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Production of Actinorhodin by *Streptomyces coelicolor* A3(2) Grown in Chemostat Culture

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Abstract: *Streptomyces coelicolor* was grown in variously limited chemostat cultures and the specific rate of extracellular actinorhodin production ($q_{\text{actinorhodin}}$) was measured. The highest $q_{\text{actinorhodin}}$ values were observed in glucose- or ammonia-limited cultures, whereas almost no actinorhodin was produced in sulfate-, phosphate-, potassium-, or magnesium-limited cultures. The effect of the dilution rate on actinorhodin production was studied in glucose-limited cultures. It was found that $q_{\text{actinorhodin}}$ was highest at $D = 0.06 \text{ h}^{-1}$, which was well below the maximal D value tested (0.14 h^{-1}). This explains why, in batch cultures, actinorhodin production starts at the onset of the stationary phase. It was also found that the use of nitrilotriacetate instead of citrate as a chelating agent had a negative effect on actinorhodin production. © 1997 John Wiley & Sons, Inc. *Biotechnol Bioeng* 54: 577–582, 1997.

Keywords: *Streptomyces coelicolor* A3(2); actinorhodin; chemostat culture

INTRODUCTION

Many microbes excrete compounds (other than carbon dioxide and water, of course) into their growth environment. Well-known examples are fermentation products such as lactate, ethanol, propionate, etc., produced when organisms are grown under anaerobic conditions. However, also under aerobic growth conditions, many heterotrophic microbes carrying out respiration, such as *Klebsiella pneumoniae*, *Escherichia coli*, or *Pseudomonas* species, will overproduce certain metabolites if they are grown under carbon-excess conditions (Neijssel et al., 1993; Neijssel and Tempest, 1975). This type of metabolism, called overflow metabolism, can be observed most easily when these organisms are grown in chemostat culture. Extensive studies have shown that the nature of the growth limitation has a profound influence on the type of products that are excreted. The rate of product formation depends on the dilution rate of the chemostat

culture (i.e., growth rate of the organism), the culture pH value, and other factors. In many cases, a physiological explanation for the production of a particular compound can be given (Neijssel et al., 1993; Neijssel and Tempest, 1975).

Whereas overflow metabolism concerns primary metabolites, the synthesis and overproduction of another class of compounds, the secondary metabolites, has been studied mostly via genetic methods. The physiological significance of the production of these compounds is still unclear (Piepersberg, 1993; Vining, 1990), which makes the development of a production strategy based on physiological principles extremely difficult. It is therefore of interest to study the effect of the growth environment, such as that created in a chemostat culture, on the production of a secondary metabolite.

In view of the widespread interest in the genetic regulation of actinorhodin production by *S. coelicolor* we have chosen to study some physiological aspects of this phenomenon. Previous studies on the production of actinorhodin and undecyl-prodigiosin by *S. coelicolor* in batch cultures were carried out by Doull and Vining (1990), Hobbs et al. (1989, 1990), and Ozergin-Ulgen and Mavituna (1993). Doull and Vining (1990) concluded, from their studies, that a decrease of the growth rate was the trigger for actinorhodin production. Hobbs et al. (1990) found that actinorhodin was mainly produced in the stationary phase and that its production was sensitive to repression by ammonium ions and to a lesser extent by phosphate. Ozergin-Ulgen and Mavituna (1994), on the other hand, claimed that actinorhodin production was growth-associated.

Recently, Bystrykh et al. (1996) reported that depletion of glucose abolished pigment synthesis, whereas depletion of other nutrients (ammonium or nitrate, phosphate, or trace elements) led to pigment synthesis. The final culture pH value also had an effect on the production of extracellular pigment; this occurred only

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when the pH value was above 6. Finally, it was shown that the extracellular blue pigment was not actinorhodin, but its lactone form (γ -actinorhodin).

We grew *S. coelicolor* in chemostat culture in a chemically defined medium under various nutrient limitations and at different dilution rates, and studied the production of extracellular (γ -) actinorhodin. We also developed a method to prevent extensive biofilm and cell pellet formation.

MATERIALS AND METHODS

Strain

Streptomyces coelicolor M145 (received from D. Hopwood) was kept as a frozen spore suspension in glycerol at -70°C .

Medium

The minimal medium described by Evans et al. (1970) was used with the modifications shown in Table I. When required, nitrilotriacetic acid (final concentration 2 mM), instead of citrate (final concentration 2 mM), was used as chelating agent.

Preculture

One hundred fifty milliliters of Evans medium (glucose 5 g/L) or Evans-plus (Evans medium without glucose, but supplemented with yeast extract [2 g/L] and monosodium glutamate 100 mM), pH 7, in a conical 500-mL flask, was inoculated with a drop of frozen spore suspension and cells were cultivated at 28°C on an orbital shaker (300 rpm) for 2 to 6 days. The pelleted culture was used as an inoculum.

Chemostat Culture

A chemostat culture (usual volume 550 mL, temperature 28°C , pH 7) was inoculated with the preculture. The dilution rate was set at 0.05 h^{-1} , CO_2 was added at 1% to 5% to the inlet air (gas flow rate 200 mL/min), and the stirring rate was set at 300 to 500 rpm. After 1 to 2 weeks, the large cell pellets had disappeared and

the cells grew in a dispersed mode. At this time the stirrer was set at 1500 rpm and air was injected at 1 vvm (usually 500 mL/min), and the desired growth condition was chosen.

Culture Purity and Stability

Every steady state was checked microscopically daily for infection. At the end of each steady state, a sample of the culture fluid was plated onto Evans-plus agar and nutrient agar. There was no indication that, during the course of these studies, a mutant organism had been selected: all culture parameters were reproducible and the appearance of the colonies on the plates remained the same throughout the different experiments.

Analyses

Biomass concentration was measured as bacterial dry weight by the method of Herbert et al. (1971).

Glucose and metabolic products like pyruvate, acetate, succinate, lactate, formate, acetoin, gluconate, etc., were determined by HPLC (LKB, Bromma, Sweden) with an Aminex HPX 87H Organic Acids Column (Bio-rad, Richmond, CA) at a temperature of 65°C with 5 mM H_2SO_4 as eluent or an Aminex A28 column (Bio-rad) at 55°C with 0.4 M formic acid adjusted to pH 5.5 with ammonia as eluent, using a 2142 Refractive Index Detector (LKB) and SP 4270 Integrator (Spectra Physics, San Jose, CA).

Oxygen consumed and carbon dioxide produced by the cultures were determined by passing the gas from the fermentor through an oxygen analyzer (Servomex Series 1100, Crowborough, England) and carbon dioxide analyzer (Servomex IR Gas Analyser PA 404).

Actinorhodin was assayed spectrophotometrically by the method of Hobbs et al. (1990).

When a culture was in a steady state this was maintained for at least 5 consecutive days and samples were taken every day. Each nutrient limitation was studied at least twice. The data shown below are the average of all measurements obtained at a particular steady state. The standard deviation of all data presented here is maximally 5%.

RESULTS

It is well known that *Streptomyces* species tend to form cell pellets in the culture fluids and biofilms on surfaces during growth. This creates several difficulties for continuous cultivation of these organisms, because sensors (temperature, pH) and the walls of the culture vessel become quickly covered by biofilms, which makes culture control almost impossible. Because cell distribution in the culture medium is not uniform, calculation of culture parameters is difficult, because a reliable estimate of the metabolically active biomass is not possible.

Table I. Concentrations of the main nutrients in the medium in relation to the type of nutrient limitation.

Nutrient	Limiting conc.	Standard conc.
Glucose	5 g/L	20 g/L
Ammonium chloride	10 mM	100 mM
Sodium phosphate	1 mM	10 mM
Sodium sulfate	0.2 mM	2 mM
Potassium chloride	1 mM	10 mM
Magnesium chloride	0.125 mM	1.25 mM

Several methods to prevent this complication have been published. For instance, Roth and Noack (1982) used a stirring rate of 3000 rpm with a glass blade stirrer, whereas Hobbs et al. (1989) added polyanions such as agar, Carbopol, or Junlon to the medium to prevent the formation of aggregates. We found that addition of agar or Junlon to the medium hindered determination of the culture dry weight (data not shown). Moreover, the presence of these compounds could have an effect on the physiological state of the cells. Although a stirrer rate of 3000 rpm was possible in our fermentors, we decided against this method because the high shearing forces could lead to extensive cell damage and lysis. We therefore used a different approach, described in detail in the Materials and Methods section, which invariably led to well-dispersed growth of the organism after about 2 weeks. The culture could be maintained in a dispersed state for several weeks. There was no indication that mutant organisms arose during cultivation.

To study the effect of the growth environment on actinorhodin production *Streptomyces coelicolor* was grown in an aerobic chemostat culture at a constant dilution rate under different nutrient limitations. After a steady state was achieved, culture dry weight, oxygen consumption, carbon dioxide production, and metabolite production were measured. From these data specific metabolic rates and carbon balances were calculated (Table II).

In agreement with the results obtained with other organisms, such as *Escherichia coli* or *Klebsiella pneumoniae* (Neijssel et al., 1993; Neijssel and Tempest, 1975), the specific rates of oxygen consumption were lowest when the organisms were grown under carbon limitation and highest under potassium limitation. The data of the other limitations (nitrogen, phosphorus, and sulfur) were between these two extremes; the data for magnesium limitation are puzzling in that they were almost identical to those for carbon limitation. Close inspection of magnesium-limited cultures showed that the bacteria did not grow fully in suspension. Thus, the steady state dry weight might have been incorrectly estimated. The color of the culture was also clearly dif-

ferent from those of the other nutrient limitations: magnesium-limited cultures were bright red, whereas the other cultures were pink to dark violet in agreement with the actinorhodin concentration.

This organism did not show extensive overflow metabolism, because the only products that were excreted were lactate, formate, and acetate. It must be noted, however, that the carbon balances of the carbon-excess cultures were not 100%, so other products must have been present in the medium. Because the main purpose of this study was to investigate the production of actinorhodin, no further search for these other products was carried out.

Actinorhodin production rates were highest under nitrogen or carbon-limited growth conditions, whereas sulfate and magnesium-limited cultures showed the lowest rates. To study the effect of dilution rate on the production of actinorhodin, we chose to grow the organism under carbon-limited conditions (Fig. 1). The specific rates of glucose consumption, oxygen consumption, and carbon dioxide production followed the usual trend in that they increased with growth rate. There was, however, a deviation from the classical linear relationship between these growth parameters and the dilution rate: above $D = 0.09 \text{ h}^{-1}$ there was a sharp increase in the q_{O_2} and q_{CO_2} values, which was also noticeable in the q_{glucose} . The specific rate of actinorhodin production, on the other hand, followed a completely different pattern. It was low at dilution rates lower than 0.05 h^{-1} , peaked at $D = 0.06 \text{ h}^{-1}$, and declined to almost 0 at the highest dilution rate tested (0.128 h^{-1}).

In the standard culture medium for this study citrate was used as chelating agent. Because citrate could influence the production of actinorhodin by its chelating properties we investigated the effect of nitrilotriacetic acid, which has been used successfully by us previously. When the effect of dilution rate on actinorhodin production by glucose-limited cultures of *S. coelicolor* was studied (Table III), again a sharp peak of production was observed at $D = 0.064 \text{ h}^{-1}$. However, the specific rate of actinorhodin production was lower than in cultures with citrate (105 vs. 415 $\mu\text{g/g} \cdot \text{h}$).

Table II. Specific rates of substrate consumption and product formation, growth yields, and carbon balances of *Streptomyces coelicolor* A3(2), grown in aerobic chemostat culture under different nutrient limitations (dilution rate = 0.06 h^{-1} , 28°C , pH 7).

Limitation	DW (g/L)	$q_{\text{act.}}$	q_{glucose}	q_{O_2}	q_{CO_2}	Y_{O_2}	Y_s	C-bal
C	2.2	415	0.8	2.5	2.5	25	78	111
N	1.5	702	1.1	2.4	2.4	26	53	74
P	2.8	35	1.2	4.0	4.0	15	51	96
S	2.5	5	1.1	3.7	3.7	17	56	99
K	1.8	71	1.8	7.8	7.7	9	36	97
Mg	4.0	2	0.8	2.3	2.4	29	77	105

The specific rate of actinorhodin production ($q_{\text{act.}}$) is expressed in $\mu\text{g}/(\text{g dry weight} \cdot \text{h})$. The other specific rates (q values) are expressed in $\text{mmol}/(\text{g dry weight} \cdot \text{h})$. Growth yield (Y) is expressed in g dry weight/mol substrate consumed. The carbon balance is given in %C recovered.

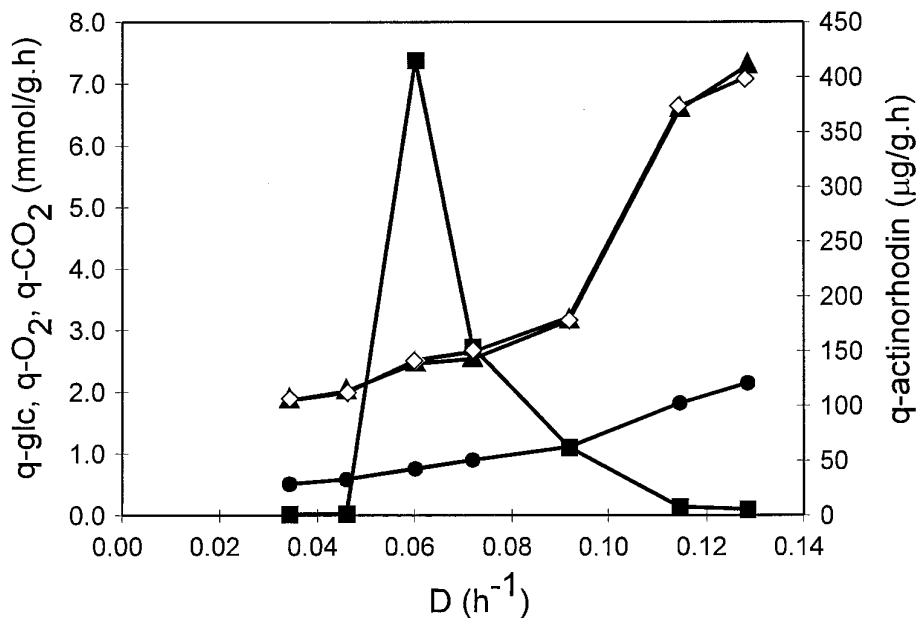


Figure 1. Effect of dilution rate (D) on the specific rates of glucose (●) and oxygen (▲) consumption and of carbon dioxide (◇) and actinorhodin (■) production by *Streptomyces coelicolor* A3(2) grown in carbon-limited chemostat culture.

DISCUSSION

Our method for growing cells in a dispersed state is relatively simple and gentle. It is probable that the initial pellets disintegrated or partly lysed and then formed small units that started to grow in a filamentous, micro-mycelial form. Microscopic observation indicated that these filaments were smaller, more spread out, and less compact than the pellets observed in the inoculum. This meant that the standard procedures for dry weight measurement could be used and reliable estimates of specific production and consumption rates could be obtained.

The relatively high rate of actinorhodin production observed in glucose-limited cultures was surprising, and showed that the physiology underlying the production of secondary metabolites is radically different from the production of primary metabolites. Excretion of primary metabolites in aerobic, carbon-limited cultures of heterotrophic organisms has rarely been observed (e.g., the production of ethanol by Crabtree-positive yeasts). The high rate of actinorhodin production in nitrogen-limited cultures is in agreement with the results of Doull

and Vining (1990) and Hobbs et al. (1990). The latter investigators found that ammonium chloride concentrations above 1 mM repressed actinorhodin synthesis. This explains the low rates of production of the other carbon-excess cultures (S, P, K, or Mg limitation) reported here. Hobbs et al. (1990) found that actinorhodin production was inhibited by phosphate concentrations greater than 24 mM. Such phosphate concentrations were never present in our media (Table I). When Hobbs et al. (1990) lowered the concentration of phosphate to 1 mM, the concentration of ammonium chloride at which the production of actinorhodin was inhibited increased from 1 mM to more than 50 mM. Except in nitrogen-limited cultures this higher ammonium chloride concentration would have been exceeded in our cultures, but our results with glucose-limited cultures indicate that actinorhodin production is not invariably inhibited by high ammonium chloride concentrations. In such cultures, the steady state ammonium chloride concentration must have been between 75 and 85 mM (the input ammonium chloride concentration was 100 mM and for every 1 g/L of dry weight approximately 10 mM ammonium is consumed). Thus, it seems that the synthesis of actinorhodin is repressed by ammonium ions, but that this repression is relieved when the carbon source is present at particular growth-limiting concentrations.

The effect of dilution rate on the rate of actinorhodin production is also different from the effects observed for primary metabolites. With primary metabolites the rate of production usually shows a linear relationship with the dilution rate. The peak of actinorhodin production at an intermediate growth rate explains the start of the production of actinorhodin at the beginning of

Table III. Specific rates of substrate consumption and product formation, growth yields, and carbon balances of *Streptomyces coelicolor* A3(2) grown in glucose-limited chemostat culture at different dilution rates.

D (h ⁻¹)	DW	q_{act}	$q_{glucose}$	q_{O_2}	q_{CO_2}	Y_{O_2}	$Y_{glucose}$	C-bal
0.04	1.7	2	0.6	1.9	1.8	19	64	101
0.06	2.5	105	0.7	2.3	2.1	29	86	109
0.16	2.2	4	2.0	4.9	4.6	32	76	92

Nitilotriacetic acid, instead of citrate, was used as chelating agent. See Table II for other units of measure.

the stationary phase in batch cultures (Bystrykh et al., 1996; Doull and Vining, 1990; Gramajo et al., 1993; Hobbs et al., 1990). In this growth phase, the growth rate of the cells rapidly declines from μ_{\max} to $\mu = 0$ and must therefore have passed the optimal value observed in our experiments. It is therefore likely that production of actinorhodin belongs to the so-called late growth events, such as the development of competence, sporulation, cell differentiation, etc. (Kolter et al., 1993).

Bystrykh et al. (1996) found that a stationary phase caused by a depletion of the nitrogen source (ammonium or nitrate), phosphate, or trace elements led to actinorhodin synthesis. The conclusions drawn from the research reported here are clearly different from those of Bystrykh et al. (1996): The nature of the growth-limiting nutrient seems to play a major role in the production of extracellular pigment. On the other hand, it has to be emphasized that it is almost impossible to compare data obtained with chemostat cultures with those derived from batch cultures. What our study shows is that continuous production of extracellular actinorhodin can be observed when the cells are growing in a specific growth environment. This is relevant in a biotechnological context: If the synthesis of other antibiotics is regulated in a way similar to that of actinorhodin, continuous and stable production is, at least in principle, possible and is therefore not confined only to time-dependent systems such as batch or fed-batch cultures.

Based on the use of the Luedeking–Piret model, Ozergin-Ulgen and Mavituna (1993) claimed that actinorhodin production was growth-associated. The data reported here show that actinorhodin is indeed produced by exponentially growing cells, but that growth rate is a dominant parameter. It is noteworthy that the kinetic parameters reported by Ozergin-Ulgen and Mavituna (1993) are very different from those found by our group. Their maximal growth rate was only 0.03 h^{-1} and the growth yield on glucose was 0.36 g/mol , whereas our data indicate a μ_{\max} above 0.16 h^{-1} and a yield on glucose of 41 to 86 g/mol . The use by Ozergin-Ulgen and Mavituna (1993) of a medium based on that of Hobbs et al. (1990), in which the nitrogen source is nitrate, could explain some of these differences, because our medium contained ammonium chloride as the nitrogen source. Nevertheless, the growth rate (0.06 h^{-1}) at which we observed a maximal rate of actinorhodin production was higher than the maximal growth rate observed by Ozergin-Ulgen and Mavituna (1993). In this connection it has to be emphasized that the production of actinorhodin in our culture system was a stable and reproducible property and we did not observe any indication of selection of mutants or loss of production when the culture was in a steady state, not even during prolonged cultivation (longer than 5 weeks) at different dilution rates.

The type of chelating agent used in the medium affected the rate of actinorhodin production, but, signifi-

cantly, again the rate of production peaked at $D = 0.06 \text{ h}^{-1}$. This indicates not only that the growth rate value is very important, but also that one should be careful with the choice of the chelating agent used in the medium. Chemostat experiments carried out by us with other organisms (e.g., *E. coli*, *K. pneumoniae*, or *Pseudomonas* species) never showed a negative effect of NTA on product formation.

To the best of our knowledge a sharp optimum of the specific rate of secondary metabolite production in chemostat cultures has not been observed previously. Roth and Noack (1982) found that turimycin production by *S. hygroscopicus* grown in a chemostat on a mineral medium was virtually absent under glucose or ammonia limitation. This was in agreement with previous observations that phosphate inhibits turimycin production (Gersch et al., 1979). Lee and Lee (1994) studied tylosin biosynthesis in ammonium-limited chemostat cultures of *Streptomyces fradiae*. They found that the specific rate of tylosin production increased linearly with the dilution rate up to a value of 0.05 h^{-1} . Between $D = 0.05 \text{ h}^{-1}$ and $D = 0.1 \text{ h}^{-1}$ the specific rate of tylosin production remained constant and declined again at $D = 0.13 \text{ h}^{-1}$. It was again observed that tylosin production was repressed by ammonium ions. In this respect, the findings reported here are clearly different, and they show that there is no general type of nutrient limitation that promotes the production of secondary metabolites.

In conclusion, we have shown that actinorhodin is produced by actively growing cells, in nitrogen- or carbon-limited cultures, and that, at least in carbon-limited cultures, the highest specific rate of production occurred at an intermediate growth rate.

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