THE EFFECT OF DISSOLVED OXYGEN CONCENTRATION CONTROL ON CELL GROWTH AND ANTIBIOTIC RETAMYCIN PRODUCTION IN *Streptomyces olindensis* So20 FERMENTATIONS

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Abstract - Cell growth and retamycin production in *Streptomyces olindensis* So20 were studied at different dissolved oxygen levels during fermentation. The profiles for cell growth and retamycin production in an experiment with dissolved oxygen control at 5% air saturation during production were similar to those in an experiment without dissolved oxygen control. However, despite slower cell growth, a twofold increase in the final retamycin concentration was achieved when dissolved oxygen was controlled at 100% during the growth phase, compared to a base experiment (no DO control). Therefore, the results revealed that retamycin production was dependent on dissolved oxygen concentration during the growth phase, even with sufficient oxygen. On the other hand, a large oxygen supply during the production phase did not contribute to an improvement in retamycin production.
Key words: *Streptomyces*, antibiotic, retamycin, oxygen transfer.

INTRODUCTION

Retamycin is an anthracycline complex produced by the wild (DAUFPe 5622) and the mutant (So20) strains of *Streptomyces olindensis*. In experiments with Walker and Yoshida sarcomas, considerable tumor inhibition occurred (Cotias et al., 1971), and positive results in the treatment of human leukemias demonstrate its potential as an anti-leukemia drug (Asfora et al., 1972).

S. olindensis So20 fermentations carried out in 10 L bioreactors by Martins (2001) showed that any combination of   specific aeration rate (φ) and rotational impeller speed (N), able to guarantee sufficient oxygen for the microorganism in the culture medium, can support good cell growth and retamycin production. However, for some antibiotics, production is dependent on dissolved oxygen concentration (DOC) in the culture medium, even under oxygen-sufficient conditions for the microorganism. In *Streptomyces clavuligerus* fermentations, Yegneswaran et al. (1991) observed an approximate threefold increase in the maximum cephamycin C yield, when dissolved oxygen was controlled at air saturation levels (100%) during the growth phase, compared to experiments without dissolved oxygen control, where DOC remained above 35% saturation throughout. Positive effects of high DOCs on virginiamycin M (Shioya *et al.*, 1999) and tylosin (Chen et al., 1990) production were also observed.

On the other hand, Kempf et al. (1997) observed

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that low DOCs (below a critical concentration) during the production phase improved gallidermin production in *Staphylococcus gallinarum* fermentations. An increase of 50% in gallidermin yields was obtained through a process strategy that allowed biomass formation in oxygen at saturation levels and antibiotic production during the stationary phase with limitation of oxygen.

Thus, the present paper aimed at studying the effect of two different DOC control modes on cell growth and retamycin production in *S. olindensis* So20 fermentations.

MATERIALS AND METHODS

Microorganism and Culture Medium

Streptomyces olindensis So20, a mutant strain, was maintained in cryotubes as 10 mL aliquots of vegetative mycelia in glycerol 20% (v/v) at −20 °C. This stock culture was prepared by submerged cultivation on a rotary shaker, using R5 modified medium (R5Mod).

The R5Mod medium (Hopwood et al., 1985) has the following composition in g.L^{-1}: glucose 10.0; K_2SO_4, 0.25; yeast extract, 5.0; casein hydrolysate, 0.10; $MgCl_2.6H_2O$, 10.12 and tris(hydroxymethyl) aminomethane, 3.09. After sterilization at 120°C for 20 minutes, the medium was complemented with the following solutions, which were sterilized separately (per 250 mL): trace elements, 0.5 mL; KH_2PO_4 (0.5% w/v), 2.5 mL and $CaCl_2$ (5M), 1.0 mL. The composition of the trace elements solution (in 1000 mL) was 40 mg $ZnCl_2$, 200 mg $FeCl_3.6H_2O$, 10 mg $CuCl_2.2H_2O$, 10 mg $MnCl_2.4H_2O$, 10 mg $Na_2B_4O_7.10H_2O$ and 10 mg $(NH_4)_6Mo_7O_{24}.4H_2O$.

Pre-Inoculum and Inoculum Preparation

Flasks of 1,000 mL containing 90 mL of the culture medium were inoculated with 10 mL of frozen mycelium in glycerol 20% (v/v). The flasks were incubated at 30 °C, 200 r.p.m., for 16 hours on a rotary shaker (pre-inoculum). Afterwards, 1,000 mL flasks, containing 180 mL of the culture medium, were inoculated with 20 mL of the pre-inoculum and cultivated on the rotary shaker for 24 hours at 30 °C, 200 r.p.m. (inoculum).

Fermentation Apparatus and Culture Conditions

The fermentations were conducted in 15 L Biolafitte bioreactors with a working volume of 10 L. The bioreactors, containing 9 L of R5Mod medium, were inoculated with 1 L of the inoculum, prepared as described previously.

Each reactor was equipped with a dissolved oxygen electrode (Mettler Toledo Ind. e Com. Ltda, São Paulo, Brazil) and mass-flow controllers (Type 5850E, Brooks Instrument, Pennsylvania, USA). The composition of the exhaust gas was monitored with a gas analyzer (News Brunswick Scientific Co Inc., New Jersey, USA) consisting of an infrared analyzer for carbon dioxide and a paramagnetic analyzer for oxygen. All the data above were transmitted to a computer for data acquisition at five-minute intervals and DOC control using a program written in LabView (National Instruments, Texas, USA), a graphics programming language for process automation. For DOC control, gas mixtures of air and nitrogen or air and oxygen were used. The total gas flow rate was kept constant and the proportion of nitrogen/oxygen to air adjusted so that the required DOC was maintained.

Four batch cultures were conducted for 48 hours at 30ºC, pH 7.0 and 500 r.p.m. Table 1 summarizes the characteristics of each experiment.

Table 1: Specific aeration rates (φ) and modes of DOC control for group I and group II experiments.

Group I		
Exp.	**φ (v.v.m.)***	**DOC**
E-10	1.0	without DOC control
E-10-C	1.0	Growth: no control Production: control at 5%
Group II		
Exp.	**φ (v.v.m.)***	**DOC**
E-2	0.2	without DOC control
E-2-C	0.2	Growth: control at 100 % Production: control at 5%

(*) volume of gas per volume of medium per minute

ANALYTICAL METHODS

Samples were taken at regular intervals to determine biomass concentration (X), residual glucose concentration (S) and extracellular (R_{extra}) and intracellular (R_{intra}) retamycin concentration.

The biomass concentration (X) was evaluated as dry cell mass in the following manner: up to 16 hours of culture the mycelium was harvested by vacuum filtration and dried in a microwave oven (180 W) for 15 minutes; after 16 hours, due to difficulties in filtration of the broth, the mycelium was harvested by centrifugation for 20 minutes at 7,300 g at 5 °C, washed with distilled water, filtrated in vacuum and dried at 85 °C for 6 hours. The residual glucose concentration (S) in the culture medium was obtained by the enzymatic method employing glucose-oxidase.

The extracellular and intracellular retamycin concentrations were obtained by spectrophotometry at 547 nm (wavelength corresponding to the purple color of the product). For determination of the extracellular retamycin concentration, the filtrate or the supernatant obtained from the cell mass separation was used, adjusting the pH to 6.3 with HCl 0.2N or NaOH 0.2N. In order to obtain the intracellular concentration, a culture broth sample was centrifuged at 7,300 g and 5 °C for 20 minutes. The harvested mycelia were then washed with distilled water, centrifuged once more, resuspended in methanol (pH 6.3) for 2 hours and the intracellular antibiotic was finally extracted in an ultrasonic bath for 15 minutes.

Both retamycin concentrations were determined with a calibration curve obtained by the Laboratory of Natural Products Chemistry (Instituto de Química da Universidade de São Paulo) using solutions of partly purified retamycin in methanol at different concentrations. The total retamycin concentration, which corresponds to the sum of the extracellular and intracellular concentrations, is depicted in the figures and expressed relative to its maximum value obtained for each group of experiments.

The volumetric ($Q_{O2}X$) and the specific (Q_{O2}) oxygen uptake rates were determined using the gas balance method (Schmidell et al., 2001)

RESULTS AND DISCUSSION

Group I

This group studied the effect of low DOC during the production phase on retamycin production.

Figure 1 shows the DOC profiles for group I experiments. Both of them were carried out at 1.0 v.v.m. throughout; in experiment E-10-C, the control at 5% saturation, brought about by supplementing air with a nitrogen stream, was initiated 17 h from the start of the culture, when the beginning of retamycin production was visually detected.

As illustrated in Figure 2, biomass concentration (X) reached 4.43 and 4.27 g L^{-1} in experiments E-10 and E-10-C, respectively, at about 36 h, when the glucose had been used up. In addition, similar values of cell productivity (P_x), about 0.12 g L^{-1} h^{-1}, were achieved, indicating that the DOC control at 5% saturation in experiment E-10-C did not interfere in cell growth.
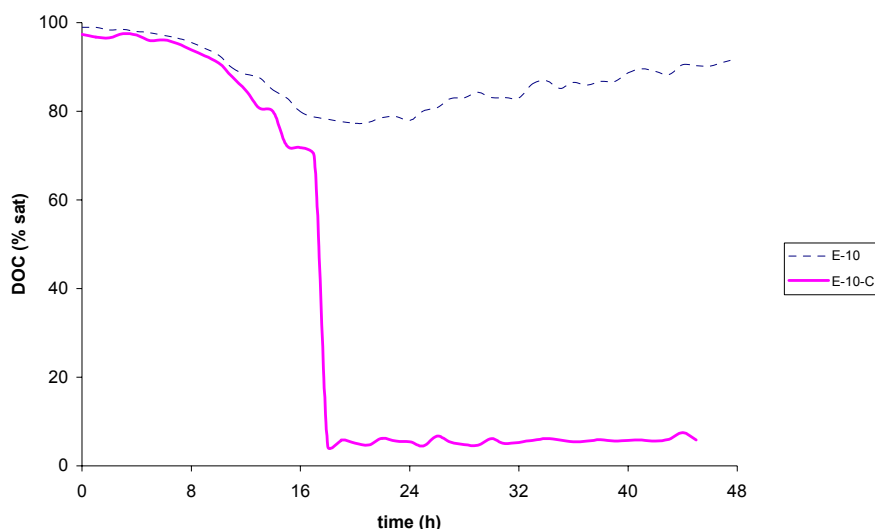

Figure1: DOC profiles (% saturation) in group I experiments (E-10: 1.0 v.v.m., no DOC control; E-10-C: 1.0 v.v.m., 5% DOC during production).

Retamycin production did not start until the end of the exponential phase and was detected at about 17 h in both experiments. Relative total retamycin concentrations (R) are given in Figure 3, depicting similar performances throughout both experiments.

The specific oxygen uptake rate (Q_{O2}) reached maximum values of 4.98 and 5.42 mmol O_2 g cell $^{-1}$ h $^{-1}$ at the end of the exponential phase (16 h) in experiments E-10 and E-10-C, respectively.   Its values decreased throughout the production phase,

from 17 h to 48 h of culture, as illustrated in Figure 4. It dropped to 1 mmol O_2 g cell $^{-1}$ h $^{-1}$ in experiment E-10, indicating a low oxygen demand for retamycin production corroborated by high DOCs during this phase, ranging from 80 to 90% saturation, in accordance with Figure 1. In E-10-C, with DOC control at 5% saturation during the production phase, Q_{O2} behaved similarly, dropping to values in the range of 0.5-1.5 mmol O_2 g cell $^{-1}$ h $^{-1}$ at the end of the culture.
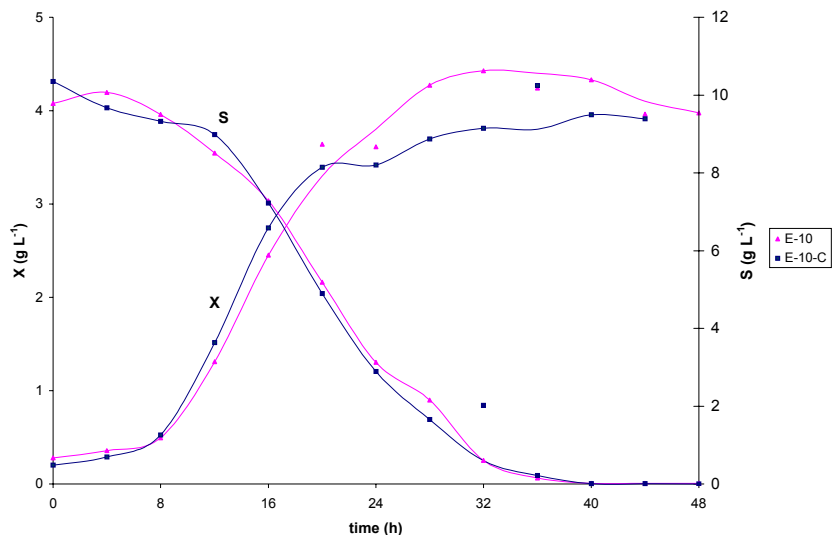

Figure 2: Biomass (X) and residual glucose (S) concentrations in group I experiments
(E-10: 1.0 v.v.m., no DOC control; E-10-C: 1.0 v.v.m., 5% DOC during production).
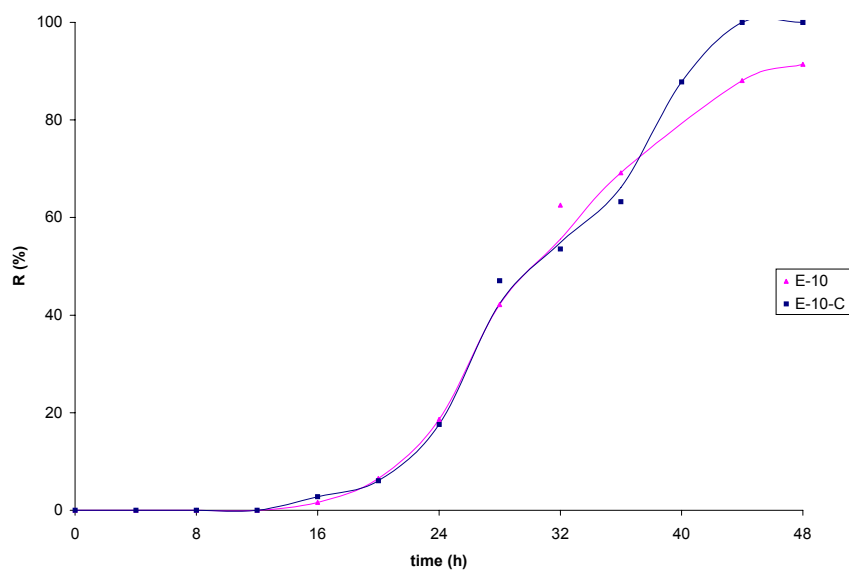

Figure 3: Relative total retamycin concentrations (R) in group I experiments (E-10: 1.0 v.v.m., no DOC
control; E-10-C: 1.0 v.v.m., 5% DOC during production). The total retamycin concentration
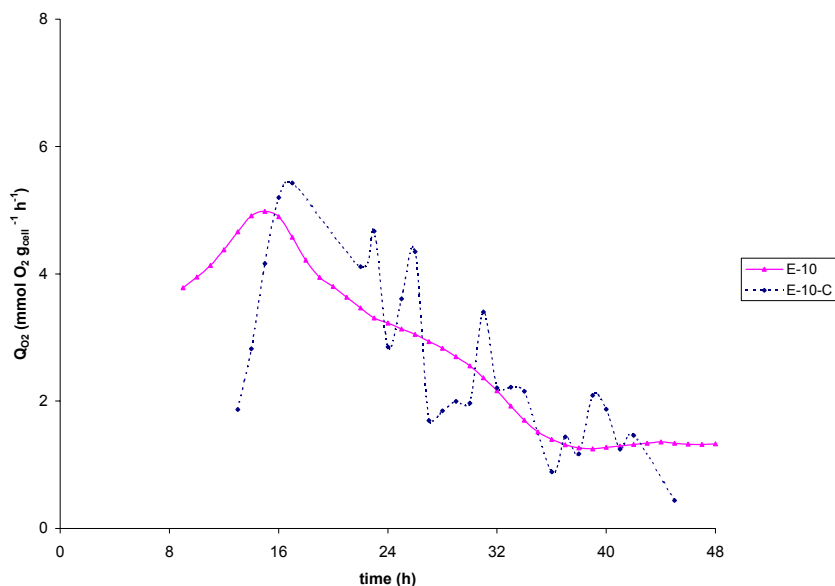was expressed relative to its maximum value obtained for group I experiments.

Figure 4: Specific oxygen uptake rates (Q_{O2}) during the production phase in group I experiments
(E-10: 1.0 v.v.m., no DOC control; E-10-C: 1.0 v.v.m., 5% DOC during production).

Group II

Experiment E-2-C was conducted at 0.2 v.v.m. and with DOC control at 100% saturation during the growth phase, brought about by supplementing air with an oxygen stream up to 21 h of culture, when retamycin was first detected. Subsequently, the set point was changed to 5% saturation and maintained throughout the production phase. The other experiment, E-2, was carried out at 0.2 v.v.m. and without DOC control. Figure 5 depicts DOC profiles for group II experiments.

According to Figure 6, biomass concentration (X) in experiment E-2, without DOC control, increased faster than in the DOC-controlled experiment, in which glucose was depleted at 28 h in the former, four hours earlier than in the latter. Nevertheless, both experiments obtained a final biomass concentration (X) of about 4 g L^{-1}. The values of cell productivity (P_x) achieved were 0.14 and 0.12 g $L^{-1}h^{-1}$, in E-2 and E-2-C, respectively. Despite similar behavior up to 12 h of culture, cell growth in the controlled experiment (E-2-C) from that time until 24 h was somehow inhibited, which led to lower cell productivity.

Concerning antibiotic production, both experiments reached maximum total retamycin concentrations at the end of the culture, as illustrated in Figure 7. The uncontrolled run, E-2, achieved higher retamycin concentrations from 16 to 30 h than those obtained in the DOC-controlled experiment. Unlike E-2, after 28 h, antibiotic yields in E-2-C increased significantly during the rest of the culture.

Figure 8 shows the profiles of specific growth and retamycin production rates in group II experiments. As illustrated, E-2 reached a maximum specific cell growth rate ($\mu_{x\ max}$) of about 0.22 h $^{-1}$. Experiment E-2-C, in contrast, achieved lower specific growth rates after 12 h, since DOC control at 100% saturation somehow inhibited cell growth. Unlike the negative effect on the growth phase, DOC control at 100% had a positive effect on retamycin production, increasing specific retamycin production rates to a maximum value 1.5 times as high as the one obtained in the uncontrolled experiment.

When DOC was controlled at 100% saturation during the growth phase in *Streptomyces clavuligerus* cultures, Yegneswaran et al. (1991) observed an average twofold increase in the specific activities of the β-lactam synthetases responsible for cephamycin C synthesis. As a result, an approximate threefold increase in the maximum cephamycin C yield was obtained in comparison to experiments without dissolved oxygen control. Similarly, Chen et al. (1990) also demonstrated that oxygen participates in the regulation of key biosynthetic enzymes and thus in the final yield of the antibiotics tylosin and macrocin by *Streptomyces fradiae*. Thus, it seems reasonable to suppose that higher oxygen concentrations in *Streptomyces olindensis* So20 fermentations increases the synthesis of enzymes involved in retamycin production.

As already observed in group I experiments, Q_{O2} in group II experiments dropped throughout retamycin production to values around 1 mmol O_2 g cell $^{-1}$h $^{-1}$ (data not shown).
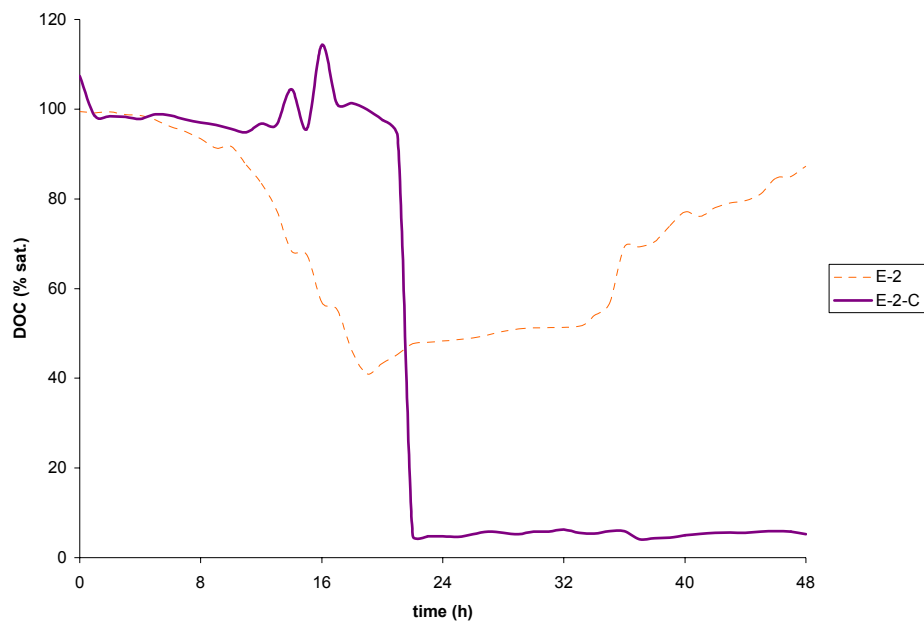
Figure 5: DOC profiles (% saturation) in group II experiments (E-2: 0.2 v.v.m., no DOC control; E-2-C: 0.2 v.v.m., 100% DOC during growth and 5% DOC during production).
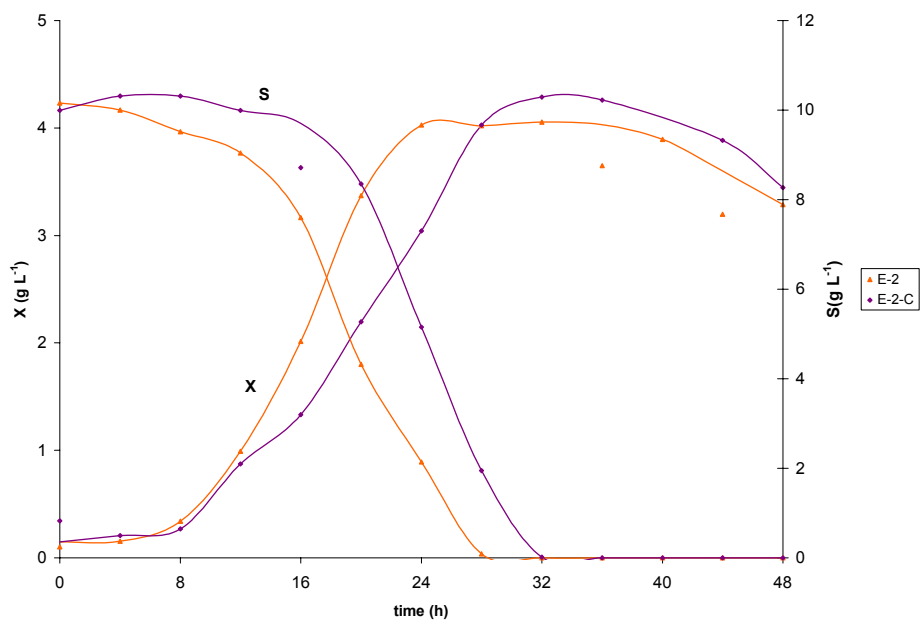
Figure 6: Biomass (X) and residual glucose (S) concentrations in group II experiments (E-2: 0.2 v.v.m., no DOC control; E-2-C: 0.2 v.v.m., 100% DOC during growth and 5% DOC during production).
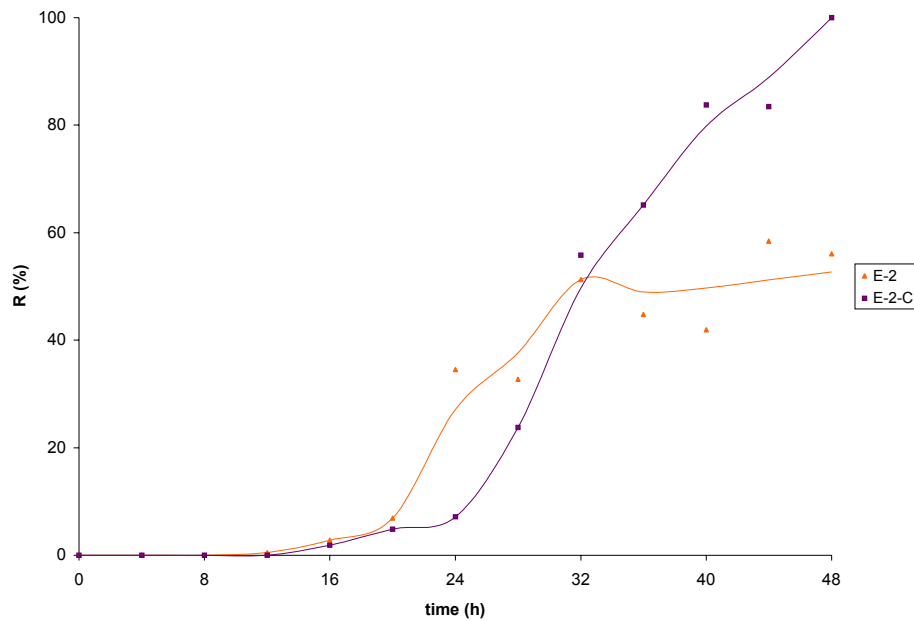
Figure 7: Relative total retamycin concentrations (R) in group II experiments (E-2: 0.2 v.v.m., no DOC control; E-2-C: 0.2 v.v.m., 100% DOC during growth and 5% DOC during production). The total retamycin concentration was expressed relative to its maximum value obtained for group II experiments.
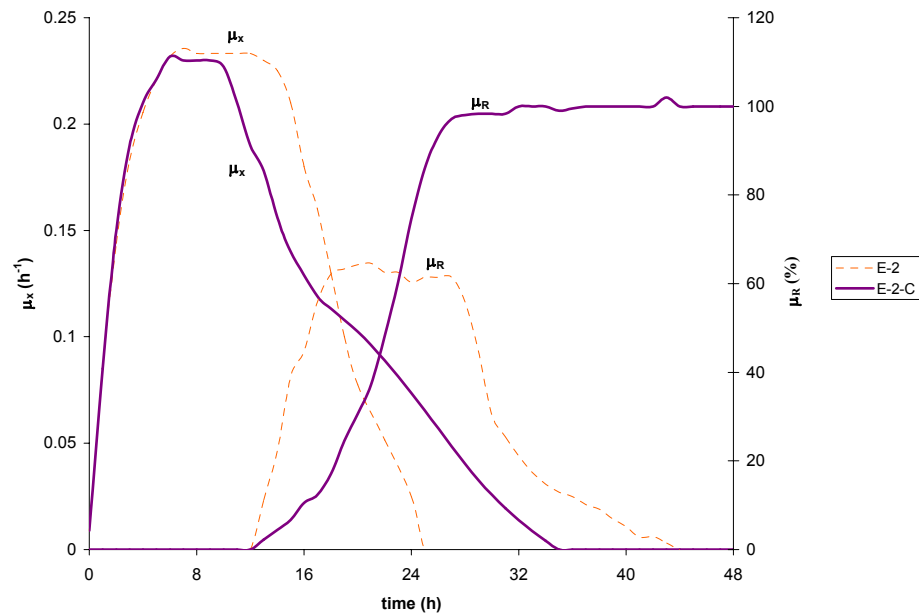

Figure 8: Profiles of the specific growth rate (μ_x) and the relative specific total retamycin production rate (μ_R) in group II experiments (E-2: 0.2 v.v.m., no DOC control; E-2-C: 0.2 v.v.m., 100% DOC during growth and 5% DOC during production). Specific total retamycin production rates were expressed relative to its maximum value obtained for group II experiments

CONCLUSIONS

The control strategy employed by Kempf et al. (1997), in which oxygen limitation during the production phase led to better gallidermin yields in *S. gallinarum* fermentations, did not succeed in *S. olindensis* So20 cultures. DOC control at 5% saturation during the production phase applied in group I experiment E-10-C had no effect on retamycin production, contrary to what occurred in the uncontrolled group I experiment. However, DOC control at 100% saturation during the growth phase in group II experiment E-2-C worked out, in spite of some cell growth inhibition, indicating production dependence on dissolved oxygen concentration in the culture medium, even under oxygen-sufficient conditions during the growth phase (Figure 5).

Therefore, a large oxygen supply in the culture medium during the growth phase was essential for achieving a better retamycin production performance, although even better performances can be obtained maintaining DOC at saturation. A large oxygen supply during the production phase, on the other hand, did not contribute to any improvement in retamycin production.

These results demonstrate the importance of studying DOC profiles in fermentations and how advantageous oxygen control can be.

NOMENCLATURE

DOC	dissolved oxygen concentration, % air saturation
N	rotational impeller speed, r.p.m.
P_x	cell productivity, $g\,L^{-1}\,h^{-1}$
Q_{O_2}	specific oxygen uptake rate, $mmol\,O_2\,g\,cell^{-1}\,h^{-1}$
$Q_{O_2}X$	volumetric oxygen uptake rate, $mmol\,O_2\,L^{-1}\,h^{-1}$
R	relative total retamycin concentration, dimensionless
S	residual glucose concentration, $g\,L^{-1}$
X	biomass concentration, $g\,L^{-1}$
ϕ	specific aeration rate, v.v.m.
μ_R	relative specific retamycin production rate, dimensionless
μ_x	specific growth rate, h^{-1}
$\mu_{x\,max}$	maximum specific cell growth rate, h^{-1}

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