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Effect of aeration on antibiotic production by *Streptomyces clavuligerus*

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SUMMARY

During the rapid growth phase of *Streptomyces clavuligerus* in a 10 litre fermentor, the level of dissolved oxygen (DO) was found to drop to almost zero for a period of approximately 10 h, delaying the appearance of and lowering the production of the antibiotic cephamycin C. Controlling the DO at either 50% or 100% throughout the fermentation did not significantly alter the specific growth rate of the culture, but did elevate final antibiotic levels two- and three-fold respectively. The improved oxygen availability affected antibiotic production both by increasing the rate of specific cephamycin C biosynthesis and by maintaining this higher rate throughout the production period. These results demonstrate that controlling dissolved oxygen levels close to saturation during periods of rapid growth markedly improves the efficiency and duration of cephamycin C biosynthesis in *S. clavuligerus*.

INTRODUCTION

In organisms that produce antibiotics after growth has ceased, the yield of secondary metabolites is influenced by the culture's previous growth history. Thus a high degree of oxygen transfer during the exponential growth phase may ultimately lead to improved antibiotic production. However, where antibiotic biosynthesis and rapid growth are taking place concurrently, and if both require oxygen as in cephamycin C production in *Streptomyces clavuligerus* [3], the much greater competition for oxygen could conceivably create severe dissolved oxygen (DO) deficiencies at a critical stage in antibiotic production.

Unfortunately, maintaining DO at a controlled level in industrial vessels during exponential growth requires constant monitoring and frequent adjustment of a number of fermentor parameters such as agitation speed, air flow rate, head pressure and the

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composition of the air intake. Moreover, localized changes in oxygen transfer rate in large fermentors can result in significant variations in dissolved gas concentrations [25]. Nevertheless, a study of the effect of these changing environmental factors on antibiotic production can be effectively carried out using small (5–15 litre) fermentors to ensure short liquid circulation times and constant environmental conditions. Indeed, small fermentor vessels have advantages over shake flasks in studying the effect of oxygen on antibiotic productivity, since the air flow rates and agitation speeds can be widely varied, permitting the DO level to be effectively monitored and controlled.

Small 7 litre fermentors have been used in an investigation of the role of DO in penicillin fermentations and established the importance of maintaining the DO tension above certain minimum levels for maximum productivity [22]. When DO was artificially cycled above and below this critical DO tension minimum for penicillin biosynthesis, a considerable decrease in specific penicillin production rate was noted, probably as a result of metabolic disturbance. However not all antibiotic fermentations are improved by maintaining DO at relatively high levels. Recently the effect of aeration and pH on the production of the antibiotic gramicidin S (GS) by *Bacillus brevis* has been studied in fermentors [21]. These workers determined that the optimum conditions for antibiotic formation were correlated with low DO tension levels during growth, and since high oxygen concentrations are known to accelerate the inactivation of GS synthetase [2,7], the low DO tension levels probably improved the efficiency of antibiotic production through an increase in GS synthetase stability.

Previous studies with small fermentors have revealed the importance of controlling aeration rates and pH in improving antibiotic productivity, and in view of the fact that several cephamycin C biosynthetic enzymes are known to be oxygen-requiring [1,20], we decided to investigate the effect of fluctuations in DO level on the biosynthesis of the antibiotic cephamycin C by *S. clavuligerus*.

MATERIALS AND METHODS

Microorganism and medium

S. clavuligerus NRRL 3585 was grown on tomato-oatmeal agar (TOA) [10] at 28°C for spore production. For fermentor culture, a modified trypticase soy broth [12] supplemented with soluble starch and trace elements (MTCS/S) was used for spore germination in shake flasks and growth in fermentors; tryptone (Difco), 17 g; soy peptone (Scott), 3 g; NaCl, 5 g; K_2HPO_4, 1.25 g; soluble starch (Difco), 10 g; 1 ml trace elements stock; $FeSO_4 \cdot 7H_2O$, 0.1 g; $MnCl_2 \cdot 4H_2O$, 0.1 g; $ZnSO_4 \cdot 7H_2O$, 0.1 g; $CaCl_2 \cdot 2H_2O$, 0.13 g; in 100 ml, per litre double-distilled water. The pH was adjusted to 6.8 with HCl prior to autoclaving.

Cultivation

(a) Seed culture preparation. A single lot of *S. clavuligerus* NRRL 3585 glycerol spore stocks was prepared and used for all the fermentor cultures. Spores were obtained using the modified method of Hopwood et al. [11]. The spores were scraped from an agar plate, resuspended in 20% glycerol in a sonicating bath, dispensed in 0.5–1.0 aliquots into vials, and kept frozen at −70°C.

Spores were incubated in 100 ml MTCS/S in 500 ml Erlenmeyer flasks at 28°C and shaken at 250 rpm until there was approximately 50% germination. This was determined by microscopic examination and the germination period was typically 6 h. If more than one fermentor was used, the germinated spore cultures were pooled first before distribution to the 10 litre fermentors. Spore counts were determined using an AO hemacytometer and the fermentor inoculation volume was adjusted to achieve a final spore density of 9×10^4 spores/ml.

(b) Growth in fermentors. S. clavuligerus NRRL 3585 was grown in MTCS/S in Microferm 10 litre fermentors equipped with a New Brunswick Scientific pH controller, with a six station stepper and Marubishi DO monitors. Each fermentor was equipped with Ingold combination pH electrodes and New Brunswick Scientific galvanic DO probes.

The pH probes were standardized against an external reference system while the DO probes were set to 100% saturation against water (400 rpm, 6 litres air/min) before inoculation. The pH was controlled by the addition of 6 N HCl and 2 N NaOH. The DO was controlled to 100% by periodic manual adjustment of a pure oxygen feed into the fermentor airstream. Control of DO to 50% was achieved with a balance of pure oxygen and nitrogen feeds into the airstream. Cultures were grown at 28°C ± 1°C, with an impeller speed of 400 rpm, and 6 l/min of air. Pure oxygen flow rates never exceeded 4.0 l/min. Foaming was controlled by periodic additions of polypropylene glycol 2000 to all fermentors (maximum final concentration 0.02%).

Cell-free extracts and analytical methods

Cell-free extracts were obtained by sonication [12]. Varying volumes of cells (10–100 ml) were filtered through Whatman No. 1 filter paper. The filtrate was retained for antibiotic assays and the cells were washed with 50 mM Tris-HCl, pH 7.0 containing 0.1 mM dithiothreitol and 0.01 mM EDTA (TDE buffer). The washed cells were resuspended in 5 ml TDE buffer and broken by sonication. Supernatant and cell extract samples were stored at −20°C and only freshly thawed samples were assayed after clarification by centrifugation. Duplicate protein assays on the cell-free extracts were carried out using the Biorad reagent and the method of Bradford [5], bovine serum albumin being used as standard. Production of β-lactam compounds (penicillin N and cephamycin C) during the fermentation was followed by bioassay in triplicate, using the super-sensitive *Escherichia coli* strain, Ess [13]. The amount of penicillin N produced was estimated from the reduced total antibiotic activity resulting from inclusion of penicillinase in the bioassay agar, and cephamycin C levels were taken as the penicillinase-stable portion. Antibiotic units per ml culture were standardized against purified penicillin N and cephalosporin C, and specific antibiotic units were expressed as μg antibiotic per mg protein.

RESULTS

Inoculum preparation

Optimal antibiotic production in batch fermentors was obtained by germinating spores of *S. clavuligerus* for 6 h in MTCS/S medium at a density of 2.7×10^5 spores per ml and inoculating the fermentors at a spore concentration of 9×10^4 spores per ml.

Effect of agitation speed and air flow rate on DO levels

At 400 rpm and 6 litres air/min, DO levels in growing *S. clavuligerus* cultures fell close to zero for approximately a 10 h period during rapid growth of *S. clavuligerus* in the batch fermentor (Fig. 1). In an attempt to prevent these rapid fluctuations in DO level, a number of fermentations were performed at different agitation speeds and air flow rates, and the subsequent effect on DO levels was determined. However, even with maximum agitation (750 rpm) and air flow rate (16 litres air/min), DO could not be prevented from falling to almost zero (data not

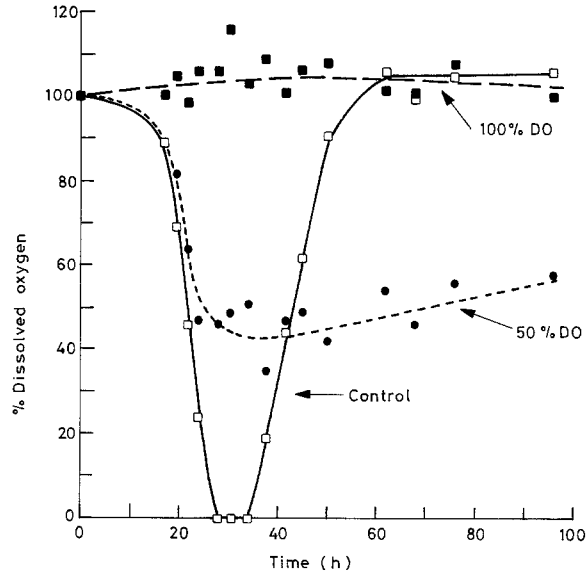

Fig. 1. Profile of DO level during batch fermentations under no DO control (□), with DO maintained at 50% (●), or with DO at saturation (■).

shown). Moreover, significant foaming problems were encountered at the higher agitation speeds, and these could only be controlled by lowering the speed, reducing the air flow rate and adding polypropylene glycol 2000 (maximum final fermentor concentration only 0.02%). Control of DO level was achieved instead by slowly feeding pure oxygen into the air stream (maximum flow 2 l/min). This method was capable of maintaining the DO at 100% even during the periods of greatest oxygen demand, such as exponential growth (Fig. 1). To maintain DO at 50% during certain fermentations, pure nitrogen was introduced into the oxygen-enriched air stream.

Effect of DO on cephamycin C production

The effects of fluctuating and controlled DO levels on cephamycin C production were examined during separate runs in 10 litre fermentors (Fig. 2). Samples for antibiotic determinations were taken at regular intervals, assayed in triplicate and, at selected times, quintuplicate samples were assayed. Fermentations were carried out at least three times under each DO condition with essentially identical results.

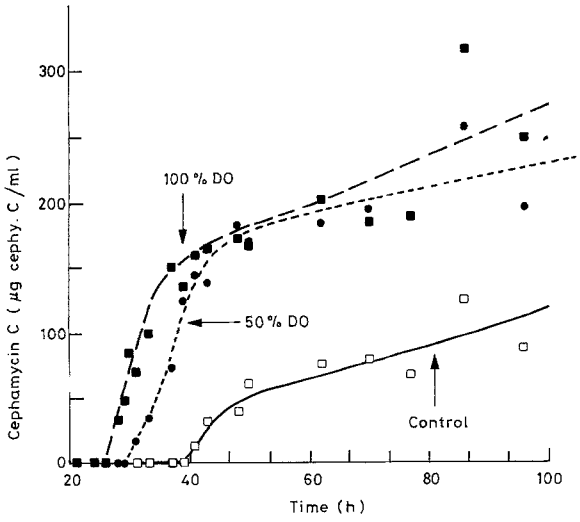

Fig. 2. Effect of DO level on cephamycin C production during batch fermentations. Cephamycin C production under no DO control (□), under DO controlled at 50% (●), and under oxygen saturation conditions (■).

Without DO control, increased oxygen demand by rapidly growing cells lowered the level of DO to zero for approximately 10 h. No antibiotic was produced until the DO level recovered to about 50% at 39–40 h (Figs. 1 and 2), which corresponds to the end of the growth phase (data not shown). Under these conditions the final cephamycin C level was around 100 μg/ml. In comparison, when the DO level was controlled to 50% throughout the fermentation, cephamycin C production began approximately 10 h sooner and the final level reached 200 μg/ml. Growth under oxygen saturation conditions produced cephamycin C levels approaching 300 μg/ml with production being initiated earlier, 25 h after inoculation (Fig. 2).

Increasing the agitation rate alone under uncontrolled DO conditions did not alter the final cephamycin C levels, indicating that antibiotic biosynthesis was indeed oxygen-limited and did not suffer from lowered mass transfer rates between culture liquid and cells.

Effect of DO on specific cephamycin C production

The specific production of cephamycin C under different DO conditions was determined as a measure of the antibiotic productivity of the fermentation. When DO was not controlled, antibiotic production first occurred after 39 h in the fermentor, and specific cephamycin C production increased linearly until approximately 60 h (Fig. 3). Thereafter, specific production was markedly reduced so that during the remainder of the fermentation there was only a marginal increase in specific cephamycin C levels. In contrast, when oxygen was maintained at 50% saturation during the batch fermentation, specific cephamycin C production began 9 h sooner, and increased faster than in the fermentor without DO control. In the 100% oxygen-saturated fermentation, specific production began 15 h sooner than in the fermentor without DO control and increased faster than in either the uncontrolled or 50% controlled DO fermentations. With improved oxygenation, specific cephamycin C production increased linearly throughout, unlike the fermentation without DO control (Fig. 3).

Finally, specific antibiotic production was in-

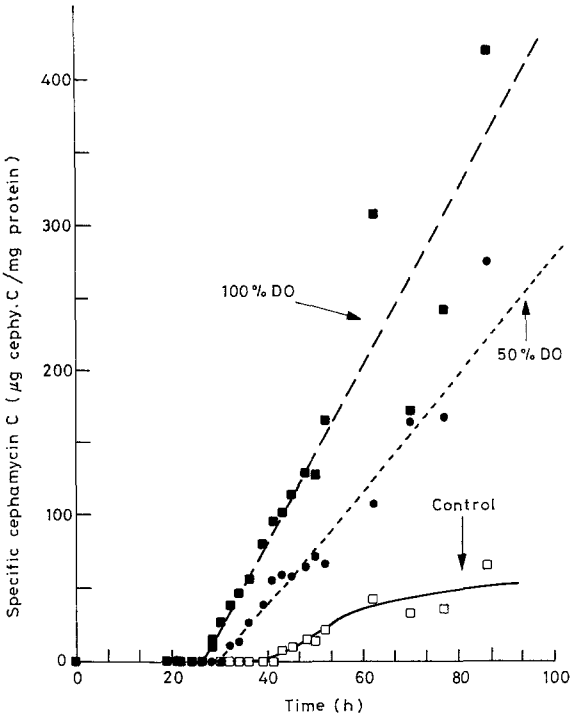

Fig. 3. Effect of DO on specific cephamycin C production during batch fermentations. Specific production (μg cephamycin C/mg protein) under no DO control (□), under 50% DO (●), and under DO controlled at 100% (■).

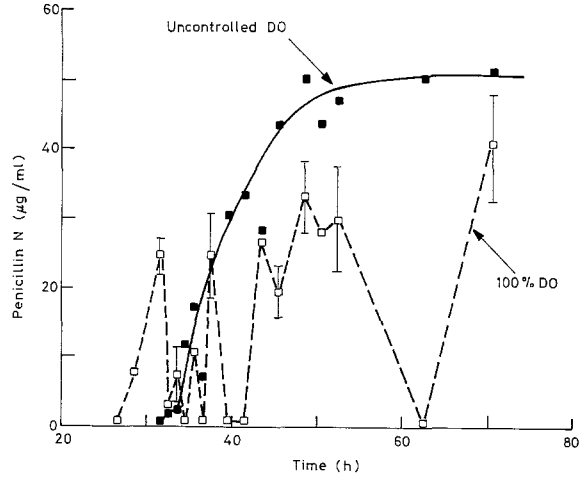

Fig. 4. Changes in penicillin N level (μg/ml culture) during DO saturated batch fermentation (□), and fermentations without DO control (■). Standard errors were calculated and are shown for each set of assays (× 5) from the DO saturated fermentation, except where error bars were smaller than the graph point.

creased three-fold under oxygen saturation, as compared to when DO was not controlled. In contrast, the specific growth rate and the maximum biomass (protein) produced were not affected by changing the oxygenation conditions (Table 1).

Penicillin N production during oxygen-saturated fermentations

Batch fermentations under oxygen-saturated conditions produced the highest levels of cephamycin C, with the antibiotic steadily accumulating over the 100 h of the fermentation (Fig. 2). Levels of the pathway transitory intermediate penicillin N, however, fluctuated widely between zero and about 50 μg/ml over the same period (Fig. 4). The changes in penicillin N levels took place despite the relatively constant environmental conditions existing in the DO controlled fermentation. In contrast, during fermentations without DO control, or DO controlled at 50%, penicillin N accumulated rapidly during growth and remained at fairly constant levels throughout the remainder of the production period (Fig. 4).

Table 1

Comparison between specific growth rates and the rate of specific cephamycin C production for different DO conditions during batch fermentations

DO condition	No DO control	50% saturated	100% saturated
Specific growth rate (μ)	0.075	0.075	0.076
Rate of specific cephamycin C production (μg/mg/h)	2.0	4.25	5.95
Maximum biomass (protein) (mg/ml)	1.905 ± 0.035	1.928 ± 0.014	2.028 ± 0.050

DISCUSSION

In recent years increasing emphasis has been placed on the genetic modification of antibiotic-producing strains in an effort to improve the efficiency of antibiotic biosynthesis. Unfortunately, less attention has been paid to the fermentation vessel design and process control necessary to best exploit this increased production potential. This is surprising since it has been established that the nutritional condition of cells during early growth is often critical in determining antibiotic yield. In addition, the rate at which growth is allowed to proceed also should be considered. Low growth rates have been shown to influence a number of different aspects of the production of many antibiotics, including bacitracin [9], gramicidin S [16], patulin [8], and cephamycin C [15]. Unfortunately, in these studies the reduction in growth rates was typically achieved by nutrient limitation and although the type of nutrient was not important, it was unclear whether antibiotic production was not simply the result of relief from catabolite repression alone. At least in the case of thienamycin production by *S. cattleya,* a specific phosphate deficiency does appear to be required in addition to the low growth rate [15].

The relationship between growth rate and antibiotic production can also be investigated by lowering the oxygen availability and interrupting oxidative metabolism. In *B. brevis* fermentations, reducing the aeration rate resulted in only linear growth as oxygen became the limiting nutrient and yields of the antibiotic gramicidin S were vastly increased [21]. It has been suggested that maintaining low DO tension levels forces these cells into 'transient growth' kinetics, with subsequent enhanced production of GS synthetase and gramicidin S [14] and increased tyrothricin biosynthesis [19]. Conversely, improving the oxygen availability during gramicidin S fermentations leads to higher growth rates and lowered antibiotic levels [21]. Interestingly, oxygen enrichment can also decrease the growth rate, as in the case of *B. licheniformis,* with the subsequent enhanced production of the peptide antibiotic, bacitracin [6]. In *S. clavuligerus,* we have found that elevating DO levels to 50% or 100% throughout the batch fermentation does not significantly alter the specific growth rate but does improve the rate of specific cephamycin C production, demonstrating that in this organism grown under non-nutrient limitation conditions, high rates of specific antibiotic production are not dependent on changes in specific growth rates.

During rapid growth of *S. clavuligerus,* DO fell quickly to zero and remained at that level for over 10 h. Although this interruption delayed cephamycin C production and final levels were reduced, antibiotic biosynthesis nonetheless recovered once growth slowed and DO levels began to rise again. However, the rate of specific cephamycin C production was much lower under these conditions than the rate in the oxygen-saturated fermentations and an even further decrease in this slow rate took place after about 20 h production. Thus, improved oxygenation elevated cephamycin C production during *S. clavuligerus* fermentations by supporting higher rates of specific production which were maintained relatively unchanged throughout the fermentation. It is possible that reduced oxygenation limited the availability of antibiotic precursors in a manner similar to that observed during the regulation of penicillin production by glucose catabolite repression in *P. chrysogenum* [17]. The penicillin biosynthetic pathway branches from the lysine pathway at the α-aminoadipate stage and oxygen limitation may result in a greater proportion of this precursor being directed through the lysine pathway into proteins, instead of to the first enzyme in the cephamycin C pathway, ACV synthetase. Alternatively, oxygen starvation may reduce the level of individual amino acid pools, which may in turn control cephamycin C biosynthesis in *S. clavuligerus* [4]. For instance, methionine has been reported to stimulate ACV synthetase [26], as well as the cyclase and expandase enzymes in *Cephalosporium acremonium* [18].

The level of the pathway intermediate penicillin N fluctuated as it was alternatively accumulated and processed through to cephamycin C during the batch fermentation under oxygen-saturated conditions. This observation is difficult to reconcile with

maximum pathway efficiency, which would require a smooth, rapid transition from penicillin N to cephamycin C. However, recent research on the biosynthesis of the antibiotic tylosin from *S. fradiae* suggests that under cyclic fed-batch conditions, the highest specific tylosin production rates were associated with fluctuations in the activities of the antibiotic biosynthetic enzymes [24]. Our results would seem to support their suggestion that the optimization of secondary metabolite production may involve a natural induction/repression cycling of biosynthetic enzymes [23], which could result in oscillations in biosynthetic intermediates such as penicillin N.

Clearly, maintaining oxygen saturation conditions during *S. clavuligerus* fermentations is a relatively simple technique to improve the rate and duration of cephamycin C biosynthesis. However, in large-scale fermentation vessels under DO control, localized depletion in oxygen levels and mass transfer difficulties can be expected. Thus, although our results demonstrate that cephamycin C biosynthesis can recover from relatively long periods of oxygen starvation, in large fermentors the culture will likely experience short, repeated interruptions in oxygen supply, and perhaps additional small-scale fermentations are necessary to accurately reflect the conditions found in industrial vessels.

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