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Anthracycline Production by *Streptomyces capoamus* in Batch Fermentation

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ABSTRACT

Anthracyclines have been intensely studied worldwide due to their importance as anticancer agents. In this work investigation was made on the production of ciclamycin, an antitumour complex of the anthracycline class, produced by Streptomyces capoamus*. The fermentation medium consisted of glucose, as the main carbon source, and soymilk, as the sole nitrogen source. Experiments were performed in a 5-liter batch bioreactor under nitrogen and carbon-limiting conditions. Increasing antibiotic activity was observed both intra and extracellularly during growth under the two conditions used. Progressive loss of activity occurred in both cases after glucose was depleted from the medium. The results obtained showed that harvest of ciclamycin in a batch process should be controlled by the level of glucose in the reactor and that nitrogen should preferably be the limiting substrate. Alternatively, results indicate that extended production might be achieved in a fed-batch process where nitrogen was the limiting substrate.*

Key words: Anthracycline; ciclamycin; *Streptomyces capoamus*; batch fermentation

INTRODUCTION

Anthracycline antibiotics are potent antitumor drugs used worldwide for the treatment of a wide variety of neoplasias. The main industrial products of this family are daunorubicin, active mainly on leukemias and lymphomas, and doxorubicin, which has the broadest spectrum of action of all antitumor drugs (El Khadem, 1982; Strohl et al., 1997). The main problem in their use is the cumulative cardiotoxicity (Semenov et al., 2001; Minotti et al., 2001) and there still remains an active search for new anthracyclines with higher efficacy to toxicity index or possessing a wider range of antineoplasic activities (Momose et al., 1998; Speitling et al., 1998; Miyamoto et al., 2000).

Anthracyclines are produced by *Streptomyces* spp. and the production process is similar to that of other secondary metabolites from these organisms. Batch fermentation and complex medium, containing slowly metabolizing carbon and/or nitrogen sources, are normally used. Even though industrial productions of daunorubicin and doxorubicin have been reported (McGuire et al., 1979; White and Stroshane, 1984), very little has been published on process and media development for maximal titer production of anthracyclines in commercial fermentations.

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Studies in our laboratories on ciclamycin, an antibiotic complex produced by *Streptomyces capoamus*, showed a highly inhibitory activity of this complex against experimental tumors (Gonçalves de Lima et al., 1964; Lyra et al., 1964). Later pharmacological and clinical studies proved its potential for treatment of human neoplasias (Santana et al., 1971; Asfora, 1978). By investigations on chemical composition, six components were identified and the composition of the complex was elucidated (Braga et al., 1967; Gonçalves de Lima et al., 1968; Bieber et al., 1982; Bieber et al., 1987).

In the present work, fermentation studies were undertaken to investigate patterns of ciclamycin production by *S. capoamus* on commercial glucose/soymilk complex medium in batch fermentation.

MATERIALS AND METHODS

Microorganism

A strain of *Streptomyces capoamus*, belonging to the Culture Collection of the Department of Antibiotics of the Federal University of Pernambuco (DAUFPE-M3123), was used. Stock culture was subcultured and maintained on solid medium containing 4 g/L glucose, 5 g/L starch, 10 g/L malt extract, 4 g/L yeast extract and 15 g/L agar (pH 7).

Fermentation

The inoculum was grown in 500 mL shake flask in liquid medium containing 50 mL of 4 g/L glucose, 10 g/L malt extract and 4 g/L yeast extract (pH 7), for 48 hours at 30ºC. This inoculum was transferred to a shake flask containing 450 mL of the fermentation medium described below, grown at the same conditions as before, and then inoculated in 4500 mL of the fermentation medium inside the bioreactor.

The fermentation medium consisted of 20 g/L glucose, 20-40 g/L soymilk, 1 g/L NaH_2PO_4, 5 g/L NaCl and 1 g/L $CaCO_3$. Growth was either carbon or nitrogen limited depending on the objective of the experiment. To estimate the appropriate glucose/nitrogen ratio, biomass yield on glucose, $Y_{X/S}$, and on nitrogen, $Y_{X/N}$, were assumed to be 0.5 g/g and 10 g/g, respectively, and these assumptions turned out to be good approximations to establish the nature of the limiting substrate.

An instrumented 5-liter-working-volume batch bioreator was utilized. Temperature, aeration and agitation were kept at 30°C, 0.5 vvm and 700 rpm, respectively; pH was not regulated. Aeration and agitation were such that oxygen was not growth limiting.

Analytical Methods

Biomass was evaluated as packed cell volume PCV (Cooney, 1981). A 10 mL sample was centrifuged in a graduated tube, at 7000rpm for 20 minutes, and the volume of sediment measured.

Reducing sugars were assayed using the dinitrosalicylic acid reagent (DNSA) method (Miller, 1959). The results were expressed as glucose concentrations using a calibration curve.

Glucose was determined using a BIOCLIN enzymatic/spectrophotometric assay kit .

Antimicrobial activity was determined by agar diffusion technique using disk papers (Martins, 1998). Centrifuged liquid sample was added directly to the disk paper to assay extracellular ciclamycin concentration. For intracellular concentration, the product was first extracted from centrifuged biomass using acetone. *Bacillus subtilis* ATCC-6633 was used as test-microorganism and results were expressed as doxorubicin concentrations using a calibration curve.

RESULTS AND DISCUSSION

Batch experiments were performed to investigate patterns of growth and antibiotic production under different growth-limiting conditions. Media were thus formulated with different amounts of soymilk, so that after exponential phase the microorganism growth was either under carbon/energy limitation or nitrogen limitation.

Growth could not be properly followed by the increase in biomass due to the presence of soymilk insoluble components; increase in centrifuged sediment was so used. However, it must be kept in mind that while cell volume increases during growth, soymilk sediment tend to deacrease due to solubilization.

Fig. 1 and Fig. 2 show the results obtained for an experiment where growth was limited by the nitrogen source. In Fig. 1, the PCV increased for about 30 hours when active growth occurred. At 30 hours of fermentation, even though more than

10 g/L reducing sugars were still present in the reactor, the PCV started to decline, indicating nitrogen depletion and soymilk components solubilization.

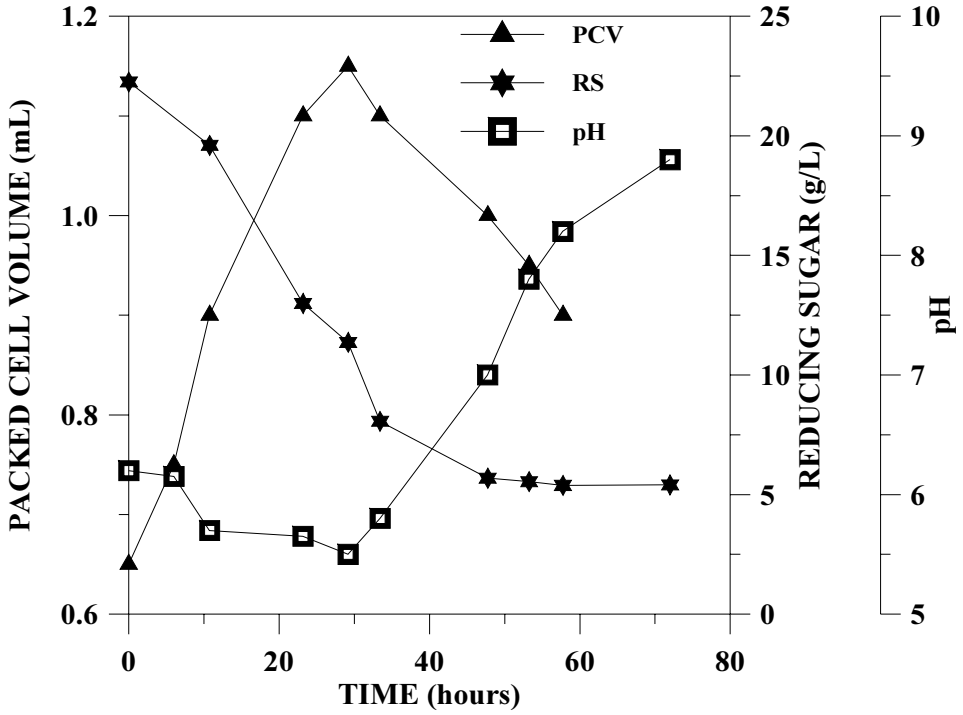

Figure 1 - Time course of biomass accumulation, reducing sugar consumption and pH variation for *Streptomyces capoamus* growth on glucose/soymilk under nitrogen-limiting condition.

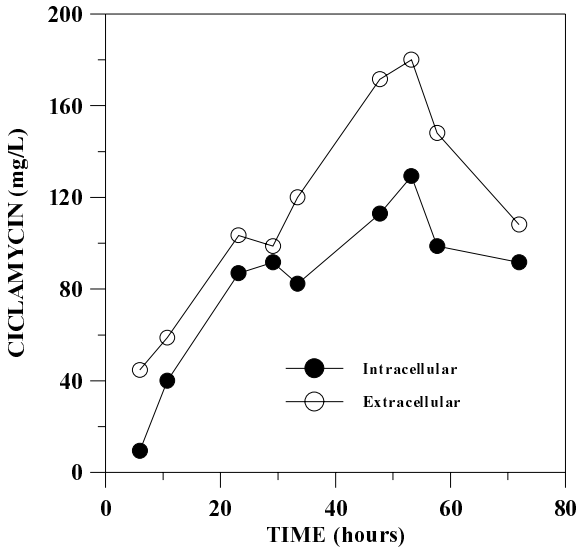

Figure 2 - Time course of ciclamycin accumulation for *Streptomyces capoamus* growth on glucose/soymilk under nitrogen-limiting condition.

The pH decreased during the active growth phase, increasing rapidly after 30 hours, another indication that growth was limited by depletion of nitrogen soluble components. Reducing compounds consumption ended at about 50 hours, even though they had not been totally utilized, what may be explained by the presence of nonfermentable reducing compounds in the broth composition.

Fig. 2 shows the results for antibiotic production. Growth and antibiotic production were mixed-associated under the condition used, in which production continues after growth has ceased (Moser, 1985).

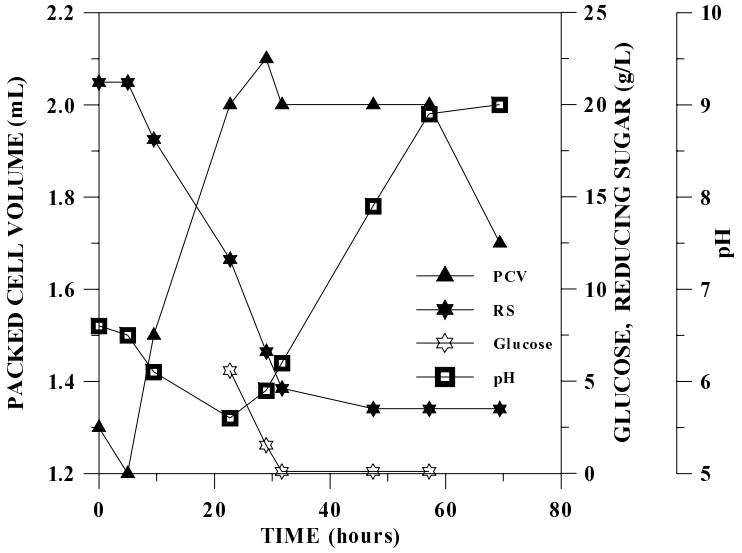

Figure 3 - Time course of biomass accumulation, reducing sugar consumption and pH variation for *Streptomyces capoamus* growth on glucose/soymilk under glucose-limiting condition.

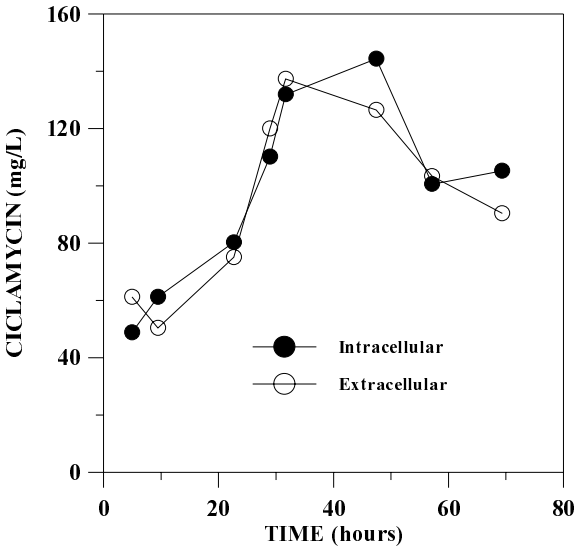

Figure 4 - Time course of ciclamycin accumulation for *Streptomyces capoamus* growth on glucose/soymilk under glucose-limiting condition.

Antibiotic activity was observed both intra and extracellularly throughout the fermentation process and intra and extracellular production followed the same pattern.

Product accumulation increased even after PCV declined and was halted only when fermentable sugars finished at about 50 hours of fermentation; progressive loss of antibiotic activity then occurred.

The results obtained for growth under carbon limitation is presented in Fig. 3. In this case, the initial soymilk concentration was duplicated, what resulted in an increase in the initial PCV.

Besides RS, glucose concentration was also determined to confirm carbon limitation. As in the first experiment, the PCV increased for about 30 hours, when glucose was still present in the medium. However, after glucose was depleted, PCV stabilized for another 30 hours and then decreased. The pH decreased during unlimited growth, increasing rapidly during restriction as in the previous case. Results for antibiotic production are shown in Fig. 4. Intra and extracellular accumulations have the same pattern; activity increases while glucose is present in the medium, after which loss of activity is observed. In this case, production was growth-associated.

It can be seen from the results above that loss of activity occurs in both cases when glucose is depleted from the medium. Greater extracellular activity production was obtained under nitrogen limiting condition, where excess of glucose was present in the environment during the stationary phase.

The loss of activity under glucose limitation might be explained by the fact that the activity of anthracyclines is related to the presence of sugar moieties in their molecules. Anthracyclinones, that is, anthracycline aglycones lacking the sugar moieties, are not biologically active (Strohl, 1997). The breakage of the glycoside bonds and release of sugars during glucose starvation might thus account for the loss of activity observed.

The chemical structure of the ciclamycin complex permitted visualizing its formation by following change in broth color during the fermentation processes. At the beginning of the fermentation, the medium was white due to soymilk; after 30 hours, it became brick, due to the formation of ciclamycin aglycone, pyrromycinone (Bieber et al., 1987); and violet at the end of the process, due to the increase in pH values, which changes pyrromycinone color. Since increasing loss of activity after glucose depletion was not followed by loss of color intensity, aglicone degradation probably did not occur.

Regarding to the fermentation process, the results obtained indicate that production of ciclamycin in a batch process should be controlled by the level of glucose in the reactor and nitrogen should preferably be the limiting substrate. Alternatively, extended production could be achieved in a fed-batch process where glucose was not allowed to attain very low levels and nitrogen was the limiting substrate.

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RESUMO

As antraciclinas têm sido estudadas intensamente devido à importância destas como agentes anticancerígenos. Neste trabalho, foi investigada a produção de ciclamicina, um complexo antitumoral da classe das antraciclinas, produzido por *Streptomyces capoamus*. O meio de fermentação consistiu de glicose, como principal fonte de carbono, e leite de soja, como única fonte de nitrogênio. Os experimentos foram realizados em um biorreator descontínuo de 5 litros, sob condições limitantes de carbono e de nitrogênio. Crescente atividade antibiótica, intra e extracelular, foi observada durante o crescimento nas duas condições de limitação nutricional. Em ambos os casos, foi observada perda progressiva de atividade antibiótica após a exaustão de glicose do meio. Os resultados obtidos mostraram que o final do processo em batelada deve ser controlado pelo nível de glicose presente no meio e o nitrogênio deve ser, preferencialmente, o substrato limitante do processo. Alternativamente, os resultados indicam que a extensão da produção poderia ser realizada em batelada alimentada com nitrogênio como substrato limitante.

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