Effect of Growth Rate on the Synthesis of Penicillin by *Penicillium chrysogenum* in Batch and Chemostat Cultures

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The kinetics of penicillin production by *Penicillium chrysogenum* Wis 54-1255 in a glucose-limited chemostat and in batch cultures are reported. The specific production rate of penicillin, q\textsubscript{pen} (units per milligram of dry weight per hour) was independent of specific growth rate over the range 0.014 to 0.086 hr\textsuperscript{-1}. Growth was stopped by restricting the glucose supply to the "maintenance ration," that is, the glucose requirement of the organism at zero growth rate with all other nutrients in excess. Under such conditions, the organism dry weight remained constant, but the q\textsubscript{pen} fell approximately linearly to zero at a rate inversely related to the previous growth rate. Glucose supplied in excess of the maintenance ration inhibited the decay of q\textsubscript{pen}. At a critical growth rate between 0.009 and 0.014 hr\textsuperscript{-1}, the decay was completely inhibited. Quantitative expressions for the q\textsubscript{pen} of growing and nongrowing cultures were derived and used to predict the steady-state concentrations of penicillin accumulating in one- and two-stage continuous processes. A rational explanation of the kinetics of penicillin accumulation in batch cultures is given, relating the rate of penicillin synthesis to growth rate. It is concluded that an important role of corn steep liquor (CSL), a heterogeneous carbon and nitrogen source commonly used in penicillin production media, is the provision of substrates which allow a high concentration of mold to be reached before the growth rate falls below the critical value. CSL had no significant effect on q\textsubscript{pen}.

In the chemostat type of continuous culture, control of the growth rate of the organism, and hence of many other metabolic rates, is possible for indefinite periods. Thus, if the chemostat is to be used for a process such as penicillin production, a thorough understanding of the relationship between the process rate and growth rate is essential.

The production of penicillin by *Penicillium* spp., like the synthesis of other secondary metabolites, is thought to be dissociated from the growth of the organism. The antibiotic is produced at its maximal rate in batch cultures after an initial period of rapid growth of the mold. The initial period of rapid growth and a second phase in which little or no growth occurs are considered necessary to the accumulation of high concentrations of penicillin. From an analysis of batch culture data, Jarvis and Johnson (4) concluded that the specific rate of production of penicillin (units of penicillin per milligram of mycelial nitrogen per hour) was at its highest when the organism growth rate was close to zero.

Maxon (5) and Gaden (2) presented penicillin fermentation as an example of a class of microbial processes in which growth and product formation are temporally separated. However, the restriction of penicillin synthesis to the period after the rapid growth of the organism may not reflect a dependency of penicillin biosynthesis on slow or no growth. In most research work, batch cultivation with a variety of carbon and nitrogen sources is practiced. It is difficult to obtain meaningful information from such processes, because numerous, inter-related parameters such as pH, growth rate, and nutritional status vary simultaneously. In contrast to the observations made on batch cultures, penicillin is produced at a high rate in growing continuous cultures (1, 9, 10).

Because in a batch culture the organism is in a constantly changing environment, behavior exactly parallel to that observed in continuous culture cannot be expected. The adaptive response...
of an organism to a change in its environment is probably always subject to a time lag. In batch cultures, therefore, it is expected that the cell is continually adapting to a past environment, whereas a steady-state chemostat culture contains the fully adapted organism. A steady state represents the state to which a cell tends under a particular set of conditions, but in batch culture the state may never be attained because the conditions are not sufficiently prolonged. This being so, observations on continuous cultures are probably of greater value in the interpretation of nonsteady-state phenomena than the reverse. We have investigated the influence of growth rate on several aspects of penicillin synthesis in chemostat culture and have used the results as the basis of a rational explanation of the kinetics of penicillin accumulation in batch cultures.

**Materials and Methods**

**Apparatus and aeration.** Batch and chemostat cultures of 1.5- to 2.0-liter volume were carried out in a vessel adapted for cultivation of filamentous microorganisms (13). Aeration was by vortex stirring with air supplied at a few inches water gauge pressure above atmospheric pressure. Measurements with an oxygen electrode revealed that, with the highest oxygen demand, the culture medium was 70% saturated with oxygen.

**Penicillin production media.** Synthetic medium M4 consisted of (grams per liter): glucose, 2.3 × mycelial dry weight required; sodium phenylacetate, 0.05 × glucose concentration; K2HPO4, 6.00; NaH2PO4, 1.86; MgSO4·7H2O, 0.25; CaCl2, 0.05; ZnSO4·7H2O, 0.02; MnSO4·4H2O, 0.02; CuSO4·5H2O, 0.005; FeSO4·7H2O, 0.10; Na2SO4, 1.00; ethylenediaminetetraacetic acid, 0.60; (NH4)2SO4, 4.72. For corn steep liquor (CSL) medium, M5, filtered CSL replaced (NH4)2SO4 in M4 to give 1.5 g/liter of CSL nitrogen. Glucose, sodium phenylacetate, and phosphates were each sterilized separately.

P. chrysogenum Wis 54-1255 was maintained on agar slopes of the sporulation medium of Moyer and Coghill (6). Spores were produced on moistened bran cultures (14). Approximately 2 × 10⁷ conidia were inoculated into 200 ml of germination medium (4) and incubated on a rotary table shaker at 25 C for 32 hr. After inoculation of the fermentor, the culture volume was brought to 1.5 liters with fresh medium. Temperature was controlled at 25 C; pH was controlled at 7.0 ± 0.1 by automatic addition of 2 N NH₄OH or 2 N H₂SO₄. Foaming was inhibited by the addition of 0.1 ml/liter medium of polypropylene glycol (P2000; Dow Chemical Co. (UK) Ltd., London, England). The air flow was adjusted such that the carbon dioxide content of the effluent air was 1 to 3%. Glucose and phenylacetate were metered into the culture independent of the rest of the medium. Glucose was the growth-limiting nutrient in M4 and M5, up to at least 20 g/liter of mycelium (dry weight). The organism concentrations used were in the range of 4 to 14 g/liter (dry weight).

Corrections were made for changes in volume due to addition of nutrients and control agents and for evaporation.

A steady state was considered to have been obtained in continuous cultures when samples taken over at least two mean residence times showed no trend in the observed parameters. To avoid the strain degeneration observed by Pirt and Callow (10) on prolonged continuous cultivation, experiments were terminated after throughputs of not more than 12 culture volumes. No evidence of strain degeneration was obtained in these short continuous cultures.

**Analytical methods.** The mycelial dry weights in 10-ml samples of culture were measured after filtration and washing twice with 15 ml of distilled water and drying at 105 to 110 C to constant weight. Penicillin was assayed by a cup method with Bacillus subtilis as the assay organism and sodium benzyl penicillin as standard.

**Results**

To establish the relationships among glucose utilization rate, growth rate, and penicillin production, a glucose-ammonia-salts medium (M4) with glucose as the growth-limiting nutrient was used. The concentration of ammonium ion was kept constant by addition of ammonia as the pH control agent.

Steady-state glucose-limited chemostat cultures were obtained at a number of growth rates between 0.023 and 0.086 hr⁻¹. Over this range, the specific production rate of penicillin (q_p; units per milligram of dry weight per hour) was approximately constant (Fig. 1). Thus, the steady-state penicillin concentration in single-stage chemostats (p₁; units/ml) rose as the reciprocal of the growth rate (μ₁):

\[ p₁ = \frac{x₁}{q_p \cdot 1/μ₁} \]

**Fig. 1. Relationship between the specific production rate of penicillin (q_p) and specific growth rate in glucose-limited chemostat cultures of Penicillium chrysogenum Wis 54-1255. Each point represents the mean of one steady state. The mean value is 1.53 ± 0.32 (p = 0.95).**
where $x_1$ is the organism concentration (milligrams of dry weight per milliliter).

At a dilution rate of 0.095 hr$^{-1}$, very slow washout occurred, indicating that the maximal specific growth rate was a little below this value. During washout, all substrates were in excess, and the $q_{\text{pen}}$ was 50 to 60% higher than under glucose-limited growth conditions.

Extrapolation of Fig. 1 suggests that no reduction of $q_{\text{pen}}$ would occur as the growth rate approaches zero. If the penicillin synthetic rate could be maintained at the level observed in the single-stage chemostat cultures when the growth rate was reduced to zero, very high titer would be obtained with the consumption of minimal quantities of substrates. Penicillin production by the nongrowing organism was investigated by stopping the flow of medium to steady-state chemostat cultures and supplying only glucose and sodium phenylacetate, the former as the maintenance ration. The maintenance coefficient (7) was found by extrapolation of the relationship between the specific growth rate and the specific utilization rate of glucose to zero growth rate (Fig. 2). It represents the organism's requirement for carbon and energy source for functions independent of growth rate. The supply of glucose only for maintenance is here termed the maintenance ration. As in the growing cultures, all other substrates were supplied in excess. The mycelial dry weight of cultures receiving the maintenance ration remained constant as long as the experiments were continued. The penicillin production rate did not remain at the value observed in growing cultures but fell approximately linearly to zero. Figure 3 shows the organism concentration and $q_{\text{pen}}$ of a typical culture during continuous growth and during a subsequent maintained, nongrowing phase. The individual estimates of $q_{\text{pen}}$ during the nongrowing phase were subject to considerable error owing to the difficulty of measuring accurately small titer changes at a high absolute penicillin concentration. In the absence of evidence of nonlinearity, a straight line was fitted through the $q_{\text{pen}}$ values; its slope is referred to as the "$q_{\text{pen}}$ decay rate." We have reported briefly that the growth rate of the continuous growth phase influenced the $q_{\text{pen}}$ decay rate in the subsequent nongrowing phase (11). This relationship is shown in Fig. 4; clearly, the higher the previous growth rate, the slower is the decay of penicillin synthetic activity. In these cultures, the $q_{\text{pen}}$ at any time ($t$) after the cessation of growth is given by

$$q_{\text{pen}}(t) = q_{\text{pen}}(0) - kt$$

where $q_{\text{pen}}(0)$ is the value of the $q_{\text{pen}}$ in glucose-limited chemostats and $k$, the $q_{\text{pen}}$ decay rate, is a constant dependent upon the previous growth rate of the mold.

Since the maintenance ration of glucose did not maintain the $q_{\text{pen}}$ at the value observed in growing cultures, higher glucose supply rates were investigated (Fig. 5). As glucose supplied at rates above the maintenance ration allowed growth to occur, frequent adjustment of the glucose feed pumps was necessary to keep the specific supply rate (grams of glucose per gram of dry weight per hour) constant. The highest glucose supply rate, 0.056 g of glucose per g of dry weight per hr, allowed a specific growth rate of 0.014 hr$^{-1}$ and completely inhibited the decay of $q_{\text{pen}}$. Thus, the range over which the $q_{\text{pen}}$ was about 1.5 can be extended to this lower limit. When the glucose supply rate was 0.038 g of glucose per g of dry weight per hr (specific growth rate = 0.009 hr$^{-1}$), the $q_{\text{pen}}$ fell.
penicillin on qpen, chemostat glucose-limited

The 95% confidence limits of each point are included. Symbols: ○, synthetic medium M4; ○, CSL medium M5.

Thus, it appears that a critical specific growth rate exists for steady-state penicillin synthesis, given by a glucose supply rate of between 0.038 and 0.056 g of glucose per g of dry weight per hr. Below the critical value, the qpen falls at a rate depending on the glucose supply and the previous growth rate of the organism; above it, the penicillin synthetic activity of the mold is independent of glucose supply and growth rate, provided glucose is the growth-limiting substrate.

The experimental system used here was unlike most penicillin fermentations, not only in the use of the chemostat principle, but also in the simplicity of the medium. The media most commonly employed for penicillin production contain CSL, a heterogeneous carbon and nitrogen source containing a variety of amino and other organic acids, sugars, trace elements, and other inorganic ions. Its adoption as a major medium component was a significant step in the history of penicillin production, giving considerably higher yields of the antibiotic. Part of the effect of CSL may be ascribed to its ability to buffer the fermentation around a neutral pH. In addition, the carbon and energy sources supplement others in the medium, allowing higher mold concentrations to develop. Our observations on fermentations carried out with glucose and lactose as the main carbohydrates have shown that CSL is particularly effective in reducing the diauxic lag between the utilization of the two sugars, presumably by supplying energy sources enabling more rapid synthesis of the new enzyme system.

The presence of CSL in numerous metabolic intermediates may enable it to modify metabolism in favor of penicillin synthesis. To test this hypothesis, the qpen of the mold was measured under the controlled conditions of the chemostat using the CSL medium (M5) and comparing it with the qpen observed on the synthetic medium (M4). No significant difference was observed in the qpen of organisms grown at 0.033 and 0.077 hr⁻¹, nor were the qpen decay rates observed after growth had stopped significantly different from those of synthetic medium-grown cultures (Fig. 4). It appears that CSL does not have a specific effect on the synthesis of penicillin by the mold. This conclusion is supported by the observations on batch cultures presented below.

The kinetics of penicillin accumulation in batch cultures on the synthetic and the CSL medium were investigated. Glucose was metered into these cultures at a constant rate and became limiting within a few hours of inoculation. The growth of the mold, the accumulation of penicillin, and qpen in one of each of these types of cultures is shown in Fig. 6. On the synthetic medium, the organism concentration increased in proportion to the glucose supply. Eventually, the growth rate (dx/dt) fell off as a greater proportion of the glucose was diverted to maintenance functions. The presence of CSL in the medium allowed more rapid initial growth, although glucose was supplied at the same rate to both cultures. This was a function of the carbon and energy sources present in the CSL; when they were exhausted, the growth rate fell to that supported by the glucose supply. Initially, the penicillin accumulation rate (dp/dt) increased until it reached a maximal value; then it remained constant for a time. The maximal linear penicillin accumulation rate was reached when the specific growth rate fell to about 0.03 hr⁻¹ (Table
Three important relationships between mycelial growth and penicillin synthesis have been observed. (i) In glucose-limited chemostat cultures, the specific production rate of penicillin, \( q_{\text{pen}} \), is independent of specific growth rate from just below the maximum to a very low growth rate (about one-eighth of the maximal growth rate). (ii) When the specific growth rate is decreased to below about one-eighth of the maximal value, the penicillin synthetic activity of the mold cannot be maintained. (iii) When the specific growth rate is decreased to below one-eighth of the maximal value, then the \( q_{\text{pen}} \) falls at a rate inversely related to the previous specific growth rate.

The relationship between growth rate and \( q_{\text{pen}} \) in glucose-limited chemostat cultures (equation 1) and between growth rate and \( q_{\text{pen}} \) decay after growth has stopped (equation 2) can be used to predict the steady-state concentrations of penicillin in single-stage and multistage chemostats (8). It is assumed that the behavior of the whole culture in the phases of the experiments in which growth was stopped by restricting the glucose supply to the maintenance ration was the same as that of a single element of culture passed from a growing first stage to a maintained second stage. Then, with the relationships described by equations 1 and 2, and by use of the expressions for the distribution of residence times in a chemostat culture, the penicillin titer in a maintained nongrowing second stage can be calculated.

The quantity of organism with a residence time of \( t \) hours is \( x_2 e^{-D_2 t} \), where \( D_2 \) is the dilution rate of the second stage and \( x_2 \) = the organism concentration.

The \( q_{\text{pen}} \) of the organism \( t \) hours after entry into the nongrowing stage is given by equation 2

\[
\frac{q_{\text{pen}}(t)}{q_{\text{pen}}(0) - kt}
\]

By summation from \( t = 0 \) to \( t = \phi \) (\( \phi \) being the age of the mold at which \( q_{\text{pen}} \) becomes zero), the penicillin titer increments in the second stage become

\[
\int_0^\phi x_2 e^{-D_2 t}(q_{\text{pen}}(t) - kt) \, dt = \left[ x_2 \frac{1}{D_2} e^{-D_2 t}(-q_{\text{pen}}(t) + kt + k/D_2) \right]_0^\phi
\]

Figure 7 shows the predicted penicillin concentrations for the observed values of \( q_{\text{pen}} \) decay rate (Fig. 4) for an organism concentration of 1 g/liter dry weight and second-stage mean residence times 0 to 200 hr. The intercept on the penicillin concentration axis indicates the titer obtained in the first stage. A test of these calcula-

**TABLE 1. Relationship between the linear accumulation of penicillin in batch cultures and the organism concentration and growth rate at its onset**

<table>
<thead>
<tr>
<th>Glucose supply rate (g per liter per hr)</th>
<th>Linear accumulation rate of penicillin (units ml/hr)</th>
<th>At onset of linear accumulation rate</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Mycelium dry wt (g/liter)</td>
<td>Specific growth rate (hr⁻¹)</td>
</tr>
<tr>
<td>Synthetic medium</td>
<td></td>
<td></td>
</tr>
<tr>
<td>0.34</td>
<td>10.5</td>
<td>4.8</td>
</tr>
<tr>
<td>0.67</td>
<td>14</td>
<td>6.3</td>
</tr>
<tr>
<td>CSL medium</td>
<td></td>
<td></td>
</tr>
<tr>
<td>0.33</td>
<td>24</td>
<td>11.4</td>
</tr>
<tr>
<td>0.67</td>
<td>21</td>
<td>9.5</td>
</tr>
</tbody>
</table>

1) Eventually, the penicillin accumulation rate fell to zero. The fall is explained by the decay of the penicillin synthetic system in the mold and not by inhibition by the medium. The difference between the initial rates of decay of the \( q_{\text{pen}} \) in the synthetic and CSL media are largely accounted for by the effect of the previous growth rate on the \( q_{\text{pen}} \) decay rate.
Fig. 7. Steady-state penicillin concentrations in the second stage of two-stage processes as a function of the mean residence time of the second stage supplied the maintenance glucose ration. Titers were calculated by the method given in the text by use of the \( q_{\text{pen}}(t) \) and \( q_{\text{pen}} \) decay rates observed for Penicillium chrysogenum Wis 54-1255 (Fig. 1 and 4) and an organism concentration of 1 g/liter of dry weight. Assuming a \( q_{\text{pen}} \) decay rate = 0.033 \( q_{\text{pen}} \) units/hr, first stage growth rate = 0.086 hr\(^{-1} \) (curve 1); = 0.053 hr\(^{-1} \) (curve 2); = 0.038 hr\(^{-1} \) (curve 3); = 0.023 hr\(^{-1} \) (curve 4); = 0.015 hr\(^{-1} \) (curve 5).

...tions is afforded by the results of Pirt and Callow (10) for penicillin production by \( P. \) chrysogenum Wis 54-1255 in a chain of two chemostats. The \( q_{\text{pen}} \) predicted from the data presented here for the second stage, when the maintenance ration of glucose is supplied to the second stage, is 0.64; Pirt and Callow (10) observed values of 0.6 and 0.7 at glucose supply rates a little above and a little below the maintenance ration, respectively.

At a growth rate close to the critical minimum for steady-state penicillin synthesis, high titers and maximal productivity can be obtained in a single-stage chemostat. However, to avoid strain degeneration, i.e., loss of penicillin synthetic ability observed on prolonged continuous cultivation, it may be necessary to use multistage cultures (8).

In the chemostat, there is constant selection for variants capable of utilizing the limiting substrate more efficiently, i.e., with a higher growth yield (weight of organism produced/weight of substrate consumed) and with a higher affinity for the substrate. Research may show that under some conditions loss of the ability to produce penicillin does not occur. These conditions may not be those that are optimal for the expression of the character. Pirt, Thackeray, and Harris-Smith (12) found that \( Pasteurella \) \textit{pestis} produced large amounts of certain antigens at 37 C but rapidly lost the ability.

However, at 28 C, although small quantities of antigen were produced, the ability to produce them was not selected against. This may be a common phenomenon, for when a gene is not expressed it will not affect the growth yield, and so the continuous culture selection mechanism will not be operative against it. It may be desirable, then, in the production of antibiotics and other compounds inessential to the growth of the organism, to operate a small first stage under conditions unfavorable to the expression of the desired character. Production would be in a second and subsequent stage operated under conditions optimal for the accumulation of the product.

In the case of penicillin production, the first stage might be sulfur or nitrogen-limited, and the second sugar-limited with a growth rate just above the minimum for steady-state penicillin synthesis.

Entry into the phase of linear increase in penicillin titer in batch cultures may be correlated with the minimal growth rate required for steady-state penicillin synthesis. A linear accumulation rate would be observed if the synthetic system ceased to be produced once the specific growth rate had fallen below a critical value.

The accumulation rate will depend on the mold concentration reached before the growth rate falls below the critical value. To achieve the maximal accumulation rate, most of the growth should occur at a rate above the critical value. The results show that the decay of the \( q_{\text{pen}} \) of nongrowing mold from chemostat cultures was slowest after growth at high rates and that the decay was inhibited by glucose supplied a little in excess of the maintenance ration. Therefore, in an optimal batch fermentation, an essential (though not sufficient) condition should be an initial fast growth phase to give a high organism concentration, followed by a phase of slow growth to minimize the \( q_{\text{pen}} \) decay rate. This type of fermentation was developed empirically early in the history of penicillin production by the adoption of CSL as a major medium component. In addition to its pH buffering capacity, CSL contains rapidly utilisable carbon and nitrogen sources which allow considerable growth at a high rate. Hence, an important criterion of the suitability of a batch of CSL for the penicillin fermentation should be its ability to support a high growth rate of the mold. The slow growth of the second phase was a result of the slow utilization of a substrate such as lactose and later an empirically chosen slow sugar feed rate (3).

The concept of “maintenance energy,” or more generally the “maintenance ration” of a nutrient.
was originally developed to account for the kinetics of carbon and energy source utilized by microbes. No real function of the maintenance requirement was demonstrated. The present work shows that the carbon and energy source maintenance ration is essential to stabilize the penicillin synthetic system and prevent its rapid decay in the absence of growth. Elsewhere (Righelato et al. in press), it is shown that the maintenance ration is an aid to the organism during conidiation.

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LITERATURE CITED