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Influence of Penicillin Instability on Interpretation of Feedback Regulation Experiments

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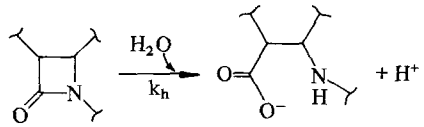
Summary. The stability of penicillin G during production is reviewed and compared to reports on end-product regulation of penicillin biosynthesis. From this analysis, it appears that penicillin control of its own synthesis when added exogenously at the beginning of the process has not been proven, since the apparent decrease of net accumulation can be explained by gradual hydrolysis of the added penicillin. It is also concluded that the maximum amount of penicillin accumulated normally is not controlled by penicillin, but rather by its stability and the ability of the cells to maintain a high synthetic rate over extended time periods.

Introduction

Although it is well known that some microbial products are unstable under fermentation conditions, this fact has frequently been ignored in studies on the kinetics of product formation. Such an omission simplifies the mathematical treatment, but may lead to data distortion and misinterpretations. As a case study, we consider here penicillin production. The inactivation of β-lactam antibiotics proceeds by general base or acid catalyzed opening of the four-membered ring:



This hydrolysis follows first order kinetics with respect to antibiotic concentration, and the observed rate of antibiotic accumulation during synthesis can accordingly be presented by:

$$dP/dt = q_p X - k_h P \quad (1)$$

(Accumulation rate, $g\,l^{-1}\,h^{-1}$) = (Synthetic rate, $g\,l^{-1}\,h^{-1}$) − (Hydrolysis rate, $g\,l^{-1}\,h^{-1}$)

X = Cell concentration, $g\,l^{-1}$
q_p = Specific synthetic rate, g product per g cell per hour
k_h = First order rate constant for product hydrolysis, $g^{-1}\,h^{-1}$
P = Product concentration, $g\,l^{-1}$

Among the β-lactam antibiotics, penicillin G (benzylpenicillin) has intermediate stability; its half-life in buffer was measured as a function of pH and temperature by Benedict et al. (1946). The rate constant for this hydrolysis (k_h) at the pH and temperature values typical for the fermentation are presented in Fig. 1. The higest stability was at pH 6.0; stability at pH 5.5 was similar to that at pH 7.5.

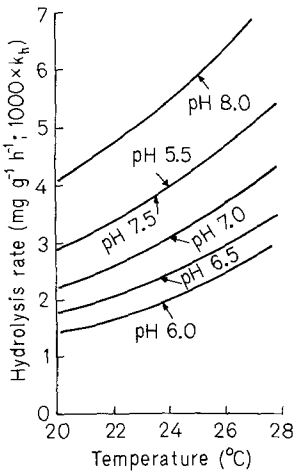


Fig. 1. First order rate constant (k_h) for penicillin G hydrolysis as a function of temperature and pH. The activation energy for hydrolysis is in this temperature region on the average 16 kcal per mole. The *curves* were constructed from half-life data published by Benedict et al. (1946). (Half-life equals ln $2/k_h$)

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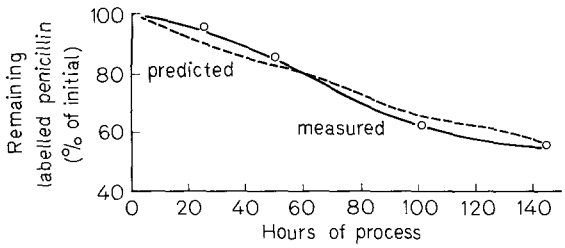

Fig. 2. Remaining deuterated penicillin G versus hours of process (as percent of initial concentration). The data-points and the *unbroken curve* represent measured values (Demain 1957), and the *dashed curve* shows the expected remaining antibiotic calculated by dynamic simulation using the hydrolysis rate constants from Fig. 1 at 25 °C and with a pH-profile as given in Fig. 3

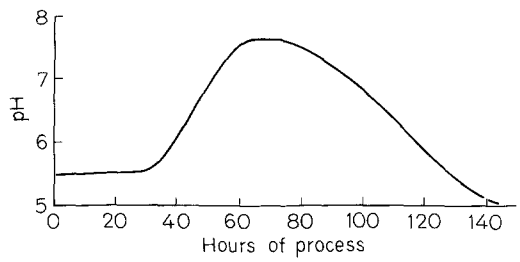

Fig. 3. pH-profile during shake flask penicillin formation

Demain (1957) measured the stability of penicillin G under actual conditions (i.e., in presence of producing cells) by adding labelled (deuterated) penicillin G to the medium before inoculation. The measured loss of labelled penicillin versus hours of fermentation is shown as the unbroken curve in Fig. 2. The pH-profile of such a shake flask fermentation (Demain, unpublished) is given in Fig. 3. Based upon this pH-profile and the data of Fig. 1, it was possible (by dynamic simulation) to calculate the loss of penicillin which would have occurred in buffer at the same temperature (25 °C) and with the same pH-history. This predicted loss is shown by the dashed curve in Fig. 2. The excellent agreement between measured and predicted hydrolysis suggests that the rate is only slightly, if at all, affected by the presence of the cells.

If penicillin has an inhibitory effect on its own synthesis, one should be able to reduce the biosynthetic rate by adding penicillin to the medium. However, in Demain's (1957) study on the inactivation rate of penicillin G, 1,740 units/ml deutero-penicillin G were added initially to the medium (about four times the antibiotic potency of the strain), yet no apparent decrease in penicillin synthesis was observed. The net production of unlabelled penicillin was 465 units per ml in six days, compared to a typical production of 450 ± 60 units per ml in the absence of added penicillin. This latter figure is an average of a number of repeated experiments.

To examine this further, we analyzed data published by Gordee and Day (1972). They added different amounts of penicillin to the medium and found that the net accumulation at the end of the six days cultivation (final concentration minus initial concentration) decreased with increasing increments added. From their data, they concluded that penicillin was regulating its own synthesis. Unfortunately, the added penicillin was not labelled in this study, and it was therefore not possible to distinguish between synthesized and added antibiotic. If we assume that the pH-profile (and accordingly the penicillin hydrolysis rate) in these experiments was similar to the pH-profile given in Fig. 3, then it is possible to calculate what the loss of penicillin (and thus the *actual* synthesis) might have been in this case. In this manner only 55% of the added penicillin should remain after the six day process. For example, if the strain produces 4 g l^{-1} in absence of added penicillin, then (provided no feedback regulation exists) the addition of 10 g l^{-1} penicillin at the beginning of the reaction would lead to a final concentration of 9.5 g l^{-1} (i.e., 0.55 · 10 g l^{-1} + 4 g l^{-1}), which is a net accumulation of −0.5 g l^{-1}.

We carried out such calculations on data from a series of experiments published by Gordee and Day. The results are shown in Table 1. The third column (A) gives the final titer actually measured. The fourth column (B) shows the predicted final titer assuming a pH-profile as in Fig. 3, a similar biosynthetic rate as in the control (3.85 g l^{-1} in six days) and no feedback effects. The last column gives the ratio of actual titer/predicted titer (A/B) for each run. Since the average value for this ratio is almost unity (0.98), it may be concluded that no feedback regulation has been demonstrated for this strain. The deviation from the control in terms of new synthesis also appears to be random and not correlated to the increments of penicillin added.

Another indication that feedback regulation was not significant involves the addition of labelled precursors by Gordee and Day. They added labelled cystine, valine and phenoxyacetate to the medium and measured the rate of label incorporation into penicillin V. They found that the incorporation rates did not decrease even after the addition of 10 g l^{-1} penicillin V to the broth. No satisfactory explanation for this observation was offered in the paper. We do not want to suggest that penicillin feedback regulation does not occur in *P. chrysogenum*. However, we want to emphasize that no clear evidence has yet been presented in the literature that the penicillin titer obtained is limited by feedback regulation. The gradual decrease in product accumulation rates typically observed during the process may therefore be explained by penicillin hydrolysis combined with the "aging" of the organism. Such "aging", or cell degeneration, has been attributed to the low specific growth rates obtained during batch culture and could be prevented during fed-batch or semi-continuous operation (Nestaas 1980).

Table 1. Effect of added penicillin G on final antibiotic titer. (Data from Gordee and Day 1972.)

Added penicillin G $g\,l^{-1}$	Time of addition (h)	A[a] Actual final titer ($g\,l^{-1}$)	B[a] Predicted final titer including hydrolysis ($g\,l^{-1}$)	A/B Ratio actual/predicted final titer
0 (Control)	–	3.85	3.85 (control)	(1.00)
1	0	4.34	4.41	0.98
5	0	5.88	6.66	0.88
10	0	10.65	9.47	1.12
1	66	4.95	4.57	1.08
5	66	6.50	7.48	0.87
10	66	10.96	11.13	0.98
15	66	15.03	14.78	1.02
10	[b]	10.72	11.51	0.93

[a] The values for *B* were predicted using penicillin hydrolysis data from Demain (1957) and assuming that new synthesis is not affected by the added penicillin (and always equal to the control; 3.85 $g\,l^{-1}$ in 144 h)

[b] Assumed linear rate of addition; 10/144 $g\,l^{-1}\,h^{-1}$

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