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CARBON CATABOLITE REPRESSION OF RETAMYCIN PRODUCTION BY *STREPTOMYCES OLINDENSIS* ICB20

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ABSTRACT

Retamycin is an anthracyclinic antitumoral complex produced by *Streptomyces olindensis* ICB20. In this work the influence of different glucose concentrations in the feed medium on the production of retamycin was studied. Chemostat cultures employing glucose concentration varying between 10 g/L and 25 g/L showed that use of high glucose concentration resulted in catabolite repression of the biosynthesis of the antitumoral. The highest specific retamycin production rate, qRTM = 7.8 mg/g.h, was obtained when glucose concentration was 10 g/L. The lowest value of qRTM, 2.5 mg/g.h, was observed when glucose concentration was 20 and 25 g/L. The residual glucose concentration varied from 0 to 13 g/L, as the glucose concentration in the feed was increased from 10 to 25 g/L.

Key words: *Streptomyces*, anthracyclines, carbon catabolite repression

INTRODUCTION

Retamycin is an anthracyclinic complex produced by *Streptomyces olindensis* (1,2). Anthracyclines are potent antitumoral agents synthesized by streptomycetes through the polyketide pathway (3). Polyketides constitute a large group of complex natural products with great structural diversity (4,5). Biosynthesis of these compounds is similar to fatty acids biosynthesis, consisting of repeated condensations of acylthioesters (e.g. acetyl-CoA, propionyl-CoA, malonyl-CoA) (5). Daunorubicin polyketide synthase (PKS) is primed with propionyl-CoA and uses malonyl-CoA as elongation unit (3).

Production of secondary metabolites, such as anthracyclines, is frequently negatively affected by the carbon source, This regulatory phenomenon, termed carbon catabolite repression (CCR), affects the production of many secondary metabolites (6), e.g., cephamycin C production by *S. clavuligerus* (7), anthracyclines by *S. peucetius* var. *caesius* (8), actinorhodin by *S. lividans* (9).

Under carbon-excess condition, other phenomena occur, such as futile cycles and overflow metabolism (10,11,12). Overflow metabolism consists of excretion of intermediate metabolites as a result of metabolic imbalance, such as organic acids (13-16).

However, the mechanisms involved in the regulation of secondary metabolism are not well understood (17). CCR appears to be exerted on secondary metabolism through glycolytic pathways intermediates, e.g. fructose 1,6-diphosphate and glucose 6-phosphate (7) or by enzymes of the catabolic pathways as glucose kinase (18).

Several previous works focused on the influence of various physical-chemical factors on the production of retamycin (19-22), however, the influence of carbon catabolite repression was not studied yet. Considering that there are relatively few data about physiology of streptomycetes, this work had the objective of studying the influence of different glucose concentrations on the production of retamycin and for this purpose, continuous cultures employing phosphate-limited

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defined medium with different glucose concentrations in the feed were run.

MATERIALS AND METHODS

Microorganism

A mutant strain of Streptomyces olindensis ICB supplied by Laboratório de Genética de Microrganismos / Instituto de Ciências Biomédicas / Universidade de São Paulo was used in this study. Mycelial suspensions in 20% glycerol solution were stored at -80ºC in 10 mL cryotubes (20).

Culture conditions

Inoculum

Inocula for the continuous cultures were prepared in a rotary shaker (New Brunswick) at 200 rpm and 30ºC following a two-step procedure as follows: 10 mL of mycelial suspension was used to inoculate a 1L Erlenmeyer flask containing 90 mL of R5M medium and after incubation for 16h, an aliquot of 20 mL of medium was used to inoculate an Erlenmeyer flask containing 180 mL of R5M medium and incubated for 24h (23).

Chemostat

Chemostat cultures were run in a 2.5 L Bioflo III (New Brunswick) bioreactor containing 1.8 L of phosphate-limited defined medium. The bioreactor was inoculated with 200 mL of R5M grown culture. The chemostat cultures were run under the following conditions: working volume 2.0 L, agitation rate 600 rpm, air flow rate 1.6 L/min, temperature 30ºC, pH 7.0, dilution rate (D) 0.65 h^{-1}.

Media composition

Inoculum

The R5M medium composition was as follows (g/L) (23): glucose (Synth) 10; yeast extract (Oxoid) 5; casaminoacid (Merck) 0.1; tris(hydroximethyl) aminomethane (Merck) 3.09; $MgCl_2.6H_2O$ (Merck) 5.06; K_2SO_4 (Merck) 0.5. The pH was adjusted to 7.2 and autoclaved for 20 min at 120ºC. After sterilization, the following sterile solutions were added: 0.5% (w/v) KH_2PO_4 (Merck) 10 mL/L; 5 mol/L $CaCl_2$ (Synth) 4.0 mL/L; trace elements 2.0 mL/L. Trace elements solution (mg/L): $ZnCl_2$ (Merck) 40; $FeCl_3.6H_2O$ (Merck) 200; $CuCl_2.2H_2O$ (Merck) 10; $MnCl_2.4H_2O$ (Synth) 10; $Na_2B_4O_7.10H_2O$ (Merck) 10; $(NH_4)_6Mo_7O_{24}.4H_2O$ (Synth) 10.

Bioreactor: The composition of phosphate-limited defined medium was as follows (g/L): $NaNO_3$ (Synth) 6.8; $MgCl_2.6H_2O$ (Merck) 2.53; K_2SO_4 (Merck) 0.25; KH_2PO_4 (Merck) 0.25. The glucose concentration in the feed medium was varied between 10 and 25 g/L. The pH of the medium was adjusted to 7.2 and autoclaved for 20 min at 120ºC. Glucose, KH_2PO_4, and 20 mL/L of trace elements solution were added after sterilization.

Analytical procedures

Steady-state samples were assayed for biomass, residual glucose, retamycin and organic acids. Biomass was evaluated by dry weight determinations: 10 mL aliquot was vacuum filtrated through 1.2 mm membrane (Schleicher & Schuell) and dried in an microwave oven for 15 min at 180 W (23).

Residual glucose was determined from the filtrate using the glucose-oxidase method.

Retamycin: The methodology for retamycin assay was adapted from the procedure proposed for anthracyclines determination (24). A 5 mL aliquot of culture broth was mixed with 10 mL of 80:20 acetonitrile/water mixture and vorticed for 10 min and filtered through filter paper. The absorbance at 495 nm of the filtrate was determined and the concentration of the antitumoral was evaluated using a calibration curve elaborated using a partially purified product.

Concentrations of pyruvate, acetate, succinate, lactate, and citrate were determined by HPLC (Waters 600E System Controller) using an UV detector (Waters 484 Tunable Absorbance Detector) at 220 nm, the samples were injected into an Aminex HPX87h column (BioRad) at 35ºC. The eluent was an 8 mmol/L H_2SO_4 solution at a flow rate of 0.6 mL/min.

Experimental values presented in this work refer to the mean of at least three samples collected during steady state.

RESULTS AND DISCUSSION

Continuous cultures with glucose concentration in the feed varying between 10 and 25g/L were carried out in order to assess the effects of carbon catabolite repression in submerged cultures of *S. olindensis* ICB20. The results at steady state are presented in Figs. 1 and 2 and in Tables 1 and 2.

Biomass concentration (X) remained approximately constant irrespective the glucose concentration in the feed, about 3.1 g/

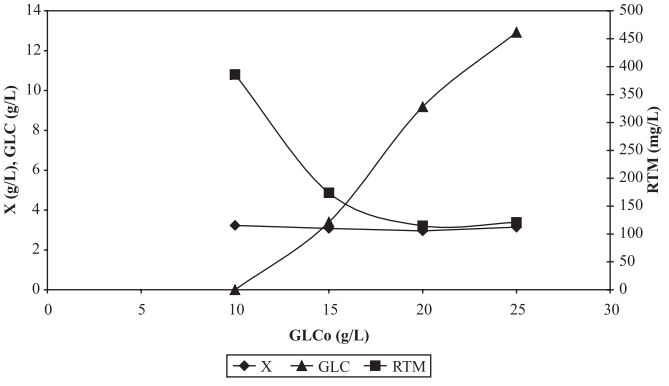

Figure 1. Steady-state values of biomass (X), residual glucose (GLC) and retamycin concentration for different glucose concentration in the feed (GLCo).

L (Fig. 1). However, the specific glucose consumption rate ($qGLC=D/Y_{X/GLC}$) increased when higher glucose concentrations were used in the feed (GLCo), indicating an increased overflow metabolism (11). As a result, the biomass yield ($Y_{X/GLC}$) was slightly lower when the glucose concentration was greater than 10 g/L, decreasing from 0.33 g/g to values around 0.27 g/g, as shown in Table 1. As expected, use of higher glucose concentration in the feed also led to higher residual glucose concentration (GLC) (Fig. 1), increasing from zero to about 13 g/L when 25 g/l of glucose was used in the feed.

Glucose concentrations higher than 10 g/L in the feed medium impaired retamycin production (Fig. 1), resulting in lower titers of the product, the highest product concentration was obtained when 10 g/L of glucose in the feed was used, approximately 386 mg/L. When glucose concentration of 15 g/L was used, the product titer reached 174 mg/L. At glucose concentrations higher than 20 g/L there seems to be no additional effect on retamycin biosynthesis, as the product concentration remained approximately constant, about 120 mg/L.

The specific retamycin production rate (qRTM=D.RTM/X) decreased when glucose concentration was increased. At 10 g/L of glucose, qRTM was about 7.8 mg/g.h, at 15 g/L of glucose, qRTM was about 3.7 mg/g.h, and at 20 and 25 g/L of glucose, qRTM was approximately 2.5 mg/g.h. The highest retamycin yield ($Y_{RTM/GLC}$), 40.2 mg/g, was obtained when glucose concentration in the feed was 10 g/L, decreasing to 15.1 mg/g, when GLC was 15 g/L, and at higher glucose concentrations $Y_{RTM/GLC}$ was about 10 mg/g (Table 1). These results are in agreement with other similar works, which observed that the biosynthesis of anthracyclines by *S. peucetius* var. *caesius*, in shaker cultures, was repressed with glucose concentration above 18 g/L (8).

The higher the glucose consumption rate, the lower the retamycin production rate, as shown in Fig. 2, indicating that the flux through glycolytic pathway possibly plays an important role in the repression of the biosynthesis of the antitumoral.

Increased glucose concentration also led to increased amounts of excreted organic acids (Table 2), indicating greater activity of overflow metabolism (10,12). Increased excretion of citrate may be related to a decrease in the activity of the a-ketoglutarate dehydrogenase activity, the enzyme that converts a-ketoglutarate to succinyl-CoA (25). This fact may indicate a problem in the generation of precursors for the biosynthesis of the antitumoral, as one of the possible sources of propionyl-CoA is the rearrangement of succinyl-CoA (26). Although a decreased TCA (tricarboxylic acids cycle) activity could mean additional supply of malonil-CoA, which can be generated by carboxylation of acetyl-CoA (27), retamycin biosynthesis was not favoured under these conditions.

The chemostat cultures results showed that when high glucose concentrations, above 10 g/L in the feed medium, were used, the retamycin biosynthesis was repressed, and concomitantly an increase in overflow metabolism was observed, as evidenced by greater organic acid excretion, resulting in lower biomass yield.

Table 1. Glucose concentration in the feed (GLCo), residual glucose (GLC), biomass yield ($Y_{X/GLC}$), and retamycin yield ($Y_{RTM/GLC}$), D=0.065 h^{-1}.

GLCo	GLC	$Y_{X/GLC}$	$Y_{RTM/GLC}$
g/L	g/L	g/g	mg/g
10	0.0	0.33	40.2
15	3.4	0.27	15.1
20	9.2	0.25	9.9
25	12.9	0.27	10.5

$$Y_{XIGLC} = \frac{X}{GLCo - GLC}$$

$$Y_{RTMIGLC} = \frac{RTM}{GLCo - GLC}$$

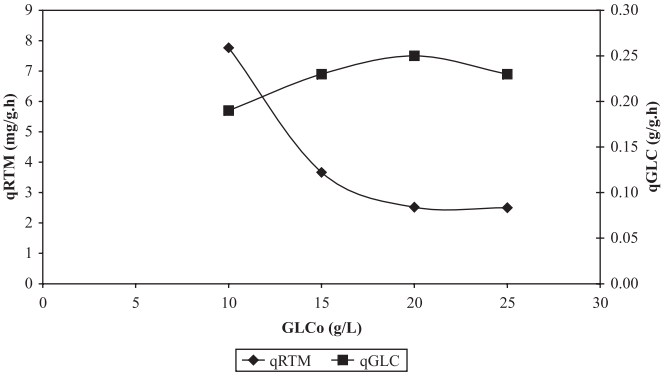

Figure 2. Steady-state values of specific retamycin production rate (qRTM) and specific glucose consumption rate (qGLC) for different glucose concentration in the feed.

Table 2. Glucose concentration in the feed (GLCo), pyruvate, citrate, succinate, lactate and acetate, D=0.065 h^{-1}.

GLCo G/L	Pyruvate mmol/L	Citrate mmol/L	Succinate mmol/L	Lactate mmol/L	Acetate mmol/L
10	-	0.11	-	0.67	-
15	*	0.44	2.88	-	10.4
20	*	0.64	7.91	-	12.1
25	0.01	0.78	4.05	-	18.9

*traces.

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RESUMO

Repressão catabólica da produção de retamicina por *Streptomyces olindensis* ICB20

A retamicina é um complexo antitumoral antraciclínico produzido por *Streptomyces olindensis* ICB20. Neste trabalho estudou-se a influência de diferentes concentrações de glicose no meio de alimentação sobre a produção de retamicina. Os resultados de cultivos contínuos mostraram que o uso de elevadas concentrações de glicose resultou em repressão catabólica da biossíntese do antitumoral. A maior velocidade específica de produção de retamicina, qRTM = 7,8 mg/g.h, foi obtida quando a concentração de glicose foi de 10 g/L. O menor valor de qRTM, 2,5 mg/g.h, foi observado quando a concentração de glicose foi de 20 e 25 g/L. A concentração de glicose residual aumentou de 0 a 13 g/L conforme a concentração de glicose na alimentação foi incrementada de 10 a 25 g/L.

Palavras-chave: *Streptomyces*, antraciclinas, repressão catabólica

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