

# The Effect of Temperature Changes on the Production of Penicillin by *Penicillium chrysogenum* W49-133<sup>1</sup>

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The optimal temperatures reported for maximum production of penicillin by *Penicillium chrysogenum* have varied between 23 C and 28 C (Peterson, 1946; Johnson, 1946; Calam *et al.*, 1951). These temperatures were established by comparing the yields obtained when the mold was held at various temperatures for the entire fermentation. Calam *et al.* (1951) obtained best yields at about 25 C.

The work reported here is an attempt to determine the effect of variations in temperature during fermentation on penicillin yield. It is well known that maximum penicillin production by a culture occurs only when well defined conditions exist for the mycelium-producing phase and equally well defined, but different, conditions exist for the penicillin-producing phase. That these two phases should have the same temperature optimum seems unlikely.

There have been relatively few published reports of the effects (on the yield of cells and cell by-products) of variations of temperature during a fermentation. Davis and Dedrick (1953) have shown that increased cell yields result when *Chlorella* cultures are grown at 25 C during the day and placed at lower temperatures during the night. Kovats (1946) found that maximum citric acid yields were obtained in sucrose surface fermentations when the temperature was held at 28 C for the first 3 days and then reduced to 20 C.

## EXPERIMENTAL METHODS

*Fermentation techniques.* *Penicillium chrysogenum* W49-133 was used exclusively in these experiments. The culture was kept in sporulated form in dry sterile soil and was used to inoculate 6-ounce bottles which contained 40 ml of the standard spore-plate medium described by Gailey *et al.* (1946).

Inoculations were made with 5 ml of vegetative inoculum. This inoculum was prepared by inoculating 100 ml of a 3 per cent (dry basis) corn steep liquor-5 per cent dextrin medium with 5 ml of spore suspension prepared from growth on the sporulation medium.

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Unless otherwise stated, all fermentation media were of the following composition: corn steep liquor, dry basis (CSL), 1.5 per cent; lactose, 2.5 per cent; CaCO<sub>3</sub>, 0.2 per cent; Na<sub>2</sub>SO<sub>4</sub>, 0.05 per cent. This medium will henceforth be referred to as "half strength" medium. (The half strength medium contains one-half the concentration of ingredients of a medium found by Tornqvist (1955) to be optimal for penicillin production in shaken flasks with strain W49-133.) The medium was adjusted to pH 5.8 to 6.0 prior to being autoclaved at 121 C for 20 minutes. Potassium phenylacetate, at a 0.05 per cent level, was added as precursor to all fermentations every 24 hours after the pH had reached 6.8 to 7.0.

The fermentation flasks incubated at 25 C and 30 C were placed on a rotary shaker which described a 2-inch circle at a speed of 250 rpm. The flasks incubated at 15 C and 20 C were placed in a water-jacketed incubator which contained a 4-place rotary shaker which described a 2-inch circle at a speed of 300 rpm. Half strength fermentations performed on the two shakers at the same temperature gave similar results.

Unless otherwise stated, all fermentations were completed in triplicate; all assays reported were from the average of 4 zones.

*Analytical procedures.* Penicillin was assayed by the Oxford cup method with the use of *Micrococcus pyogenes* var. *aureus* H as the test organism and penicillin G as the standard.

The pH of samples was determined immediately after collection by means of a glass electrode.

Residual sugar was determined by the method of Shaffer and Somogyi (1933). When lactose was present, the samples were hydrolyzed by heating at 120 C for 20 minutes in 1 N HCl. Titrations were referred to a standard curve obtained with glucose or hydrolyzed lactose.

Soluble Kjeldahl nitrogen was determined by the method described by Johnson (1941). The mycelial nitrogen was calculated by subtracting the soluble nitrogen present at the time of sampling from the soluble nitrogen present at the time of inoculation. Nitrogen in the washed and dried mycelium was determined by a modified Kjeldahl method described by Hiller *et al.* (1948).

## RESULTS AND DISCUSSION

It was decided that a half strength medium should be used for the fermentations conducted to determine the effect of variations of temperature on penicillin production. It is known that for particular aeration conditions and a particular culture, there is an optimum concentration of medium for the production of penicillin at 25 C (Tornqvist 1955). This optimum medium is one in which the nutrient concentration is sufficient to give an amount of mycelium which uses all of the air available to it. A higher mycelium concentration results in oxygen starvation with lower yields, while a lower mycelium concentration results in lowered yields

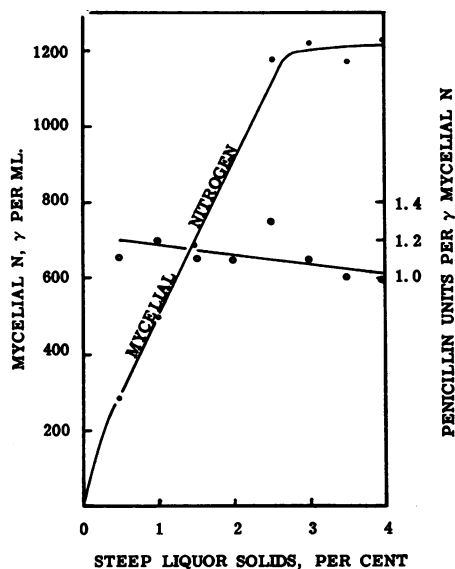


FIG. 1. Effect of concentration of steep liquor on mycelial nitrogen and its effect on penicillin production as related to mycelial nitrogen. Temperature: 25 C. Ratio of steep liquor solids to lactose was 0.6.

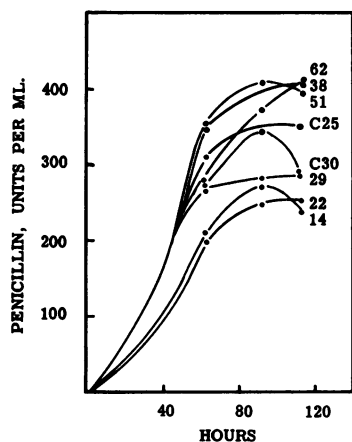


FIG. 2. Effect of change of incubation temperature on penicillin production. The numbers to the right of each curve represent the time, in hours after inoculation, at which the fermentation temperature was changed from 30 C to 25 C. Curves C25 and C30 represent control fermentations which were incubated throughout the fermentation at the respective temperatures.

because, in general, penicillin production is proportional to mycelium concentration. If the medium is optimal for fermentations conducted at 25 C, then air might become limiting in fermentations conducted in the same medium at 30 C, since metabolism might be more rapid at the higher temperature. The use of half strength medium should circumvent this difficulty.

*Effect of concentration of corn steep liquor.* In order to establish that air might be a limiting factor in penicillin production in shaken flasks, eight separate fermentations (completed in duplicate) were carried out on a rotary shaker at 25 C. The percentages of CSL in the various fermentations were: 0.5, 1.0, 1.5, 2.0, 2.5, 3.0, 3.5 and 4.0. The ratio of CSL to lactose in all fermentation media was 0.6. This ratio of CSL to lactose was reported by Tornqvist (1955) to give optimal penicillin yields in shaken flasks when used with strain W49-133.  $\text{CaCO}_3$ ,  $\text{Na}_2\text{SO}_4$ , and precursor were added with the CSL and lactose in proportions similar to those in the half strength medium described in *Experimental Methods*.

The results obtained from this experiment are shown in figure 1. As may be seen from the graph, the mycelial nitrogen formed was proportional to the concentration of CSL in the medium except at concentrations of CSL above 3 per cent. At the 3 per cent CSL concentration air probably became limiting because the ratio of mycelial nitrogen to CSL concentration decreased. The penicillin produced per microgram of mycelial nitrogen was less at high CSL concentrations. The results from this experiment indicate that some factor in shaken flask fermentations at high CSL concentrations limits penicillin production. The factor was not pH, since the pH at maximum yield varied from 6.9 at low steep liquor concentrations to 7.5 at the highest concentration. If the factor is the lack of air then use of half strength medium is justified.

*Effects of temperature change during incubation.* At the outset of this problem, the effect of changing the temperature during incubation on penicillin production was not known. It was decided first to investigate the effects on penicillin production of growing the mycelium at 30 C and completing the penicillin-forming phase at 25 C. If, in a penicillin fermentation, the growth phase is to be conducted at one temperature and the penicillin-producing phase at another temperature, the age of the culture at the time of the temperature change might be an important variable. Therefore, a series of fermentations were conducted in which the incubation temperature was changed from 30 C to 25 C at various times.

Twenty-four flasks were inoculated and at 14, 22, 29, 38, 51 and 62 hours, respectively, three flasks were transferred from the 30 C shaker to the 25 C shaker. Controls were run at 25 C and 30 C for the entire

fermentation period. The results of the experiment are shown in figure 2.

The curves show that when fermentations were changed to the lower temperature at 14, 22 or 29 hours, the final yields of penicillin were well below those of the controls, or of the fermentations changed after 29 hours. The 30 C control flask decreased in titer after 92 hours. A sugar analysis on the fermentation broth showed that the sugar had been completely used and autolysis had occurred. The differences in the fermentations were not due to variations in pH, since the pH values, at 92 hours, of all flasks were between 7.6 and 7.9.

The three curves plotted in figure 3 show the sugar remaining in the flasks at 38, 62 and 92 hours respectively. It can be seen that fermentations that were changed from 30 C to 25 C prior to 38 hours did not use sugar as rapidly as those fermentations changed at or after 38 hours. The fermentations in which temperature changes occurred before 38 hours had the lowest penicillin titers. The decreased utilization of sugar might be explained by assuming that the mold is sensitive to temperature changes which take place during the period of rapid mycelium formation.

Studies with bacteria have shown that the physical and chemical organization of the cell is not fixed through the growth period. For example, during the late lag and much of the exponential growth, bacteria appear to be most sensitive to sudden changes of environment. They are easily killed by heat and cold and by transfer into solutions of high salt concentration (Sherman and Albus, 1923; Hegarty and Weeks, 1940).

It is not unlikely that a similar phenomenon occurs in molds, which would explain, in part, why low yields were obtained from fermentations changed from 30 C to 25 C before 38 hours.

From the experiment just described it is evident that when mycelium is produced in a fermentation at a temperature of 30 C and it is desired to continue the penicillin-producing phase at 25 C, the change of temperature should not take place until 35 to 40 hours after inoculation. In this series of fermentations the growth phase lasted approximately 35 to 45 hours. Hence in these fermentations, temperature changes made before the end of the growth phase resulted in lower penicillin yields.

*Optimum temperatures for the two phases.* In the next series of experiments a number of flasks were inoculated and placed at 20 C, 25 C and 30 C respectively, for the first 42 hours of the fermentation. At 42 hours (shown in the previous experiment to be a satisfactory time for the change) some of the flasks at each of the three temperatures were placed at a different temperature for the remainder of the fermentation. Control flasks were kept at 20 C, 25 C, and 30 C for the entire fermentation.

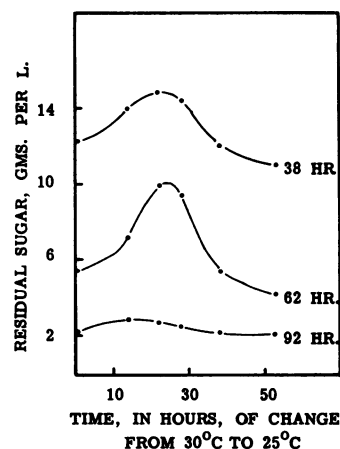


FIG. 3. Effect of temperature change on residual sugar. The three curves show the residual sugar in the flasks of figure 2 at 38, 62 and 92 hours, respectively. Each point on a particular curve represents the residual sugar in a flask which had been transferred from 30 C to 25 C at the particular time given on the abscissa.

TABLE 1. Summary of results obtained from the fermentations shown in figure 4

Fermentation Temperature		Lactose Used from 42 to 92 hr	Nitrogen in Mycelium		Mycelial Dry Weight		Maximum Penicillin Production	Time of Maximum Yield	pH at Time of Maximum Yield	Penicillin Production Rate*
Before 42 hr	After 42 hr		42 hr.	116 hr.	42 hr.	116 hr.				
C	C	g/l/hr	%	%	%	%	units/ml	hours		units/ml/hr
20	20	0.27	7.3	4.8	8.2	13.8	450	141	7.8	3.2
25	25	0.23	5.8	4.8	11.6	13.5	420	92	7.6	4.6
30	30	0.17	5.3	4.6	12.5	9.2	555	92	7.7	6.0
30	20	0.15	5.3	4.5	12.5	13.8	635	141	7.7	4.5
30	25	0.17	5.3	4.8	12.5	12.4	570	92	7.5	6.2
25	30	0.23	5.8	4.7	11.6	12.7	480†	68†	7.5	—
20	30	0.37	7.3	4.6	8.2	12.8	350	92	7.5	3.8

\* Penicillin production rate = 
$$\frac{\text{maximum penicillin production, units/ml}}{\text{time to reach maximum yield, hours}}$$

† The 68-hour assay value is questionable.

The results obtained in this series of fermentations are summarized in table 1 and figure 4. A number of conclusions may be drawn from the data. The fermentations in which the growth phase took place at 30 C yielded the most penicillin, regardless of the temperature of incubation during the penicillin-forming phase. Further, 20 C proved to be the best temperature for the penicillin-forming phase for mycelium grown at 20 C as well as for mycelium grown at 30 C. When the best growth temperature (30 C) was combined with the best temperature for the penicillin-forming phase (20 C), yields were 50 per cent higher than in the 25 C controls.

It will be noted that the mycelium grown at 30 C had, at the beginning of the penicillin-producing period, a lower nitrogen content than mycelium grown at lower

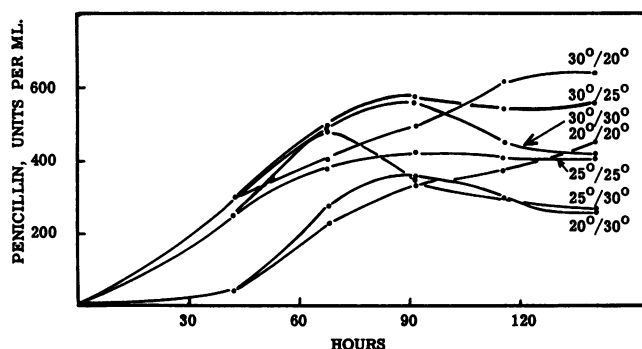


FIG. 4. Effect of change of temperature of incubation on penicillin yields. Temperatures used: 20 C, 25 C, 30 C. Time of temperature change: 42 hours after inoculation. The figures referring to each curve indicate the 2 temperatures at which the fermentation was studied. For example: 30/20 indicates that the fermentation was incubated at 30 C for 42 hours and then transferred to 20 C for the remainder of the fermentation.

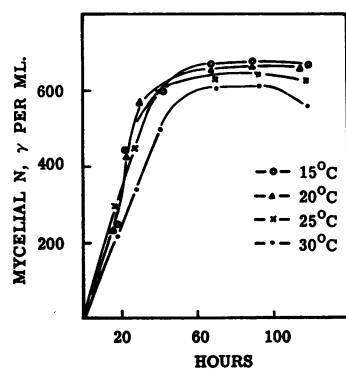


FIG. 5. Effect of temperature of incubation on mycelial nitrogen production. Temperatures of incubation: 15 C, 20 C, 25 C, 30 C.

temperatures. Its weight, however, was greater. It is also significant that the mycelium grown at 30 C used sugar more slowly during the penicillin-forming phase, regardless of the temperature during that phase. The reason for the superiority of the mycelium grown at 30 C is not clear, but it is probable that its low nitrogen content and its low rate of sugar utilization are significant.

The results obtained from the experiment of figure 4 indicate that mycelium grown at 30 C for 42 hours produced more penicillin than mycelium grown at 25 C or 20 C for the same time. However, the time of change of temperature (42 hours) was selected on the basis of an experiment in which the mycelium was grown at 30 C. It was thought possible that the mycelium grown at 20 C and 25 C, and changed to other temperatures at 42 hours after inoculation, had not completed the growth phase (maximum mycelium production) at the time of the temperature change. If maximum mycelial production was not attained at 42 hours then this fact might have been the reason for the low penicillin yields obtained from fermentations initiated at 20 C and 25 C. It was therefore decided to investigate the

rate of formation and production of mycelium in fermentations incubated at 15 C, 20 C, 25 C and 30 C. The mycelial nitrogen values obtained at the four temperatures are shown in figure 5. The 25 C and 30 C fermentations were similar in all respects except temperature. The 20 C and 15 C fermentations were conducted at different times and in the 300 rpm shaker. The pH during fermentation was similar in all flasks. At 67 hours it was between 7.2 and 7.4 in all flasks. It is evident from the graph that the rate of formation of mycelium was greatest in the fermentations conducted at the lower temperatures. No satisfactory explanation can be offered for this observation. It was expected that slow growth would be observed at the lowest temperatures. From figure 5 it is also evident that at 42 hours (the time of temperature change in experiments of table 1) the mycelial nitrogen values of fermentations incubated at 15 C, 20 C and 25 C were somewhat higher than those incubated at 30 C. The differences, however, are small compared to the differences in penicillin production caused by differences in growth temperature.

The production of higher mycelium concentrations at lower incubation temperatures has also been reported by Ryan, Beadle and Tatum (1943). They observed a heavier growth of mycelium at lower temperatures when *Neurospora crassa* was grown on a sucrose-mineral salts-agar medium.

The work described above indicates that there are two temperature optima for the penicillin fermentation. One of the temperatures, in the vicinity of 30 C, is best for the mycelium-producing phase; the other temperature, in the vicinity of 20 C is best for the penicillin-forming phase.

It should be emphasized that these results were obtained by the use of a dilute medium, in which, it is believed, an excess of oxygen was always available to the mycelium. Fermentations carried out with more concentrated media might yield different results. It should also be recognized that the results may hold only for the strain (W49-1333) used in these experiments.

#### SUMMARY

The effects of the concentration of corn steep liquor on production of mycelial nitrogen and penicillin were investigated. It was found that, at the aeration level used, penicillin production closely paralleled mycelial nitrogen production up to concentrations of 3.5 and 4 per cent corn steep liquor (dry basis). At these concentrations the amount of penicillin produced per microgram of mycelial nitrogen was lowest. It appeared that air was a limiting factor in the production of penicillin at the high steep liquor concentrations. Therefore, a medium was used containing only 1.5 per cent steep

liquor to ensure that the air supply was not the limiting factor.

Experiments were carried out to determine the age, after the time of inoculation, at which temperature changes, from 30 C to 25 C, should be made. It was found that if mycelium was grown in a fermentation incubated at 30 C, and it was desired to continue the penicillin-producing phase at 25 C, the change of temperature should not be made until 35 to 40 hours after inoculation. Temperature changes made before that time resulted in lowered penicillin yields.

From the experiments completed it was realized that there are two temperature optima connected with penicillin production. One, about 30 C, was best for the mycelium-producing phase; the other, about 20 C, was best for the penicillin-producing phase.

It was shown that 50 per cent more penicillin was produced by fermentations which were started at 30 C and transferred to 20 C, 42 hours after inoculation, than was produced in controls incubated at 25 C for the entire fermentation.

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