the proper R-acid to the fermentation. The mould will use the added acid as a constituent of the penicillin molecule. As will be seen, however, not all acids can be used.

If a penicillin fermentation is carried out in a synthetic medium, and if no precursor acid is added, the so-called "natural" penicillins are produced. In fig. 6, from the work of Thorn & Johnson,¹² the upper diagram shows the relative amounts of these. The diagram is from the quantitative analysis of a filter-paper chromatogram.⁷ The chief penicillins are F, the R-acid of which is β,γ -hexenoic; dihydro-F, from hexanoic acid; and K, from octanoic acid. Variable amounts of other K-like penicillins are produced,

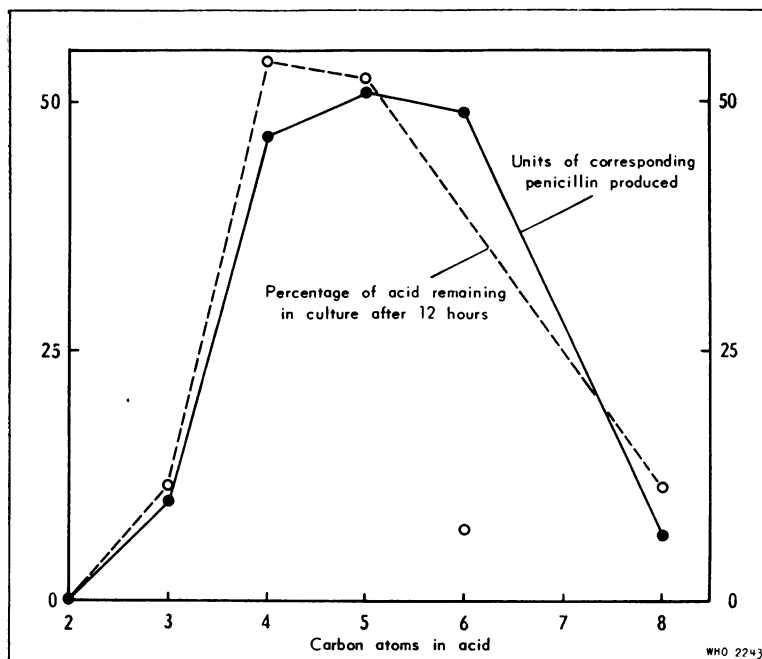
FIG. 6. EFFECT OF AN ALIPHATIC PRECURSOR ON PENICILLINS SYNTHESIZED



the structure of which is not known with certainty, and then there are more polar penicillins (moving more slowly on a buffer-ether chromatogram), which in fig. 6 are called I, III, and IV. A component II also often occurs in small amounts. If butyric acid or tributyrin is added to the synthetic medium, considerable amounts of a penicillin occurring in the "II" position are found. This has been chemically characterized as being, as one would expect, a butyryl penicillin. In the same way, valeric acid may be added to the medium, to cause the formation of a characteristic penicillin. The amount of new penicillin formed is related to the rate of oxidation by the

mould. Fig. 7 shows the relation, for simple straight-chain fatty acids, between oxidation-rate and precursor efficiency.⁸ One would suppose, since oxidation of fatty acids by moulds is known to proceed by β -oxidation, that a chemical structure which would prevent ready oxidation at the β -carbon atom would increase precursor activity. This is found to be the case. Behrens and co-workers² found that an etheric oxygen or sulfur atom attached to the β -carbon atom of the precursor acid gave good precursor efficiency. It is well known that phenylacetic acid is a very good precursor.

FIG. 7. RELATION BETWEEN PRECURSOR EFFICIENCY AND OXIDATION-RATE



In table II it is shown that higher percentages of the characteristic penicillins are given by acids with phenyl or cyclohexyl substitution at the β -carbon atom.⁸

Another relationship of structure to precursor activity is easy to demonstrate. Acids which are substituted at the α -carbon atom are not effective. We and others have tried many such acids, but the only one with detectable activity was α -methylbutyric acid. Substitution of an ethyl or larger group eliminated activity. Simple steric hindrance may be responsible for the effect. It can be concluded from the data of Behrens and co-workers that ortho-substitution of phenylacetic acid derivatives also eliminates precursor activity. Such ortho-substituents, as is apparent from models, occupy the same space as an α -substituent.

An important property a good precursor must have is lack of toxicity. We have found no correlation of structure with toxicity in the limited number of experiments we have run.

All the data we have indicate that the precursor efficiency of an acid which is not α -substituted depends primarily on the concentration of that acid in the cell. Rapidly oxidized acids are effective only if added often and in high concentrations. Acids which, because of their structure, are more slowly oxidized are more effective, unless their toxicity precludes their use in appreciable concentrations. The best precursor we have found is phenyl-

TABLE II. PRECURSOR EFFICIENCY OF VARIOUS ACIDS

Precursor acid used	Percentage of corresponding penicillin formed
Propionic	18
Butyric	34
Valeric	51
Phenoxyacetic	90
Phenylacetic	90
Phenylpropionic	50
Cyclohexylacetic	83
Cyclohexylpropionic	50

acetic acid. This is not surprising. Corn steep liquor contains probably thousands of compounds. When, in the early days of penicillin production, low-yielding penicillin fermentations were carried out in a corn steep liquor medium, most of the penicillin produced was penicillin G, the phenylacetyl penicillin. In other words, the mould itself was allowed to select the most effective precursor from all the compounds present.

Precursors which are efficient in the sense that they bring about formation of a large percentage of characteristic penicillin, and which have low toxicity, have another surprising characteristic. They greatly increase the total amount of penicillin produced by the culture. Illustrative data are given in table III. It will be seen that even on a steep liquor medium, which contains some precursor, addition of phenylacetic acid approximately doubles the yield. Data obtained with a number of precursors used at different levels indicate that availability of precursor is often the limiting factor in penicillin synthesis.

Turning now to the use of various derivatives of phenylacetic acid as precursors for penicillin G, it can be said that a large number of derivatives of this acid are active. Compounds like β -phenylethylamine, which can be oxidized to phenylacetic acid, or phenylacetyl glycine, which can be hydro-

lysed to phenylacetic acid, are effective. Time does not permit presentation of the experimental results, but a considerable amount of work has been done on G precursors in our laboratory¹⁰ and elsewhere.

The conclusions we have drawn from the work are easily summarized. Phenylacetic acid is oxidized by the mould, and to maintain an effective concentration in the medium, the acid should be added to the fermentation at intervals of 12 hours or less. The amount necessary varies considerably with the mould strain used, and with the medium and other environmental conditions. Normally, addition of 0.5 g per litre every 12 hours will give

TABLE III. EFFECT OF PHENYLACETIC ACID ON TOTAL PENICILLIN YIELD

Culture	Average yield* (units/ml)	
	without precursor	with precursor
Q 176	358	735
48-749	254	407
49-133	648	1,037
49-2105	450	1,030

* The shake-flasks contained steep liquor—lactose medium to which 0.05 % phenylacetic acid was added at 12-hour intervals.

an excess of precursor. Phenylacetic acid is much more toxic to the mould at low pH values than at high values; therefore, less than 1 g per litre should be added at the time of inoculation. Derivatives of phenylacetic acid are often more effective than phenylacetic acid itself, but only when all the precursor is added at once. In such a case, a derivative which will be gradually transformed into phenylacetic acid, at a rate such that an adequate level of phenylacetic acid is maintained, will be found better than phenylacetic acid itself. If the precursor is added at intervals throughout the fermentation, however, it is our experience that phenylacetic acid is a better precursor than any of the considerable number of derivatives we have tried.

Environmental Factors

I have, up to this point, omitted discussion of one of the most important factors influencing penicillin production: the nutritive state of the mould and the pH of the medium. Knowledge of the nutritional factors influencing mycelial growth and penicillin synthesis is of course far from complete, but the most important points have, I believe, been clarified.

The requirements for good penicillin production, as far as is known, are the following: First, there must be mycelium. Methods for growing

the mycelium will be discussed later, but the method of growth does not appear to be important as long as an adequate air-supply is available. Secondly, this mycelium, for optimum penicillin synthesis, should be maintained, under adequate aeration and in the presence of precursor, at a pH between 7.0 and 7.5. The pH optimum apparently depends upon the culture used and on other less well-known factors, so perhaps it would be

TABLE IV. MINERAL REQUIREMENTS FOR OPTIMUM GROWTH AND PENICILLIN PRODUCTION *

Element †	mg per litre required	
	for optimum growth	for optimum penicillin production
Potassium.....	40	40
Magnesium ...	8	8
Phosphorus ..	80	200
Iron	0.2	7
Sulfur	70	100

* In these fermentations, growth was limited by sugar-supply to 0.05 moles mycelial nitrogen per litre.

† Copper, zinc, and manganese are also required.

better to say between 6.8 and 7.8. Thirdly, this mycelium must be supplied with carbohydrate at a near-starvation level. This may be done by using a slowly-fermenting sugar, such as lactose, or it may be accomplished by slow addition of other, more readily utilized sugars. The nutrient level should be such that the mycelium, as measured by the mycelial nitrogen, remains stationary or, better, shows a slow increase. If growth is rapid, no penicillin is formed. If absolute starvation occurs, rapid autolysis takes place, and the period during which penicillin is formed is very short.

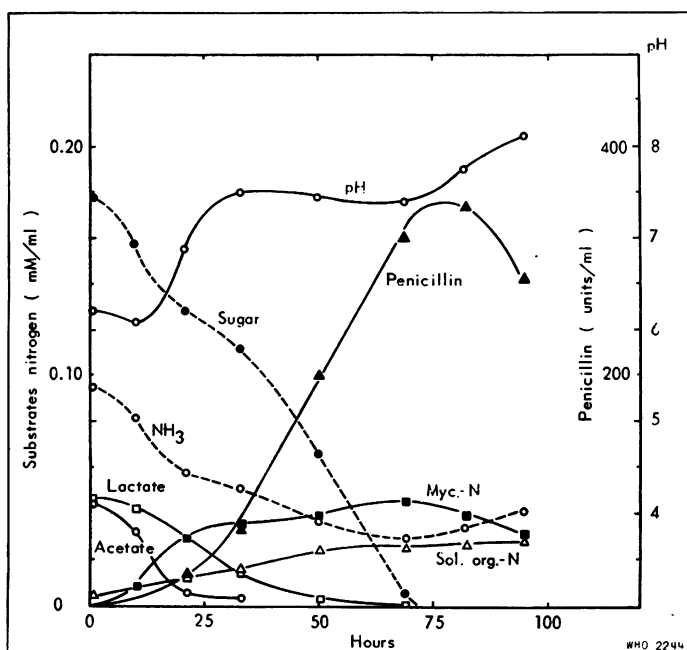
I shall discuss first the growth of the mycelium. An ideal nitrogen source is ammonium ion, although nitrate ion, or amino-acids, proteins, and other nitrogen sources can be used. Any rapidly-available carbon and energy source, such as glucose, sucrose, or amino-acids, can be used. The pH during growth is not too important. We have obtained good growth at automatically controlled pH values from 3.5 to 6.0. In shake-flasks, automatic pH control is not practicable, and may be controlled by the presence in the medium of acetate ion. The mechanism of this control has been discussed elsewhere.⁵ Acetate, however, is toxic at pH values where appreciable amounts of undissociated acetic acid are present.⁵ When urea is used as a nitrogen source, so that the use of acetate as a pH-controlling agent is not necessary, growth is better at pH values below 6.0. In the

presence of acetate, no growth takes place at low pH values. In both media, growth is slow at high pH values.

We customarily grow mycelium, in our work on penicillin production, under controlled conditions, at pH 4.5-5.0. We have no data to indicate that this pH is in any way optimal.

We have carried out some work⁶ on mineral-element requirements for growth and penicillin production. Table IV summarizes our findings. It will be noted that the requirements potassium and magnesium are the same for growth and penicillin synthesis, while more iron and phosphorus are needed for penicillin synthesis than for growth. More sulfur is apparently needed for penicillin synthesis than for growth, but calculation shows that the extra amount needed is equal to the sulfur in the penicillin molecule itself.

FIG. 8. PENICILLIN FERMENTATION IN A SYNTHETIC MEDIUM



Having grown the mycelium, preferably at pH 6 or below, to obtain penicillin synthesis one need merely hold the pH at 7 or above, and supply a limited amount of carbohydrate. The traditional, and the most convenient, way of doing this is by the use of lactose, which is oxidized only slowly by the mould. Data on such a fermentation,⁵ in a shake-flask, are given in fig. 8. The mycelium was grown on glucose (0.75%). After a lag phase, the glucose was used rapidly, and rapid mycelial growth occurred.

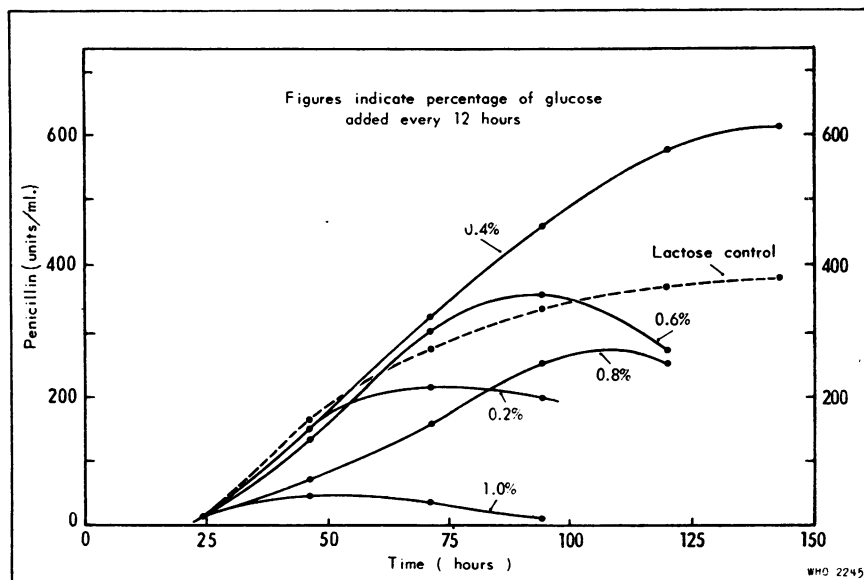
Ammonium ion was used to furnish the nitrogen for this growth. The pH remained at about 6 in spite of this utilization of ammonia, since acetate, which is as readily used as glucose at this pH, was also rapidly used. The advantages of acetate as a pH-controlling agent in the region of pH 6 have already been mentioned. After the acetate and glucose have been used, the lactate present is the most available carbon source remaining. It is then oxidized, resulting in a rise in pH from about 6 to about 7.5. The amount of pH rise obtained is again more or less automatically controlled, since, as can be seen, once the pH has risen, lactate is more slowly used. After an acclimatization period, lactose utilization begins. However, it is much slower than the utilization of glucose, and is apparently only rapid enough to supply the energy demands of the mycelium, for very little growth takes place. It will be noted that less mycelial growth occurred accompanying the oxidation of 2.25% lactose than accompanying the oxidation of 0.75% glucose. During this semi-starvation phase, when the pH is above 7, penicillin is produced. When the lactose is exhausted, autolysis occurs, mycelial nitrogen decreases, ammonia increases, and the pH rises. No more penicillin is formed, and some penicillin destruction takes place.

If glucose is substituted for lactose, rapid growth continues, even at pH 7, and very little penicillin is formed. If no carbohydrate whatever is present, rapid autolysis takes place.

If the function of lactose is purely that of a slowly-available carbon source, it should be possible to substitute a rapidly-available carbon source, such as glucose, for lactose, if it is made slowly available by slowly adding it to the culture. The feed-rate, one would guess, should be approximately the rate at which lactose is utilized. It will be seen from fig. 8 that 2.25% lactose was used in about 48 hours, or a little more than 1% every 24 hours. It will also be seen that the growth phase of the fermentation is completed in 24 hours. One might predict then that if a medium was used which contained about 1% glucose, for mycelial growth, the acetate and lactate necessary for pH control, but no lactose, and if a fermentation on such a medium were allowed to proceed for 24 hours (or until the pH rise and sugar analyses showed that the growth phase was complete), and if glucose were then slowly added to such a fermentation, at a rate of about 1% per day, penicillin should be formed.

Fig. 9 summarizes the results of experiments, again in shake-flasks, where exactly this was done. Glucose was fed, for convenience, only every 12 hours. It will be seen that a feed-rate of 0.4% every 12 hours, or 0.8% per day, was found to be optimal, and that the yield exceeded that of the lactose control. Sugar analyses showed that the sugar added was completely fermented in less than 4 hours when the fermentation was young, but was barely utilized before the next addition when the fermentation was old, that is, at the time of the penicillin maximum. Other rapidly-available

FIG. 9. PENICILLIN PRODUCTION FROM GLUCOSE



sugar sources can be substituted for glucose. Sucrose, starch, galactose, xylose, and sorbose—all gave good results.

In order to determine whether continuous feeding of glucose was superior to intermittent feeding, a number of experiments have been run with apparatus arranged to feed glucose solution continuously to shake flasks. The yields have been somewhat better with this procedure, equalling or exceeding the best shake-flask yields we have obtained on steep liquor media with the organism used (Q 176). Fig. 10 shows a plot of feed-rate versus yield on a series of such fermentations. It will be seen that well over 1,000 units per ml were obtained on a synthetic medium in shake-flasks at feed-rates of approximately 0.03% per hour.

In shake-flask fermentations, one cannot obtain entire independence of pH from other variables. It will be seen that at the higher feed-rates, the pH tended to plateau at lower values. Although this effect was probably not sufficient to have a great effect on the curve of feed-rate versus yield, it is certainly a disturbing factor.

In order to have more freedom from pH changes, a jar fermenter similar to those described elsewhere⁹ was equipped with a glass electrode and an amplifier so arranged that sodium hydroxide solution was added to the fermenter whenever the pH fell below any desired value. With such automatic pH control, it is possible to dispense with acetate or lactate as pH-controlling agents, and to have great freedom in growing mycelium or producing penicillin at any desired pH. The basal medium used contains

glucose, ammonium sulfate, and the essential inorganic salts. Since any utilization of nitrogen causes the pH to drop, sodium hydroxide is added automatically and frequently to control the pH. With such an arrangement, mycelium can be grown at any desired pH. Then, when the growth is complete, the pH can be shifted to another value for penicillin production. Fermentations to which glucose is slowly fed (once per hour) have been carried out with this arrangement. A typical fermentation with a suitable feed-rate is shown in fig. 11. The mycelium was grown on 1% glucose and, near the end of the growth period, feeding was begun at the rate of 0.042% per hour. The pH was held close to 7 during the penicillin-forming phase. It will be seen that there was a rapid increase in mycelium during the growth phase, and a slower increase during the penicillin-producing phase. The penicillin yield was a little over 1,500 units per ml.

It will be noted that the fermentation was very long, about 150 hours, and that there is an apparent "adaptation" period after feeding begins, and before penicillin formation becomes rapid. This lag period is characteristic of the fermentations run with automatic pH control, but does not occur in shake-flask fermentations with glucose feeding. It may be related to the pH at which the mycelium is grown, or to other factors. We have as yet had no opportunity to investigate it.

The yields of these jar fermenters are equal, at optimal feed-rates, to those produced by the same organism on corn steep liquor media, but the fermentation times are longer. At high feed-rates, there is of course continued rapid growth of mycelium, and at low feed-rates, as in the case of the shake-flask fermentations, there is autolysis.

As has been mentioned, we have as yet no systematic data on the effect of the pH at which the mycelium is grown on its subsequent ability to produce penicillin. However, we have obtained penicillin production from mycelium grown at pH values ranging from 4.5 to 6.5. We have no data on the air requirements of the growth phase and the penicillin-producing phase separately. It is conceivable that a reduced air-supply during penicillin formation would not be harmful, and it is also conceivable that optimum feed-rate is a function of aeration-rate. We already have evidence that it is a function of pH. The fragmentary data we do have, however, certainly indicate that the most important conditions for penicillin formation are a pH somewhat above 7, adequate air- and precursor-supply, and a limited carbohydrate-supply.^a

Our conclusions with regard to the environmental factors involved in penicillin formation can very conveniently be summarized by describing the properties of a typical steep liquor medium. During the early days of penicillin production, Czapek-Dox medium and other synthetic media were

^aThe work on the use of glucose feeding and of pH control in establishing the optimum conditions for penicillin synthesis has been done in our laboratory by Peter Hosler, Fred Soltero, and Marshall Bautz.

FIG. 10. EFFECT OF GLUCOSE FEED-RATE ON PENICILLIN PRODUCTION

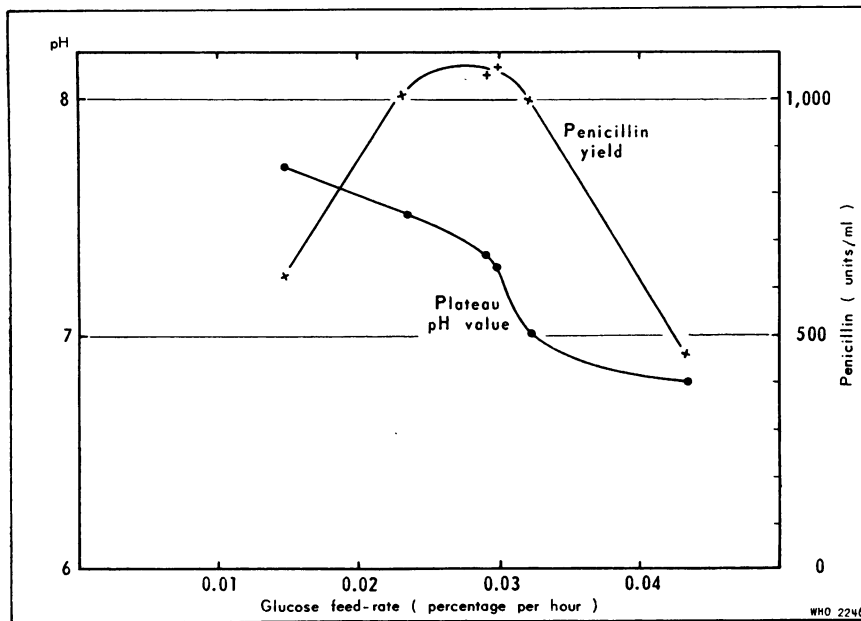
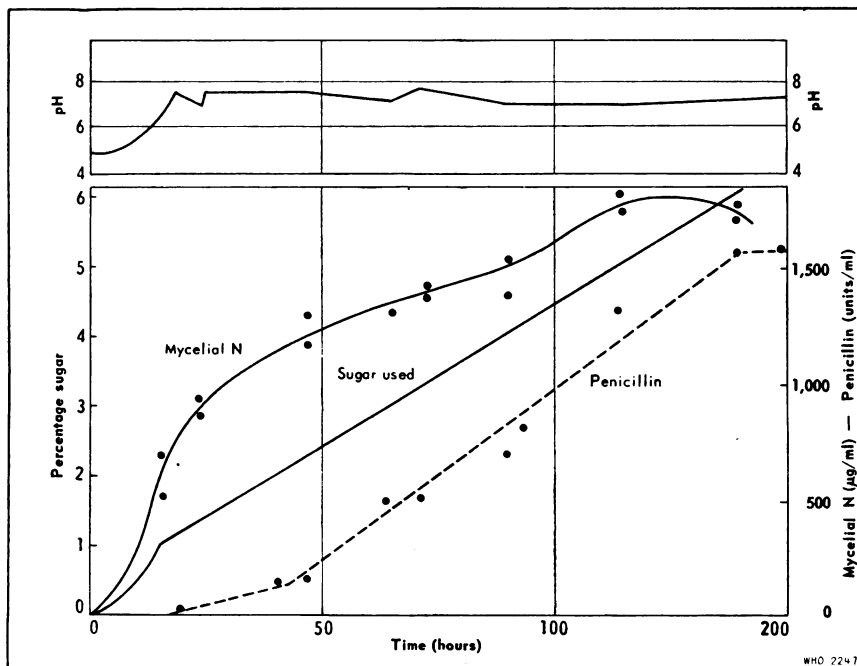


FIG. 11. PENICILLIN PRODUCTION FROM GLUCOSE IN A 30-LITRE JAR FERMENTER



used. The requirements for good penicillin production being what they are, it is no wonder that the yields were low. During the second World War, however, a medium consisting of corn steep liquor and lactose was found to be greatly superior to other media.³ The reason for this was not known. Many laboratories, including ours, wasted a good deal of time fractionating steep liquor in an attempt to isolate the magical compound or compounds responsible for its stimulation of penicillin production. Today, one can readily see exactly why a steep liquor—lactose medium is a very good medium. Corn steep liquor is a material produced during the processing of maize, and is, roughly, the water-soluble constituents of the maize, subjected to anaerobic fermentation by a mixed flora. It contains a little sugar, but most of the sugar has been converted to lactic acid. It contains amino-acids and peptides. It contains bacterial degradation products of amino-acids, including phenylethylamine and phenylacetic acid as degradation products of phenylalanine.

When a penicillin-producing mould is grown on a steep liquor—lactose medium, the peptides and amino-acids of steep liquor are readily oxidized by the mould at the relatively low pH of the steep liquor, and rapid mycelial growth occurs. Since compounds high in nitrogen are being oxidized, ammonia is liberated, and the pH rises somewhat. When the readily-available carbon sources of the steep liquor have become exhausted, the lactic acid, being more readily oxidized than the lactose, is attacked, and the pH rises to 7 or somewhat above. The mycelium is now at the proper pH for penicillin production, just as it was at a suitable pH for growth before the lactic acid was utilized. As will be remembered, growth is slow at pH 7. Precursor, sufficient for the needs of the low-yielding cultures used in the early days, is present in the steep liquor. The lactose is oxidized at a slow rate, giving the semi-starvation conditions necessary for penicillin production. The ammonium ion liberated during the growth phase furnishes a nitrogen-supply during the penicillin-forming phase, and all inorganic elements necessary are present in great excess in the steep liquor.

The use of corn steep liquor and lactose made efficient penicillin production technologically possible many years before the biochemical factors involved were even recognized. Inasmuch as science should lead technology, rather than lag behind it by many years, the recent advance in knowledge of the biochemistry of penicillin fermentation can hardly be regarded as a noteworthy achievement.

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SUMMARY

Advances in knowledge of the fundamental processes involved in penicillin fermentation, and in the technological application of this knowledge, may be classified under such general headings as : mutation studies on mould cultures ; investigation of variables involved in aeration and agitation ; research on the relationships between structure and activity of precursor compounds ; and work on the composition of the culture medium.

Since 1946, the standard culture for penicillin production has been *Penicillium chrysogenum* Q 176. Under many environmental conditions, this culture is still the best for commercial penicillin production. A number of useful new cultures, mutants derived from Q 176, have been developed in both industrial and university laboratories. Most of them do not produce yellow pigment in the medium. The pigmentless strains developed at the University of Wisconsin, Madison, Wis., USA, vary considerably in their properties, but are in general characterized by efficient precursor utilization, rapid oxidation of lactose, and, often, lower air-requirement. Reports on yields are conflicting. In some laboratories yields are higher than those with Q 176, and in others lower.

Comparison of ultra-violet light, x-rays, and nitrogen mustard (MBA) as mutagenic agents has shown that all are effective, and that the choice of agent is determined by convenience. The testing of spores surviving mutagenic treatment presents a difficult problem. Since thousands of tests must be made, a simple shake-flask fermentation procedure, with the use of only one medium, appears the only practical testing method. It has repeatedly

RÉSUMÉ

Les recherches récentes sur les processus fondamentaux de la fermentation de *Penicillium* et sur leur application pratique à la production de la pénicilline peuvent être classées sous les rubriques générales suivantes : mutation des cultures de moisissures ; influence des conditions d'aération et d'agitation ; relation entre la structure et l'activité des précurseurs ; composition du milieu de culture.

Depuis 1946, on utilise *Penicillium chrysogenum* Q 176 comme souche standard pour la production de pénicilline. Dans diverses conditions de culture, cette souche demeure la meilleure pour la fabrication commerciale de la pénicilline. Certains laboratoires industriels et universitaires ont mis au point une série de nouvelles cultures intéressantes, mutants dérivés de Q 176. La plupart d'entre elles ne donnent pas de pigment jaune dans le milieu. Les souches sans pigment cultivées dans les laboratoires de l'Université de Wisconsin, Madison, Wis. (Etats Unis d'Amérique), diffèrent considérablement par leurs propriétés ; mais elles sont caractérisées en général par une utilisation à bon rendement du précurseur, par leur aptitude à oxyder rapidement le lactose, et, souvent, par le fait qu'elles exigent une aération moins importante. Les chiffres de rendement sont contradictoires. Suivant les laboratoires, ils sont supérieurs ou inférieurs à ceux que l'on observe pour Q 176.

Les rayons ultra-violet, les rayons X et la moutarde à l'azote (MBA) se sont tous révélés d'efficaces agents de mutation ; selon les circonstances on utilisera l'un ou l'autre d'entre eux. Il est difficile d'étudier les propriétés des spores qui survivent à la mutation. Comme on est obligé de procéder à des milliers de tests, une simple fermentation en milieu unique dans des flacons soumis à l'agitation semble être la seule méthode pratique.

been found, however, that mutants are thus selected which are peculiarly adapted to the conditions of the test, but do not necessarily give good performance in large fermentation equipment.

There has been some progress in knowledge of the relation of aeration and agitation to growth and yields in penicillin fermentation. It is known that effective aeration (as distinguished from gross aeration-rate) is often a limiting factor. Recent work from a number of laboratories indicates that approximate determination of effective aeration is feasible, and that a promising field is the study of aeration in large fermenters.

Recent work on precursors has shown that the structural requirements for activity (i.e., conversion to a corresponding penicillin) of a carboxylic acid are an unsubstituted α -carbon atom, and a configuration around the β -carbon atom not conducive to β -oxidation. Lack of toxicity is, of course, also a requisite. The indications are that the important factor in precursor activity is the concentration of the precursor in the cell. Of possible precursors for penicillin G, phenylacetic acid, added at intervals throughout the fermentation, appears to be at least as good as any of its derivatives.

The necessary constituents, apart from precursors, in a penicillin medium are : (1) an adequate supply of essential elements, such as phosphorus, sulfur, potassium, zinc, magnesium, and others ; (2) a source of available nitrogen, such as ammonium ion ; (3) a rapidly-available carbon source to supply material and energy for mycelial growth during the first phase of the fermentation ; (4) a slowly-available carbon source, such as lactose, for provision of a low level of energy nutrient during the penicillin-forming phase ; and (5) provision for pH control such that the pH is in a region suitable for rapid growth during the growth phase, and in a region

Cependant on a constaté à plusieurs reprises que les mutants choisis de la sorte peuvent satisfaire aux exigences de cette épreuve, mais ne pas donner nécessairement de bons résultats lorsque la fermentation porte sur un volume plus important.

On connaît maintenant un peu mieux l'influence de l'aération et de l'agitation sur les cultures et sur le rendement. On sait que l'aération effective (qu'il y a lieu de distinguer de l'aération brute) est souvent un facteur limitatif. Les travaux récemment poursuivis par un certain nombre de laboratoires montrent que l'on peut déterminer approximativement l'aération effective et que l'étude de l'aération dans les grandes cuves de fermentation est susceptible de donner des résultats intéressants.

Les recherches récentes sur les précurseurs ont révélé que, pour être efficace (l'efficacité étant déterminée par la transformation en pénicilline correspondante), un acide carboxylique doit présenter un atome de carbone en position α non substitué, et une structure telle autour de l'atome de carbone β , qu'elle exclue la possibilité d'oxydation en β . Le précurseur doit, bien entendu, se caractériser par son atoxicité. Son activité paraît dépendre principalement de sa concentration dans la cellule. Parmi les précurseurs utilisables pour la pénicilline G, l'acide phénylacétique, ajouté à intervalles réguliers pendant la fermentation, semble donner des résultats au moins aussi bons que n'importe lequel de ses dérivés.

Outre les précurseurs, le milieu doit nécessairement contenir : 1) une quantité appropriée d'éléments essentiels, tels que le phosphore, le soufre, le potassium, le zinc, le magnésium, etc. ; 2) une source d'azote telle que l'ion ammonium ; 3) une source de carbone aisément assimilable assurant les matériaux et l'énergie nécessaires pendant la première phase de la fermentation (croissance du mycélium) ; 4) une source de carbone plus lentement assimilable, telle que le lactose, qui fournisse la faible quantité d'énergie nécessaire pendant la phase de formation de la pénicilline. Il faut en outre pouvoir contrôler le pH pour s'assurer qu'il permet un

suitable for rapid penicillin production during the second phase.

A medium consisting of corn steep liquor and lactose satisfies all the above requirements. It is perfectly possible, however, to obtain good penicillin yields (1,500 units per ml in 30-litre jars) without the use of either steep liquor or lactose. In such fermentations, pH is controlled by automatic addition of sodium hydroxide, ammonium ion is the nitrogen source, glucose is the readily-available carbon source, and glucose added at a slow and controlled rate is the slowly-available carbon source.

développement rapide pendant la phase de culture et une production rapide de pénicilline pendant la seconde phase.

Un milieu à base de macération de maïs (corn-steep liquor) et de lactose répond parfaitement aux conditions indiquées ci-dessus. Il est cependant tout à fait possible d'obtenir de bons rendements (1.500 unités par millilitre dans des ballons de 30 litres) sans macération de maïs ou sans lactose. En pareil cas, le pH est ajusté par addition automatique d'hydroxyde de sodium, l'ion ammonium est la source d'azote, le glucose assure la mise à disposition rapide de carbone, la source de production lente étant constituée par le glucose ajouté lentement et à une vitesse contrôlée.

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